Medical Aspects of Chemical and Biological Terrorism

Biological Terrorism and Traumatism

Edited by
ALEXANDER MONOV
CHRISTOPHOR DISHOVSKY
Contributors

Robert M. DeBell, Ph.D.
Senior Research Leader
Vice President
Battelle Memorial Institute
1725 Jefferson Davis Highway, Suite 501
Arlington, Virginia 22202-4172
USA

Christophor Dishovsky, M.D., Ph.D., D.Sc.
Department Military Toxicology
Military Medical Academy
3, St.G.Sofiiski Str.
1606 Sofia
Bulgaria

Philip H. Elzer
LSU AgCenter and School of Veterinary Medicine
Departments of Veterinary Science and Pathobiological Sciences
111 Dalrymple Building
Baton Rouge, LA 70803
USA

Paul N. Florin, M.D., Ph.D., M.PH.
Medical Directorate
Ministry of National Defense
Romania

Lieve M. F. Herman
Agricultural Research Centre
Department of Animal Product Quality
B-9090 Melle
Belgium

Ivan Ivanov
Department of Microbiology
National Center of Infectious and Parasitic Diseases
26, Yanko Sakazov Blv.
1504 Sofia
Bulgaria

Todor Kantardjiev, M.D., Ph.D.
Head of Microbiology Department
National Center of Infectious and Parasitic Diseases
26, Yanko Sakazov Blv.
1504 Sofia
Bulgaria

Alexander Monov, Professor of Toxicology
Scientific Consultant of Clinical Toxicology
President “Medical Sciences” Section at the Union of Scientists in Bulgaria
24, Midjur Str.,
1421 Sofia
Bulgaria
Hristo M. Najdenski, Ph.D.  
Deputy Director  
Institute of Microbiology  
Bulgarian Academy of Sciences  
1113 Sofia  
Bulgaria

Plamen Nenkov, M.D., D.Sc.  
Professor, National Center of Infectious and Parasitic Diseases  
BulBio – NCIPD  
Sofia  
Bulgaria

Elena Ryabchikova, Ph.D., D.Sc.  
Head of Laboratory of Ultrastructural Researches and Pathomorphology  
State Research Center of Virology and Biotechnology ”Vector”  
Koltsovo, Novosibirsk region  
630559  
Russia

Eva K. Tcherneva  
Central Veterinary Research Institute  
1606 Sofia  
Bulgaria

Bogdan Petrunov, M.D., D.Sc.  
Professor, Director  
National Center of Infectious and Parasitic Diseases  
BulBio – NCIPD  
Sofia  
Bulgaria

Olga Timoshenko,  
State Research Center of Virology and Biotechnology ”Vector”  
Koltsovo, Novosibirsk region  
630559  
Russia

Nancy P. Rijpens  
Agricultural Research Centre  
Department of Animal Product Quality  
B-9090 Melle  
Belgium

Il’ya Vinogradov  
State Research Center of Virology and Biotechnology ”Vector”  
Koltsovo, Novosibirsk region  
630559  
Russia
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Preface

The fight against terrorism, including its medical aspects is one of the most pressing issues of the new century. Terrorism definitely has many aspects.

The use of Sarin during the terrorist act in Tokyo and the city of Matsumoto in Japan, as well as the use of Anthrax and other agents for terrorist goals showed that the weapons of mass destruction can also be used in times of peace. Nuclear weapons when used by terrorists are endangering them, and, on the contrary, chemical and biological agents could be used for terrorist actions with no danger for the terrorists. Moreover, chemical and biological weapons can be created quite easily. The importance of chemical weapons also increases due to the fact that during terrorist acts on plants and other industrial facilities, a lot of toxic chemicals, including such chemicals that could be synthesized under the conditions of the terrorist act and that could cause fire or explosion, could be released. In such an accident, the act of terrorism turns into mass traumatism. Lastly, chemical and biological weapons are becoming easier to be carried around and more difficult to be found.

Scientists from all over the world believe that the danger of use of chemical and biological substances for terrorist goals is increasing. The continuous increase of the number of synthesized chemical substances (including highly toxic ones), as well as the development of gene engineering and other high technologies, require a lot of attention on the part of the scientists. This is necessitated by the possibility of these substances and technologies to be used for development of new biological and chemical weapons. Yet, the problems related to the indication, protection against these biological and chemical substances, and their destruction increase alongside. Last but not least, the attention paid to the medical treatment the victims of these substances should receive is greater than ever before. And this attention is based on the increased chances for forming of new pathologies in the human organism, which respectively require new diagnostics and medical approaches.

The terrorist acts in the USA of 11 September 2001 led both to a change in the danger assessing criteria and in the extent of readiness to prevent terrorism on a global level. New guidelines came forth in research encompassing chemical, biological and radiological defense.
The series *Medical Aspects of chemical and biological terrorism* aims to give the Bulgarian and international authors the opportunity to reflect on current scientific aspects of chemical and biological terrorism issues and mass traumatism. Considering the multidimensional issues and problems related to the above topics, our series will be divided in topical areas.

The first issue will be devoted to the biological terrorism and traumatism, because of the rapidly increasing interest for this area. The editors did their best to form a highly competent international team of authors to mirror a variety of hot topics illustrating scientific and applied aspects of the problem of biological terrorism and traumatism.

The chapter contributors are experts in science of the biological and toxic chemicals agents. Their contributions are summarized as follows:

Ryabchikova, Timoshenko and Vinogradov analyze the possibilities electron microscopy provides to investigate outbreaks of unknown aetiology diseases caused by the intentional use of viruses.

Najdenski, Tcherneva, Rijpens and Herman describe the subtractive hybridization/amplification procedure used in an attempt to develop PCR primers, specific for *B. canis* and subsequently for *Brucella* spp.

Petrunov and Nenkov demonstrated the capacity of one polybacterial immunostimulant in the treatment of viral diseases and in modulation of the immune reactivity based on interferon production.

Kantardjiev and Ivanov experimental data show that the environment protozoa may serve as important reservoirs and sources of *F. tularensis*.

Florin offers a review of the biological warfare and bio-terrorism. He presents his own concept as to the steps to be taken by medical authorities in the event of BW attack in order to reduce the morbidity and mortality in the population to the utmost degree possible.

Elzer provides a truly comprehensive review of zoonotic bacteria as biological and agroterrorist weapons.

DeBell shows a future perspective on the manipulation of *Bacillus anthracis* and the progress in its therapies.

Monov presents a unified doctrine of the fight against biological terrorism and traumatism and modern clinical concepts of effective behaviour in conditions posing immediate life-threat to the injured, such as acute respiratory insufficiency, acute cardio-vascular insufficiency, acute cerebral insufficiency including loss of consciousness and seizures, acute immune deficiency. The author suggests emergency effective diagnostic and therapeutic methods for speedy control of the above
mentioned critical conditions which manifest in all severe forms of traumatism and in terrorism making use of highly damaging agents. Monov characterizes food poisoning as a model of biologic terrorism and traumatism. An original presentation is given of amanitin intoxications as a model of biologic terrorism and traumatism.

Dishovsky reviews some basic aspects and draws attention to certain differences in the preparation to fight chemical and biological terrorism.

Finally, the editors wish to thank all contributors for their participation. They all took part in this series creation on a voluntary basis and were not paid anything for their efforts. The same refers to the editors. The latter have the ambition to further on this scientific series with the issues to follow. The editors strongly hope that the authors’ pains will be a contribution to the world’s struggle against terrorism.

Alexander Monov, Christophor Dishovsky
About the Editors

Alexander Monov is born in Pleven, Bulgaria in 1921. He studied medicine, graduated the medical faculty and took “doctor of medicine” degree in 1945 at the “St. Kliment Ohridsky” state university in Sofia, Bulgaria. Dr. Alexander Monov was elected assistant professor (1968), professor -research associate 1st degree of toxicology (1973) and university professor of internal medicine and clinical toxicology (1976). Prof. Monov was director of the Clinic of Urgent Internal Medicine. He is founder, director and present patron of the National Clinical Poisoning Centre at the “Pirogov” Institute, Sofia, of the Bulgarian School of Clinical Toxicology and Chief republican toxicologist, more than 40 years. He is also honorary president of the Bulgarian Association of Clinical Toxicology, constant member of the Bulgarian National Academy of Medicine, founder-member of the European Association of Poison Control Centre, founder-member and ex-regional secretary for Europe of the World Federation of Clinical Toxicology, member of the Bulgarian Toxicological Society (EUROTOX and IUTOX); president of the “Medical Sciences” Section of the Union of Scientists in Bulgaria. Prof. Monov is one of the fathers of clinical toxicology as a separate discipline on a world scale. He is more than 50 years scientific researcher, clinicist and university lecturer in the field of toxicology, urgent medicine, mass traumatism and terrorism. In his books and publications he is author of doctrines treating all aspects of toxicology, important issues of the coma states, shock states, immunity pathology, clinical ecology and others. He is laureate of prestigious national and international awards.

Christopher Dlshovsky was born in Sofia, Bulgaria, in 1940. He graduated in 1966 from Sofia Medical University. He obtained a PhD- (Kiev Medical Institute, Kiev, former USSR, 1971) and D.Sc. (Military Medical Academy, Sofia, 1989) degrees in toxicology for research of mechanism of action and development of new antidotes of nerve agents intoxications. Professor of Toxicology (1989) and Pharmacology (1996), with extensive experience of almost 36 years in Military Toxicology, Pharmacology and Chemical Defense. Included in the Golden Book of Bulgarian Discoverers and Inventors. President of Bulgarian Toxicological Society (member of EUROTOX and IUTOX).
1 The Problems of Chemical and Biological Terrorism

Christophor D. Dishovsky

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I. INTRODUCTION

The recent use of sarin in the terrorist acts in Matsumoto city and Tokyo underground was considered by a number of specialists a new era in terrorism. It removed any doubts about the possibility of using chemical weapons by terrorists. The members of the Japanese cult Aum Shinrikyo produced also anthrax and botulinum toxin and tried to spread it in Tokyo using sprayer systems. Fortunately the attacks with them were not successful.

The events of September 11, 2001 showed that a serious reassessment of the means for battling terrorism is needed. The recent anthrax and ricin attacks on the mail supply system in the US emphasized the need for advances on countering the effects of biological and chemical terrorism.

Today, it is clear that terrorism can not only be a state policy, but can also be realized by separate individuals, groups or organizations. Considering the world globalization, the anti terrorist measures could be effective if only they are the result of all countries and international organizations participation. Even if terrorist acts occur on an individual country or regional level only, the response system in case of chemical and biological terrorist acts is a problem of all state institutions.
In the recent years, the preparation for anti terrorist actions has improved quantitatively and qualitatively both on individual countries' level and on the level of numerous international organizations. It could be considered that overall, in some countries, the main conceptual points of organization are cleared and that plans are developed on country, local and institutional level [1, 2, 3, 18, 25, 27]. The main issue is that the "how to's" of organization are only conceptually cleared so far. The actual process of implementation, if it has started at all, has different speed in the various countries. And we also should not forget that there are some countries that tend to support and spread terrorism. Therefore, it is too early to make conclusions on the real and major success of the fight against terrorism.

II. THE CHARACTERISTICS OF THE CHEMICAL AND BIOLOGICAL WEAPONS

Due to their characteristics, the biological and chemical agents that can be used for the spread of terrorism, have huge importance on international scale. The human kind has used biological and chemical agents for military and terrorist purposes since long ago [4, 19, 20, 23,]. Regardless, these agents possess numerous qualities that make them useful for terrorists at present.

Chemical and biological weapons have a number of advantages that make them a priority among terrorist agents [7, 8, 12, 20]:

- relatively easy and cheap production;
- available and easy to access precursors of chemical weapons, which are routinely used in industry and in life;
- precursors and toxic chemicals are easy to carry and transport because of difficulties to monitor and control their movement;
- resulting damage are considerable in amount and content;
- their fast effect requires emergency response which makes difficult the rescue operation;
- with time their psychological impact will extend far beyond the actual size of damage or number of casualties;
- the arsenal of chemical agents likely to be used for terrorist purpose is practically unlimited.

A very important feature of biological agents is that apart from being relatively easy and cheap to make, these agents can also have very significant effect in very small dosages. Unfortunately, genetic modifications of these agents could lead to the creation of biological weapons with unknown qualities. In such a sce-
nario, it may turn out that the supplies of vaccines and antibiotics may not be
enough to successfully treat the resulting damage [6, 13, 14, 24].

Amongst other advantages of the biological agents is the fact that they
could self recreate and spread, including outside the area of initial damage. More-
over, some of these agents cause illnesses that have symptoms similar to the en-
demic illnesses of the area of damage. Further, it is also hard to detect biological
agents, due both to the available techno-logy and the difference in their incubation
time [18, 19, 23]. Many researchers mention as biological weapons, advantage
their weaker effect on the environment, especially compared to the nuclear and
other weapons.

Thus, in light of all of the above, the preparation of anti terrorist defense
should include the optimal clarification of what chemical and biological weapons
have been used by the terrorists. Amongst the chemical agents that might be used
by terrorists the following could be considered [7, 8]:
– chemical weapons that are already known;
– chemical agents, which may be used as chemical weapons with the
help of a new technology;
– unknown chemical agent, which may be used as chemical weapons;
– chemical agent, which cause public panic and social disruption;
– chemicals commonly used in the industry [8, 10, 15];
– pesticides;
– toxins;
– chemicals, which may be obtained after explosion, fire or other inci-
dents in the industry or transportation of chemicals.

In addition to chemical weapons, terrorists can use different toxic chemi-
cals from che-mical industry, agriculture or products released from terrorist acts
on industrial facilities [8, 9, 10, 15]. There are some differences in use between
chemical weapons and any terrorist act employing deliberate destruction of chemi-
cal plants resulting in toxic chemicals release:
– both can be directed against military forces and or civil population,
– military or terrorist attacks against an industrial facility, cause eco-
   nomic damage, civilian casual-ties and environmental damages;
– industrial chemicals are less toxic than CW, but will be some times
   available in much higher quantity;
– contamination with the hazardous industrial chemicals some times
   will cover a big area;
– chemical weapons are relatively small number of potential agents;
  toxic industrial chemicals- tens of thousand;
– decontamination some times will be long lasting and expensive process;
– the products of decontamination can also damage the environment;
– relatively simple detection and identification equipment and methods have been deve-loped for specified chemical weapons; the potential variety of industrial chemicals makes detection problem very diffi-
cult;
– military protective filters are optimized against chemical weapons and biological weapons; some hazardous industrial chemicals are not adequately filtered by military filters.

Chemical Accident Database (United Nations Awareness and Preparedness for Emergencies at the Local Level - APELL) showed that hydrocarbon production, storage, transportation and distribution facilities are at the top of the list of potential targets. Considerable accidents occurred with explosive industrial chemicals, chlorine, ammonia, industrial acids and bases, pesticides and chemical inter-
mediates, polychlorinated biphenyls, unspecified chemicals and others.

Analysis on biological agents shows many bacteria, fungi, viruses, rickettsial agents, and toxins have been mentioned in various literature sources as possible biological warfare agents. Those mentioned most often include Bacillus anthracis (anthrax), botulinum toxin, Yersinia pestis (plague), ricin, Staphylo-
coccal enterotoxin B (SEB), and Venezuelan equine encephalitis virus (VEE) [5, 12, 20, 23]. Even minor genetic changes or the use of new technologies to spread the toxins could lead to development and usage of new biological agents. It should also be considered that many biologic agents actually possess higher toxicity than the chemical agents. Table 1 shows examples.

The use of the biological agents as weapons or for terrorist purposes has a few specific characteristics. Mainly this is the huge number of the injured, secondly, these agent spread via contagion, which differs them from the chemical agents. Therefore, it is difficult to isolate and treat the affected people, as the therapy could be very lengthy and have many side effects [13, 24,25]. In addition, the timely self aid and buddy aid is not as critical as in cases of use of chemical weapons.

At the same time, the incubation period of such agents allows the spread of the conta-gion and raises the criticality of the issue of indication of the biological agents. Overall, there is a need for means of indication, which would allow for the correct and fast diagnosing and the adequate treatment of the contagion with biological agents. When diagnosing the effect of the biological agents, it is neces-sary to consider the similarities and differences between some of the toxins and some chemical agents [12 ].
TABLE 1
Comparative Lethality of Some Current Toxins and Chemical Agents

<table>
<thead>
<tr>
<th>AGENT</th>
<th>LD$_{50}$(µg/kg)</th>
<th>SPECIES</th>
<th>SOURCE</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TOXINS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Botulinum Toxin</td>
<td>0.001</td>
<td>Mice</td>
<td>Bacterium</td>
<td>12</td>
</tr>
<tr>
<td>Tetanus Toxin</td>
<td>0.002</td>
<td>Mice</td>
<td>Bacterium</td>
<td>12</td>
</tr>
<tr>
<td>Diphtheria Toxin</td>
<td>0.10</td>
<td>Mice</td>
<td>Bacterium</td>
<td>12</td>
</tr>
<tr>
<td>Batrachotoxin</td>
<td>2.0</td>
<td>Mice</td>
<td>Arrow-Poison Frog</td>
<td>12</td>
</tr>
<tr>
<td>Ricin</td>
<td>3.0</td>
<td>Mice</td>
<td>Plant (Castor Bean)</td>
<td>12</td>
</tr>
<tr>
<td>Tetrodotoxin</td>
<td>8.0</td>
<td>Mice</td>
<td>Puffer Fish</td>
<td>12</td>
</tr>
<tr>
<td>Aconitine</td>
<td>100.0</td>
<td>Mice</td>
<td>Plant (Monkshood)</td>
<td>12</td>
</tr>
<tr>
<td>T-2 Toxin</td>
<td>1,210.0</td>
<td>Mice</td>
<td>Fungal Mycotoxin</td>
<td>12</td>
</tr>
<tr>
<td><strong>CHEMICAL AGENT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VX</td>
<td>14.1</td>
<td>Mice</td>
<td>Man-made</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>Human</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Soman (GD)</td>
<td>42.0</td>
<td>Mice</td>
<td>Man-made</td>
<td>26</td>
</tr>
<tr>
<td>Sarin (GB)</td>
<td>70.0-113.0</td>
<td>Mice</td>
<td>Man-made</td>
<td>22, 26</td>
</tr>
<tr>
<td></td>
<td>14.0</td>
<td>Human</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>Tabun</td>
<td>311.0</td>
<td>Mice</td>
<td>Man-made</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>14.0</td>
<td>Human</td>
<td></td>
<td>22</td>
</tr>
</tbody>
</table>

Routes of administration for Biological agents are intraperitoneal or intravenous (12). For Chemical agent – i.v. (22, 26, 28).
The intoxication with the most commonly used chemical weapons - the nerve agents is recognized due to the following characteristics [23]:

- numerous cases of inhibition of the cholinesterase activity;
- the direction of wind blowing affects the direction of spreading the agents;
- the fast occurrence and development of the symptoms;
- the dependence of the extent of intoxication on the distance from the center of release of the chemical agent and its way of penetration into the human organism.

Overall the intoxication with chemical agents is very fast, while the effect of the biological agents is much slower. Toxins differ also from classical chemical agents by source and physical characteristics [12].

Furthermore, the progress of damage with biological agents depends on whether active vaccination and prophylactic with antibiotics has been undertaken. Therefore, the preparedness with the respective vaccines, antibiotics and anti-virus means determines the response ability to the biological agents action. It is known that the availability of anti-virus medication at the right place is still an issue. Alongside new antibiotics are being developed continuously. For the most widely known biological weapons there are vaccines and anti-toxins or such are on the way to be developed. The basic issue is to prepare scientists to develop vaccines against modified pathogens [16].

Moreover, the latent period of the development of the symptoms of damages with biological agents leads to the critical question of their indication. It is important therefore to create a network of analytical labs and to improve the systems of indication. The perspectives are for development of fluorescence particle detectors, highly portable manual antibody-assay systems and detectors based on mass-spectrometry technologies.

The prophylactic and treatment of chemical agents induced intoxication during and after terrorist acts is another issue to be resolved. On the one hand, there is a large scale of hard to predict toxic agents, and on the other hand the concepts of use of chemical weapons are changing a lot. Other important issues are the identification of the problems of small dosages, the occurrence of delayed effects, the treatment of convulsions, and handling combined damage case. Thus, the issue of optimization of the prophylactic and therapy of chemical agent intoxication is foremost to be resolved. In many countries, new antidotes for treatment of nerve agents, such as the reactivators of the cholinesterase activity HI-6, are being implemented.

The research of new anti-convulsion means becomes very up-to-date due to
the relation between the convulsions and the occurrence of delayed neurological effects. The issue of cyanide intoxication management still remains. We should not forget that the number of toxic agents that could be used for the purposes of terrorism is very large, which makes it very hard to develop specific means for prophylactic and treatment.

III. ORGANIZING THE FIGHT AGAINST CHEMICAL AND BIOLOGICAL TERRORISM

Today it is clear that terrorism can be not only a state policy, but can also be carried out by separate individuals, groups or organizations. The response system in case of chemical and biological terrorist acts is a problem of all state institutions [7, 8, 11, 13, 19]. The following factors can serve as a basis of a well-organized fight against biological and chemical terrorism:

– well structured civil defense;
– identification and classification of dangerous substances and chemical facilities;
– documentation of industrial processes and products;
– development of risk;
– environmental monitoring;
– well organized health system and sufficient number of hospitals, physicians and pharmacists;
– well organized epidemiology of infectious diseases and information system;
– development of environmental protection and information;
– developed communication system;
– experience in studying and producing antidotes, antibiotics, vaccines and individual protective equipment.

In some countries civilian institutions have some disadvantages:

– absence of effective control and modernization of devices for detection of toxic chemical and biological agents (including mobile devices);
– absence of effective control of the quality and quantity of decontamination equipment, individual protection equipment, and medicines (antidotes, vaccines and other life-saving aids);
– lack of stockpiling of antidotes, antibiotics, vaccines and other life-saving aids;
– insufficient teaching and training activity for fight against chemical and biological terrorism at all levels and structures of society (including physicians);
– lack of identification and designation of specific hospitals and alternative health care facilities for managing of large number of patients after chemical and biological terrorist acts.

The army can play an important role in the fight against chemical and biological terrorism. The issue of chemical and biological terrorism has direct relevance on the army. On one hand, a military unit can be the target of a terrorist act. On the other, as practical experience shows, army units and the army medical corps are involved and take active part in coping with industrial accidents and natural disasters and the management of their consequences.

The army, in general, is in a better position than civilians to act in chemical and biological counter-terrorist operations for the following advantages: the higher level of training, availability of chemical and biological defense equipment, antidotes, and devices for indication and decontamination. The problem is, however, that all this potential and readiness is prepared for effective use in conditions of chemical and biological warfare.

The question arises whether the army units involved have prior developed action plans in case of chemical and biological terrorist acts and attacks either on them or in the vicinity of their dislocation. Such plans for the army should be an integral element in the government counter-terrorist policies and the politics of each state.

According to the conclusions reached by a panel of international experts, many countries still lack an overall concept and view on the army's participation in fighting and eliminating the outcome of chemical and biological terrorism.

Within the overall counter-terrorist coordination among different state agencies and units, the planning and preparation of the country should be focused on the following specific points [21, 24, 25, 27]:

– risk assessment for the use of chemical and biological agents as terrorist agents with particular attention to toxic industrial chemicals and toxins;
– update assessment of the effective toxic levels that should cover both the known chemical weapons in view of the modern technologies of their use and toxic compounds and chemicals of industrial origin;
– assessment of the available capability and contemporary technologi-
cal devices for the detection and identification of a broad range of chemical and biological compounds;
– modernization and optimization of individual protection with particular focus on respiratory protection and protective clothing;
– inventory and assessment of the available means for medical treatment of chemical intoxications. Assessment of the required amounts and types of antidotes (in view of the broader range of potentially toxic agents) and their update with development and introduction of new compounds;
– assessment of the available means for indication and control of chemical contamination and the effectiveness of decontamination. This should consider the broader range of potentially toxic agents and the available state-of-the-art technologies.

Personnel's training acquires particular significance in the preparation of the army, its medical corps and all first responders to counteract chemical and biological terrorism. It should incorporate and implement the latest achievements of computer simulation and virtual reality technologies.

The effective preparation of the country for action in chemical and other terrorist attacks is an expensive and long-lasting continuous process. That can be improved and made more productive with the joint coordinated efforts of all neighboring countries. There are a number of opportunities and unused potential in this respect:

– coordination of resource utilization and trans-border mutual aid in terrorist acts near the borders of neighboring countries;
– use of available special military medical and civil defense units in emergency situations;
– shared stockpiling of antidotes, antibiotics, vaccines and other life-saving aids [21];
– unified notification and information system for the applied primary medical treatment and incidence of specified and unspecified illnesses in a particular country;
– effective triage and transport to the nearest specialized medical facility;
– joint exercises of the medical corps and civil defense of the co neighboring countries in managing and eliminating the consequences of chemical and biological terrorism.
IV. SUMMARY AND CONCLUSIONS

To conclude, we can say that regardless of the advance in the fight against chemical and biological terrorism, there are many issues yet to be resolved. One of the critical tasks is to improve the level of preparedness in many countries. The medical bodies and the epidemiologic network should provide the respective timely and most up-to-date information on the potential terrorist acts that could use chemical and biological weapons. The hospitals and medical units should be ready to treat large quantities of injured. The level of education, especially of the medical workers should be raised. It is necessary to create national stockpiling of antidotes, antibiotics, vaccines and other life-saving aids.

The scientific research related to the indication, decontamination, and defense of the population and the army against chemical and biological weapons is becoming crucial. It is necessary to create a dense network of analytical labs and increase the portable equipment support for research on both biological and chemical agents. It is also imperative to reassess many concepts for the evaluation of the risk and toxicity of the chemical agents, as well as the approach towards the development of new biological agents and the use of both. New technologies could be used to facilitate the research in that area.

The research of and the development of new means for prophylactic and treatment of the increasing number of toxic agents, new anti-bacterial and anti-virus agents, as well as vaccines is becoming a priority for scientific investigation.

REFERENCES

In the beginning of the new century the modern human civilized countries and their societies are strongly alarmed by the information about a new type of aggression on people’s health, named hereafter biological terrorism (Table 1).

In some cases it is due to sudden accidents, in others - to terrorist or other criminal actions. It is caused by biologic substances with strong damaging effect on human body. The extensive development of various branches of organic chemistry, biology and bacteriology in recent years, created opportunities for synthesizing strongly damaging biologic agents adapted for foods, household activities,
textile and other industrial products, which by accident or intentionally in the hands of particularly inventive criminals are capable for a short time to cause different widespread biologic damages. The first problem of this pathology is the type and the classification of causative agents. They are currently divided into two main groups: biologic poisons and pathogenic microorganisms (Table 2).

**I. TYPES OF BIOLOGIC POISONS**

There are two types of biologic poisons:

1. *Toxic substances of plant origin*

They include several types of substances. The mycotic or fungal poisons are found in a number of poisonous fungi. The consumption of food, prepared with these fungi frequently leads to group biologic toxic traumatism, known as Mycetismus.

Among them the group of Amanitin, the poison of the mushrooms of the flyagaric kindred can be of special importance as a means of biologic toxic terrorism. By means of new technologies it is possible to obtain industrial-laboratory
forms of the amanitine poison for terrorist purposes. Here should be emphasized that the amanitine fractions (contained in the amanita falloides and other species), particularly the alfa-amanitin are some of the most powerful poisons on earth with strong toxic effect mainly on the liver, on immunity and on the brain structures. The ricin group of plant poisons which can cause biologic traumatism are toxic proteins (which affect the central nervous system, the bulbar centers and the immune processes) and a hemotoxic glucoside (which causes biologic traumatic damage with severe hemolytic, renotoxic and shock effects). Recently to the toxic causes of biologic traumatism of plant origin has been included a more special group provisionally named chemical-oil-products. These are an associate of non-saturated fatty acids in various plant-oils with chemical denaturants used in the industry for processing these oils. The chemical-oil end-products are strongly toxic – particularly the representatives of the anilide group synthesized by using aniline as denaturant. When criminals mix such substances with plant-oils in the food industry for an adulteration or profiting, the consumers develop severe toxic traumatism with polyorgan damages and failure.

2. Toxic substances of viral and bacterial origin

They are biologic toxins, emitted by various microorganisms under the laboratory-industrial conditions. The treating by special technologies makes the toxin more aggressive and increases its stability and activity in the human body. In special packages these toxins can be distributed for terrorist purposes in various regions and by air, water and food to cause damages to many people. Representatives of this group – causative agents of biologic traumatism are the toxins of Clostridium botulinum, Corynebacterium diphtheria, some Staphylococcus strains, Bact. Coli, Proteus vulgaris, etc. Their list is gradually extended with the improvement of the laboratory-industrial technologies for cultivating new types of microorganisms.

II. STRONGLY PATHOGENIC MICROORGANISMS

With the use of special technologies various viruses and bacteria are cultured in laboratory- and industrial conditions which increases their pathogenic activity, their contagiousness and steadiness. Changed in this way a number of microorganisms, transported in special packages and disseminated by air, water and food in destined regions can cause dangerous epidemic bursts in the population. More famous representatives are the smallpox virus, hemorrhagic fever virus, various types of gut bacteria, typhoid fever bacillus, anthracs bacillus, tula-
remia agents, etc.

A particularity of the damaging mechanisms in biologic traumatism (especially by terrorism) is the destroying effect on the victims, depending mainly on the specific properties of the biologic agent, the entering site and the conditions of contact with the person. The group or mass character of most of cases of biologic traumatism has a severe social burden. It is also important that the noxious agents, used for biologic terrorist traumatism destroy in greater extent mainly the basic functions with vital importance and the defensive mechanisms, such as breathing, blood circulation, immunity, etc.

The clinical characteristics of this type of pathology are of strategic importance to the diagnosis and treatment. Studies carried out by the author give grounds to form a universal model of the clinical picture of biological traumatism and terrorism consisting of the following elements (Table 3):

### Table 3

<table>
<thead>
<tr>
<th>Universal clinical model of biologic traumatism and terrorism (Al. Monov)</th>
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<td>Systemic symptoms and syndromes</td>
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<td>Respiratory syndrome</td>
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<td>Cardiovascular syndrome</td>
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<td>Febrile syndrome</td>
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<td>Immunodeficiency syndrome</td>
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<tr>
<td>Water-salt dysbalance syndrome</td>
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### III. SYSTEMIC SYNDROMES AND SYMPTOMS

*Respiratory syndrome*

Expressed by partial or complete damage of the ventilation due to bulbar paralysis, various extents of ventilation blocking with corresponding dyspnea,
partial or complete cellular hypoxia due to enzymatic toxic block and various extents of respiratory failure. This syndrome occurs under particular conditions in all the severe forms of biological traumatism and terrorism, deteriorating the general conditions of the victims.

**Cardiovascular syndrome**

Expressed by state of shock and/or various forms of cardiac rhythm disorders; there occur various types of heart or cardiovascular insufficiency with deteriorated general condition.

**Febrile syndrome**

Expressed in various extent, duration and with different type of body temperature increase in biologic terrorism, caused by toxins with viral or bacterial origin and directly caused by viral or bacterial strains.

**Immunodeficiency syndrome**

Characterized by various extent of damage of different parts of the immune system, expressed by various forms of immune reactions disorders and with immune insufficiency. It occurs in all forms of damages by viral or bacterial toxins and by direct actions of viruses and bacteria into the body. It is observed in some forms of this pathology, caused by toxic substances of plant origin: amanitin, chemical-oil products (anilides), neurotoxic ricinic protein, etc.

**Water-salt dysbalance syndrome**

Expressed by damage of the balance between the water component and the main electrolytes and protein fractions of the humoral media of the body. In other cases there occurs acid-base balance. These two types of dislocations are clinically expressed by different changes in the homeostasis of the poisoned person: dehydration, hypo- and hyperkalemia, hypo- and hypernatriemia, acidosis, alkalosis, hypoproteinemia, etc. These changes occur in all kinds of biologic traumatism and severely deteriorate the general condition of the victims.

**Haemostasis dysbalance syndrome**

Characterized by various forms of hemorrhagic diathesis occurred as a result of the blood clotting damage by the causal factors of biologic traumatism and terrorism.

**IV. ORGAN LESION SYMPTOMS AND SYNDROMES**

The different causal factors of biologic traumatism depending on their physical and biologic properties make specific damages on particular organs, toward
which they reveal affinity or with which have been in direct contact (respiratory tract and lungs, digestive tract, skin and mucosas, etc.).

In all situations of biological poisons usage for terrorist purposes the organ damages are severe and maximally expressed. They express their polyorgan characteristic initially or gradually. This is due to the “improved” in advance action of the biologic “terrorist-poison”. The live microorganisms directly used for the same purpose are also maximal in their damaging action due to the preliminary “Improvement”. In all cases of biologic terrorism the universal clinical model described above expresses itself with two main features:

1. The generalized group of symptoms and syndromes on all types of its forms and causative agents is expressed in complete set simultaneously or in short consecutive intervals. In the extremely severe forms the rapid damage of the respiration and circulation leads to fast lethal end – then there is no time for the other symptoms to develop. In many of the severely affected by plant poisons the febrile syndrome may be expressed late or not at all. In all cases however the immune deficiency syndrome is rapidly developed which is almost the main specific background of the systemic damaging effects.

2. The organ-motivated symptoms and syndromes are expressed in almost all forms of the polyorgan-deficiency form. Quite rarely the onset is with a monoorgan leading syndrome which rapidly expands to polyorgan type on the background of systemic clinical signs. In the cases with amanitine terrorist poisoning, for example, initial or dominating clinically expressed damage will be this to the liver, with the corresponding syndromes and laboratory findings; in botulinic toxic terrorism the cerebral-neuroparalytic damage will be the leading organ pathology with the corresponding symptoms; in case of aggression by gut bacteria or their products the leading organ signs will be the gastro-enterocolitic ones and in case of antrax products inhalation the lung damage will be the leading one, etc.

For the early and rapid diagnosis of the damages by up-to-date biologic terrorism a medical constellation is used, based on the following criteria (Table 4):

1. Large scale simultaneous or sequential unanticipated sudden damage to the population within a definite region. Single sudden damage is a major exception, and its diagnosis is determined according to the criteria that follow.

2. Acute simultaneous occurrence of several generalized clinical syndromes with no motivation in the information available to that moment. Major part of the victims quickly develop an acute immunodeficiency syndrome within the dynamic processes of biological traumatism. Initial severe febrile syndrome is established in cases of viral or bacterial biological terrorism.
3. Organ-dominated clinical syndrome is established within the background of generalized symptoms and syndromes; this syndrome characterizes the adverse effect of a definite biological agent.

The joint use of the above criteria following the initial clinical examination of all or part of the victims presents a real chance for early diagnostic orientation. For most toxic substances utilized for terrorist purposes, there are no laboratory tests to express identification of the biological poison in materials taken from the victims. There are chances to determine viral or bacterial causal agents as well as the dynamics of their serological titer following a time period defined for every distinct microorganism. Of special value for the diagnosis of the damage in a major part of the victims are the positive tests for acute immune lesions of rapid onset. Medical technical and laboratory examinations show the type of the generalized and organ lesions. These are important for the more integral diagnostic decision.

The course of biological traumatism is a two-way process depending on whether it was due to casual accidents or to a criminal act. In the first case, the clinical picture is more varied: moderate and mild forms occur together with eventual severe cases. Rapid lethal outcome may occur in the severest cases while the victims in the other disease stages experience a favorable therapeutic effect following a time period of various duration. In case that biological traumatism is an act of criminals, they may use biological agents of purposefully created maximal aggressiveness. The course of the disease in many cases is short without the modern strategic rescue activity. The integral treatment of the survivors from biological terrorist traumatism is continuous.
Treatment is the following problem of extraordinary significance in cases of biological traumatism and terrorism. The aforementioned data regarding agents, clinical features and forms of lesion require the administration of a special universal state strategy adequate to the features of this pathology in order to fight successfully biological traumatism (Table 5).

According to the author’s concepts based on his profound studies, this strategy encompasses two compartments: medical and social on the one hand and informative on the other. The medical compartment represents a universal diagnostic and therapeutic as well as organizational and medical program. To the diagnosis, this program offers an adequate universal clinical model of biological traumatism with the aforementioned content that should be made widely known to medical specialists in view of utilizing prompt diagnostic decisions. Besides, this compartment requires introduction of most recent laboratory and other medical technical methods and technologies for determination of immune, biological, hematogenous and chemical deviations within the victims as well as for determination of structural and functional disturbances of various organs. Such technical laboratory findings will improve and accelerate diagnostic decision in various
cases of biological traumatism (Table 6).

To treat the latter, four major groups of therapeutic means should be obligatorily included by all countries into the universal diagnostic and therapeutic program:

Reanimation, correlative and substitutive means. This group encompasses means and methods for respiratory and cardiovascular reanimation as well as corrective solutions and preparations to restore basic vital equilibria disturbed by the aggression. This therapy helps to the avoidance of quick lethal outcome for the severest cases and establishes conditions to continue life and develop an adequate therapeutic regime.

Establishment of a potential for a prompt large-scale administration of immunoprotective and immunosubstitutive therapy. This is necessary because of the wide involvement and dominant role of immune lesions in cases of biological traumatism. This immune-motivated therapy has an indirect lifesaving activity in
most forms of this traumatism with viral and bacterial toxins or living microorganisms as agents against which no specific means exist till present. The immunomotivated therapy realized an indirect lifesaving action. The means and methods from this group are elements of a new trend in contemporary medicine dealing with the immune determination of pathology and its treatment.

Round-the-clock alertness regarding the use of antibiotics against microorganisms as well as antidotes or their analogues against bacterial toxins provided that such products are available (sera against botulin, diphtherial and other poisons; silibinin and legalon against amanitine, etc.) These accomplish a powerful etiopathogenetically grounded lifesaving therapy.

Organoprotective means and methods. In this aspect, the valuable achievements of modern pharmacology and medical technology (hyperbaric chambers and other technical means) prevent the functional failure and destruction of the organs affected by the biological aggressor.

The administration of the therapeutic means and methods from the outlined groups is done in a complex way according to the indications, and priority is given to reanimation methods as well as to the means and methods with maximum number of indicators.

The organizational and medical universal program of the medical compartment within the universal strategy against biological traumatism and terrorism outlines the mechanisms for its accomplishment and determination of its strategic consideration.

This program includes (Table 6):

1. Determination by the means of administrative acts a provision with drugs and other therapeutic means of all the medical structures within the country relating to emergency and large-scale disease; giving them a chance to be involved within the therapeutic process at any time of the day.

2. Preliminary account and determination of health institutions and hospitals (titular and reserve) and medical personnel who in case of occurrence of the specified pathology should immediately initiate and accomplish the therapeutic process in three stages: at the site of the accident or diversion, in the transportation vehicle, and at the health institution.

3. Establishment within the country of laboratory bases equipped with specialists, required material and introduced technologies that should be able to diagnose the victims immediately and at any time if needed. Establishment of regional specialized laboratories that should be immediately involved into the examination of objects from the environment: air, water, food, clothes, etc. in order to identify
a suspected biological “aggressor”.

4. Organization within the country of qualification courses for medical personnel and their collaborators aimed at the study of the features, method of treatment and prevention from the aggression of biological traumatism. (The present chapter also performs such a task).

5. Involvement of the mass media into information about the nature of the biological traumatism activities in an adequate non-traumatizing way, the methods of prevention and the civil reactions and behavior in case of its terrorist forms. Such knowledge should be included in an adequate manner for various age groups as well as into the program of the institutes of education.

This strategy against biological traumatism in its large-scale and terrorist varieties will guarantee the maximum effectiveness in the fight against this new insidious and extremely dangerous form of violence and terror against which contemporary state is not adequately prepared. This strategy should become an important component of the state policy for all civilized countries in the modern world. Only in such a case would science become a generator of ideas and activities to save society from this great potential evil of the new century.

V. BASIC THERAPEUTIC TRENDS AGAINST THE BIOLOGIC TERRORISM (THERAPEUTIC MEANS, METHODS AND PROGRAMS)

The outlined features of etiology, mechanisms of lesions and clinical manifestations of biological terrorism show that its lesions within human organism cannot be overcome with the therapeutic means, methods and forms used till present. The strategy presented within the current universal doctrine requires administration of new means, methods and programs in the following directions: reanimation-substitution and correction, detoxication, antidotes, immunobiological activity, antiviral and antibacterial activity, and organoprotective activity.

1. Reanimation and correction-substitution

The following means and methods are administered:

1.1. By victims with affected homeostasis and vital functions that are imme diately life-threatening. Inclusions:

1.1.1. In case of severely disturbed respiration: respiration reanimation by means of respiratory equipment with or without intratracheal intuba tion and introduction of oxygen mixture into the respiratory tracts: this establishes a basic reanimation background drug stimulation of bulbar respiratory centers (Micoren, Bemegrid, Cardiamin, etc.
i.m. or i.v. according to the indications in case of acute paralytic effect upon these structures; transfusion of blood and erythrocytes in case of acute demolition of the erythrocytes through acute toxic or immunogenic hemolysis; enzymeprotective preparations in case of acutely developing immunopathy of enzymatic groups regulating cell oxidation (glucocorticoids); Coenzymatic factors (Coenzyme Q10), cocarboxylase, etc. (vitamins from group C and B, etc.)

1.1.2. In case of acute disorder of blood circulation. Cardiovascular reanimation should be administered using the following means: in case of shock: intravenous infusion of water saline and high molecular solutions in adequate ratios and quantities for hypovolemia; vasopressor preparations should be included if data against the vasomotor bulbar depression are present; in case of heart lesions and rhythm disorders: strophanthin, ampules with monosaccharide solutions, (contraindicated for rhythm disorders) eventually an add-in of potassium preparations and/or antiarrhythmic medications.

1.1.3. In case of acute disturbance of the equilibrium of water versus electrolytes and of proteins versus electrolytes. Solutions of water and electrolytes, protein preparations or their analogues are administered according to the present indications, most frequently combined. In case of disturbed acid and alkaline equilibrium: corrective and substitute alkaline or acid solutions with adequate vitamins and trace elements are included.

1.2. In case of disturbed homeostasis and vital function with slower development. The aforementioned means and measures are administered at other doses and methods. In such cases of widespread hypoxia of multiple organs, favorable effect is reached through hyperbaric oxidation in a baric chamber adequately to the lesions and in absence of contraindication.

The activities described within section 1.1. are administered in the beginning of the therapy and continue during the three basic stages of evacuation and treatment at the site of the accident, in the transportation vehicles and at the clinic. The activities described in 1.2. are done in full scope most often at the clinic or at home provided that no contraindications were present.

2. Detoxication

The following means and methods are administered:
2.1. Antidotes. Detoxifying effect against biological weapon (the amanitine poison) possess the preparations containing Silibinin (Legalon, etc.) Antidote effect against the botulin poison is accomplished using the botulin antitoxin (Botulismus Antitoxin, etc.). It is used right in the beginning of the treatment according to the established doses and present indications. Antidotal sera are administered against the diphtherial, gas-ematous and tetanic toxins as well as against certain modified poisons of poisonous snakes according to the established methods for any of them. All antidotes are obligatory combined with antihistamine preparations against acute allergic reaction or serum disease.

2.2. Detoxifying depuration of the organism. It is accomplished in case that the poison has penetrated through the digestive tracts as food intoxication. Stomach lavage, intestinal depuration and subsequent oral intake of adsorbent according to the determined methods are accomplished. Endogenous toxic products formed during the occurrence and development of a severe lesion in the methods of detoxifying depuration (forced diuresis, dialysis) in cases of other types of intoxication with biological toxic weapon.

3. Immunobiological activity

This is a major strategic direction because the immunity-injuring mechanisms are the most important feature of the biological weapons of all groups. The activities here are preventive and therapeutic.

3.1. Preventive immune activities. These are:

3.1.1. Nonspecific immune prevention. It is performed by a large-scale use by the population within different regions of various preparations of vegetable or animal origin as well as of pharmacological forms with immunopreventive effect: Echinacea; Esberotox-N tabl.; Deodan pulv. These are used most often in the form of food additives (help food).

3.2. Therapeutic immune activities. Immune substitutes, immune protectors, immune modulators are used in the course of treatment: Gamma Venin amp.; Gammaglobulin amp., Azimexon. Other medicaments: Levamisol, Thiabendazol, etc.

4. Antiviral and antibacterial activity

Chemotherapeutic means including antibiotics from the newest generations are used when biological terrorism against the victims has been performed with the use of various very aggressive and pathogenic viruses and other microorgan-
isms that are highly resistant to the existing chemotherapeutic means. This feature is the reason for a major new qualitative requirement that the treatment of this type of biological terrorism should be performed using a combination of new antibiotics and a potent immunotherapeutic program. In this aspect, effective may be the following groups and types of chemotherapeutic means including antibiotic medications: antiviral medications, avyclovir group: Virolex amp.; Zovirax amp.; inosine group: Isoprenozine; antibiotics: cefuroxin group; Zinnat; ceftriaxene group: Rocephine, Roch fl.; quinolone group: Cyprobay flac, etc.

5. Organoprotective activity

All forms of biological terrorism occur as multiple organ pathology with rapid destructive development. That is why in case of acute lesions prompt and adequate treatment is required at the background of adequate reanimation, as follows:

5.1. Pulmoprotective means and methods. They should be administered very quickly in case that the biological weapon is penetrating through the lungs. Included are: broncholitic preparations, antiedematous preparations (new generations of glucocorticoids, calcium preparations, diuretics; acetylcystein; antiinflammatory means, antibiotics, sulfonamides, etc.).

5.2. Cerebroprotective means and methods. Used are: antihypoxic and antidysmetabolic medications: nootropic preparations (Pyramem, Nootropil, Pyracetamum, etc.); meclophenocate preparations: Centrophenoxin, etc., vitamin B group, antiedematous means, etc. Hyperbaric oxidation (baric chamber) in case that contraindications are not present.

5.3. Hepatoprotective means and methods. Included are enzymeprotective, membraneprotective, antihypoxic and antidysmetabolic means and activities: Essentiale, Hepa-Merz, Trans-methil Heparegen, Orocetam and other preparations, antioxidants in case that indications are present.

5.4. Other organoprotective activities. These are performed using pharmacological and other means of contemporary clinical medicine in case that indications are present.

The therapeutic means and methods within the described activities against biological terrorism should be administered in a complex way under the present indications while commencing with the most motivated ones according to the conditions of the victims and the type of the biological “aggressor”.
Biological Warfare and Bioterrorism. Epidemiological Approach. Impacts on Public Health Services

Florin N. Paul

In the book *Genetics: the Clash Between the New Genetics and Human Value*, by David Suzuki and Peter Knudtson, biological warfare is defined as “the deliberate use of microorganisms or toxic substance derived from living cells for hostile purposes”.

Many of the diseases most easily adapted to military use are infectious diseases that have ravaged the human population for the centuries. Respiratory anthrax, pneumonic plague, smallpox, tularemia or botulism are diseases of which etiological agents are suitable to be weaponized.

Biological warfare is generally regarded as highly unethical and morally repulsive.

The attraction for biological weapons, as well as for chemical or nuclear weapons in war and in terrorist attack is attributed to their devastating effects, due to their common property of wreaking mass destruction.

The nature of terrorism is such that it uses fear as a means of intimidation. Thus, bioterrorism uses biological agents to instill that fear. The incitement of extreme fear can lead to political and economic destabilization. The identification of symbolic or random targets further contributes to people’s fear and anxiety.

The agents of terror include small arms/light weapons, explosives, incendiaries, chemical weapons, and biological weapons.

The particular attention focused on biological weapons is attributed to their low production costs and easier access to a wide-range of microorganisms that can be used in criminal purposes.

DaSilva considers that one of the main goals of biological warfare is the undetermining and destruction of economic progress and stability. The emergence
of bio-economic warfare as a weapon of mass destruction can be traced to the development and use of biological agents against economic targets, such as crops, livestock and ecosystems.

Anticrop warfare, involving biological agents and herbicides, results in debilitating famines, severe malnutrition, and destruction of the agriculture production. Defoliants in the Vietnam War have been widely used as agents of anticrop warfare. Cash crops that have been targeted in anticrop warfare are sweet potatoes, soybeans, sugar beets, cotton, wheat and rice.

Puccinia graminis tritici and Piricularia oryzae, fungus Tilletia caries and Tilletia foetida were used as biological weapons against the targets mentioned above. Fungus Fusarium have been used as a source of the mycotoxin warfare in Southeast and Central Asia.

The use of such warfare agents in order to destroy the national economy of the targeted country, area or population is followed by serious health disorders in all population, in addition with economic crash. In the same area of food warfare agents there are very well known the bacterial and viral agent that contaminate food and cause a wide range of foodborne disease, like dysentery, cholera, hepatitis A, typhoid fever etc.

Such warfare can always be carried out under the pretexts that their effects are caused by natural circumstances as epidemics, with plausible denial. Therefore, from the natural to manmade biological crisis is only an imaginative matter.

Biological weapons have many features that make them suitable for military or terrorist purposes. They have a large area of application, from incapacitating guerilla attacks to fatal epidemics that sweep enemy population.

Disadvantages of using biological weapons must be considered when we are thinking about a biological attack. The evolution of epidemics is difficult to be predicted and in the same time to be controlled, especially if the agents is human to human transmissible. Many external factors such as wind direction, temperature, humidity may influence the result of the attack. The threat of spreading the infection at long distance including the population that launched the attack is real.

Looking at particularities of biological warfare we must understand that the model is the nature itself. The natural evolution of a disease is the best teacher we have to learn and to understand the disease, how to diagnose, to treat and to prevent it. History of epidemic of plague, cholera, influenza, anthrax or smallpox witnesses that epidemics, especially with lethal agents are the most frightening events, may be similarly with earthquake or hurricanes, except that mortality is
quite different.

Thinking about the SARS in Asia and all over the world, or about the West Nile epidemic in United States and Europe, or Bovine Spongiform Encephalitis or the most recent zoonose in Europe, that cause not only economic disorders but confusion in population too, we must accept that anytime, anywhere a biological crisis could occur.

In the last twenty years new problems occurred. Progress in biology, medicine, and immunology and genetic opened large and optimistic doors to treat and to save human lives. Genetic research brought a new and effective therapeutic arsenal in fighting with diseases, including infectious diseases too.

Genetic engineering techniques like DNA recombinant technologies are used to obtain vary effective vaccines, as hepatitis A and B vaccines; they are also used to develop new diagnosis methods and techniques.

Unfortunately the same scientific discoveries could be used to develop modified warfare agents. Genetic techniques help to produce vaccine resistant strains for terrorist and warfare purposes; also they are usefull to modify the susceptibility of the germs to antibiotics or to enhance their invasiveness and pathogenity. Genetic engineered commensals became redoubtable pathogens against the “virgin” immune defense system of the host body. The end of this fight is very ease to be predicted.

Genetic modified organisms can be used to produce a wide variety of potential biological weapons such as:

- organisms resistant to antibiotics, vaccines or immunotherapy;
- organisms with modified antigenic profile that do not match known identification and diagnosis standard procedures;
- organisms with enhanced resistance in hostile environment or to disinfectants;
- organisms producing modified toxin, venom or enzyme;
- organisms with modified targets and pathogenity.

Having in mind all the facts mentioned above, a new concept needs to be defined, as an epidemiological concept to approach the diseases caused by criminal dissemination of biologic warfare agents.

The new clinical entities could be named Biological Weapon Borne Disease (BWBD).

This approach helps the public health authorities and the medical personnel involved in response to biological attack or bioterrorist act to organize and to react properly. The concept is necessary to achieve the goals of medical response,
to decrease as much as possible the effects of the attack in the targeted population, to reduce the mortality, to minimize the damages of the environment.

A unique concept will help all the governmental agencies, local authorities, medical professionals, other organizations involved in response to crisis, to understand and to develop together the policy and the strategy of response, and specifically the medical response.

Having the generic name, **case definition of BWBD** must be defined.

The definition should notice the following details:

- the pathogen with its specific reservoir in the nature, vectors, natural way of transmission, way of entrance in the human body, pathogenesis and natural clinical symptoms, and treatment of natural borne disease;
- the methods used for dissemination of the pathogen in the environment or in the targeted population;
- the epidemiological profile of the disease
- probable changes of the clinical and epidemiological profiles of the BWBD, new treatment and prophylaxis methods;
- impact of the pathogen in the environment, livestock, agriculture (crops) etc.

All of these data, that are not the exclusive list and probably do not cover all the details and needs for a comprehensive plan of action, are useful to sketch the plan of proper medical response to biological attack or bioterrorist act.

The basic epidemiological approach of **biological weapon born disease** is similar with the response to natural epidemic, in spite of difference in clinical manifestations.

Diagnosis, especially differential diagnosis is essential to identify the source and the pathogen. Laboratory methods are used to identify and to characterize the strain and its trace, if is natural or manmade.

Any small or large outbreak of disease should be evaluated as a potential biological attack.

Unusual disease or high rates of illness should be considered as a biological attack if natural way of occurrence can’t be proved.

Everyone involved in response activities must very well know epidemiological particularities of each disease.

Some of disease is individual, meaning that no human-to-human transmission occurred, as anthrax or botulism, but other develops epidemics because they’re high contagious.
The aspect is important for quarantine measures and for planning the medical facilities and the stock of medicine and vaccines, for disinfection and sanitation of the affected area.

A very sensitive matter is to identify if an epidemic is naturally occurred or not.

Practical aspects are closely connected with the clinical form and evolution and treatment requirements. For example respiratory anthrax is rare in the nature, and the most common for human is cutaneous form. Anthrax spore used as biological weapons to be effective must be aerosolized in order to induce pulmonary syndrome. Clinical evolution is dramatic and the mortality is very high despite the sophisticated medical treatment.

Unlike other mass destruction weapons, biological attack or terrorism is not immediately obvious but may appear insidiously. The first notice could be a hospital laboratory or an epidemiologist, a pharmacist distributing more antibiotics then usual etc. At least all epidemic data could be tracked if continuously surveillance of the area is organized.

A very important issue is the way of dissemination and the release mechanisms of the pathogen in the environment, with the criminal purpose.

Two basic types of disseminators are possible for both chemical and biological weapons. The aerosolizer is a spraying device that comes in many forms and can be used to spray a liquid or a powder into the air. Microorganisms in liquid form would be aerosolized by such a mechanism. Toxins in powdered or liquid form could also be aerosolized, provided they are ground to sufficiently small sizes.

Aerosolizers can be powered by pressurized gas, such as CO2 or air, to produce vapors. They can also use techniques such as ultrasonic atomizers or “spinning top” aerosolizers to produce clouds of vapor. Although atomization might reduce water to molecules, microbes would be reduced to elementary bacteria, viruses, or clumps of the same. The aerosolization process can destroy some or all of the microbes, depending the species fragility, pressure, and nozzle type.

Most chemicals tend to evaporate under normal room temperature and pressure, so pressurized mechanisms may not be necessary. anyway. Such was the approach used by the Aum Shinrikyo cult in Japan. The potential for mass destruction inherent in the use of chemical weapons is eclipsed by the potential of biological weapons. Although more difficult to create, biological weapons have an order of magnitude more destructive potential on a per mass basis.

Bombs for disseminating chemicals or biological agents can be driven by
two mechanisms – explosives or pressurized gas. The main problem with using explosives is that explosives tend to destroy the BW agents. The approach used by Iraq, the Soviets, and the Japanese when they constructed anthrax bombs was to use a slow detonating explosive.

Dissemination in the outdoor air is considered the least likely scenario due to the quantities that would be required, and the fact that wind would tend to disperse airborne agents to harmless concentrations in short order. Such was the case with the Aum Shinrikyo cult; their attempts to disperse anthrax outdoors all failed.

The most likely approach that would be used by terrorists would be to employ an aerosolizer to release the agents, either into the ventilation duct or into general areas of the building. Release of a BW agent on a single floor would heavily contaminate that floor, but the remaining floors would see much lower concentrations since the agent would arrive from the supply duct in lower concentrations. Both the mixture of normal outside air and the passage through the air handling unit would tend to reduce contaminant levels in the supply air. Passive dissemination of toxins and pathogens is another possibility. An agent like anthrax could be dusted on interior surfaces where aerial dispersion would result. Terrorists would be likely to contaminate themselves by such an act, but may not care.

Explosive dispersion inside a building is one possible approach that terrorists may use, but this would cause immediate alarm and mitigate the effects. The most likely approach would be to use an aerosolizer to release the agents into the return air ventilation duct or air handling unit.

The first step in epidemiology of biological weapon borne disease is “case definition”. This should be constructed to determine the number of casualties, clinical symptoms, rate of mortality and morbidity, external factors that could influence the results of attack, rate of epidemic extension etc. The basic treatment and the minimal requirements for medical care must be included in case definition.

The main aim of medical support is life saving. In case of biological warfare casualties a huge questions are rising: which is the most effective treatment? What stock of medicine we must have to face with a large number of victims? Do we choose the best solution? Could we treat all casualties? Who is first?

These questions and other many additional are the clues in response to biological attack.

For example, in Romania we treat the anthrax with antibiotics and, if the
condition of the patient is poor we administrate specific horse hyper immune serum. We are very aware about the many and vary risks that patient is exposed but first of all we try to save his life. It is well, ethical or not to expose the patient to an unpredictable risk?

In case of a natural disaster the intervention is influenced by several factors, including the disorganization of medical services. Despite that intervention is effective and safe for the emergency team. Biological attack means identification of the pathogen and until it is not characterized the external support is highly risky.

The education of all-medical personnel and people involved in emergency situation, primary care providers and emergency personnel in dealing with biological weapon victims is essential. Training should include basic epidemiology principles, like way of pathogen transmission from human to human, primary preventive measures, disinfection and self-protection against secondary contamination, clinical information on diagnosis and basic treatment.

Preparedness activities must be conducted from the public authorities level, including emergency network, facilities for medical assistance, communication and transportation. Reserves of medicine are crucial and involve huge funding that probably will be never reimbursed. Antibiotics and vaccines are short live products that require refreshing from time to time. Community must pay for that, otherwise all efforts are useless.

Medical response in biological crisis is a very sensitive issue due to large variety of pathogen agents, clinical evolution and capabilities of the medical facilities, number of casualties etc.

In my opinion medical response in biological attack or bioterrorist act must cover the following areas:

- medical treatment of casualties;
- medical surveillance of the population in the targeted area;
- veterinarian surveillance of the livestock if situation requires that;
- cleaning and disinfection of the contaminated area;
- active and/or passive immune prophylaxis of the contact population

Medical support is a very large and complex issue that includes not only the medicines and pharmaceuticals. There are also included the equipment for vital functions support, number of available beds in case of mass casualties, evacuation capabilities and the strength of medical personnel.

In peace time condition an outbreak in adult population with Shigela flexnery resistant to almost all available antibiotics at that time, except colimicicine
(polimixine B) was very difficult to be controlled and stopped because the lack of the specific antimicrobials in the stockpiles. This antibiotic is used only in pediatric practice and the dose for the adult is ten times more than for the children. How to treat simultaneously 600-700 people when the stock is for about 200 and for only 1 day? In biological crisis such situation could often occur!

Speaking about the prophylaxis of the infectious diseases there many limits. They consist in efficacy of vaccination, the last of immune status, the cost of mass immunization etc.

The etiologic agent used as biological weapon may differ from naturally found in the environment. Even in the absence of genetic engineering modified pathogen, the agent used as biological weapon could differ in recognizable way from the natural/endemic strains in the area.

In the environment natural selection and genetic drift cause continually diverge of the strains. Presence of a previous isolated or different strain could raise the suspicion of a criminal act.

Approximately 70 different types of germs can be “weaponized” for use as agents of biological warfare. The term “weaponized” refers to packaging or treating an agent so that it becomes easier to distribute to a large area. For example, manufacturing anthrax spores as a fine powder increases the ability of the spores to become airborne and be inhaled.

Approximately 30% of the diseases that would be caused by the known available agents can be treated However, this percentage does not take into account the potential for genetic engineering and its potential contribution to exacerbating problems caused by currently treatable organisms.

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War and terrorist acts create large numbers of casualties, refugees and displaced persons, drains financial and human resources, poisons the environment, and supports violence and military engagement as a legitimate approach to settling disputes. In a seminar entitled “War and Public Health”, Barry Levy described some of the long-term physical and mental effects of war, listing the issues that need immediate attention from the public health community:

- improving the public health infrastructure so that it is sufficient to combat terrorism;
- improving the public health infrastructure’s ability to address a wide range of public health issues;
- educating and informing the public so that it can deal appropriately with threats;
- supporting policy and programs aimed at providing adequate mental health services;
- ensuring protection of the environment, particularly food and water;
- preventing hate crimes and promoting civil liberties;
- protecting civilian communities;
- reducing poverty and disparity throughout the world;
- controlling and eliminating biological and nuclear weapons.

In spite of the great progress made in the diagnosis, treatment and prophylaxis of infectious diseases, the threat of using biological agents as weapons has grown up, especially as terrorism activity. Bacteria, viruses, or toxins (of microbial, plant, or animal origin) may be used as biological weapon agents.

A biological attack has some specific aspects, that make it very dangerous: a large number of victims, contamination of the environment, significant impacts on the health care system.

Practically the effect of biological attack or bioterrorist act is unpredictable and very difficult to be controled.

The effects are always amplified if the public health authorities are purposely missed in such cases.

The large number of victims, the delay in etiological diagnosis, and the loose of the confidence in the medical service capabilities may induce the panic in the population.
A crisis situation disorganizes the economic and social life and deeply changes human behaviors.

Biological attack induces long-term ecosystem disturbances (high average of mortality in the people, contamination of plants, water, soil, high rate of mortality and morbidity in animals and plants, destroying of food resources).

Epidemiological characterization of the area of responsibility and infection control and surveillance in humans and veterinary are essential to organize the public health and medical response to biological attack. These data are useful to identify and to define if an outbreak naturally occurs or is manmade.

An unusual outbreak of disease may be defined as an outbreak that is unexpected within the prevailing context of environmental and epidemiological parameters.

There are at least three profiles of the epidemics that can be identified:

– airborne and human-to-human transmissible disease;
– airborne disease without human-to-human transmission;
– vector transmission disease.

Identification of all variables that could influence the evolution of the epidemic is crucial.

The most important factors affecting the evolution of the disease in exposed population in the target area, after a biological attack are:

– the meteorological conditions that influenced the physical decay of infective particle and stability of the pathogen (thermal stability, relative humidity, wind speed and direction across the targeted area);
– pathogenity and virulence of the strain used as weapon;
– population density;
– health status in population (natural immunity, artificial immunity, death rate, recovery rate etc);
– rate of transmission of the infection in connection with the crowd, different in urban and rural areas;
– rate of secondary transmission and incubation time;
– immunization programm;
– availability of efficient medication (post exposure prophylaxis and treatment) and medical facilities.

Focusing on the effects of biological weapons in the human population, the following aspects that require immediate action have to be mentioned:

– reducing the direct - immediate effects in the health of the people exposed to the biological attack;
– minimizing the impact on the health care services due to increasing medical;
– attendance requirements, absence or lack of specific antidotes or vac-
cines, can overwhelm of medical resources.

Unfortunately this problem is so complex and, so many and different things
involved in evolution of the process that no unique solution could be suggested.

The most common medical and epidemiological aspects that rise in a bio-
logical attack are:
– latency in the debut of the clinical disease (from hours for toxin till
days if virus or bacteria are used);
– the absence of external trace of using BW which hides the contami-
nated area and population;
– different clinical aspects due to immune status and the dose received
by each victim may cause confusion and waste critical time for treat-
ment;
– risk of spreading infection in other areas and secondary cases or
outbreak at long distance from the area hit by the (BW).

Requiring Plans for Bioterrorist Events

To ensure that a sufficient number of health-care providers, laboratory tech-
nicians, public health epidemiologists, and administrative support workers show
up for work during a bioterrorist attack, appropriate personal protection (e.g.,
respiratory protection, vaccination, or chemoprophylaxis) for the worker and,
probably, for household members of the worker are essential. When performing
nonstandard work, the worker may also need legal protection, as discussed above.
Plans for a bioterrorist attack should include these factors and be written by the
employer who knows how the agency operates and is staffed because people work
for an agency, hospital, or institution, not a region. Nonetheless, it makes sense to
develop mutual aid agreements with neighboring jurisdictions and integrate single
institution or agency plans into community, regional, or statewide plans.

Disease Reporting and Surveillance

Disease reporting requires specification of what to report in what manner
and timeframe to which parties. A first legal step in this process is to require
immediate reporting of any suspected or confirmed illness, syndrome, or outbreak
caused by any potential bioterrorist agent.

Disease surveillance systems are critical not only for the initial detection of
an outbreak but also for monitoring the extent and spread of the outbreak and for
determining when it is over. Managing a large outbreak would require gathering information from contact tracing and source-of-exposure investigations as well as information about the availability of critical medicine, medical equipment, and the handling of corpses. These information needs are much different than those needed for early detection of an attack. Therefore, legal authority for surveillance should be modified as necessary to ensure collection of all information that could be needed by the public health agency to fulfill its duties throughout the epidemic. This legal authority may include requirements for groups that do not commonly report information, such as pharmacists, to provide it.

**Restrictive Measures, Isolation, and Quarantine**

Administrative public health orders restricting personal behavior of persons with certain diseases, such as tuberculosis, are relatively common in this country.\textsuperscript{[15]} Such orders are usually hand-delivered to a specific person(s), and the restrictions are removed after a specified period, such as after one incubation period or when an ill person is no longer infectious. Another type of public health order might involve work restriction, e.g., health-care providers who cannot demonstrate evidence of immunity to a vaccine-preventable disease are not permitted to work during an outbreak of such disease.

Medical authorities must face complex aspects in dealing with the effects of BW attack. Their objective is to reduce as much as possible the mortality and the morbidity in the population. To achieve this goal it is necessary to organize the chain of reaction.

In our opinion the following steps are required:

A. Before The Event (Attack):
   A1. Continuous area surveillance regarding the germs circulation and morbidity in people and animals. Special attention to water, air and food, if natural or technological incidents occur (flooding, drought, epizootic, damage of potable water system or waste disposal, etc.)

   This is a common part of epidemiological and hygienically surveillance made by public health services, focusing on:
   - respiratory transmitted diseases (influenza, respiratory viral diseases),
   - fecal transmitted diseases as dysentery, viral hepatitis, and cholera;
   - human morbidity by virtual BW agents;
   - domestic animal morbidity and the circulation of germs involved in their pathology (especially toxin producing strains);
   - vectors circulation;
– potable water system and water quality.

This is routine activity and any changes (increasing of morbidity or detection of new strain) must be investigated immediately. Sentinel posts are in charge to perform the surveillance;

A2. Production and storage of protective drugs and vaccines for the most dangerous BW agents. This is a very expensive activity and requires high technology.

It is also a very delicate problem and international community control is required to build the confidence between countries.

A3. Preparing activities before a possible disaster, that means Emergency Medical Care Framework. This consist of:
– extra places for hospitalization in case of disaster;
– diagnosis capabilities to be operationally immediate and to be able to identify a large spectrum of biological agents, including toxins, viruses, spores;
– reserve of drug and vaccine to be available in the shortest time;
– medical team specially trained for this purpose;
– communication and transport facilities;

A4. Training, that has two special topics: the first is the training of people to act in disaster situation and, the second to convince the mass media people to communicate a very accurately the correct information to the people and thus avoid panic.

B. After Disaster (Ba):

B1. Alert of all system involved in management of biological crisis. Public authorities will coordinate all activities to isolate the casualties (illness), to organize the emergency teams, to conduct the fight with biological agent and its effects.

B2. Surveillance of the consequences of the BA, especially for long term effects. The main targets are:
– the exposed population
– the environmental control (water, soil, animals, plants, air)
– veterinary surveillance of domestic animals

B3. Evaluation of the efficiency of the activities done during the crisis and to reconsidered the strategy for the future.

Validity of this concept could be proved only in real situation, unfortunately. More or less all of four efforts are directed to reduce the tragedy. How much we will succeed in our enterprise nobody could predict.
REFERENCES


I. INTRODUCTION

In the last years, terrorism has become rampant. States and international agencies are compelled to develop efficient measures for rescuing human population. This set of measures includes prevention of bioterrorism as an essential component. Until recently, the mere idea of use infectious agent for act of terrorism would seem preposterous. However, the outbreak of anthrax in the USA caused by agent sent by post was a flagrant case demonstrating that bioterorists can exploit any means for their ill purposes [64]. Explosions, acts of terrorism, are detonating all over the world. The number of victims is related to the amount of
energy released by the explosive and to the number of individuals nearby. As for spread of infectious agents by terrorists, each and every victim can disperse disease and, in such a case, events become out of control [10; 11; 34; 55]. Precisely this specificity of bioterrorism makes the international community to take resort to an arsenal of methods allowing to identify morbific agents, which include viruses. At the present time more than 30,000 different viruses comprising 56 families are known, and a human to host 21 of the 26 viral families specific for vertebrates, and many are pathogenic [65].

Viruses cause a wide variety of diseases, affecting humans regardless of age, sex, race and social status. Many of the viral diseases nowadays do not frighten the public at large, despite their high death toll. They are perceived as the commonplace attendants of our everyday life. These include viral hepatitis, influenza, herpesvirus caused diseases, viral colds, and gastrointestinal viral infections. Certain other diseases, such as hemorrhagic fevers and smallpox, in contrast, horrify, although single individuals encounter the causative agents in real life. Obviously, terrorists can use any infectious agent to make people panic stricken. The SARS virus, which has spread from China to many countries, caused considerable economic losses and interfered with the routine course of life. Events related to this infection showed how hazardous an epidemic is capable to be in today’s world.

Monitoring of all infectious diseases is of great importance for timely detection of disease outbreaks caused by malevolent or uncontrollable use of infectious agent. Monitoring is performed so as to meet national regulations, but in any event speed and certainty are decisive for an investigation of outbreaks of infectious disease. Identification of an infectious agent is essential not only for a particular affected subject, but also for an appropriate choice of antiepidemic measures.

A diverse toolkit of various diagnostic methods, which are continually improving, are now available to use in virology. Among the methods, electron microscopy is of a great diagnostic service because it allows to see what the viruses look like on and thus to identify them. This is possible because viral families have a characteristic morphology and sizes. The eye, the sense of sight, is most essential for recognizing the surrounding world and thus also for the understanding of causal relationships (greek: “dia gnosis” means “look through). Electron microscopic diagnostics for viral agents offer an open, undirected view; a catch-all method; and speed. More detailed reasons for usage of electron microscopy for diagnostics of viral disease and comparison with other diagnostic methods may
be found in many publications [7; 8; 9; 25; 42; 33]. The aim of the present work is to analyze the possibilities of the electron microscopy in investigation of outbreaks of diseases of unknown etiology caused by intentional use of viruses.

II. METHODS OF ELECTRON MICROSCOPIC STUDY OF VIRUSES

The size of viruses is beyond of resolving capacity of the light microscope. Only the largest, poxviruses, are discernable as small points after special staining. The electron microscope is the sole device making possible to see how viruses look like, their identification, and detailed examination of their structural features. For this merits, especial role is accredited to electron microscopy in virology [9; 8; 33; 51; 52]. It will be recalled that viruses are organized as complexes of macromolecules built up by self-assembly using cellular biosynthesis. Electron microscopy allows to visualize the assembly of viral particles and the virus-cellular interactions. Morphology underlies the classification of viruses and, therefore, electron microscopic investigation ensures their identification [65].

Three basic electron microscopic methods are used, depending on the aim of research:

- the negative staining technique;
- ultrathin sectioning;
- immune electron microscopy.

The negative staining technique is most widely applied in virology because it is the simplest and quickest. The gist of the method is to immerse a virus in solution of high electron density material. Viruses consist of atoms of light “biological” elements, that are electron transparent and, consequently, stand out against the background of dark contrasting material [2; 30; 37; 51]. It takes a few minutes to make preparation by the negative staining technique, and this makes the technique indispensable for an emergency diagnostics of viral infections. The technique is also applied for analysis of the purity of virus preparations, determination of the concentration of virus particles and study of the fine structure of the virion surface.

Ultrathin sectioning is a method requiring more time for making preparations and needs additional equipment. The method, however, is also applicable to the diagnostics of viral infections, especially when the available specimens are not suitable for studying by negative staining. Ultrathin sectioning permits to visualize virus particles and structures relevant to viral replication in cells, and to examine pathological changes of cells and organs. The most information about
virus-cell interactions is provided precisely by this method.

Immune electron microscopy increases the specificity and sensitivity of electron microscopic diagnostics. The identification of an infectious agent is feasible to the accuracy of species in the presence of virus-specific antibodies. To render the binding visible, additional labeling by secondary antibodies bound to electron dense label (ferritin, colloidal gold) is used. There are many modifications for making preparations for viral diagnostics using immune electron microscopy [3; 12; 18; 28; 61; 63]. Wide application of this method is limited by need of virus-specific antibodies, and electron microscopist should be taught special skill for a successful application of the method.

The methods for diagnostic work should be unsophisticated, reproducible and applicable to biological material of various kind. This overview is focused on methods that can aid the identification of viral agents in the case of their uncontrolled use or outbreak of unknown disease in any laboratory performing electron microscopic examinations and not specialized in studies of viruses.

### III. IDENTIFICATION OF VIRUSES BY THE NEGATIVE STAINING TECHNIQUE

It is a common laboratory practice to apply negative staining to the study of virus containing suspension (a medium in which infected cells are cultured, human or animal serum etc.). The technique has a limitation: the concentration of virus particles must be rather high, not less than $10^7$ particles per ml, and there must be no extraneous admixtures. However, only one found in electron microscope virus particle can provide a diagnosis and identification of an agent. The concentration of virus particles can be increased by ultracentrifugation. Fluid specimen may be centrifuged for 1-2 h at 10 - 20,000 g with resuspension of the resulting pellet in a drop of distilled water [33; 46; 63]. The procedure is very efficient, enabling to detect virus particles at their low initial concentration. The virions can be precipitated onto a grid by ultracentrifugation, thereby trapping virtually all the particles in the specimen. This method increases the yield of viral particles by three or more orders of magnitude and is more efficient than ultracentrifugation in a tube [29]. Another simple procedure for increasing virus concentration in a specimen is the addition of a virus-specific antibodies that glue the virions into aggregates increasing their adsorptivity. This procedure is applicable only in the case when the corresponding antibodies are available [3]. Very simple and effective approaches are application of agar-diffusion (pseudoreplica tech-
nique) and serum-in-agar techniques [5; 12; 22; 23; 38; 39]. Agar-diffusion will result in an enrichment factor of approximately 5x [26].

Should bioterrorists use without control and maliciously pathogenic viruses, it is interest to directly detect virus particles in powders, washes from various surfaces, and in fluids of unknown composition. Main problem for such studies is low concentration of virus particles in a sample, and contamination with mechanical and chemical substances. There are no current publications describing such studies, although some common recommendations may be extracted from published papers. It is recommended to suspend carefully the powder in saline, best by using a sonicator, followed by low speed centrifugation, then to collect the supernatant that presumably contains virus particles. The preparation is further processed as a usual virus-containing suspension using ultracentrifugation (see below). If the wash is obtained by a wad of cotton, the wad is washed in saline, and the saline is further processed as a virus-containing suspension. If the preparation contains mechanical admixuters, it is purified by low speed centrifugation (at 1,000 – 2,500 g for 10-30 min.). Before studying any virus containing fluid it is expedient to subject this to sonication that disrupts aggregated particles [33; 63]. A task to indicate microbes and their spores by negative staining looks more practicable. Electron microscopy was successfully applied by Tom Geisbert and Peter Jahrling (U.S. Army Medical Research Institute of Infectious Disease) for rapid detection and quantification of spores in the B. anthracis bioterrorist letter attack upon USA Senate Majority Leader Daschle and this investigation showed abilities of electron microscopy to detect and identify unexpected and unknown agents [33].

The negative staining technique is most useful and powerful in the identification of viral agents in specimens obtained from patients. Such examinations are most important in the cases of infections of unknown aetiology. Appropriate are: serum, nasopharyngeal washes, urine, feces, discharge of the conjunctiva, the cerebro-spinal fluid, the content of vesicles, or in other words any biological fluid. Fluid samples may be collected by a tuberculin syringe and transported to electron microscopic laboratory. It is highly recommended to prepare specimens for electron microscopic examination directly by gentle touching of coated electron microscope grids to the vesicle fluid, lesion base, or both; allowed to air dry (direct touch preparation); or to prepare at least two grids when the specimen is collected [33]. It is well to remember that the virus is not consistently present in the blood, even in the acute feverish condition. A failed first attempt to detect virus should not discourage from the repeatedly obtaining of specimens for 3-4
days with intervals of 24 hours.

It is desirable to use unfixed specimens to get the best out of negative staining technique; however, a researcher is then at high risk of infection. Abidance by rules of biosafety is very important in electron microscopy facilities. Routine reagents used for negative staining are unable to inactivate many viruses, and spores are resistant even in vacuum of column of electron microscope [33]. Consequently, it is advisable to fix all virus-containing specimens. The procedure is deadly for the potentially hazardous, infectious agents in a specimen, and enables to work “on the table” with no special protection of the personnel. To fix (inactivate) a virus, solutions of paraformaldehyde (formalin) or glutaraldehyde in final concentrations 2% and 0.5% suffices. In order to inactivate external surface, the test tubes are washed with a solution of disinfectant (for example, 0.5% sodium hypochlorite (10% household bleach), and then transported to the electron microscopy facilities [33; 49].

The negative staining technique involves the use of 200-400 meshes grids covered with the specimen support plastic film. The preparation of the films has been described in many papers [7; 26; 30; 36; 40]. It is advisable to check the quality of each batch of support films before starting to work by examining several grids in electron microscope. An important step is treatment of the support films to increase their hydrophilic properties and achieve maximum sorption of virus particles. Glow discharge or treatment with chemical agents can be applied to improve hydrophilicity and particle adherence [1; 26].

The procedure of negative staining is as follows: A droplet of electron-dense stain is placed on a strip of parafilm (or any clean water-repellent surface). A droplet of virus suspension (about 10-20 ml) is placed next to the stain droplet. The most common negative stains are 1% (60 mM) aqueousuranyl acetate, pH 2-4.5, and 1% (2.5 mM) phospho-tungstic acid, pH adjusted to 7.0 with NaOH. The grids are firstly applied to a droplet of virus suspension, then dried with filter paper and placed on a droplet of a stain, thereafter the virus is left again to dry. Period of adsorption of virus may vary from 10-30 sec. to minutes and to a hour depending on sample. The preparation is ready for examination. This version exemplifies the basic principle of the technique, being the simplest. In practice, there are many modifications of the negative staining technique [2; 7; 12; 23; 30; 33; 37; 51]. Importantly, the maximum number of grids should be prepared by a single droplet of specimen by varying the time of virus adsorption and staining. These variations are very useful to obtain good and most informative images of a virus. The prepared grids are examined in the electron microscope and the de-
ected virus particles are photographed. Despite its seeming simplicity the negative staining technique requires skills and foremost experience to well identify viruses on the basis of their morphological appearance.

In the classic version of the negative staining virus particles are electron-transparent, with a distinctive surface relief, well distinguished against the background of dark stain. A contrast substance can penetrate into the virion, staining its inner structures. The virus particles infrequently adhere to each other gathering into aggregates, and in this case virus morphology can be examined only at the aggregate edges. A question arises: how long a grid should be examined to make negative conclusion? Drs. P. Hazelton and H. Gelderblom, who are very experienced in electron microscopic diagnostics, think that examination of 10 meshes is enough to conclude that “no etiological agent identified” [33]. Electron microscopy can yield false-negative results because of the law content of a virus in a specimen. Conversely, a positive result is consistently unequivocal. Sometimes viruses can be identified at the first glance, if not - photographic records and measurements of virus size are often required. Spherical viruses, ranging from 80-130 nm in size, are difficult to identify by the negative staining technique. Such viruses often do not resemble their beautiful “portraits” in textbooks, and high skill and training required for their identification. When examining such preparations, it is advisable to photograph all the detected structural versions of virus. This will provide the most representative sample for analysis and measurement. There are many books and articles describing morphology of viruses, which can be used for identification of found particles [7; 14; 51 and many others]. The most useful practically is Virus Taxonomy, providing brief, but comprehensive information about sizes and morphology of viruses [65].

Ebola and Marburg filoviruses, and smallpox virus are referred to agents which may be used by terrorists, so need a special consideration [11; 44; 55; 64]. It is no problem to identify Marburg and Ebola filoviruses possessing a unique morphological appearance in negatively stained preparations [41; 60]. Virus particles with a pronounced shepherd’s crook, a club shape, loopy, ring and branchy shape are compelling evidence of filovirus infection (Fig. 1). Particles of Marburg and Ebola viruses may appear cross-striated and showing empty central tube. A considerable part of a filovirus population is composed of rod- or thread-like virions, and a number of these kind of virions depends on period of infection. The diameter of a filovirus is about 80 nm; however, preparations of Marburg virus contain comparatively more ring-like particles, and a length of thread-like virions is shorter than in Ebola virus preparations. Highly skilled researcher can distin-
guish filoviruses to the precision of a species.

Smallpox (variola) virus belongs to Orthopoxvirus genus which includes many viruses pathogenic and non-pathogenic for a human. The identification of orthopoxviruses in electron microscope also possess no difficulties thanks to their size and surface relief covered with randomly scattered tubular structures. Clinically smallpox can be mixed with chickenpox, various skin pustular lesions, generalized vaccinia virus infection, cowpox and monkeypox infections [19; 20; 53]. Respectively, smallpox virus firstly should be recognized from herpes- and parapoxviruses (Fig. 2). The unique structure of orthopoxviruses allows to distinguish them from rounded herpesviruses easily [14; 21; 65]. This is the key feature in the differential diagnostics of smallpox from chickenpox, which is the most important for investigation of outbreaks. Herpesviruses in negatively stained preparations look definitely different from orthopoxviruses. Parapoxviruses are smaller in size than orthopoxviruses, more ovoid, and clearly differ from orthopoxviruses by the surface structure having the long parallel surface tubules surrounding a virion [65]. This makes possible to identify both orthopox- and parapoxviruses easily in negatively stained preparations (Fig. ). It is necessary to see characteristic surface structure of orthopoxviruses to be sure in diagnosis to avoid false positive result based only shape and size of found virus particles. It should be noted that electron microscopic examination is unable to distinguish avi- and mollusci-poxviruses from orthopoxviruses, as well to recognize different orthopoxviruses. Such differentiation is possible if appropriate species-specific antibodies are on hand which are able to recognize species-specific viral proteins.

At the time when smallpox was affecting human populations, its differentiation from chickenpox and other skin pustular infections was required. Analysis of the pustular fluid by negative staining technique takes 10-30 min, provided that ready grids are available, and allows to decide how to enfold antiepidemic measures. Such cases have been reported during the waning years of smallpox, providing the weightiest evidence for the efficiency of electron microscopy in the urgent diagnostics of smallpox. In fact, non of the current methods allows so quickly and certainty to identify the virus in pustular fluid [45; 47]. Quickness of diagnosis of smallpox should correspond to quickness of its spreading among humans, and electron microscopy provides such diagnostics of smallpox. Electron microscopic examination can provide a definite answer “This is a poxvirus” or “This is a herpesvirus” in 20-30 minutes from delivery of a sample to laboratory. Bearing in mind that terrorists are potentially capable of malevolently utilizing smallpox virus, many researchers believe that methods of the emergency diag-
nostics of orthopoxviruses are to be more widely taught to the staff of electron microscopic laboratories. It should be noted, that several methods of collecting of pustular fluid exist, and direct touch of opened vesicle by electron microscopic grid provides most effective examination. Crusts also can be used for negative staining examination: crusts should be powdered in a drop of distilled water, and this drop is used for preparation of a grid for negative staining [27; 33; 48].

Negative staining is a highly efficient approach to the diagnostic of viral infections. Its greatest advantage is speed that can be decisive in an adequate response to a terrorist attack. This circumstance place electron microscopic diagnostics at a very significant position in a system of preventive measures against bioterrorism.

IV. THE ULTRATHIN SECTIONING METHOD

The diagnostic value of ultrathin sectioning method for urgent diagnostics may appear, at the first glance, small. It is more time-consuming than the negative staining technique and requires additional equipment. Nevertheless, the method is advantageous for studying the samples which are unsuitable for negative staining technique, and also specimens containing cells, bacteria and other microorganisms. Good examples are the sputum of patients, biopitates and aspirates. Ultrathin sectioning allow to identify not only viral agents, but also other microorganisms, in particular bacteria, fungi, chlamydia, mycoplasma. Thus, for example, sections of the cells present in bronchopulmonary lavage from patients with atypical pneumonia contained chlamydia along with coronavirus particles [35].

Ultrathin sectioning is suitable for cell cultures, pieces of human and animal organs, scrapings of the skin surface and epithelium, and also for preparations of any biological fluid. Choice of organs and tissues for sampling depends on the clinical manifestations of a disease. If orthopoxvirus is suspected, and there exists objective evidences of the disease, crusts are taken for the examination. The size of samples taken by biopsy conforms in every patient the accepted medical norms and standards. The size of pieces cut from different organs of cadavers or experimental animals should be about 1 cm for electron microscopy. The volume of the cellular precipitate can vary widely. It is expedient to use more than 1.5x10^6 cells (in the case of cell culture). If the volume of fluid specimen is small and the concentration of the causative agent is presumably low, special beam-capsules may be used, which allow to examine very small amount of cells [15]. Precipitation is quantitative in these capsules and the material is further
processed in them.

All the specimens for studying by ultrathin sectioning are fixed in a 4% solution of paraformaldehyde (pH 7.2-7.4) best prepared using Hanks, Eagle or Earle mediums as a buffer solutions. Phosphate or cacodylate buffer solutions can be used as well. Prior to fixation, it is desirable to precipitate cell suspension by low speed centrifugation, then carefully discard the supernatant and layer the fixative, filling the tube to the brim to provide inactivation of the infectious agent. If centrifugation is impracticable, the specimen containing cells (cell culture, lavage, sputum) is placed into a 1/5 ml plastic tube and then the tube is filled with the fixative. As a rule, the fixative should be at least 10 times greater in volume than the specimen. The vials are chosen for fixation taking into account the number and volume of the pieces, and completely filled with the fixative. For an emergency diagnostics, pieces of organs no large than 1 cm in size are each of them placed onto a drop of fixative, cut into many small 1-2 mm pieces with a sharp razor blade, then placed into 2-2.5 ml plastic tube which then should be filled with fixative [15; 17; 32]. The tubes allowed to stay for 1h or more gently shaken at regular intervals. Large pieces of organs (up to some cm in size) are placed in the fixative for not less than 48 hours, thereafter the pieces are processed for light and routine electron microscopy. Thus, for quick processing of samples for ultrathin sectioning the following samples are suitable:

- cell precipitate (or suspension) in microtubes;
- small pieces of organs in microtubes;
- small precipitates in beam-capsules.

All tubules should be provided with a close lid. The period of the dehydration of specimens, penetration, embedding in epoxy resin, and polymerization are chosen depending on the size and type of the specimen. Our experience showed that even pieces of organs, such as liver and kidney, can be processed during 10-12 hours. Some publications reported methods of rapid processing of samples requiring more short time. The relevant recipes may be found in following publications [13; 15; 17; 32; 43]. To accelerate the procedure we recommend to omit postfixation in osmium tetraoxide. Fixation in paraformaldehyde provides good preservation of cell structures, and the right staining of the ultrathin sections ensures high contrast. A laboratory should train to perform rapid embedding for electron microscopic diagnostics using ultrathin sectioning to avoid mistakes in a case of incident.

The next step is ultrathin sectioning of the hard blocks resulting from polymerization. The cell precipitates are promptly cut. For organ specimens, 1-2
semithin sections should be prepared for assessment of pathological changes and the right choice of the area for making a pyramid. The procedure allows a targeted choice of the organ site most suspected of harboring viral infection. Suspicious are based on presence of leukocyte infiltration, accumulation of destroyed cells, the presence of inclusions in nucleus and cytoplasm, even just a unusual appearance of cells against background of intact tissue. We believe that an azur-2 solution is the most satisfactory stain for semithin sections. It is stored better and provides more clear staining than toluidine blue. Both semithin and ultrathin sections are made by the standard method with the use of the available laboratory equipment. A high degree of contrast is achieved in the ultrathin sections by the standard methods. We use a saturated alcohol solution of uranylacetate and 2-5% solution of lead citrate [56; 66]. For the detection and identification of a virus, the ultrathin sections in the electron microscope are examined at magnification about 15 000. This allows the visualization of virus and cellular structures.

As in the case of negative staining technique, the filoviruses are most easily identified due to the characteristic structure of virus particles and cytoplasmic inclusions [16; 24; 60]. First diagnostic feature is a shape of Marburg and Ebola virus particles. The particles in ultrathin sections look different from those stained negatively. Accumulations of Marburg virus always contain many empty bubbles and vesicles having nucleocapsid inside, while Ebola virus usually us represented by filamentous forms (Fig. 3). A section may be oriented at different plans providing various shapes of viruses. Cross- and longitudinal sections should be examined to identify Marburg and Ebola viruses. Sometimes filovirus particles are not visible in organs, while other diagnostic features may be found: virus-specific inclusion bodies in cytoplasm, and unusual membranous structures between cells.

Marburg virus usually produce polymorphous inclusion bodies having irregular shape (Fig. 4). The inclusions are composed of nucleocapsids, chains and sheets of nucleocapsids, and tightly packed tubules. Composition of these elements provide all morphological varieties of Marburg virus inclusion bodies [60]. Size and electron density of the virus inclusion bodies increase during the infection. In animal organs inclusions usually have dense appearance and contain crystallloid-like structures. Inclusion bodies produced by Ebola virus (Fig. 5) usually contain distinct nucleocapsids separated by granular substance of middle electron density. The nucleocapsids mostly are located in parallel to each other and form rectangular blocks, however may be dispersed in granular material of inclusion. To find several cells containing filovirus-specific viral inclusion bodies should be enough for diagnostics.
Both Marburg and Ebola viruses form on cellular plasma membrane by budding. Process of budding provides all known morphological varieties of filovirus particles. A characteristic feature of Ebola virus replication is formation of large net-like structures representing proliferation of plasma membrane of infected cell (Fig. 6). Sometimes these structures are the only sign of Ebola virus infection in animal organs, and thereby serve as a good diagnostic feature of Ebola virus infection. Marburg virus is unable to produce large net-like structures, and only few membrane foldings may be found in animal organs.

Comparison of ultrastructural features of Marburg and Ebola virus replication show that they are alike, but differ in details which make possible to distinguish two viruses using all diagnostic features: morphology of virions, inclusions and presence of net-like structures [60].

In analysis of postmortem material, liver and spleen containing large number of filovirus infected cells are analyzed first and foremost. It is noteworthy that Marburg and Ebola infections are not associated with any noticeable leukocyte infiltration even of heavily infected liver [6; 50; 57; 58; 59; 60]. For this reason, choice of the “suspicious” cells in the semithin sections should rely firstly on the presence of cytoplasmic inclusions.

If smallpox is suspected, crusts and scrapings should be examined by ultrathin sectioning first of all. Clumps of fibrin and cellular debris can be seen in the crust sections. Skin cells remaining intact to various degrees of destruction, blood cells, fibrin strands are seen in the scrapings. Orthopoxvirus particles are well visualized and identified in the sectioned material due to their characteristic morphology (Fig. 7). Viroplasm, spherical immature and ovoid mature virions are seen in infected cells. Herpesviruses are easily recognized by presence of particles in cellular nuclei, while orthopoxviruses reproduce in cytoplasm [65]. Different orthopoxviruses produce similar structures in infected cells, but there are some specific features which may be helpful for identification of these viruses. Thus, replication of cowpox virus is related to formation of large spherical or oval homogeneous inclusions of middle electron density (Fig. 8). Presence of such inclusions provide definite diagnosis of cowpox virus infection from vaccinia or smallpox, because last two viruses do not produce such inclusions [19]. Cases of generalized cowpox virus infection have been reported, so such differential diagnostics may be necessary in practice [4; 31; 62; 67]. Rapid diagnostics of smallpox by ultrathin sectioning looks less effective in comparison with negative staining, but the method can be successfully applied for diagnostics of other viral disease particularly when a culprit agent is unknown. The method of ul-
thin sectioning allows, as a rule, to unequivocally identify the viral agent because of presence both virus particles and virus-related structures in cell sections [52].

V. CONCLUSION

Well-established responses to chance or abuse of hazardous infectious agent emergence imply its most speedy identification. It is critical for timely deployment of quarantine measures, vaccination and other related activities. Electron microscopic study may play crucial role in this, provided with information that would have been difficult, that impossible, to obtain otherwise. Fast urgent diagnostics may be a matter of the first importance in case of smallpox incident, and no other means exist to identify a poxvirus during 10-30 minutes at the present time. There is no doubt that appropriate equipment and well-trained personnel are needed for electron microscopic studies. However, the fact remains that the virus is visible and can be identified by electron microscopy, and this is a high point in the case of usage of unknown virus agent. Electron microscopy should be available as a constituent part of diagnostic serves in various countries providing more safety of a life.

ACKNOWLEDGMENTS

The authors are grateful for the excellent technique assistance to Ms. Julia Filippova. We feather acknowledge the support by INTAS (grant # INTAS 00 750).

REFERENCES

Fig. 1. Negative staining of filoviruses with 1% aqueous uranyl acetate.

A – Marburg virus particles in serum of a guinea pig, day 7 postinfection. Note variety of shapes. *Bar corresponds to 200 nm.*
Fig. 1. Negative staining of filoviruses with 1% aqueous uranyl acetate.

B and C – Ebola virus particles in Vero cell culture fluid, day 4 postinfection. Fig. 1B represent different sizes of the virus particles. Bar corresponds to 100 nm. Fig. 1C shows two long filamentous virions. Bar corresponds to 100 nm.
Fig 2. Negative staining of orthopox-, parapox- and herpesviruses with 1 % aqueous uranyl acetate (A, B), and 1% phospho-tungstic acid (C-G).

Fig 2. Negative staining of orthopox-, parapox- and herpesviruses with 1 % aqueous uranyl acetate (A, B), and 1% phospho-tungstic acid (C-G).

B – the same preparation at higher magnification showing surface structure of the virus. Bar corresponds to 150 nm.

C – a particle of mousepox virus showing surface tubular structures scattered on surface. BHK-21 cell culture fluid. Bar corresponds to 60 nm.
Fig 2. Negative staining of orthopox-, parapox- and herpesviruses with 1 % aqueous uranyl acetate (A, B), and 1% phospho-tungstic acid (C-G).

D – parapoxvirus particles showing typical for this family surface structure. Note differences of shape and surface structures of orthopoxviruses presented on fig. A-C. Bar corresponds to 100 nm.

E, F - different appearance of herpes virus in negatively stained preparation. Vero cell culture fluid. Bars correspond to 100 nm.

G - herpes virus particles with additional envelope. Such particles may be found in clinical samples. Bar corresponds to 100 nm.
Fig. 3. Ebola and Marburg filoviruses on ultrathin sections.

A – Ebola virus particles in green monkey connective tissue. Note predominant filamentous shape of viral particles. *Bar corresponds to 400 nm.*
Fig. 3. Ebola and Marburg filoviruses on ultrathin sections.

Fig. 3. Ebola and Marburg filoviruses on ultrathin sections.

C – D – Marburg virus particles in green monkey spleen (C) and Vero cell culture (D). Note many polymorphous viral particles.

Bars correspond to 400 nm.
Fig. 4. Different appearance of Marburg virus inclusion bodies in ultrathin sections of infected Vero cells.

A shows chains and sheets of nucleocapsids.

B shows dense conglomerates of tubular structures.

*Bars correspond to 200 nm.*
Fig. 5. Different appearance of Ebola virus inclusion bodies in ultrathin sections of infected Vero cells.

A represents regular arrays of straight tubular nucleocapsids separated from each other. B shows chaotic distribution of nucleocapsids, some are cross-sectioned. Bars correspond to 200 nm.
Fig. 6. Net-like membranous structure related to Ebola virus infected macrophage cell in spleen of a guinea pig.
   Note right-hand dense inclusion body in cytoplasm.
   *Bar corresponds to 400 nm.*
Fig. 7. **Orthopoxvirus in ultrathin sections.**

A – a cell of Vero culture infected with variola virus. Viral factory composed of granular material and spherical immature particles is located near nucleus. Many mature virions are seen at the periphery of a cell.

*Bar corresponds to 600 nm.*
Fig. 7. **Orthopoxvirus in ultrathin sections.**

**B** – spherical immature particles of variola virus in cytoplasm of a cell of Vero culture. Note different appearance of virions.  
*Bar corresponds to 300 nm.*
Fig. 7. **Orthopoxvirus in ultrathin sections.**

C – Mature viral particles of variola virus in cytoplasm of a cell of CV-1 culture. Virions are sectioned at various plans, and so show different appearances. *Bar corresponds to 150 nm.*

D – Fine structure of variola virus at high magnification. Oval mature particle and spherical immature particle. *Bar corresponds to 60 nm.*
Fig. 8. Reproduction of cowpox virus in cells of mouse spleen.

A – Electron dense spherical inclusion is seen in a center of infected cell. The inclusion contains virus particles inside. *Bar corresponds to 750 nm.*
Fig. 8. Reproduction of cowpox virus in cells of mouse spleen.

B – Large and small inclusions having middle electron density are seen at the foot of photo. Numerous immature particle are located in left upper corner.

*Bar corresponds to 750 nm*
Subtractive hybridization and PCR amplification for detection of *Brucella* spp.

Hristo M. Najdenski, Eva K. Tcherneva, Nancy P. Rijpens, Lieve M. F. Herman

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I. INTRODUCTION

Brucellosis is an important zoonosis causing significant economic and social problems especially in the countries with transitional economic structure. All six *Brucella* species are potentially pathogenic for humans. Primary diagnosis of brucellosis is problematic because the representatives of the genus *Brucella* are relatively slow growing bacteria and the serodiagnosis is not always reliable. This is especially true for *B. canis* (Carmichael and Shin 1961). *B. canis* is similar to *B. suis* in its biochemical reactions but it is antigenically similar to *B. ovis*, lacking the somatic lipopolysaccharide side chains of S-*Brucella* (Carmichael and Bruner 1968; Carmichael 1990).

Since the *Brucella* genome is highly conserved among the different species (O’Hara *et al.* 1985; Verger *et al.* 1987; Allardet-Servent *et al.* 1988) most PCR tests (Fekete *et al.* 1990a; Fekete *et al.* 1990b; Fekete 1992; Baily *et al.* 1992; Herman and De Ridder 1992; Romero *et al.* 1995) identify *Brucella* spp. at the genus level. It is observed that the repetitive genetic element IS711 (previously
reported as IS6501) (Halling et al. 1993; Ouahrani et al. 1993) is unique for Brucella species. For most Brucellae at least one copy of the element is inserted at an unique species- or biovar-specific chromosomal locus. The unique locations of these elements are used in diagnostic assays for Brucella spp. (Ouahrani et al. 1993; Bricker and Halling 1994). Unfortunately, IS711 is not inserted in the B. canis genome in a species specific manner and makes the diagnosis of B. canis by PCR unusually difficult.

Therefore, we tried to develop a PCR test, specific for B. canis. To isolate B. canis specific DNA fragments, a combined subtractive hybridization and amplification procedure was carried out. The subtractive hybridization allows the removal of homologous sequences of two (or more) different strains so that unique sequences for the target organism can be isolated (Bjourson et al. 1992; Herman et al. 1997). In this paper the subtractive hybridization/amplification procedure was used in an attempt to develop PCR primers, specific for B. canis and subsequently for Brucella spp.

II. MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are summarized in Table 1. Bacteria were cultivated on Tryptic soy agar supplemented with 1 g/l Yeast extract (Oxoid Ltd, London, England) at 37°C for 72 h. Each strain was subsequently inoculated into Tryptic soy broth/Yeast extract (Oxoid) and cultivated with shaking at 37°C for 24 h.

DNA extraction from cultured cells. Cultures were centrifuged for 2 min at 13 000 x g to collect the bacteria. Bacterial chromosomal DNA was extracted by 2 different methods: i) as described previously by Flamm et al. (1984) in the case of Coxiella burnetti and Rickettsia spp., and ii) by the method of Pitcher et al. (1989), for the other strains. For the application of the method of Pitcher et al. (1989) on Brucella strains a modified lysis procedure was used. Crude bacterial cell lysates were prepared by adding 50 ml of 0.1 M NaOH and 50 ml of 0.25% sodium dodecyl sulphate to the bacterial pellet and subsequently heating for 17 min at 90°C. The DNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nm.

Subtractive hybridization. The subtractive hybridization between probe strain DNA and subtracter DNA was basically performed as described by Bjourson et al. (1992). Sau3A-digested DNA from the probe strain (B. canis RM6/66) was ligated to linker L-P, obtained after hybridization of primers TB7006 (5’ HO-
AGCGGATAAACATTTCCACACAGGA-OH 3’) and TB7008 (5’ P-GATCTCCTGTGTGAATTTGTTATCCGCT-OH 3’). Sau3A-digested DNA from the subtracter strains (B. suis 1330, bt.1; B. abortus B95/1, bt.3; B. abortus AF93/4, bt.3; B. melitensis MB274, bt.1; B. melitensis MB276, bt.3; and B. ovis MB272) was ligated to the biotinylated linker L-S, obtained after hybridization of primers TB7007 (5’ Biotin-CGCCAGGGUUUUCCAGUCACGAC-OH 3’) and TB7009 (5’ P-GAUCGUCGUGACUGGGAAAACCCUGGCG-OH 3’). The ligated DNAs were purified by phenol-chloroform extraction and concentrated by precipitation (Sambrook et al. 1989). Probe strain DNA was prepared by PCR amplification of the ligated DNA with primer TB7006 according to the PCR protocol described below with an annealing temperature of 55°C and 45 amplification cycles. Subtracter DNA was prepared by PCR amplification of a mixture of the ligated subtracter DNAs with the biotinylated primer TB7007 according to a PCR protocol basically as described below. Instead of dTTP, 200 mM dUTP were added, an annealing temperature of 55°C was applied, and 45 cycles were performed. After purification by phenol-chloroform extraction, denatured probe strain DNA and subtracter DNA were hybridized in solution with an excess of subtracter DNA. The hybridization was performed at 55°C and at 50°C for 6 subsequent subtraction/amplification rounds. Subtracter DNA and hybrids between probe DNA and subtracter DNA were removed by 150 ml Dynabeads M-280 Streptavidin (Dynal A.S., Oslo, Norway) according to the supplier’s instructions. The supernatants were purified by phenol-chloroform extraction and dissolved in 20 ml H2O. Five microliters were applied in the next round of subtraction. One microliter was diluted 10 and 100 times. From each dilution 10 ml were treated with 15 U uracil DNA glycosylase (Life Technologies Inc., Paisley, United Kingdom) at 37°C for 4h to remove traces of subtracter DNA. Finally, the remaining sequences were amplified by PCR with the TB7006 primer following the PCR program, described below, with an annealing temperature of 55°C and 45 cycles.

Cloning of subtracted probe DNA fragments. Subtracted probe DNA fragments, obtained after 6 rounds of subtraction/amplification at 50°C and 55°C, were cloned in the E. coli plasmid vector pMOS (Amersham Life Sci., UK). The subtracted probe DNA was purified from agarose gel by GenClean Kit Bio 101 (Bio 100 Inc., La Jolla, CA), ligated in the pMOSBlue T-vector and transformed into XL1-Blue competent E.coli cells according to the description of the manufacturer (Amersham, Life Sci.). The recombinant pMOS DNAs were checked by BamHI/HindIII restriction digestion.
$^{32}P$ labelling of DNA. The recombinant pMOS DNAs and the subtracted probe DNAs were radiolabelled by using the Random primers DNA labelling system according to the supplier’s instructions (Life Technologies Inc.). Unincorporated nucleotides were removed after separation on a Sephadex G-50 fine (DNA) grade column (Sambrook et al. 1989).

Analysis of DNA dot blots. Total genomic target DNA (1mg) was denatured and spotted onto a Hybond-N membrane (Amersham) by a DOT blot manifold (Bio-Rad, Richmond, CA). The DNA was fixed onto the filter by baking for 2 h at 80°C. Filters were hybridized at 68°C in 6 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M tri-sodium citrate - 5.5 hydrate) according to the general protocol (Sambrook et al. 1989). Filters were washed at 68°C once with 6 x SSC, once with 3 x SSC, once with 1.5 x SSC and once with 0.75 x SSC, each time for 1 h and subjected to autoradiography for 24 h.

DNA sequencing. The sequence analysis of the subtracted probe DNAs inserted in the pMOS vector was performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) in both directions using primers T7 and U19 (pMOSBlue T-vector kit, Amersham). Sequence data were analysed with the Genetics Computer Group sequence analysis package (Inc., 1995, Program manual for the GCG package, version 8.1, University of Wisconsin, Madison). Homology between the different subtracted B. canis DNA fragments was analyzed with the BESTFIT (Smith and Waterman 1981) and GAP (Needleman and Wunsch 1970) programs in both directions. Homology with bacterial sequences of the EMBL database was investigated with the FASTA program (Pearson and Lipman 1988).

PCR amplification. 25 ng purified DNA or 1µl of crude cell lysate were used as template in the PCR. PCR was performed in a final volume of 25 ml containing 2.5 ml 10 x PCR buffer (200 mM Tris with pH 8.3, 10 mM MgCl$_2$, 500 mM KCl), 0.25 mM each dNTP (Pharmacia Biotech Inc.), 1 U DNA polymerase (Cetus - Perkin Elmer) and 0.2 mg of each appropriate primer. The mixture was subjected to 30 cycles of amplification in a PCR 9600 thermal cycler (Perkin Elmer Corporation, Branchburg, N.J.). The first cycle was preceded by initial denaturation at 95°C for 15 sec., annealing at the appropriate temperature for 15 sec. and extension at 72°C for 30 sec. The last cycle was followed by a final extension step at 72°C for 8 min.

Agarose gel electrophoresis and plasmid analysis. For preparative purposes, the subtracted probe strain DNA was separated on 3% (wt/vol) NuSieve GTG agarose (FMC BioProducts, Rockland, Maine) according to a standard pro-
tocol (Sambrook et al. 1989). 10 ml of the PCR products were analyzed on a 1.5% (wt/vol)SeaKem ME agarose gel (FMC BioProducts).

III. RESULTS AND DISCUSSION

Taking into consideration the high degree of homology between subtracter and probe strain DNA, subtraction/amplification was carried out two times at different hybridization temperatures: first, in 5 rounds (four at 60°C and one at 55°C) and second in 6 rounds (four at 60°C and two at 50°C). Each subsequent round of subtraction/amplification resulted in an increasing purification of a DNA fragment of about 270 bp and the attendant smear (Fig. 1).

Southern blot analysis revealed that the radiolabeled probes of either the subtracted 270 bp fragment or smear, failed to hybridize with B. ovis MB272 (Fig. 2A, lane 6) and hybridized to low degree with both strains of B. abortus and B. melitensis (Fig. 2A, lanes 2, 3, 7, 8 and Fig. 2B, lanes 2, 3, 6, 7). The subtracted probe DNA did hybridize strongly with B. suis 1330 and B. suis Thomsen (Fig. 2A, lanes 4 and 5; Fig. 2B lanes 4 and 5), suggesting an extensive DNA homology between DNAs isolated from B. canis and B. suis.

The DNA fragment and the smear generated by 6 rounds of subtraction/amplification at final temperature 55°C and the DNA fragment obtained at a final hybridization temperature of 50°C were cloned in the pMOS vector resulted in 14, 15 and 15 clones, respectively. All 44 recombinant pMOS clones were hybridized to DNA of the different Brucella spp. Forty clones hybridized equally to the different Brucella DNAs. Only 4 clones (2 with an insert from the subtracted DNA fragment obtained at 55°C and 2 with an insert from the subtracted smear at 55°C) showed a weaker (but not missing) hybridization signal with DNA of B. abortus and B. melitensis compared to B. canis and B. suis DNAs. All of the 12 DNAs isolated from the a-2 subdivision of the class Proteobacteria did not hybridize.

The DNA inserts of the 4 clones were sequenced. Three different DNA sequences (F18, F26 and S33) were obtained without mutual sequence homology (Fig.3). No homology was found with bacterial sequences of the EMBL database.

One forward and one reverse primer derived from each of the 3 sequences (Fig.3) were tested for specific identification of B. canis by PCR (the following annealing temperatures were used: F26-F1/F26-R2: 50°C; F18-F1/F18-R2: 55°C and S33-F1/S33-R2: 58°C. All three primer pairs reacted with all the Brucella spp. included in this study. No primer pair cross-reacted with any species belonging to the a-2 subdivision of Proteobacteria (Table 1). All the three pairs of prim-
ers reacted positively in PCR with crude bacterial lysates from *Brucella* spp. cells in dilutions up to $10^5$ CFU/ml.

The method of subtractive hybridization and PCR amplification was used after Sau3A digestion of bacterial DNA aiming the isolation of genomic sequences specific for *B. canis*. Although stringent hybridization conditions were used and a total of 6 rounds of subtraction/amplification were applied, the obtained DNA fraction was only partially specific for *B. canis*. It recognized as well the other *Brucella* spp. Only a weak cross hybridization signal was noticed with *B. melitensis* and *B. abortus* and no signal was obtained with *B. ovis*. *B. ovis* is known to hybridize differently compared to the other *Brucella* spp. with a BCSP31 probe (Halling and Zehr 1990). All *B. ovis* strains, tested in a RFLP characterization of the omp-31 locus, were characterized as well by the absence of a Sau3A site (Vizcaino et al. 1997). On the other hand Sau3A digestion can give a species-specific marker in *B. ovis* DNA, which makes these strains distinguishable from the other *Brucella* spp. by RFLP. A strong cross hybridization signal was noticed with *B. suis* DNA. *B. canis* and *B. suis* (esp. bt.3 and 4) have been reported to be closely related on the basis of phenotypic characteristics (Meyer 1990) which can explain the difficulties found to differentiate them at the molecular level. In spite of the high degree of DNA homology detected in the *Brucella* genus (Hoyer and McCollough 1968a; Hoyer and McCollough 1968b; Verger et al. 1985). Vizcaíno et al. (1997) reported significant differences at the genetic level in *Brucella* spp., which could be involved in some of the phenotypic and/or pathogenic characteristics that differentiate the *Brucella* species. There are some successful techniques to discriminate *Brucella* spp. and strains using RAPD (Fekete et al. 1992), the position of the IS711 insertion element (Bricker and Halling, 1994; Bricker and Halling, 1995), rep-PCR (Tcherneva et al. 1996) and PCR-RFLP (Vizcaíno et al. 1997).

The endonuclease Sau3A, used in the first step of the subtraction hybridization probably cuts the *Brucella* DNAs so that the received fragments were all partially homologous which can be expected on the basis of the high degree of homology between the different *Brucella* spp. Taking into account that the IS711 elements are located downstream of the omp-31 protein gene sequence in *Brucella* spp., and that the IS711 based differentiation of *Brucellae* is possible after endonuclease digestion (Bricker and Halling, 1994), we can imagine that other endonucleases could be more suitable for the subtractive hybridization of *B. canis* DNA. It could be assumed that the AvaII site in the omp-31 locus of *B. canis*, which is located at position 399, should be more useful.
Because the omp-gene is suspected of playing a major role in the pathogenicity of Brucella, it is used as a suitable target for development of PCR system for differentiation between each of the Brucella spp. (Wilborn et al. 1997). The results revealed that on the one hand several of the tested pathogens could not be detected (B. melitensis bt.2; B. suis bts.2-4) and on the other hand two of the three tested B. ovis isolates, which have no relevans as a human pathogens gave a positive result. Therefore, a successful approach could be the selection of larger sequence segments from omp-gene region and utilization of the method of single stranded conformation polymorphysm with which the smallest variations in the DNA sequence (e.g. point mutations) should be determined.

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**TABLE 1.**
Strains, included in the investigation

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Biotype</th>
<th>Origin</th>
<th>Method of DNA preparation</th>
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**CVI** - Central Veterinary Research Institute, Sofia, Bulgaria;  
**DVK** - Department of Animal Product Quality and Transformation Technology, Melle, Belgium;  
**VAR** – Veterinary and Agriculture Research Centre, Brussels, Belgium
Fig. 1

*B. canis* DNA remaining after subtraction/amplification.
Molecular size marker X (Boehringer GmbH)
Fig. 2
Specificity of DNA sequences from *B. canis* RM6/66, generated by subtractive hybridization.

DNA from probe strain isolated after six rounds of subtraction/amplification was hybridized against DNA from other *Brucella* spp. The figure is a reversed autoradiogram.


B: The smear is used as probe. 1.*B. canis* RM6/66; 2. *B. abortus* B95/1 bt.3; 3. *B. abortus* AF 93/4 bt.3; 4. *B. suis* 1330 bt.1; 5. *B. suis* Thomsen bt.2; 6. *B. melitensis* MB274 bt.1; 7. *B. melitensis* MB276 bt.3.
Fig. 3. DNA sequences of three different DNA clones obtained by subtraction-hybridization/amplification. One forward (F) and one reverse (R) primer are indicated in each fragment.
I. INTRODUCTION

It has been well established that *Bacillus anthracis* is an ideal organism with which to initiate and develop an offensive biological weapons program. Nature has perhaps unintentionally, but obviously facilitated its use among terrorists and states with malicious intent by the many characteristics that make *B. anthracis* easily employed as a biological weapons agent. To mention but a few of these attributes, this organism can be easily isolated from soil and is distributed worldwide; it is quite easy to culture and grow in large quantities; and, its spores are
very stable and can be stored at ambient temperatures either in wet or dry form. In dry form, the spores of this organism are highly resistant to temperature, radiation, and chemical disinfection. Using an appropriate agent preparation, a cloud of aerosolized spores of *B. anthracis* can be extremely dangerous in that inhalation anthrax is considered almost 100% lethal.

Beyond this description of *B. anthracis*, however, may be more serious considerations regarding this organism because of ongoing research directed at understanding the genome of this organism and how it can be manipulated for an expanded spectrum of capabilities including the expression of new virulence factors. Although these studies may be academic and valuable from a microbiology perspective, there is no way to control the use of such knowledge disposable to those who choose to develop biological weapons. To manage such potential threats, new therapies are needed to effectively prevent and treat anthrax in all its forms, especially inhalation anthrax because of the typical short time to death following an individual’s exposure coupled to the popularity of *B. anthracis* as a biological weapons agent.

Of the more than 50 species within the genus *Bacillus*, those closely related to *B. anthracis* include *B. cereus*, *B. thuringiensis*, and *B. mycoides*.(21) These species are categorized in *Bacillus* subgroup 1 – large Gram-positive rods with a cell width >1 µm and cylindrical or ellipsoid spores that do not distend the sporangia.(21,11) Among the common characteristics of these species is the fact that three species produce toxins as a basis for disease. *B. thuringiensis* with its array of crystal proteins (*i.e.*, Cry toxins) is entomopathogenic to insect larvae.(34) *B. cereus* produces a wide array of toxins, but the emetic toxin is most noteworthy and is responsible for gastroenteritis caused by this opportunistic pathogen.(2) Two toxins, lethal factor (LF), and edema factor (EF), are produced by *B. anthracis* along with a third protein, protective antigen (PA), which facilitates the entry of the toxins into cells resulting in anthrax, a disease of herbivores and humans.

Although immunological, metabolic, and genetic similarities have been observed within this group, results from molecular studies have shown the high degree of relatedness among the species.(39) Multilocus enzyme electrophoresis suggested these organisms should be considered the same species, and functional differences among these organisms depend largely on the expression of plasmid genes.(16) DNA base composition determinations and DNA-DNA hybridization experiments have shown that three species – *anthracis*, *cereus*, and *thuringiensis* – have a high degree of relatedness characterized by a 36.4±0.6% guanine + cytosine content.(20,18) Of more than 2900 nucleotides comprising the base
sequences of the 23S rRNAs of both *B. anthracis* and *B. cereus*, only two small differences, a nucleotide change at one position and a nucleotide insertion in the *B. cereus* RNA, were found.(4) On the basis that these species are such close “molecular” relatives, researchers have used these relationships to investigate new ways to manipulate *B. anthracis* to express new factors, and in some cases new virulence factors, which could potentially affect vaccine efficacy.(31,25,30) In other words, expression of these factors may increase the ability of *B. anthracis* to cause and sustain an infection even in the presence of recognized therapies.

II. ANTHRAX: BASIC PATHOGENESIS

Anthrax is a disease caused by the secretion of toxins following infection and germination of *B. anthracis* spores. A suitable host is exposed by ingestion of spores or the introduction of spores through open cuts or sores on the skin. If the spores are aerosolized as with a biological weapon, a highly lethal form of the disease results through the inhalation of these spores. The spores are carried from the lungs to the lymphatic system where the spores germinate, and the resulting vegetative form of the organism elaborates toxins. Sufficient lethal toxin (LF), in the presence of protective antigen (PA), ultimately causes death of the host, especially if the infection is left untreated. Although it is possible for anthrax to be lethal through any portal of infection, inhalation anthrax is, by far, the most lethal form of the disease with an especially short incubation period from 24 to 72 hours.

Mikesell, *et al.* demonstrated toxin production in *B. anthracis* is plasmid-mediated. (26) The major virulence factors for *B. anthracis* consist of the production of both the toxins and the capsule. The genes expressing the toxin proteins are located on the pXO1 plasmid, and expression of the capsule is sourced on the plasmid designated pXO2. *B. anthracis*, Sterne strain, although capable of making toxins, lacks the pXO2 plasmid, and, therefore, is incapable of making the glycopolysaccharide capsule. Lacking the benefit of this major virulence factor, *B. anthracis* most often cannot cause anthrax in most animal hosts, but the organism remains immunogenic, the basis for the Sterne strain vaccine.

The pXO1 plasmid contains the genes that encode for the three-part toxin complex expressed by *B. anthracis*. These proteins consist of lethal factor, edema factor (EF) and protective antigen. EF and LF are intracellular toxins but require the intervention of PA to be effective. A biochemical interaction between PA and specific cell-surface receptors permits the attachment of PA to these receptors.
Following attachment, PA mediates the transfer of EF and LF from the exterior to the interior of the cells. The U.S. and U.K. vaccines induce anti-PA antibodies that eliminate the intracellular toxic activities of EF and LF by eliminating the possibility that toxins can gain entry into cells. LF, in the presence of PA, is primarily responsible for the lethality often associated with anthrax. Without the intervention of PA, LF cannot access the interior of cells eliminating the potential for anthrax to develop.

III. THE PlcR REGULON

In 1997 Russian researchers described the main virulence factors for *B. anthracis*, the capsular polysaccharide that coats the exterior of vegetative forms of the organism and the tripartite toxin secreted by the organism during infection, and then remarked that other factors may play a role in the organism’s pathogenesis.(31) In particular, they focused on the ability of *B. anthracis* to lyse red blood cells. The ability to lyse red blood cells has been a factor used to differentiate *B. anthracis* from *B. cereus* and *B. thuringiensis*, species that are almost always hemolytic. Because this diagnostic trait is used commonly to differentiate species, there appears to be a lack of interest in the ability to lyse red blood cells as an actual virulence factor of *B. anthracis*. Some strains of *B. anthracis*, however, have demonstrated hemolytic activity expressed under appropriate, but limited, conditions.(13,27)

Although the mechanism responsible for the observed hemolytic activity in *B. anthracis* has not been explained, the hemolytic property of *B. cereus* is attributed to the expression of two genes, the cereolysin AB genes which are under the control of a global regulator PlcR. These genes are located in tandem on the *B. cereus* chromosome, and the expression of both genes is important for effective hemolytic activity.(14) More specifically, the cereolysin AB are the *plc* and *sph* genes producing phospholipase C and sphingomyelinase, respectively.(14,30) Although *plc* and *sph* exist in *B. anthracis* with other genes known to be under the control of PlcR in *B. cereus*, these genes are not expressed in *B. anthracis* because the *plcR* gene in *B. anthracis* contains a nonsense mutation (25) causing the production of a truncated PlcR (1) that does not function as a transcriptional activator necessary for the expression of many genes, including *plc* and *sph.*

In their 1997 report (31), however, the Russians described the insertion of the cereolysin AB genes from *B. cereus* into several strains of *B. anthracis* consisting of the following: a competent pathogen strain (H-7) capable of causing
typical anthrax, a live vaccine strain (STI-1), and a control strain designated 221. Each of these strains differs with respect to their genetic make-up responsible for expression of the major virulence factors. H-7 has both the pXO1 and pXO2 plasmids responsible for the toxin complement and capsule, respectively. STI-1, lacking the pXO2 plasmid, is incapable of making a capsular polysaccharide but has the toxin complement. Strain 221 lacks both plasmids and, therefore, lacks the ability to produce the major virulence factors.

Genetic engineering was employed to achieve the transfer of the cereolysin genes from *B. cereus* to each of these strains, and clones were selected on the basis of lecithinase activity, an enzyme test indicating that the cereolysin AB genes were transferred successfully into *B. anthracis*. Spores for each of the recombinant clones were made and tested in mice for virulence, and the spent supernatant medium from culturing each clone was tested for hemolytic activity. Each of these recombinant strains indicated the inserted cereolysin AB genes functioned successfully via effective hemolytic activity for each clone tested. To compare the new genetically engineered strains and the original or parent strains from which they were derived, isolates of the recombinant strains were selected with infectivities similar to those of the parent strains. Recombinant and parent H-7 strains with similar mouse LD$_{50}$ values were used to determine protection provided by the recombinant and parent STI-1 strains.

Each of the original and each of the recombinant vaccine and control strains were injected into Gold hamsters to determine the level of protection when the hamsters were challenged with either the original or recombinant H-7 pathogen strains. The results are shown in Table 1.

**Table 1. Protection by Parent and Genetically Engineered Strains of *Bacillus anthracis* in Gold Hamsters.**

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<td></td>
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<td>H-7</td>
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<tr>
<td></td>
<td></td>
<td>H-7+</td>
<td>YES</td>
</tr>
<tr>
<td>CONTROL</td>
<td>221</td>
<td>H-7</td>
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<tr>
<td></td>
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<td>H-7</td>
<td>NO</td>
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<td></td>
<td></td>
<td>H-7+</td>
<td>NO</td>
</tr>
<tr>
<td>NONE</td>
<td>not immunized</td>
<td>H-7+</td>
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</tr>
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</table>

N.B. Transformed strains (i.e. with cereolysin AB genes) are designated with “+”.
The results in Table 1 with non-immunized animals demonstrate the pathogenicity of both the H-7 parent and H-7+ recombinant strains and provide a control with which to assess the other test cases. When hamsters were immunized with the control strains or not immunized at all, no protection was afforded against the challenge H-7 or H-7+ strains.

Results become complicated with the animals vaccinated with vaccine strains. The data indicate a recombinant vaccine strain is required to protect against a recombinant pathogen. The parent vaccine strain, STI-1, protected against the typical pathogen, H-7, as would be expected, but did not protect against the genetically engineered pathogen, H-7+. Such a finding would not be anticipated and is important because it demonstrates that a traditional vaccine could not protect against a modified pathogen.

Although there has not yet been a convincing explanation as to why STI-1 did not protect the hamsters against H-7+, the Russian researchers did not ignore this fact and suggested a few reasons for the results. Since the appearance of this work, no follow-on research within their own facility has been published to explain these results. The most significant rationale offered was a citation for work performed in the U.S. that described the insertion of the hemolysin gene from *Listeria monocytogenes* into a non-spore forming *Bacillus subtilis* permitting the organism to grow within mammalian cells. This citation was found to be of profound significance.

*Listeria monocytogenes* typically contains a hemolysin gene called “listeriolysin O” that appears to be associated with the ability of this organism to enter a host cell and multiply. Mutants lacking the listeriolysin O gene fail to become established within cells suggesting the gene is essential to establish infection. In 1990, the study published in the journal *Nature* showed that the transfer of the listeriolysin O gene into *B. subtilis* changed the character of this organism to that of a pathogen. The transformed organism produced listeriolysin O, disrupted phagosomal membranes, and multiplied within the cytoplasm of cells. (See Figure 1.) In essence, it transformed the “non-pathogen” *B. subtilis* to an organism that could be characterized as a pathogen because of its new ability to escape phagocytosis and multiply within mammalian cells.

If the cereolysin AB genes introduced into *B. anthracis* produced a response analogous to that observed with the listeriolysin O gene inserted into *B. subtilis*, the end result could be a modified *B. anthracis* that would cause anthrax even in animals previously vaccinated.

Anthrax is considered a toxigenic disease. Although acquiring spores may
initiate the infection, the spores must germinate into vegetative cells capable of multiplication and expression of the appropriate virulence factors. The protective capsular polysaccharide coating is considered an essential virulence factor in mammals. During an infection this coating provides a protective barrier to the organisms. In the absence the polysaccharide capsule or the toxin molecules, mammals do not contract anthrax. It should be noted that anthrax infections in mice are possible even if the offending organisms cannot produce the capsular polysaccharide.(41)

Although the “toxin complement” consists of a group of three different proteins secreted by the organism, two of the proteins are toxins, edema factor (EF) and lethal factor (LF). EF (i.e., a calmodulin-dependent adenylate cyclase) causes fluid accumulation, and LF, described as a zinc metalloprotease, inactivates a protein kinase and stimulates macrophages to release cytokines (i.e., TNF-α and IL-1β) resulting in sudden death in systemic anthrax. The precise molecular mechanisms for the toxic activity of LF have yet to be determined.

The third protein, protective antigen or PA, is required for the translocation of EF and LF from the exterior to the interior of cells where EF and LF are functional. Individually, these three proteins are considered generally harmless for mammals, but together are considered the root cause of anthrax providing the capsular polysaccharide is produced by the infecting organisms. These phenomena are demonstrated with B. anthracis, Sterne strain, which lacks the genetic material (i.e., plasmid pXO2) for capsule formation, but does possess the genes for the toxin proteins (i.e., plasmid pXO1). In mice where the capsular polysaccharide is not required for infection, large numbers of spores

Fig. 1. Light micrographs of B. subtilis with listeriolysin O gene growing in J774 cells. Top: 2 hr after infection; Bottom: 5 hr after infection. (from Bielecki, et al., 1990)
of Sterne strain injected in mice cause anthrax indistinguishable from the infection with a fully virulent, encapsulated strain. (41)

In the absence of PA, EF and LF, by themselves, are incapable of gaining entry into cells and incapable of causing disease. Alterations to the gene for PA in B. anthracis have eliminated the potential for infection in mice. (8) Alternatively, it is suspected that if there were a way to bypass PA, that is, if EF and LF could get into cells without the intervention of PA, it is likely that anthrax would result.

It was suggested previously that the insertion of the cereolysin AB genes into B. anthracis might produce a result similar to that observed with B. subtilis (31) with the listeriolysin O gene – multiplication of the organism within the cell. This manipulation would cause a change in the pathogenic character of B. anthracis in that its vegetative form could multiply in mammalian cells. Unfortunately, such a change would likely have dire consequences because of the nature of the anthrax disease process.

Typical anthrax pathophysiology is characterized by the uptake of spores by macrophages that migrate to the lymph nodes. Spores evade the phagocytic effects of macrophages. The spores germinate into the vegetative forms that multiply and are subsequently released from the macrophages. (9) Although subject to environmental factors such as temperature and CO₂ concentration, the toxin proteins are actively secreted from the vegetative cells resulting in characteristic anthrax. (10) Vegetative forms are apparently protected from phagocytosis by virtue of the capsule, secrete the toxin proteins outside of cells, and do not multiply within cells.

If the cereolysin AB genes inserted into the pathogenic B. anthracis conferred the ability to multiply within cells following entry, it is probable that these internalized organisms secreted toxins, namely EF and LF. This, in turn, eliminated the need for PA to translocate the toxins into cells. Since EF and LF must, under typical conditions, be transported into cells to cause anthrax, it might be difficult to discern the type of organism responsible. That is, if EF and LF are functional when secreted by internalized organisms that multiply within cells, it may not be possible to discriminate whether anthrax is mediated by toxins internalized by PA or secreted by intracellular organisms.

Since the 1997 publication by the Russians, much has been done to determine what genes are controlled by the transcriptional activator PlcR, the mechanism responsible for control of plcR, and what happens when a functionally complete PlcR is present in B. anthracis. PlcR is the first pleiotropic regulator in pathogenic Bacillus species found to control the expression of several virulence
factors on genes widely dispersed on the chromosome. The product of the plcR gene in both *B. cereus* and *B. thuringiensis* is functionally equivalent unlike the non-functional PlcR encoded in *B. anthracis*.

In 1996, a study by Lereclus, *et. al.*, showed that transcription of the plcA gene responsible for producing a phospholipase C (PI-PLC) to cleave phosphatidylinositol in *B. thuringiensis* required a trans activator encoded by the plcR gene in *B. thuringiensis*. PlcR was found to regulate its own expression and the expression of PI-PLC. A conserved 17 base pair DNA sequence upstream of the plcR and plcA transcriptional start sites was suspected of being the specific recognition sites for PlcR activation. Because the promoter regions of the plcA genes in both *B. cereus* and *B. thuringiensis* had previously been shown to be identical, it was concluded that PlcR likely controlled expression of plcA in *B. cereus*, as well.

When the plcR gene was disrupted to produce mutant strains of *B. cereus* and *B thuringiensis* Cry which are both normally pathogenic for insect larvae and mice, the mortality of either the insect larvae or mice was reduced by at least 90% when infected with organisms containing the mutant plcR gene. The reduction in mortality occurred with infections caused by the introduction of spores or vegetative cells. Also, the cytolytic activities of either of the mutant strains also greatly decreased when tested against sheep, human, and horse erythrocytes. This work suggested that the pathogenicity of either of the tested organisms was controlled by PlcR.

This work eventually led to the determination that PlcR controls a large regulon of at least 14 genes that encode degradative enzymes, cell surface proteins, and both hemolytic and non-hemolytic enterotoxins. The promoter region of PlcR-regulated genes revealed the presence of a highly conserved palindromic region (TATGNAN₄TNCATA) thought to be the PlcR recognition site for gene activation.

Although PlcR is active in both *B. cereus* and *B. thuringiensis*, *B. anthracis* contains a nonsense mutation in the plcR gene which yields an inactive PlcR consisting of approximately 213 amino acids (Fig. 2), and this truncated PlcR is likely responsible for the lack of hemolytic activity found with *B. anthracis* as opposed to that of the other members of the *Bacillus cereus* group. Although a PlcR regulon exists in *B. anthracis*, it is silenced by the nonsense mutation of the plcR gene, and expression of a functional PlcR in *B. anthracis* produces expression, albeit weak, of a complete regulon.

The expression of plcR is controlled by SpoA−P, a regulator protein for
sporulation that likely prevents the PlcR activator from binding to sites necessary for plcR expression.\(^{22,36}\) In addition, Slamti and Lereclus, 2002, determined PlcR activation of genes in the regulon is under control of a small 48-amino acid peptide, PapR, and disruption of the papR gene abolished expression of the regulon. They found that PapR was secreted from the bacteria, taken back into the bacterial cell by means of the activity of oligopeptide permease (Opp), and processed to a pentapeptide that activated the PlcR regulon by activating PlcR to bind to the appropriate DNA sites. The mechanism was found to be strain specific and did not function, as expected, in \(B.\) \textit{anthracis} because of the lack of a functional plcR gene.

The nucleotide sequences of the \(plcR\) and \(papR\) genes from different strains of \(Bacillus\) species including \textit{cereus}, \textit{thuringiensis}, and \textit{anthracis} were aligned with published sequences of the genes, and no differences were observed for the PlcR operon among the strains except for the truncated 212 amino acid PlcR encoded by \(plcR\) in \(B.\) \textit{anthracis}.\(^{30}\) PlcR from the other species consisted of 285 amino acids, and the \(papR\) gene from all species encoded a polypeptide of 48 amino acids.

Recently, Pomerantsev, \textit{et al.}, 2003, performed gene replacements to determine the effects on expression within the PlcR regulon. When the \(B.\) \textit{cereus} \(plcR\) gene was exchanged with the \(B.\) \textit{anthracis} \(plcR\) gene that can only produce a PlcR activator truncated at the C-terminus (Fig. 2), the activities of phosphatidylcholine-specific phospholipase C (PC-PLC) and sphingomyelinase (SPH) were eliminated. This result was consistent with the previous observations that inactivation of PlcR greatly reduced PC-PLC and SPH expression in \(B.\) \textit{cereus}. If, however,
plcR from *B. cereus* was transferred to *B. anthracis* either on a multicopy plasmid under control of the *B. anthracis* protective antigen gene promoter or containing the entire *B. cereus plc-sph* operon, *B. anthracis* was found to produce hemolytic activity.

In light of the work published in 1997 by Pomerantsev, *et al.*, the work being performed with the PlcR regulon presently is suggestive that it may be possible to eventually produce modified forms of *B. anthracis* that will have altered virulence attributed to the expression of genes presently silent in this organism. Ultimately, just as with the STI-1 vaccine in the golden hamsters (31), these studies may lead to an altered pathogenesis in *B. anthracis* which may alter the efficacy or circumvent vaccines now in use. New approaches to vaccine development may be necessitated if hemolytic activity or other potential virulence factors not usually associated with *B. anthracis* are now produced through genetic engineering methods.

**IV. HISTORY OF ANTHRAX VACCINES**

Louis Pasteur has long been credited with initiating the history of anthrax vaccines with the development of heat-attenuated cells used to protect animals against *Bacillus anthracis*. (29) Pasteur’s vaccine schedule consisted of two phases whereby two inoculations were given two weeks apart. (40)

- **Phase I.** A vaccine that consisted of cells (*n.b.*, pathogenic only for mice and young guinea pigs) incubated at 42 to 43°C for 15 to 20 days.
- **Phase II.** A vaccine that consisted of less attenuated cells incubated at 42 to 43°C for 10 to 12 days.

This vaccine was used widely in Europe and South America for the next 50 years. It was replaced in the 1920’s with a single-dose vaccine consisting of spores suspended in 50% glycerol which improved the stability of the vaccine preparation and improved immunizing efficiency over the previous preparation. In the 1930’s new vaccine formulations involved adding between 1 and 10% saponin to the Phase II Pasteur vaccine or other pathogenic forms of *B. anthracis*. (15) The saponin contributed to a violent inflammatory reaction at the inoculation site. The reaction was thought to limit the extent of the infection.

The next major breakthrough in anthrax vaccine development resulted from the isolation of a form of *B. anthracis* that lacked the ability to form a capsule. Max Sterne discovered this in 1939 (38), and the Sterne strain (designated 34F₂) is still, today, widely used as a veterinary vaccine. The final formulation for the
Sterne strain consisted of between 0.6 and 1.2 x 10^6 spores per ml (n.b., today the formulation consists of ~10^7 spores/ml) suspended in 0.5% saponin and 50% glycerine-saline. (38)

With regard to human vaccines, a live spore vaccine was used in the USSR and is still in use in Russia. This is the STI-1 strain that is similar to the Sterne strain, but it is administered by skin scarification. (35) The vaccine contains 2 to 2.5 x 10^9 spores per ml and is applied by aseptic inoculation of two drops onto the skin of the arm. The skin is scarified by scratching the skin with a stylet to produce four scratches 1.5 to 2.0 cm long. When fluid is observed at the inoculation site, the vaccine material is rubbed into the skin. The scarification doses range from 1.3 to 1.6 x 10^8 spores.

In contrast to Russia, the U.S. and U.K. have avoided the use of live spore vaccines. Instead, the U.K. employs an alum-precipitated cell-free filtrate of Sterne strain, and the U.S. employs an adsorbed cell-free filtrate of cultures of a non-encapsulating, non-proteolytic derivative of a B. anthracis strain derived from a case of bovine anthrax. The mainstay of both of these vaccines is primarily the production of protective antigen (PA). This non-toxic immunogen is capable of providing protection against B. anthracis in humans. Although the vaccine preparations contain small amounts of the EF and LF toxins, these essentially monovalent PA-based vaccines are in use in both the U.S. and U.K. for service-wide vaccination programs. The U.S. vaccine was named originally MDPH-PA or AVA. In January 2002, it was renamed BioThrax. This vaccine has been administered to approximately 900,000 members of the armed forces and some civilians. Six inoculations – scheduled at 0.5, 1, 6, 12 and 18 months following the first inoculation – are required for a complete vaccine regimen. This action by the U.S. was the first time in history a vaccine is being administered for the purpose of protecting personnel from potential attack with a biological weapon agent.

V. NEW VACCINE CONCEPTS

Much work has been accomplished recently to eliminate the potential for B. anthracis to establish infection and to ensure that vaccines are more efficient in protecting against the disease. Although these are still largely experimental, they offer much potential for the future even in the event that genetic engineering is employed to alter the virulence and pathogenesis of B. anthracis.

Perhaps on the forefront of novel vaccines is genetic immunization shown in one study to be very effective in mice. (32) Gold particles (diameter of one
micron) coated with plasmids containing cloned sequences that encoded either a biologically active portion of PA or a large fragment of LF were injected into mice using a gene gun three times at two week intervals. Antibody titers to PA and LF were four to five times higher in animals injected with both types of plasmids as compared to those injected with only one type of plasmid. Immunized mice were challenged (i.e. intravenous tail vein injection) with five LD$_{50}$ doses of lethal toxin (i.e., PA plus LF), and all mice immunized with either one type of plasmid or both plasmids survived whereas all mice not immunized died.

The importance of multi-valent vaccines that induce the production of antibodies against several antigens may eventually become a greater necessity should genetic engineering alter the virulence in *B. anthracis* as in the case where the PlcR regulon is made active. Stepanov, et. al., 1996, stated that the induction of a strong and stable immunity requires the entire antigen complex to include spore, surface, and toxin components of *B. anthracis.*(37) Although spore infection may lead eventually to anthrax, germination is essential to toxin production and disease pathogenesis. Since antibodies to the toxin (i.e., PA plus LF) can inhibit spore germination, the presence of antibodies to both PA and LF are synergistically more effective than the presence of antibodies to either of these factors alone. Also, the virulence of encapsulated *B. anthracis* is similar for both vaccinated and non-vaccinated animals.(37)

Spore antigens may also contribute to the efficacy of existing vaccines which rely mainly on antibodies to PA. In both mice and guinea pigs, formaldehyde-inactivated spores with PA produced total protection against virulent *B. anthracis.*(7) In another study with either PA or live Sterne strain spore vaccine, guinea pigs were challenged with a variety of *B. anthracis* isolates.(24) Nine of the 27 isolates tested were resistant to the PA-based vaccine, but none were resistant to the spore vaccine demonstrating the importance of spore antigens in active immunity to *B. anthracis* infection.

In spite of these ongoing studies to provide improved vaccines for anthrax, PA-based vaccines already administered to hundreds of thousands of individuals within the defense communities continue to be the vaccines of choice for human immunizations in the U.S. and U.K. For even more widespread immunization, a recombinant PA-based vaccine has been developed and tested and is now in production.(12) PA-based vaccines, including the recombinant, have been shown to be very effective against virulent *B. anthracis* infections in non-human primates. The benefits of the recombinant vaccine will include more dependable production of a product with great consistency, and it is planned to reduce the number of
injections from 6 to 3. Although there may be a need to expand the array of antigens needed to protect against potential recombinants of *B. anthracis* in the future, the present vaccines are both very safe and effective.

**VI. MOLECULAR MECHANISMS AND FUTURE THERAPIES**

The fact that anthrax can be described as a “toxigenic disease” whereby it is the production of the toxin components that are responsible for damaging phagocytes leading to death of the organism as circulating toxins ultimately debilitate the immune system. The potential that bacterial resistance can be developed to antibiotics (37) and vaccines (31) has promoted investigations into anti-toxins that may help in the treatment of anthrax. Fortunately, work which will certainly help development of an anti-toxin is the description of the LF crystal structure with its functional domains. (28) LF is a protease (molecular weight: ~90 kDa) that cleaves mitogen-activated protein kinase kinase (MAPKK) inhibiting cell signaling pathways. It was shown to have four domains – the PA binding domain and the other three domains that are responsible for the binding and cleavage of MAPKK.

At the molecular level, the mechanism to introduce the toxin molecules into cells has been elucidated. The 83 kDa holoprotein “PA83” binds to a specific cell receptor called the “Anthrax Toxin Receptor” (ATR) found on a variety of mammalian cells (6). PA83 is then cleaved by furin (17) releasing a 20 kDa molecule (*i.e.*, PA20) and leaving a 63 kDa molecule (*i.e.*, PA63) bound to the ATR. This initiates the aggregation of seven PA63 molecules into a heptameric pore structure which permits the binding of up three molecules of EF, LF, or a combination of the two. (42) Once the PA63 heptamer is complexed with EF, LF, or both, it is internalized by virtue of lipid rafts (*i.e.*, cholesterol and glycosphingolipid rich regions of the plasma membrane on a cell). (3) From the lipid raft, EF and LF toxins are released from the complex causing specific deleterious effects associated with anthrax.

A thorough understanding of these mechanisms offers a variety of modalities for future therapies. Many novel approaches are being attempted as described in the review by Young and Collier, 2002. (42) For example, it was found that the ATR spans and protrudes from the cell membrane, and the protrusion actually serves as the attachment site for PA. Thus, there is a search for inhibitors for the ATR, as well as molecules to mimic ATR to bind PA to prevent it from attaching to cells. Mutant forms of PA that form heptamers, but are unable to introduce
toxins into cells, are also being investigated. Drugs are being sought to “plug” the PA63 heptamer and prevent the attachment of actual toxins. Continuing investigations not only into new ways to treat anthrax but also into the molecular mechanisms that provide insight as to how to best develop new therapeutic approaches will certainly yield advantages to manage complicated and often deadly infections.

VII. CONCLUSION

The use of *B. anthracis* as a biological weapon is an issue of grave concern. This was amply demonstrated by the deaths in the United States during the anthrax mailings that started in September 2001. Simply because of the bioterrorism threat that now exists worldwide, it is most important that new and improved therapies be developed against *B. anthracis*, probably the most widespread BW agent of concern.

Attempts to improve therapies will provide the opportunity to mitigate the effects of an anthrax attack and probably show marked improvement in the ability to survive. For example, in contrast to the widespread belief that inhalation anthrax is not curable following the appearance of symptoms, six of the first ten victims from the U.S. anthrax attacks were saved probably by the administration of effective combinations of antibiotics during the early phase of the disease before typical late phase serious symptoms appeared.(19)

New, effective vaccines must be developed to be more practical for human use than those presently employed. Although the present vaccines are very safe and effective against typical *B. anthracis*, these vaccines, because of the series of six shots during an 18 month course, are impractical for broad, public use. There is an effort to improve the existing vaccines by using a nucleic acid recombinant to produce “synthetic” PA – a vaccine now being produced to replace BioThrax, the vaccine produced from live *B. anthracis*. Using this “rPA” with a new adjuvant should decrease the number of shots required and also the total time required to complete the vaccination.

The use of genetic engineering to investigate the genome of *B. anthracis* is certainly important to be able to address a variety of issues relating to this organism as well as the entire *Bacillus* genus. These investigations, however, have shown the potential for *B. anthracis* to exhibit virulence factors which typically cannot be expressed by this organism. DNA recombinants of *B. anthracis* may, however, be organisms that could cause disease in previously vaccinated ani-
Although this information may lead to the development of better anthrax vaccines, it is most unfortunate that this has to be done because of concerns regarding offensive BW programs and bioterrorism.

The fact that nations must now vaccinate against anthrax not because it is a natural threat but rather because of the fear that other individuals or states could use such organisms as weapons is thought to be a sad commentary on both the state of global peace and health. By virtue of the rapid progress in our understanding of the molecular and clinical intricacies of the disease, however, it is apparent that effective therapies for anthrax will continue to improve. Hopefully, the value of these therapies will never be demonstrated against the spread of anthrax by intentional use.

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Zoonotic Bacteria as Biological and Agroterrorist Weapons

Philip H. Elzer

Zoonosis is the transmission of disease from animals to man. Typically zoonotic agents are associated with the care, products and processing of livestock. Most individuals at risk for zoonotic infections are farmers, abattoir workers, and veterinarians. The illegitimate use of zoonotic bacterial organisms makes them potential weapons of mass destruction in that they can be used for biological and agricultural terrorism. As biological weapons, these agents can be used to infect military or even civilian personnel on a large-scale basis. As agricultural terrorism agents, these organisms can destroy livestock and their unborn offspring, leading to massive economic losses impacting on agricultural production. The use of a zoonotic agent compounds these effects in that public and veterinary health officials need to worry about the horizontal spread of these infections to the general public. The four primary bacterial agents focused upon will be: *Bacillus anthracis* (Anthrax), *Coxiella burnetti* (Q Fever), *Francisella tularensis* (Tularemia), and *Brucella* spp. (Undulant fever).

The tragedy that occurred in the US on September 11, 2001 has heightened our awareness of terrorism and our vulnerability to terrorist activities. Question, concerns, deliberations and dialogue about the possibility of future terrorist attacks, the forms they may take and precautions that will be necessary to prevent/counter these dangers are on everyone’s mind. One form of terrorism that has been largely overlooked until recently is agroterrorism. Agroterrorism, the attack on the nation’s agricultural infrastructure and food supply, would have direct and immediate consequences on the safety of the nation’s food supply and be an effective means of quickly crippling our country. The average US city has a five day supply of fresh meat, fruit and vegetables. In the aftermath of an agroterrorism agents (ATA’s) attack, food supplies would rapidly disappear as a result of panic/hysteria buying. Furthermore, intended effects of agroterrorism using catastrophic ATA’s in ready-for-market foods, plants and livestock would be demoralizing to the population,
result in tremendous human mental and physical hardship, and also devastate the nation’s economy [1].

The US agricultural sector is highly vulnerable to terrorist assault and the following factors contribute to our nation’s agriculture vulnerability: (1) livestock industries are concentrated in only a few geographical areas; (2) farms are becoming increasingly larger and integrated, consequently poultry and livestock farms are more vulnerable to spread of disease; (3) increased international travel has exacerbated our vulnerability to accidental or intentional disease transmission; (4) reliance on pesticides, herbicides and antibiotics could result in pathogen resistance that could decimate our crops and livestock; (5) lack of crop diversity renders US farmlands vulnerable; (6) a notable percentage of imported hybrid seeds used for US crop production comes from only four countries – Mexico, Chile, Iran and China (Iran and China are suspected of having significant covert bio-agricultural weapons development programs); and (7) a variety of pathogenic agents that can infect livestock and crops as well as contaminate our food supply are easily obtainable.

The properties of the ideal biological weapon are that the agent should be highly contagious and consistently produce a known disease or syndrome. It is best if it can be disseminated throughout the environment, i.e. aerosolized; and it needs to be stable under production, storage, and delivery to target. However the organism should have a short survival time in the target area so it is not a threat at later time points to delivery personnel. It is preferable that the target populations have little or no natural resistance.

If used in an agroterrorist attack, these organisms could decimate a generation of livestock and companion animals [7]. With the concentration of livestock, lack of genetic diversity, increased farm sizes, importation of animals, and increased international travel, agriculture around the world is very vulnerable to a terrorism attack. The effects of agroterrorism would be immediate in that there would be mass hysteria manifested by panic buying of the stocked products. There would be demoralization due to the loss of food, plants, companion animals, and economic devastation. This economic destruction could become worldwide if a country’s main agricultural export product or products were boycotted by numerous nations [8].

Due to the potential use of biowarfare agents, public health officials need to be constantly aware of possible intentional exposures to infectious agents. There are needs for rapid and specific detection devices for environmental releases and medical samples. Improved surveillance and diagnostics with effective communication amongst numerous regulatory and emergency agencies is necessary to contain and control an exposure or outbreak. An example of this is illustrated by
a case report of a 38 years old woman from New Hampshire, USA, who was thought to have been exposed to Brucella spp. This report highlights several aspects of the needed public health response to a possible biowarfare agent [10].

Zoonosis is the transmission of an infectious agent from animals to man. Therefore if a zoonotic agent is used as a bio or agro-weapon, a new wrinkle is added to the above scenario. Now one has to worry about public health concerns including human, animal health and safety as well as food safety issues, including product and production. Public health concerns would deal with awareness of agents, early and rapid detection methods, effective communications between government agencies and coordinated responses [1].

Bacillus anthracis, the causative agent of anthrax, has a worldwide distribution. This Gram-positive, spore-forming, capsulated rod survives by killing its primary host-ruminants. Cattle, which graze on spore-infested pastures, become infected and the resulting bacteremia kills the cattle and allows the organisms to produce more spores to contaminate more pastures. Humans, unlike cattle, do not die from the bacterial infections but are highly sensitive to the toxins these organisms produce which frequently lead to death. Humans are exposed to this organism via inhalation of spores, either naturally through handling of soil, carcasses, or skins, or via deliberate terrorist attacks. Humans can be treated with penicillin, doxycycline or Cipro during early exposure to spores to prevent death. There is a vaccine available for human anthrax; however it generates a short-lived immune response (18 months), and it requires numerous boosters to achieve any level of immunity [2].

Coxiella burnetti is the causative agent of Q-fever in man. This organism is of the Rickettsia family and causes flu-like symptoms in man. The primary hosts for Coxiiella are sheep, cattle and goats; and the routes of exposure for man are airborne, exposure to infected reproductive tissues, and ingestion of unpasteurized milk. The treatment of choice for this debilitating agent is tetracycline and chloramphenicol. A live and killed vaccine for Q-fever exists for animals; however, they are not used in humans due to numerous side effects [3].

Francisella tularensis is the causative agent of Tularemia/Hunters disease in man. The natural hosts for this debilitating granulomatous disease are rabbits and sheep. The primary routes of exposure are aerosol, exposure to contaminated water, exposure to contaminated carcasses, or penetration through unbroken skin. Treatment includes streptomycin, gentamicin or kanamycin. There is a live, avirulent strain which is used to vaccinate military personnel [4].

All of the brucellae are Gram negative, facultative intracellular pathogens, which survive and replicate in host macrophages [5,6]. In man, brucellosis, also known as undulant fever or Malta fever, is caused by only four species of brucellae
(B. abortus, B. melitensis, B. suis, and B. canis). Human infection is caused by ingestion of infected raw milk products, exposure to infected animals, and aerosolization of the organism. Brucellosis in man is characterized by a cyclical fever that starts two to three weeks post-exposure. Night sweats, headaches, backaches, and general malaise are symptoms associated with acute infection. Chronic brucellosis can lead to a debilitating condition, including arthritis, dementia and even death. Patients with chronic brucellosis have frequent relapses, and 2/3 of these individuals develop psychoneurosis. Human brucellosis can be treated with the administration of tetracycline or doxycycline in combination therapy with rifampin or gentamicin [9].

Since there are no human vaccines against brucellosis, most, if not all populations, have little or no natural immunity to this organism. Brucella species were weaponized in the United States following WWII. These species were field tested in cluster bombs in 1955. However all of the munitions using this agent were destroyed in 1969.

The CDC classifies B. abortus, B. melitensis and B. suis as “agents of mass destruction” and as category B organisms. Brucella canis, a less virulent strain, can cause human disease but only when contracted by an immuno-suppressed individual. B. melitensis is the most infectious to man in that 1-10 colony forming units (cfu) are thought to cause disease followed by B. suis (1000-10,000 cfu), B. abortus (100,000 cfu), and finally B. canis (>1,000,000 cfu) in immuno-compromised individuals (Table 1). Brucellae are characterized as BSL-3 organisms due to their ability to infect humans through aerosol exposure, which makes them an ideal bacterial agent of mass destruction [11]. If the general public were exposed to this biowarfare agent, medical resources would be stretched 10 fold to take care of the large number of people that would be debilitated by this organism. Currently there is no approved vaccine for human use, and a vast majority of the animal vaccines

Table 1. Human Susceptibility to Brucella spp.

<table>
<thead>
<tr>
<th>Brucella spp.</th>
<th>Natural Host</th>
<th>Human Virulence</th>
<th>Number of organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. melitensis</td>
<td>goats, sheep</td>
<td>HIGH</td>
<td>1-10</td>
</tr>
<tr>
<td>B. suis</td>
<td>swine</td>
<td>High-Moderate</td>
<td>1,000-10,000</td>
</tr>
<tr>
<td>B. abortus</td>
<td>cattle</td>
<td>Moderate</td>
<td>100,000</td>
</tr>
<tr>
<td>B. canis</td>
<td>dogs</td>
<td>Low/immunosuppressed</td>
<td>&gt;1,000,000</td>
</tr>
</tbody>
</table>
are virulent to man. Thus, there is a need to find a safe and efficacious vaccine that can be used in humans [11].

REFERENCES

Study of Bulgarian Tularemia Outbreaks: Diagnosis and Epidemiology

Todor Kantardjiev, Ivan Ivanov

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I. INTRODUCTION

The genus Francisella comprises of two species: *F. tularensis* and *F. philomiragia*. Only *F. tularensis* with its four subspecies (subsp. *tularensis*, *holarctica*, *mediasiatica* and *novicida*) is capable of causing a severe infectious disease known as tularemia (1). The manifestation of the disease includes mainly six forms: ulceroglandular, glandular, oculoglandular, oropharyngeal, intestinal, pneumonic.

A significant number of confirmed cases of tularemia (about 261; see table 1) have been registered in two Bulgarian regions since January 1998. 76% of the patients have developed tonsillitis and their cervical and submandibular lymph nodes have been affected. 25% of the patients had suppuration, that led up to either a spontaneous fistulisation or necessity for an incision. The treatment with penicillin and cephalosporines (in 1997) was not successful, but the patients’ state improved quickly after a course of streptomycin, gentamicin or chloramphenicol.
II. DIAGNOSIS

The following serologic studies have been performed to prove the ethiological diagnosis:

The hemagglutination test with an antigenic commercial test for tularemia (produced at National Center of Infectious and Parasitic Diseases). 1858 serum samples have been studied and diagnostic titers have been detected in 261 cases. Second serum sample was taken 25 days later from each patient. Most of the samples showed a fourfold increase or decrease of the titers. Specific antibodies were proven with titers 1:25 000 and higher in some patients’ samples. All re-examined patients have kept titers of 1:12 800 for two years. Agglutination test (exploratory and stage) have been performed parallel to the hemagglutination test. The data obtained by the three methods have shown a total correlation. The inhibition of the hemagglutination test performed with the tularemic antigen (produced at the National Center of Infectious and Parasitic Diseases) proved the specificity of the aforementioned reaction.

The indirect immunofluorescence reaction has proven antibody titers against the tularemic antigen higher than 1: 1600. The specific nature of the reaction has been demonstrated by stopping of the fluorescence caused by the tularemic antigen. Blood cells containing the tularemic antigen have been observed microscopically in bubo punctates from patients by DFA reaction with an anti-tularemic horse serum. Since 1998 several strains have been isolated from patients and water reservoirs.

Table 1

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of confirmed cases</th>
<th>Studied serum samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998</td>
<td>96 region of Slivnitza</td>
<td>721</td>
</tr>
<tr>
<td>1999</td>
<td>44 region of Pernik</td>
<td>273</td>
</tr>
<tr>
<td>2000</td>
<td>29 sporadic cases</td>
<td>218</td>
</tr>
<tr>
<td>2001</td>
<td>7 sporadic cases</td>
<td>165</td>
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<tr>
<td>2002</td>
<td>9 sporadic cases</td>
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<tr>
<td>2003</td>
<td>76 region of Meshtitza</td>
<td>335</td>
</tr>
<tr>
<td>total</td>
<td>241</td>
<td>1858</td>
</tr>
</tbody>
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Further confirmation of tularemia was performed by application of genetic methods. We have developed a reliable and specific PCR tests for diagnostics and identification of *F. tularensis* on the basis of two primer pairs. Primer pair TUL1/TUL2 amplify DNA fragment derived from *F. tularensis fopA* (ferredoxin) gene, and TUL-435/TUL-863 amplify fragment derived from *tul4* gene (2). The developed PCR tests were applied for investigation of the last tularemia outbreak that has occurred 40 km Southwest from Sofia in 2003. There were tested about 200 clinical specimens as well as 100 natural water samples from suspected endemic regions and a good correlation with serological diagnosis was found. Newly isolated strains were obtained from 2 patients and from 4 water samples (in the endemic region of Meshtitza see table 2) and confirmed by culture and serological assays as well as PCR. These strains were then characterized genetically by means of 16S PCR-RFLP with three different restriction enzymes. Two of the investigated strains (strain Krastowa bara and Lalintzi) exhibited extraordinary 16S PCR-RFLP profiles in comparison with all other and reference strains. Further genetic investigations will show if these strains are actually *F. tularensis* subsp. *holarctica*.

After a treatment course with streptomycin, gentamicin, doxycyclin a successful recovery of the patients has been observed.

### III. MOLECULAR TYPING

*Francisella tularensis* has been described as a genetically homogeneous species with a limited diversity among isolates. However recent phylogenetic studies have successfully demonstrated variability in subspecies and in individual strain level, applying typing methods with strong discriminatory power (3). For molecular typing of *F. tularensis* we developed and applied AFLP (Amplified Fragment Length

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Source (strain)</th>
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<tbody>
<tr>
<td>2003</td>
<td>Pernik region</td>
<td>Open water reservoirs (4 strains)</td>
</tr>
<tr>
<td>2003</td>
<td>Pernik region</td>
<td>Patient (strain Dragan)</td>
</tr>
<tr>
<td>2003</td>
<td>Pernik region</td>
<td>Patient (strain Galia)</td>
</tr>
<tr>
<td>1998</td>
<td>Slivnitza region</td>
<td>Open water reservoir (strain Aldomirovrtzi)</td>
</tr>
<tr>
<td>1998</td>
<td>Slivnitza region</td>
<td>Hare (strain Lalintzi)</td>
</tr>
<tr>
<td>1953</td>
<td>Srebarna Lake</td>
<td>Natural water (strain Srebarna)</td>
</tr>
</tbody>
</table>
Polymorphism) method allowing the discrimination among different strains *F. tularensis*. At first the isolated bacterial genomic DNA was digested completely with *BamHI* and *Pst I* restriction enzymes. Specific double-stranded adaptors were subsequently ligated to the ends of the obtained restriction fragments. PCR reaction with primers specific to the ligated adaptors and end-restriction sites was performed. One of the primers was $^{32}$P labelled. After electrophoresis and autoradiography the obtained profiles were scanned and submitted to computer clustering analysis from which a dendrogram was constructed on the basis of the similarity coefficients among the strains. Two software packages were applied – Cross Checker 2.91 developed by J.B.Buntjer and TreeCon 1.3 developed by Yves Van de Peer.

Twenty-four *F. tularensis* strains originating from Asia, Europe and North America collected during the last 80 years and available in the Bulgarian Type Culture Collection were typed by AFLP. The AFLP profile of a strain *F. tularensis* “Srebarna” isolated in 1962 during an outbreak near the lake of Srebarna, north of Bulgaria near the Danube river, shows high genetic similarity with a Norwegian strain isolated in 1953. In general all 24 AFLP profiles showed little variability. On the basis of our experience we could conclude that *F. tularensis* undergoes slow molecular evolution and the exploration of new genetic markers with fast molecular clock is crucial for the discrimination among *F. tularensis* strains.

Our group has experience investigating the interaction between *F. tularensis* and some protozoa. The presence of viable protozoa (genus Acanthamoeba) in the experimental environment permits a long persistence and multiplication of *F. tularensis*. *F. tularensis* replication in co-cultures with protozoa reached higher bacterial concentrations compared to cultivation in liquid media only. These experimental data show the possibility that in the hostile conditions of the environment protozoa may serve as important reservoirs and sources of *F. tularensis*.

We concorded a national consensus strategy for treatment and prophylaxis of tularemia.

REFERENCES


Nutritional Poisonings by Epidemic Incidents and Biological Terrorism

Alexander Monov

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Food* poisonings are a priority pathology in modern society due to the wide spectrum of possibilities for their spring up, their mass character in most of the cases, the difficulties in their diagnosis and treatment (1, 2). These characteristics open possibilities for them to be used in biological terrorist acts. The author’s several years of research shows that a specific strategy should be applied based on an unified doctrine for maximum effectiveness in struggling against food intoxication and its marginal areas. Only thus, can today’s issues on its etiology, pathogenesis, clinical peculiarities, diagnosis, treatment and prophylaxis, be solved (1, 4, 5).

I. RESEARCH IN THE FIELD OF ETIOLOGY

Research in the field of etiology gave the possibility to elaborate a unified

* Including the samely potable water and different kinds of drinks
classification in which food poisonings are grouped according to the cause of the poisoning. Thus the medics are substantially assisted for a quick diagnosis orientation and therapeutic actions (3, 5).

1. Food poisonings by non-bacterial source poisons. Here belong:
   a) Poisoning by actually poisonous food from plant (mushrooms, the nuts of some fruits, etc.) and animal (fish, mussels, etc.) sources;
   b) Poisoning by food having temporary toxic effect: potatoes (solanin), some fish etc.;
   c) Poisoning by exterior toxic compounds in food: chemical compounds – arsine preparations, talium, pesticides, etc., secale cornutum, biological matter (toxic oil, toxic phyto- and zoo-extracts) etc.

2. Food poisoning by toxins of bacterial origin: staphylococcus, botulin, etc. (6).

3. Food toxic infections, caused by salmonella, conditionally pathogenic bacteria (esherihia colli, proteus etc.) and other kinds of bacteria and viruses (6).

During the hot and warm seasons, intoxication of the second and third group is more often met with as epidemic incident, than of the first group. In Bulgaria, in summer most often staphylococcus intoxication and salmonella toxic infections are spread. The sea aquatoria of different regions can be a potential birthplace of food intoxication by enteric bacteria from contact with water and badly prepared seafood – fish, mussel, shrimps. This can happen under worsened ecological circumstances, due to the kept alive bacterial flora in sea-water (as a consequence of the disordered bactericide characteristics resulted from the ecological disbalance). They often appear as biological traumatism and terrorism, when chemical and biological poisons are put in food. During the mentioned seasons, food intoxication of the first group is most often caused by pesticides and nitrates, because of the more abundant consummation of fresh fruits and vegetables, which have not been washed properly.

**II. REFERRING TO PATHOGENESIS**

Referring to pathogenesis, in the light of the unified concept for the spring up of food poisoning, the following harming factors develop in the patient’s organism:

1. Exogenous nutritional poisons and bacteria, imported in the stomach-intestinal tract by the food – they cause local disturbances in the struc-
ture and functions of the stomach and the intestines, hyperemia and increased exudation of the mucous membranes, dyskinesia with hypermobility or atonia. However they often inflict other organs and cell structures in the organism depending on their chemical or biological characteristics – heart and blood vessels, liver, kidney, blood and others, most often on the basis of enzyme malfunction and cell metabolic disorder, combined with intracellular hypoxia in the severe cases.

2. Stomach intestinal-colon mechanisms: disorders in the excretion of biliary liquids, digestive pancreatic and other enzymes, activation of the saprophyte intestinal flora and its transformation into pathogenic, its passing through the intestine mucous membranes and penetration into the lymph- and blood circulation, different degrees of stomach-intestine biochemical disorders.

3. Balance and homeostatic disorders – most often expressed in dehydration, water – electrolytic and acid-alkaline disbalance, arisen mainly from the stomach-intestinal processes. These mechanisms function in medium severe forms.

The mentioned damaging processes take place irrespective of the factor’s etiology group and the symptoms depend on the degree of intoxication.

**III. CLINICAL CHARACTERISTICS**

In the light of the proposed unified concept, the clinical characteristics of all kinds of food poisonings are determined by the following symptoms and syndromes:

1. Gastric-intestinal symptoms. These are the first evidences of the food disease and begin after different latent periods. When the cause is chemical noxa this period is usually short – 1-4 hours. For some infections – salmonella and others, this period is 6-8 hours, for the action of some biological poisons – the toxins of the mushroom amanita falloides, amanitine and others – approximately 10-14 hours. The clinical picture is as follows: vomiting at the beginning of the intoxication for the mild forms, diarrhea – for the enteric and colitis changes. The presence of mucus and blood in the excrements is most often due to the toxic infection (salmonellosis, dysentery). The atonic intestine and stomach processes often point to botulism. The gastrointestinal colon symptoms are the most frequent evidences for food poisonings.
2. Poly-organic symptoms and syndromes. Depending on the kind of the noxa they are manifested by different organs and systems: cerebral and polyneuritic – for the action of neurotropic substances (botulism, fish and mushroom toxins, etc.); cardiovascular (shock conditions – in the presence of staphylococcus and other toxins); hepatic – with hepatotropic toxins (*amanita falloides* mushrooms), hematogene-hemolytic and other types; kidney – arsine etc. This group of manifestations is characteristics for the severe forms of the intoxication or with specific organotropicity of the inflicting agent.

3. General toxic symptoms and syndromes. They are observed for the severe and terminally severe forms of all kinds of food intoxication and do not have specific importance for the kind of the poisoning. Here belong the homeostatic and disbalance processes such as general weakness, shock state, dehydration, breathing disorder, acidosis, febrility etc.

**IV. THE DIAGNOSIS OF FOOD POISONING**

The diagnosis of food poisoning is based on the following anamnesis: consumption of food (mushrooms, herbs, canned meet, food of dubious quality or improperly preserved during the hot months food) - leading to this kind of diseases; presence of the disease or similar clinical manifestations in several individuals simultaneously or at short intervals; suspicion and proof that highly toxic substances have been purposefully introduced in the food or the water. The presence of gastric, gastric-enteric syndrome and colitis-like pain also speak for food poisoning. Beside the above mentioned characteristics of the stomach-intestine state, some specificities of the polyorganic and general-toxic symptoms and syndromes point also to the kind of the food poisoning. For example the combination of gastric-enteric with hepatic processes when they continue for a longer period, point to a falloid mushroom or amanitine terrorist poisoning; gastroenteric syndrome with febrility is most often due to bacterial intoxication or food toxic infections. The diagnosis decision for a timely and correct treatment requires to establish beside the kind of the intoxication, the degree of the inflicted damage. An isolated gastroenteric syndrome with a good general condition points to a mild degree of food poisoning. The presence of polyorganic symptoms, disorders in blood circulation, breathing, conscience, convulsions, very high febrility, heavy dehydration speak of severe and terminally severe degrees. Mass intoxication requires that it is thought of and acted upon as a purposeful aggressive act. For the
quick identification of the kind of the author, it is necessary before treatment begins to take biological samples (excrements, urine, blood) as well as material from the vomited content and water for a bacterial and chemical analysis depending on the clinical direction.

V. TREATMENT OF FOOD POISONING

Treatment of food poisoning, taking into consideration the above circumstances, is carried out after a complex therapeutic program. It is described as follows (4, 5):

1. Nonspecific treatment means and methods:
   a) Reanimation and replacement-corrective procedures – antishock, cardioprotective therapy, breathing reanimation, water-electrolyte and other solutions infusion (drops-venous application, adequate to the observed general disorders). They are applied in the severe and terminally severe cases according to the manifested indications and they create the main treatment background of these patients. They begin already on the site of the incident and continue during transportation.

   b) Detoxic depuration of the stomach-intestinal tract following the traditional methods – it is applied on all patients during the first hours of the intoxication; after finishing with the washing out of the stomach and the absorption of intestine purgative, every 1-3 days an absorbent is administered perorally – medical charcoal or adsorgan 2-3, 1 coffee-spoon with water daily.

Food intoxication with chemical and biochemical substances, that have penetrated in big quantities into the blood, detoxic blood depuration is carried out by the method of forced diuresis, partial blood transfusion and dialysis methods as described in the complex program. They are carried out in clinical conditions.

2. Specific means and methods:
   a) When anamnesis and clinical data show bacterial etiology of the food poisoning and when there is a significantly expressed enterocolitis syndrome, chlorchinaldol or a suitable sulfonamide with a local enteric effect is included at the beginning of the treatment perorally. If vomiting is strenuous and persists and there is high temperature and bad condition, eventually an antibiotic – chlorocid or other is administered perorally or venously in sufficiently high
doses. After the result of the antibiogram and the sensitivity to antibiotics, the therapy is adapted to it.

b) Anti-poisons – antidotes and others to chemical or other kinds of toxic substances – administered with food. At suspicion for botulism, anti-botulin serum in prophylactic doses is applied already on the first day and if results are positive – is continued in treatment doses. At staphylococcus noxa, anti-staphylococcus serum is included. At suspicion or proof for another kind of poison – consult the information data on Antidotes and the Chapter on Amanitine poisoning.

3. Organoprotective methods:

Depending on the damaged organs and systems, the following medication is included: glucose and levulose solutions, vitamins of the B group and other hepatoprotective drugs – for liver manifestations; nootropic preparations, tranquilizers, vitamins of the B group – for cerebral damage; vitapiracen and others, gluco-corticosteroids, calcium gluconate, vitamin C and others – for hemolytic incidents.

4. Dietary treatment:

During the first hours of an acute gastric enterocolitis, liquids should be taken – tea, rice water, later – potato soup, diluted yogurt, toast, rusk, water are advised. Other foods are included gradually. Liquids are taken in sufficient quantities to compensate the loss. If other organs are damaged, other suitable diets should be observed.

According to the indications, other treatment procedures can be applied. Patients with food toxic infections are hospitalized in the infectious sections or in other specialized hospitals. Irrespective of where the treatment is carried out, current disinfecting and hygienic activities are obligatory.

VI. PROPHYLAXIS

Prophylaxis of food intoxication, especially during the hot seasons, requires strict observation of the rules for obtaining and conservation in refrigerators of the nutritive products. It is necessary to apply modern culinary technologies in food preparation. For example: eggs and egg dishes without sufficient thermal processing, creams and sweets including eggs, fish, mayonnaise etc., should not be consumed during hot seasons. Regular sanitary and microbiological control should be carried out. Regular health education courses should be organized by medical authorities. Obligatory chemical and biological laboratory analysis should
be made at adequate intervals using urgent methods to establish the quality of water and mass food. 24-hour readiness should exist to carry out such analysis at national and regional levels.

MAIN REFERENCES

The periodic spring up of single and group lethal falloid and lepiotic mushroom poisoning and the possibilities due to biological technologies for its main group of poisons – the amanitin poisons, to be laboratory-industrially produced demand solutions to the important issues, presented in this review (1, 2, 3, 4, 5).

I. ETIOLOGICAL ASPECTS

There are possibilities, by special technologies and in laboratory and industrial conditions, fractions of the amanitin poison to be included in products, that penetrating the human organism as terrorist poisons can cause lethal amanitin intoxication. The pointed substances are contained in the *Ammanita falloides* mushrooms and in some kinds of *Lepiota*. 
II. INJURING MECHANISMS

The processes through which the said etiological factors injure the human organism are an important issue to this kind of lethal intoxication (Table 1). These highly toxic substances are thermostable. They represent amanita-toxins – polypeptides with low molecular weight (3, 4).

Table 1

<table>
<thead>
<tr>
<th>Injuring mechanisms of the amaniture poison (after Al. Monov)</th>
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<tbody>
<tr>
<td>Hepatalgic mechanisms</td>
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<tr>
<td>Direct injuries of the hepatocytes</td>
</tr>
<tr>
<td>Cell and organelle membranes</td>
</tr>
<tr>
<td>Cell nucleus</td>
</tr>
<tr>
<td>Mitochondriae</td>
</tr>
<tr>
<td>Lysosomes</td>
</tr>
<tr>
<td>Indirect injuries of the hepatocytes</td>
</tr>
<tr>
<td>Glycogenic synthesis</td>
</tr>
<tr>
<td>Protein synthesis</td>
</tr>
<tr>
<td>Phospho-lipid synthesis</td>
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<tr>
<td>Glyco- and phospho-lipid synthesis</td>
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<tr>
<td>Cell water-electrolytic balance</td>
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<tr>
<td>Extrahepatalgic mechanisms</td>
</tr>
<tr>
<td>Pancreas</td>
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<tr>
<td>Kidneys</td>
</tr>
<tr>
<td>Main brain</td>
</tr>
<tr>
<td>Generalized life-supporting balances</td>
</tr>
</tbody>
</table>

The amanin group
This group includes the following representatives:
1. Alpha-amanitin – 100 g of fresh mushroom substance contain 8 mg of alpha amanitin, lethal dose – 0,10 mg/kg body weight;
2. Beta-amanitin - 100 g of fresh mushroom substance contain 5 mg of beta amanitin, lethal dose – 0,4 mg/kg body weight;
3. Gama-amanitin - 100 g of fresh mushroom substance contain 5 mg of gama amanitin, lethal dose – 0,25 mg/kg body weight.

The three groups of thermostable poisons have a hepatropic and a strong hepatotoxic effect. Under certain conditions other organs are also injured through the mechanisms pointed hereafter. Among them the amanin fractions, especially the alpha- and gama-amanin are among the strongest poisons in the world.
The mentioned poisons penetrate the organism mainly through the food in which they are contained. The special laboratory and industrial forms of the amanitin fractions penetrate also as toxic aerosols through the inhalatory system during terrorist or military acts in certain regions. A direct target of the toxic aggression in the organism are the liver cells; upon aerosol penetration, the different groups of cells in the main brain can also be affected.

The mentioned toxins affect the liver cells through two groups of mechanisms:

1. Direct mechanisms, including toxic injury of the:
   1.1. Cell and organelle membranes. By injuring the selective pervasibility of cell membranes for potassium and sodium ions, their metabolism is disturbed due to a decrease in a different degree of the potassium ion concentration in the intracellular space, at the expense of sodium ion concentration.
   1.2. Cell nucleus. Poisons suppress the migration of information and transport ribonucleic acids from the nucleus to the protoplasm. The former copulate with the aminoacid groups, the latter transport the newly formed complex in the microsomes, where different specialized proteins are synthesized. Consequently through such suppression of the migration, the amanitin poison provokes malfunction of the protein synthesis during the mentioned intermediary phase.
   1.3. Mitochondriae. Here the amanitin and its metabolite poisons inhibit the redox-enzyme systems and harm the oxidation processes in these organelles - life-significant for the hepatocyte functioning. The mentioned toxic substances also disturb the process of transformation of adenosine-monophosphates into adenosine-triphosphates in the mitochondriae. Adenosine-triphosphates are the main energy donors to the basic cytobiological processes. This kind of malfunction provokes a sharp decrease of their concentration in the hepatocyte, which results in serious biochemical intracellular concussions.
   1.4. Lysosomae. The amanitin poison strikes the single membranes of these organelles in the cells, meanwhile strongly aggressive enzymes penetrate the protoplasm, and destroy basic protoplasm compositions and by lymph and blood ways pass into other organs (kidneys and other), where they act in a similar way.

2. Indirect injuries of the hepatocytes – as a result of the above-listed direct amanitin effects, namely disturbances of the:
2.1. Glycogenic synthesis. The monosaccharides penetrating the hepatocytes, to synthesize glycogen, need energy from (ATPh) adenosine-tri-phosphate, whose production is strongly suppressed. An acute glycogenic need of the hepatocytes occurs with all the catastrophic consequences for the liver.

2.2. Protein synthesis. It is affected during its two phases, namely:
   2.2.1. During copulation of amino-acids with ribonucleic acids – due to direct suppression of the latter’s migration from the poison.
   2.2.2. During the synthesis of polypeptides in protein molecules in the microsomes due to insufficient energy by ATP, the produced of the latter is strongly damaged by the amantin in the mitochondriae. A strong hunger is felt by the organism for specialized proteins, which are mainly produced by the hepatocyte (hemostatic and other factors).

2.3. Phospholipid synthesis. The fat acids invading the hepatocytes cannot synthesize phospholipids for lack of the needed energy, provided by the diminished adenosine-triphosphates (ATPh). The organism feels a strong deficit for phospholipids, whose main supplier are hepatocytes. These turn into bubbles, full of inassimilated fat acids.

2.4. Glyco- and lipoprotein synthesis. This is due to the toxically suppressed ATP production, which supplies energy for this kind of synthesis in hepatocytes. This results in a strong violation of the cytoarchitectonics of hepatocytes, where lipoproteins and glycoproteins participate.

2.5. Intracellular water-electrolytic balance of hepatocytes. The latter are impoverished in sodium and potassium ions and retain an increased quantity of water with potassium ions.

As result of the above listed processes taking place in the liver of the poisoned, the carbohydrate, protein and fat metabolism is severely violated followed by diffuse fat degeneration, high water-electrolytic dysbalance and significant membrane destruction. Thus, for a short period of time, the natural exo- and endotoxic function of this organ is destroyed and a chemical hepatectomy occurs.

Extrahepatic injuries take effect in the pointed target organs, by means of the following mechanisms:

1. Pancreas. Toxins affect directly mainly cells A type in the Langerhans islands. Compensating this process, the activity of cells B type increases. As a result of this disturbance, a series of alternating hypoglycaemic
and hyperglycaemic conditions occur.

2. Kidneys. The cells in the tubular structures are damaged, less often the glomerules, both due to the amanitin poison direct influence – less often, or to the severe injury of the liver – more often. In some cases a pathological hepatorenal associate is formed.

3. Main brain. This organ can be directly damaged by some laboratory-industrial forms of amanitin poison in special extreme conditions and by the mushrooms amanitin molecules in early infant age. In any case upon developing a severe mushroom poisoning this organ is severely affected by the endotoxic substances, obtained after the catastrophic decay of the liver caused by the poisoning.

4. Main generalized life-supporting balances. The mentioned mushroom poisons and their laboratory-industrial analogues violate some basic life-significant balances in the organism by means of the following mechanisms:

4.1. Water-electrolytic dysbalance. It happens after the large degradation and loss of sodium, potassium and calcium ions and proteins caused by the direct damage of the stomach-intestine ligaments by the mushroom poisons. This dysbalance gives start to mechanisms of a metabolite type injuring several organs and systems – heart, kidneys, endocrine glands, etc. and to hypoproteinemia.

4.2. Hemostatic dysbalance. The mentioned poisons, by inhibition of the specialized protein fractions in the hepatocytes, participating in supporting the hemostatic balance, secondarily determine multiple hemorrhages in different parts and organs of the poisoned individual, some of which with lethal end.

4.3. Acute immune deficit. The amanitin fractions show damaging effects on immune units throughout the entire organism, by direct destructive and indirect dismetabolic mechanisms. As a result, acute immune deficiency appears with all the dramatic consequences for the patient.

The above mentioned mechanisms determine the amanitin poisons as one of the most toxic substances, consequently the amanitin poisoning – as one of the severest aggressions on human organism. These mechanisms express themselves in multiple directions simultaneously or in short time intervals – from a few hours to several days.
III. PHASE DYNAMICS OF THE CLINICAL PHENOMENA

The results of different authors’ studies in recent years gave grounds to establish a six-phase dynamics of the start and flow of the clinical events of the lethal amanitin poisoning by *falloides* and *lepiota* mushroom types. This dynamics is the next important aspect of this pathology, determining a new state-of-the-art strategy of diagnosis and treatment (3, 4).

**Phase ONE.** Includes the latent period, without clinical evidence. Continues in the average about 6-10 hours after the consummation of mushrooms of the *falloides* group and 12 and more hours after absorption of mushroom food of the poisonous *lepiota* type. After inhalation of terrorist amanitin poison this period is much shorter – on the average 1-4 hours. This phase includes the entero-hepatic and the pulmo-cerebral-hepatic circulation depending on the peroral or inhalatory absorption of the poison. During this phase the amanitin forms a complex with the plasma proteins and is biotransformed by enzyme groups in the hepatocyte whereby strongly toxic metabolites appear. Its industrial-laboratory analogues reveal associative interrelation with protein fractions in the plasma and enzyme groups in the brain neurons.

**Phase TWO.** The entero-hepatic and the pulmo-cerebral-hepatic circulation continues for - on the average - 2 to 7 days. It is expressed by a heavy gastrointestinal syndrome for the peroral absorption of the poison: vomiting, per-fuse diarrhea of a truly choleriform type, severe hemoconcentration, oliguria, shock condition. After inhalatory absorption of the amanitin poison, severe catarrhal changes occur in the lungs followed by lung inflammation and acute breathing insufficiency in different degrees. Cerebral disturbances are felt; dizziness, often inadequacy, sometimes bulbar paralysis (especially in young children and elderly patients).

**Phase THREE.** It is usually expressed during the second or third day. Very short and unstable. Evidences of the second phase continue. Biochemical laboratory data show that liver destruction begins: increased values of the enzyme groups: transaminase fractions (SGTP, SGOT, etc.)

**Phase FOUR.** Most often it begins after the third day of intoxication and continues about 10 – 14 days. The present gastrointestinal and pulmo-toxic changes decrease in the clinical picture and gradually disappear. A severe polyorgan deficiency is present. The liver processes are prevailing, due to the heavy fat degeneration and the cytolysis in the liver, namely: icter in a different degree, hepatome-galy turning into a severe liver insufficiency accompanied by hepatic pain and heavy brain damage, reaching hepatic coma. Multiple hemorrhages on mucoses,
skin and organs expressing severe hemorrhagic diathesis. Data is established for a severe kidney damage with oligo-anuria and increased values of nitrogen-containing substances and sodium in the blood. During this period the immune deficiency develops stormily accompanied by quick acute septic complications. Laboratory analysis also shows high values of the bilirubin in the serum, more on the direct fraction, hypoproteinemia, hyper-ammoniemia, low values of the prothrombin index and the V factor, data for metabolite acidose and heavy immunosuppressive constellation with decrease of cytologic and humoral immune indexes is found. In many of the cases during this phase high febrility appears due to the superposed stormy inflammatory processes. Inadequate and unsuccessful treatment might lead to lethal end during this phase.

**Phase FIVE.** It is expressed by data showing improvement of the liver damage. The immune deficiency and septic complications arisen during phase four are still significantly present during this phase. It includes slowing down symptoms of changing character of the liver lesion and the other organ deficiency. This phase largely varies in duration – 3 to 4 weeks on the average. The paraclinical, laboratory-clinical, immune bacterial and bacterial laboratory data persist in this phase with a changing character but gradually subsiding clinical expressions.

**Phase SIX.** It is observed in healed from the intoxication patients mainly from their overcome clinical and mainly laboratory data, but showing or expressing periodical symptoms of cytological, cholestatic, steatose character or immune type hepatopathy and immunopathy. This phase includes the established earlier by the author ondulating hepatopathy, which is probably mainly due to autoimmune mechanisms. This phase can continue for a period between several months to about 3-4 years. Sometimes it leaves permanent traces, determining in a different degree a post-intoxication invalidity.

The presented phase dynamics after A. Monov, of the clinical flow of falloid and lepiotic intoxication, called with due reason the amanitin “plague”, represents a strategic frame for successful implementation of a comprehensive state-of-the-art treatment program.

**IV. UNIFIED PHASE-INDICATED TREATMENT PROGRAM (AFTER A. MONOV)**

Due to its state-of-the-art contents, it represents the next actual issue to be reviewed regarding this lethal pathology. Following A. Monov’s concept (Table
2), elaborated after continuous research and based on modern scientific methods, this program can be formulated into the following basic stages:

**Table 2**

Unified phase-indicated treatment program (after A. Monov)

- Removing the toxins from the organism
- Overcoming the amanitine-hepatalic and the amanitine-cerebral conflict
- Membrane-protective and immuno-corrective procedures
- Hyperbar oxigenation
- Urgent liver and hepatocites transplantation

1. Removing the toxins from the organism. It is put into effect, as follows (3, 4):

   1.1. At the gastrointestinal level. The procedure is carried out during the first, second and sometimes the third phase by administering absorptive drugs (medical charcoal), followed by stomach-intestine purgatives with continuous laxative effect. Thus by absorbing the amanitin and throwing it away from the organism with the faeces, a significant quantity of the poison is removed, before it penetrates through the intestinal wall into the blood and completes the entero-hepatal circle.

   1.2. At the pulmonary level. Upon inhalation of industrial aerosol product provoked by terrorist or other aggressive act, the following fractionated inhalation is prescribed: Acetylcystein-aerosol – in generally applicable methods on the average, alternated with inhaling of oxygen mixture and spasmolytics and becotid – aerosol form. Thus the alveolar air, containing the poison is taken out and a direct local neutralizing effect of the poison is obtained.

   1.3. At the hematogenic level. This treatment is carried out throughout the second stage of the intoxication and consist in applying dialysis methods (carbo-hemo-perfusion, peritoneal dialysis, plasmaphoresis etc.), forced diuresis and blood transfusion. It is specialized by including penicillin preparation, suitable for venous application, after checking for supersensitivity. It is applied by special methods concurrently with the hemo-depurating procedures. The penicillin disassociates in the blood the amanitin poison from the amanitin-protein
plasma complex and contributes to a more effective removal of the poison from the blood by means of the described purgative methods. Concurrently to these procedures, glyco-corticoids and antihistamine preparations are included in the treatment taken parenterally for the antiallergic prophylaxis against penicillin, venously applied.

2. Avoid the amanitin-hepatocyte and amanitin-cerebral conflict. This conflict leads to severe destruction of the liver with heavy consequences for the organism, as described earlier. Upon inhalation of the amanitin product, such destruction can occur directly in the brain structures due to the special new properties developed for the industrial amanitin poison. The following procedures are applied:

2.1. Inclusion in the second phase of Silibinin and its derivatives Legalon, Silimarmin etc. in liquid forms. 20-30 mg/kg of body weight are applied on the average for 24 hours, distributed in 4 series infused by drops venously. The duration of the application is 2 hours and it is applied for 3-6 days. The preparation breaks the entero-hepatic circle and suppresses the amanitin penetration into the hepatocyte. There are reasons to believe that similar anti-amanitin effect is obtained also in the neuron after poison penetration in the organism by inhalation. It is advised to combine this antidote with Orocetam or Nootropil preparations. The combination increases the therapeutic effect, since the Orocetam and the Nootropil help overcome the hypoxia and the metabolic disturbances occurring in the hepatocytes and the brain cells.

3. Membraneprotective and immune-corrective procedures. They are applied during the second, third and the beginning of the fourth phase: The immuneprotective therapy continues also in the fifth and sixth phases if evidence is present. It includes admission of glyco-corticosteroids at adequate treatment doses, vitamins of the B, E, K, C groups at generally applicable doses, levulose at 10% dilution and other hepatic-immune-protective combinations, hemostasis protectors applied according to evidence and in adequate doses and hepatoprotective medicaments.

4. Hyperbaric oxygen therapy. It is applied most often with a baric chamber usually after the second phase and at lack of contraindication. Under the supervision of Prof. Monov and following his concept with the participation of his associates, a Antifalloid hyperbaric therapeutic complex was created combining the hyperbaric oxygenation with the other
mentioned methods. Its timely application leads to decreasing the lethal outcome of the acute amanitin poisoning from approximately 90% to 30%.

5. Urgent liver and hepatocytes transplantation. Investigations demonstrate that the big contingent of patients undergo the described specialized treatment quite late – during the third or the following phases of intoxication, when changes in the liver are irreversible and incompatible to life and the described traditional treatment procedures are poorly effective. In such cases modern surgery and toxicology apply under special conditions, urgent infusion of hepatocyte cultures or liver transplantation. It is done during the fourth phase of the clinical dynamics of poisoning. A lifesaving effect at this point of the treatment program has been established, observing specific evidence and criteria.

Based on own investigations on clinical cases as well as on literature data, and in correspondence with state-of-the-art research on this issue, the author is presenting hereafter, in correspondence to his own doctrine on this pathology, a system of criteria and solutions for such intervention.

5.1. Clinical group. It includes acute deficit for liver functions, overcome shock condition and recovery of the main life balances; not occurred yet acute polyorganic deficiency and “cerebral death”.

5.2. Paraclinical technical group. It includes electro-encephalographic measurements showing data for I to II degree encephalopathy; electrocardiogram, excluding heavy myocardial damages.

5.3. Clinical-laboratory group. It includes all available indications showing severe hepatic lesion and insufficiency, protrombin time – approximately 10-12% in the hemostatic constellation factor V – on the average 10-8%.

6. Other criteria – they are composed according to individual and other clinical data up to the moment of the intervention discussion.

The presented phase-indicated treatment program should be carried out on the background of adequate reanimation and substitute therapy.

The unified doctrine for amanitin intoxication that has been presented here, has been applied except for liver transplantation by the author and his pupils with very good results and beneficial effects against one of the most lethal group and single toxic pathologies.
V. HEPATIC AND IMMUNE PROTECTIVE COMPLEX AGAINST AMANITIN-RELATED BIOLOGICAL POISONING (AFTER AL. MONOV)

1. Antiamanitin hepatic protection
   1.1. Silibenin 15-20 mg/kg round-the-clock administered at 6-hour equal doses.
   1.2. Levulose 10% solution 2 x 500 ml i.v. drops for 24 hours.
   1.3. Antihypoxic and antidysmetabolic combinations: Pyramem, Centrophenoxin and Vitamin B₆ in cocktails, other hepatoprotective medicaments
   1.4. Baric chamber in case that concentrations are not present.

2. Immune protection
   2.1. Gammaglobulin i.m.; Gamma Venin i.v.
   2.2. Immunocytological activators: Azimexon, Levamisol, Thiabendazol, Bestatin, Tuftisin, Deodan, etc.

The complex is administered in the beginning of the treatment and continues at a composition and doses according to the indices for hepatic and immune lesions.

MAIN REFERENCES

Clinical Aspects of the Main Critical States in Human Organism in Biological Traumatism and Terrorism

Alexander Monov

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Biological traumatism is essentially an urgent pathology and biological terrorism purposefully provokes quick acute damages to human organism, directly endangering the life. In current medicine, they are classified as critical states with a bad prognosis. This is especially valid for damages incurred by biological terrorist means. Due to the specific nature of these agents, the resulting critical states require special diagnostical and treatment methods, as well as specific organizational clinical approaches. The many years of investigations on critical states in man in general and their etiology, pathogenesis and clinical forms and the accumulated experience on their treatment, allows the author to present in this book specific concepts for their treatment in biological terrorist cases. More specifically, these issues will be dealt with in relation to the following critical states (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13):
- Acute respiratory insufficiency;
- Acute cardiovascular insufficiency (shock states, etc.);
- Acute cerebral insufficiency (comatose, convulsion);
- Acute immune deficiency;

The listed critical states will be discussed in their respective chapters.

I. ACUTE RESPIRATORY INSUFFICIENCY

These critical states can be provoked by most of the existing biological agents of mass traumatism and terrorist acts. The mechanisms provoking malfunction of the respiratory processes can be classified in four different groups, depending on the biological aggressor type (Table 1): paralysis of the respiratory center situated in the medullary brain, affection of the respiratory system, damage of blood hemoglobin and enzyme structures, providing for the cell oxidation processes (in the mitochondria, etc.) (2, 4, 5).

1. Paralysis of the respiratory center

Paralysis can occur after absorption of bigger doses of neurotropic biologi-
cal poisons and microorganisms, which have been specially produced to be strongly aggressive. From among the phytotoxins, such agents could be the different groups of amanitins, the neurotoxic ricin protein, etc. From the microbe toxins, such agents are botulin- and diphtheria-toxins, etc. and a number of strongly pathogenic bacteria and viruses.

Partial depression or complete paralysis of the respiratory center can express the damage. Clinically it is manifested by disturbance of the rhythm of respiratory excursions, decrease of their volume – occurrence of superficial breathing or complete apnoea.

The toxic affection of the respiratory center determines the malfunction of the respiratory neural regulation and system.

### Table 1

<table>
<thead>
<tr>
<th>Toxic disorders of the respiratory system</th>
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<tbody>
<tr>
<td>Affection of the respiratory center</td>
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### 2. Affection of the respiratory system

The most often met with damages are oedema of the larynx, pulmonary oedema with inflammatory process, pulmonary oedema, asthmatic bronchitis, respiratory disorders in children.

**Oedema of the larynx**

Clinically it is manifested by abrupt or gradual occurrence of asphyxia together with severe psychomotor reaction of the patient, resulting in quickly degrading condition into cyanoses and convulsions, hoarse voice or aphonia.

**Pulmonary oedema and inflammatory processes**

This condition is most often provoked by specific viruses and bacteria pur-
posefully mutated after laboratory intervention for highly increased pathogenicity. Such agents can arise spontaneously in the human organism provoked by unfavourable regional environmental conditions (TORS would eventually be an example) or can be created for terrorist purposes. Clinically this damage is manifested, beside evidences, laboratory and roentgen data for pulmonary inflammation (it can be from bronchopneumonic areas, interstitial pneumonia or acute diffuse bronchitis or bronchiolitis), also by data for bilateral generalized pulmonary oedema, combined sometimes with brochospasm and atelectasic areas.

**Pulmonary oedema**

It is observed in cases of

a) Acute poisonings by phytopoisons with cholinomimetic effects. They are characterized by abundant secretion from the bronchia and alveolus’ mucus membranes; as a result of their increased permeability, hyperaemia and lavish transudation develop;

b) Some severe poisonings with neurotropic poisons and comatose states – the cortex regulation of the pulmonary blood circulation is disordered followed by metabolite changes with oedema, for example severe barbiturate coma, alcohol coma, other exotic coma etc.;

c) Severe generalized bacterial and viral infections or inflammatory pulmonary focus, preceded by mucus damage and secondary layered infection;

d) Acute cardiac insufficiency, exotoxic shock (see below).

In the mentioned conditions, pulmonary oedema can appear immediately after the noxa effect or after a definite latent period. Clinically it is manifested by dyspnea and orthopnea, coughing with watery expectoration, sometimes mixed with blood and combined with snoring breathing. When the patient is unconscious, the breathing is noisy with abundant bronchia secretion. In the cases of severe pulmonary oedema, cyanoses of the face and the extremities quickly occur. The physical examination finds slight tympanic pulmonary tone, bilaterally weakened respiratory mobility, vesicular breathing with added numerous small and medium moist bilateral crepitations. The pulmonary roentgenography shows characteristic spot-like shadows in both pulmonary areas and increased hilus shadows. Clinically negative forms also exist, where the physical examination does not establish the changes characteristic of the pulmonary oedema process, and the roentgenography shows only the significantly increased hilus areas.

**Asthmatic bronchitis**

It occurs after inhalation of phytotoxic, microbial and zoo-toxins. Acute
asthmatic bronchitis with destruction or oedema of the mucous membrane and spasm of the bronchioles appears. Clinically it is manifested by a strong feeling of dyspnea, expiratory dyspnea, noisy whistling breathing. The physical examination establishes acute vesicular breathing with diffuse whistling crepitations bilaterally. In the most severe cases, cyanosis on the lips, the face and the toes of the extremities appears. Similar clinical manifestations can be also observed in some cases of bacterial or viral pulmonary affections.

The described direct respiratory system damages lead to the restriction of air inflow in the pulmonary alveolar area, resulting in acute disorders of the alveolar ventilation and the gas metabolism.

Some toxic damages of the heart and the blood vessels, especially the toxic myocarditis, toxic infarction and exotoxic shock provoke malfunction of the respiratory system by restricting and decreasing the pulmonary blood supply. A direct effect of this disorder is the break down of gas metabolism in the alveolus.

The three basic mechanisms affecting the respiratory system in cases of exogenous biological intoxication described so far finally result in respiratory insufficiency. Our investigations show that in most of the intoxicated patients, it is manifested in a combined form – as cardiac-pulmonary insufficiency.

**Respiratory disorders in children**

In childhood, the toxic respiratory disorder is mainly due to two processes: affection of the respiratory centre or direct damage to the respiratory system. The exotoxic shock is seldom the cause for respiratory disorders. These peculiarities are determined by the type of the poison, with which intoxication occurs in childhood, namely with neurotropic poisons and less so with cardiotropic and inhalation noxae.

The respiratory centre damage is most often from early childhood poisonings due to the vulnerability of the child’s central nervous system to smaller doses of neurotropic poisons, and especially due to the still functional lability of this centre. In most cases it is manifested by isolated disorders in the respiratory system neural regulation, expressed by irregular and superficial breathing or with bradypneic periods. Even in comparatively mild cases, apnoea, convulsions and clinical death occur very quickly.

From among the direct damages of the respiratory system, the most often observed, especially in early childhood is oedema of the larynx; on the second place is pulmonary oedema. The latter is determined, more often than in grown ups, by intoxication with unconscious states. Asthmatic toxic bronchitis in chil-
dren is very rare because of the small frequency of inhalation intoxication at this age.

Acute respiratory insufficiency in children sets in very fast and is characterized by very severely expressed hypoxia and metabolite acidosis. This is due to the greater lability of enzyme systems in the cells, providing for the oxidation processes in children.

3. Blood damage

In this case, acute respiratory insufficiency appears after severe damage of the erythrocytes and the haemoglobin due to the following etiological factor: hemolysis, carboxihemoglobinaemia, metahemoglobinaemia.

In terrorist acts the biological aggressor often provokes acute hemolyses with extreme anaemic syndrome (phytotoxic substances, highly aggressive hemotropic microorganisms). The oxygen transportation from the lungs to the cells is quickly blocked and severe generalized hypoxia of the organism occurs.

4. Damage of the intracellular enzyme oxidation system

In cases of biological terrorist aggression, this process is observed very often. Mainly the enzymes of the mitochondria respiratory chain are affected. Basic cell metabolite processes, connected to the intracellular oxidation are broken down.

The mentioned disease states of the human organism in the conditions of mass traumatism and terrorism quickly result in acute respiratory insufficiency through direct affection of the whole human respiratory system by different units of its sophisticated chain.

Acute respiratory insufficiency can result also from some indirect mechanisms in cases of severe blood circulation disorders and malfunction of the oxygen supply to tissues and cells due to acute severe cardiovascular insufficiency.

During the described shock states the acute vascular insufficiency develops in two phases: compensated or initial and decompensated phase.

The compensated phase of acute vascular insufficiency is determined by a 25% loss of the circulating blood volume. It is characterized by adaptive variable vasoconstriction and vasodilatation of the different blood circulation sectors, by variable volemia and by gradual restriction of the vasodilatation mainly in the capillaries blood circulation, by draw back of blood flow and increased tissue transudation. Clinically it is manifested by general condition disorders – excitement, depression, faintness, cold extremities, starting cyanosis. The arterial systolic pressure is slightly decreased – about 13,3 kPa (100 mm Hg); evidence is
present for moderate tachycardia and mildish pulse. The central venal pressure is normal or with moderate deviations from the norm.

The decompensated phase of the acute vascular insufficiency is characterized by atonia of the blood bed, with considerable hypovolemia in the big blood circulation circle, with strong stagnation and delay of the blood flow in the micro-circulation area (in the capillaries net), with severely deteriorating tissue perfusion.

5. Treatment procedures for acute respiratory insufficiency

The therapy of acute respiratory insufficiency resulting from biological traumatism and terrorism damages should correspond to the noxa type, the respiration affecting mechanism and the clinical forms of its manifestations. The treatment is carried out in the following directions, in accordance with the main principles of our unified doctrine:

- Respiratory reanimation. It is always the first step and includes medication and methods adequate to the type and degree of respiratory affection. In bulbar and pulmonary forms of respiratory damage, oxygen mixture is urgently introduced in the organism by adequate techniques (intratracheal intubation, assisted breathing, etc.). In cases when hematogen and enzyme mechanisms are applied for the respiratory reanimation, the oxygen mixture can be introduced in fractions, for the very severe cases – with barocamera if not contraindicated.

- Stimulation of the respiratory centre in cases of bulbar paralysis, combined with cerebroprotective medication. Stimulators of the respiratory centre are Coramin, ampulla 2 ml are injected in the muscle or subcutaneously; Micoren, ampoule 1,5 ml depending on the pharmaceutical type are introduced muscually or venally, and others.

- Pulmoprotective therapy. Larynx oedema treatment includes glucocorticoids, ampoule (for example Urbasan, etc.) applied venally in adequate doses, adrenaline ampoule 0,5 mg injected subcutaneously etc. Pulmonary oedema is treated with glucocorticoids, introduced in higher doses by fractions at short intervals, calcium preparations – venally in big doses, inhalation of oxygen under pressure, a diuretic preparation only once and other medications according to indications. Bronchospasm and astmatic bronchitis – in addition to the above indicated preparations, antiallergic and purine preparations and expectorating combinations are introduced.
II. ACUTE CARDIO-VASCULAR INSUFFICIENCY

This critical state of the affected by biological traumas and terrorist acts human organism is provoked by a number of phytotoxins, zootoxins, strong pathogenic bacteria, viruses and their cultures.

The mentioned aggressors act on the organism mainly by four damaging mechanisms affecting directly: the vasomotor centres in the medullary, the heart, the vessels and severe dehydration and hemorrhage resulting in serious hypovolemia. The mentioned mechanisms have single manifestation or in combination – this depends on the type of the biological aggressor and its damaging power. Biological traumatism and terrorism provoke critical states in blood circulation and cardiovascular system functions that are expressed by the following most frequent forms of diseases: acute myocarditis and acute myocardosis, toxic infarction of the myocardium, shock states such as cardiogenic, hypovolemic, vasomotor, allergic shocks (Table 2). The mentioned clinical forms result in the following critical states: acute cardiovascular insufficiency, acute cardiopulmonary insufficiency, acute vascular deficiency (4, 5).

1. Acute myocarditis

It is provoked by strongly virulent microorganisms, in the first place mutan virus groups with increased pathogenicity. It is manifested as part of the whole inflammatory process clinical picture of the organism. Its clinical picture is presented by rhythmus and frequency disturbance and acute cardiac insufficiency.

2. Acute myocardose

It is caused by poison intoxication of underlined cardiotropic effect. Such properties are characteristic for digitoxins and the other cardiac glucosides, veratrin, etc. The toxic effect of the noxa is manifested directly on the myocardium in the following several directions:

   a) Affection of the potassium-sodium myofibrilla metabolism by damaging the permeability of the myofibril membrane, the potassium ions are
imprisoned in the extracellular area and the potassium concentration in
the myocardial cells abruptly falls;
b) Abrupt disorder of the oxidation processes in the myofibrillaes mito-
chondria on the account of the anaerobic phase, at which milk acid is
accumulated in the intracellular area;
c) Glycogen synthesis disorder and decrease of its quantity in myofibrillaes;
d) Spasm of the coronary arteries;
e) Disorders in the transmission system processes;
   The clinically acute toxic myocardose and acute myocarditis are mani-
fested by changes in the QRS-complex and the ST-segments or with the
ECG-image of bundle branch block and extrasystoles and also by dif-
ferent rhythm disorders depending on the type of noxa and the absorbed
quantity.

3. Toxic myocardial infarction

   It occurs after poison intoxication, provoking acute ischaemia of a specific
area of the myocardium by means of coronary spasm and myofibril enzyme com-
plexes disorders (phytoergotamine preparations etc.). Changes in the patient state,
infarctus-like are established clinically and by electrocardiogram. According to
some authors during the acute phase, the Erythrocyte Sedimentation Rate (ESR)
does not show substantial changes and the biochemical constellation in the blood
resembles the acute ischaemia of a myocardic area (Table 2).

4. Acute pulmo-cardiac syndrome

   It is observed in severe biological poisoning cases, which run with severe
pulmonary oedema or bronchial spasm or with acute left-ventricle cardiac insuf-
siciency. Due to the complications that arise in the small blood circulation circle,
some patients also manifest severe right-side cardiac insufficiency with deterio-
rating oedema. This syndrome is most often observed in patients that were previ-
ously suffering from damaged heart, that has been compensation before the bio-
logically aggressive incident.

5. Acute cardiac insufficiency

   In cases of acute myocarditis and toxic myocardoses, irrespective of the
organic heart damage type, if the myocardium has been affected severely, than
acute cardiac insufficiency may occur. It is due to the fact that the heart cannot
catch up with the blood needs of the organism. In biological terrorist incidents,
this insufficiency may happen as an isolated event and a lethal outcome may
quickly come (Table 2).

Clinically it is manifested by left-sided insufficiency phenomena – dyspnea, pulmonary oedema or degradation, if it was previously existing. Cases with prolonged decompensation result in acute cardiac insufficiency of the cardiogenic type.

The blood vessels damage is expressed by their functional state disorders – acute cardiac insufficiency and restricted damage of the vessel wall.

6. Acute vascular insufficiency

The different types of acute vascular insufficiency in severe biological aggression flow in two main phases: compensated or initial and decompensated (Table 2).

The compensated phase of acute vascular insufficiency is determined by a 25% loss of the circulating blood volume. It is characterized by adaptive variable vasoconstriction and vasodilatation of the different blood circulation sectors, by variable volemia and by gradual restriction of the vasodilatation mainly in the
capillaries blood circulation, by draw back of blood flow and increased tissue transudation. Clinically it is manifested by general condition disorders – excitement, depression, faintness, cold extremities, starting cyanosis. The arterial systolic pressure is slightly decreased – about 13,3 kPa (100 mm Hg); evidence is present for moderate tachycardia and mildish pulse. The central venal pressure is normal or with moderate deviations from the norm.

The decompensated phase of the acute vascular insufficiency is characterized by atonia of the blood bed, with considerable hypovolemia in the big blood circulation circle, with strong stagnation and delay of the blood flow in the microcirculation area (in the capillaries net), with severely deteriorating tissue perfusion. During this phase, the organs are affected due to:

a) Intracellular hypoxia, caused by decreased oxygen supply – the intracellular oxidation turns from aerobic to anaerobic form.

b) Disorders in tissue metabolism. Due to hypoxia, the intermediary product of anaerobe glucolysis – pyruvate, cannot be transformed in the next phases and is reduced to milk acid, which accumulates in the tissues. Further occur suppression of protein synthesis, loss of plasma protein and proteolysis through activation of the plasmin enzyme system, resulting in hypoproteinemia and lack of albumin, fibrinogen, prothrombin and gamma-globulin. Hypoxia upsets the adenosine triphosphatase in the mitochondria and the absorption and the metabolism of fat acids in the parenchymal organs cells, resulting in fat degeneration of the latter. The affection of water-electrolyte and acid-alkaline balance in the complex of metabolite disorders is represented by dehydration and low electrolyte values, mainly of sodium, accumulation of milk acid and pyruvate and decrease of standard bicarbonates that provoke occurrence of severe tissue metabolite acidosis.

c) Thromboembolic processes in the organ small vessels due to the delayed blood flow and increased blood coagulation capacity and hemoconcentration.

The mentioned disorder types occurred during the shock decompensation phase determine severe dystrophic damages to vital organs such as cerebrum, heart, liver, kidneys, etc. Clinically this phase of the shock is manifested by a marginally severe general condition, cold cyanotic extremities, pale, halogen face, collapsed veins (except for the cardiogenic shock), low systolic arterial pressure – 10,7-0 kPa (80-0mm Hg), very mild filiform pulse, strong tachycardia (sometimes absent). In most of the patients, severe hypovolemia with low central venal
pressure is observed; sometimes the pressure is normal or increased (in occurring cardiac weakness). In most patients, the hematocrit shows normal or increased values. Diuresis is feeble or missing.

The acute vascular insufficiency by biological aggression is manifested by different kinds of shocks (Table 3).

**Cardiogenic shock**

It is caused by biological toxic noxes, directly affecting the cardiac muscle. It reveals to be a complication of the acute cardiac insufficiency and its mechanism represents a decreased strike and minute volume of the heart. It determines the hypoxia of tissues and vital organs as well as other important cell metabolite disorders and vascular wall low vitality. Usually this type of shock runs with normo- or hypovolemia with high central venous pressure. Hypervolemia can be observed in very rare cases, in very advanced phases of damaged whole function of blood circulation. Clinically it is manifested, beside the cardiac decompensation events, by fall of the arterial pressure under 13,3 kPa (100 mm Hg), with cyanosis of the extremities, cold sticky sweat on the face and the body, etc. In biological terrorist incidents, this kind of disease turns very quickly into asystolia due to the high aggressiveness of the damaging factor.

**Vasomotor shock**

It occurs at neurotropic poisons intoxication, that cause depressions or paralysis of the vasomotor centres in the medullary. Direct mechanisms are the low vitality of arterial wall and a short paresis of the big blood circulation circle vessels with following compensatory venulae spasm and decreased strike volume.

**Table 3**

<table>
<thead>
<tr>
<th>Acute vascular insufficiency in biological traumatism and terrorism</th>
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<tbody>
<tr>
<td>Cardiogenic shock</td>
</tr>
<tr>
<td>Vasomotor shock</td>
</tr>
<tr>
<td>Allergic shock</td>
</tr>
<tr>
<td>Hypovolemic shock</td>
</tr>
<tr>
<td>Hypoxia</td>
</tr>
<tr>
<td>Metabolite disorders</td>
</tr>
<tr>
<td>Thromboembolic disorders</td>
</tr>
</tbody>
</table>

Dystrophic and degenerative changes in cerebrium, heart, liver, kidneys and other organs and tissues
Very soon, if the disorder is not overcome, the compensatory vasoconstriction phase gradually sets in, followed by hypovolemia and shock decompensation.

*Hypovolemic shock*

It happens mainly in cases of acute biological poisonings, accompanied by significant losses of liquids and salts, provoked by persistent vomiting and diarrhoea, by voluminous secretion of the lungs (in children age), by massive hemorrhages (hemorrhagic shock – very seldom), by massive transudation of liquids from vessels to tissues, etc. The direct damaging mechanism is the loss liquids by the intravascular area and the appearance of hypovolemia with acute disorders in vital organs’ blood supply. Clinically the shock is manifested by lowering of the arterial pressure – under 13,3 kPa (100 mm Hg), low or fluctuating CVP (Central venous pressure), cold extremities, cyanotic face and extremities, cold sticky sweat on the face, general indisposition, etc.

*Allergic shock*

In this type of shock, in relation to the abruptly appeared liberation of significant quantities of mediators, mainly histamine, under the influence of allergen – antiallergenic reaction, we observe generalized vasodilatation and increased permeability of the capillaries with transudation of liquid in the tissues; longer persistence of the process leads to hypovolemia and irreversible paresis of vessels.

In the mentioned acute vascular insufficiency forms, the main disorder in the hemodynamics consists of a drop in the volume of the circulating blood mass, stasis and liquid transudation from the capillaries to the tissues, severe paresis of the whole bed and deep disorder of tissue perfusion. The described blood circulation abnormalities arise with the direct participation both of the central nervous system and of endocrine units, caused by the direct effect of the biological noxe and the respective aggressive mechanism (damage of the myocardium, loss of liquids or affection of the vasomotor centres, allergic reaction). In acute poisonings and biological aggression provoking allergic reaction in the affected human organism, the different phases of the basic hemodynamics disorder manifest themselves more slowly with some specificities as compared to changes in hemorrhagic and traumatic shocks. They are determined by the differences and peculiarities of the etiopathogenetic factors.

7. *Treatment directions for severe cardiac and vascular insufficiency*

The mentioned above critical states require therapeutic activity conformed to the existing leading damages of the cardiovascular system, which can be systematized as follows:
- **Cardiovascular reanimation.** It is carried out with cardiotonic and cardioprotective medications: most often Strofanthin is prescribed, administered venally in dosage of 1/8 to 1/4 mg with glucose solution by the established methods if not contraindicated, or digitalis preparations parenterally – upon indications. Cardiac glycosides can be applied in acute toxic myocardose cases, unless the toxic noxe has cardiotropic cardiotoxic effect. In presence of such contraindications, other preparations with cardiotonic effect, such as caffeine preparations etc., are taken parenterally. In rhythm disorders adequate anti-rhythm medications are applied parenterally. Most patients with tachyarhythmia or tachycardia are beneficially affected by lidocaine preparations, applied parenterally after the established methods. In bradycardia or bradyarhythmia patients a good immediate effect is obtained by a combination of atropine with caffeine preparations, applied parenterally if not contraindicated. The effect is very quick. Very often the cardiovascular reanimation is combined with respiratory reanimation, if the breathing is disturbed, by means of fractionated input of oxygen with adequate equipment. Severe left-ventricle insufficiency and pulmonary oedema are positively affected by a onetime application of diuretic preparation is applied parenterally in order to relieve the heart. Bronchia spasms are treated with broncholytics (purine group and other drugs).

- **Antishock therapy.** Medications and methods are applied that correspond to the type of the shock of the cardiovascular insufficiency mechanism. Cardiogenic shock is treated by fully adequate cardiotonic and/or anti-arrhythmic preparations according specific indications (see above). For left-ventricle insufficiency and dramatically appeared cardiopulmonary insufficiency cases, the treatment includes also adequate forms of respiratory reanimation and glucocorticoid preparations are introduced parenterally to fight the developing pulmonary oedema. In very severe pulmonary oedema manifestations, a onetime application parenterally of a diuretic is advised. In patients with previous hypertonic disease combined with abruptly appeared cardiogenic shock, special attention should be paid to arterial pressure, which should be brought to moderate values by adequate medication. Infusion venal therapy is regulated according to the activity of hemodynamic indications including hourly diuresis dynamics and extrarenal liquid secretion. Vasomotor shock is treated by stimulating the bulbar vasomotor centres by means of venal
drop infusion of water-electrolyte solutions and plasma substitutes with hourly diuresis and hemodynamic indications control. The hypovolemic shock appearing after acute dehydration or other mechanisms is treated by adequate rehydration by means of venal drop infusion of water-electrolyte solutions and plasma substitutes. Allergic shock is treated by antiallergic and glucocorticoid preparations, used parenterally in adequate doses.

- **Vasoprotective therapy.** When the vascular wall is directly damaged by the toxic or virus noxe, the treatment includes ascorbic acid (vitamin C) and rutin preparations parenterally. For thromboembolic disorders useful would be different kinds of anticoagulant and antiaggregate preparations after indications.

- **Specific therapy against the main aggressive agent.** Three main groups of treatment preparations are applied, depending on the most frequent aggressive substances: antidotes – for biotoxic incidents, antibacterial and antiviral preparations (antibiotics, etc.) – for microbacterial aggressors and immune protective and stimulating medications – if decided by the physician.

- **Symptomatic and organoprotective drugs** – according to indications in the process of therapy.

The above described treatment process should be in accordance with A. Monov’s unified doctrine for biological traumatism and terrorism (see the respective chapter).

III. ACUTE CEREBRAL INSUFFICIENCY

1. **Unconscious states**

A unified doctrine created by Prof. Al. Monov has been adapted for victims of biological terrorist acts. This doctrine has proved to be effective in a number of applications (4, 5, 7).

1.1. **Etiology and pathogenesis of unconscious states**

Disorders of consciousness are observed in a number of diseases of various character, exogenous poisonings, different mechanic and physical traumas and impacts on the cerebrum. According to the unified doctrine these are considered to be etiological factors, creating conditions for disorders of consciousness. Depending on the way they affect the brain, they can be defined as:
1. **Factors affecting directly the Central Nervous System (CNS).** Here belong the different neurotropic biochemical, bacterial and other agents.

2. **Factors affecting indirectly the CNS.** They first provoke disease changes in the organism outside the cerebrum, and then these changes damage the brain and result in unconscious states. Different metabolic disorders, liver and kidney, circulation and breathing malfunctioning might be significant in this respect. These disorders result in a progressive worsening of the summary balance of the organism, especially the water-electrolyte, alkaline-acid, hemostatic balances, blood circulation, breathing etc.

The pathogenetic mechanisms for consciousness disorders, though conditionally, may be classified as outer-cerebral, disrupting the physiological conditions in which operate the neuron and some cerebral cells, related to processes upsetting the metabolism and the function of namely the cerebral cells. What is important here is that the specific neuron microstructures (organelles) are affected directly or indirectly by the terrorist aggression agents, and manifest deviations in their specific functions.

**Cell membranes.** Protein phospholipid complexes in the cell membrane structures are damaged. The enzymes, which by supplying energy (adenosine triphosphatase etc.), assist the electrolyte, water and other chemical substances transportation through the membrane, are suppressed. As a result of the changed penetrability of the membrane, the neuron water-electrolyte balance mainly of the potassium ion is disrupted. Conditions are created for penetration of a number of damaging for the cell processes noxa, for retention of more water in the intracellular media, resulting in intracellular watering.

**Mitochondria.** Damaging agents such as exogenous, endogenous and bacterial toxins, penetrate the double lipoprotein membranes and violate the oxidizing phosphorylase and the formation of ATPh (adenosine triphosphate), as well as the oxygenating processes consequence of upsetting the activity of the

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Table 4

<table>
<thead>
<tr>
<th>General etiology and pathogenesis</th>
<th>Unified classification</th>
<th>Unified diagnostic system</th>
<th>Unified treatment program</th>
</tr>
</thead>
</table>

**Unified doctrine for unconscious states due to biological terrorism (after Al. Monov)**

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redox-enzyme system and the Crebs cycle. As a result, primary intracellular hypoxia occur and basic metabolite processes of the neuron are disordered due to insufficient energy for their operation.

Neuron nucleus. Due to the effect of the noxa on the nucleus and the penetrability malfunction of the double membrane, the passage of information and transport ribonucleic acids in the cytoplasm and the assistance of the plasmatic processes in the neuron are limited.

Neuroplasma. The contents of the different enzyme types, which catalyze the glycogenolysis and other metabolite processes is upset. Deviations occur in the neuron cytophysiology, mainly due to decreased energy input by the monosaccharides destruction and to the damaged cytomorphology of the different lipid complexes.

Nissl corpuscles. They build the tigroid structure of the neuron. During consciousness state disorders, the damaging agents upset the participation of the Nissl corpuscles in the exciting and withholding processes of the neuron cell.

Lysosomes. Upon severe aggression of the etiological agent on the neuron, the lysozyme monomembrane structure is broken and the neuroplasm is penetrated by cytolysing enzyme groups, which severely damages, often irreversibly, the neuron cell and preconditions heavy consciousness breakdown.

Neurofibrils and their inter-neuron synapses. The etiological agents that provoke consciousness derangement harm the protein spherical molecules situated on the axis of the neuro-filaments or damage the chemical mediation of the neural excitement in the interneural synapses, accomplished by acetylcholine and catecholamines, but mainly noradrenaline.

The mentioned cerebral cell affections, respectively their structural components, are preconditioned both by the direct effects of the noxa and by some other organism malfunctions: breathing upset – for asthmatic states and other severe break down of pulmonary ventilation; blood circulation disorders – for shock states, heart diseases with acute coronary deficiency, especially for very low erythrocyte number and severe anaemia.

All these disorders can be provoked by substances, activated for mass toxic and biological traumatism and terrorist purposes.

The described neuron disorders, as well as of other parts of the organism, caused by different disease agents and resulting in consciousness breakdown, precondition the occurrence in the brain of biochemical processes that directly provoke unconsciousness – hypo- or anoxia in the neuron, cerebral oedema and sup-
pression of the excitement process in the neuron (Table 5). Irrespective of the type of the existing disease or the acting noxa, three types of phenomena representing a complex biochemical unconsciousness substrate should be present for the occurrence of the unconscious state.

### Table 5

Pathogenetic mechanisms of unconsciousness in terrorist disorders

- Brain hypoxia
- Brain oedema
- Suppression of the excitement processes in the neuron

### Table 6

Factors for cerebral hypoxia

- Malfunction of oxidation in the mitochondria
- Limited transfer of monosaccharides towards the neurone
- Limited transportation of oxygen towards the cerebral cells

Cerebral hypoxia or anoxia appear after disorder of the biological oxidation in the neuron. This disorder is mainly due to a sharp breakdown of the oxidation reactions in the mitochondria, especially the redox-enzyme system, resulting in acute malfunction of energy production for the specific cytophysiological operation of the neuron. The disturbance of the oxidation reactions in these cells, can be provoked by direct damage to the enzyme systems, providing for the intracellular breathing, by impeding the oxygen supply in the neuron and by limiting the contribution of the monosaccharides – the main substance subject to oxidation in the neuron cell (Table 6). The first mechanism lies is the basis of most exotoxic diseases, leading to consciousness disorders. This type of hypoxia represents primary intracellular hypoxia. The second mechanism leads in an indirect way to hypoxia of the neuron, by limiting oxygen and monosaccharides supply to the cerebrum. It is observed in a number of different diseases of the organism with
unfavorable flow, to which also belong virus or bacterial diseases provoked by terrorist acts. This type of hypoxia is indirect or secondary hypoxia of the cerebral cells.

Table 7

<table>
<thead>
<tr>
<th>Exotoxic processes</th>
<th>Endotoxic processes</th>
<th>Bacterial toxins</th>
<th>Severe cardiovascular deficiency</th>
<th>Severe respiratory deficiency</th>
<th>Physical and mechanical factors</th>
<th>Neuro-psychic diseases</th>
<th>Cerebral insult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular cerebral oedema</td>
<td>Intercellular cerebral oedema</td>
<td></td>
<td></td>
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</tbody>
</table>

The next process in the cerebrum that preconditions consciousness disorders is cerebral oedema (Table 7). It represents a direct reaction of the cerebral tissue against hypoxia. Cerebral oedema can be intracellular or intercellular. For some specific diseases (inflammatory processes, blood circulation troubles, effect of different physical agents on the brain, etc.) it can be primary and in a mechanical way determine the appearance of hypoxia.

Hypoxia and cerebral oedema participate in all cases of comatose state of different types of diseases. Even when the organism is saturated with the necessary quantity of oxygen, proven for cerebral blood circulation, hypoxia can still occur even in the conditions, when the cortex structures that execute the intracellular oxidation are disturbed. Such break down can be provoked by a number of substances disseminated by terrorist acts. Cerebral hypoxia and cerebral oedema form a vicious cycle where the operation of one process worsens the other.

The next process that predetermines consciousness disorders is the suppression of the excitation in the neurons and their transfer through the interneuron synapses (Table 8). It is a consequence of the previous two processes. They impede the excitation process in its three phases: spring up, transfer and restoration of the neural cell rest.
The spring up of excitation in the neurone requires a big amount of oxygen and chemical energy. The etiological factors determining consciousness disorders, suppress the emergence of excitation in the following two directions: referring to oxygen and its absorption – by disruption of the supply mechanisms and the main neurone enzyme systems supporting the oxidation process; referring to energy – by suppressing the hexokinases, the aldolasis, the phosphatase and other enzymes, provoking glucose and glycogen glycolysis. A significant role here plays the suppression of the acetyl dehydrogenase, the adenosine triphosphatase etc., which participate in the metabolism and disintegration of phosphatides. At the excitation stage, due to disorders in the cell membrane permeability, its functions change too, which results in a change of the neurone cell status and the electrolyte content.

The transfer of the neural excitation and its penetration through the interneural synapses in consciousness disorders is damaged due to malfunction of its chemical mediation. Acetylcholine participates in the transfer of cholinergic impulses, noradrenaline – of adrenergic impulses. According to D. Nachmansohn’s theory upon emergence of neural excitation around the neurone, the axone and the

**Table 8**

Factors disturbing the cerebral excitement processes (after A. Monov)

- Initiation of excitation suppression in the cell
- Suppression of excitation transfer by various biological factors
- Suppression of cell processes restoration by various toxic and other substances

<table>
<thead>
<tr>
<th>Malfunction of oxygen supply in the cells</th>
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</thead>
<tbody>
<tr>
<td>Malfunction of monosaccharides supply in the cells</td>
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<tr>
<td>Damage of oxidation enzymes and glycogenolysis in the cells</td>
</tr>
<tr>
<td>Disorders in cell membrane permeability</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disorders in synapses chemical mediation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disorders in the repolarization of the neuron</td>
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<tr>
<td>Disorders in synapses metabolite dynamics</td>
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<tr>
<td>Deepening of intracellular hypoxia</td>
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</tbody>
</table>

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synapses and under the effect of choline acetase and trans acetase, acetylcholine is synthesized from choline and acetate in the presence of the coenzymes A and ATP (adenosine triphosphate). Acetylcholine depolarizes the neurone surface in the synapses area, which is charged negatively against the surrounding parts of the neural filament that remain unexcited. At the next moment, the same process occurs in a nearby area of the axone, thus carrying out transmission of the impulse. Under the effect of the acetyl cholinesterase, hydrolysis and disintegration of the acetylcholine into choline and acetate take place.

The cell membrane is repolarized, the permeability changes again and a number of potassium ions invade back, while sodium ions exit the cell. The Nissl corpuscles, which have disappeared during the excitation phase, reappear in the neural plasma. The etiological factors and the pathogenetic mechanisms that lead to consciousness disorders, provoke disruption of excitation spring up and transmission, by suppressing it in the already mentioned phases of its operation and the related biochemical processes. For example a number of chemical and biochemical exogenous metabolite toxic substances upset the acetyl choline and other mediators’ synthesis, deviate the excitation process along the axone and its transmission to the intercellular synapses. Others block the cholinesterase and provoke severe damage to acetylcholine metabolism by accumulating the latter in the cerebral tissue. In the most severe cases of manifestation of this process, the correct operation of neurone and synapses excitation is blocked.

A number of disease processes provoke consciousness disorders by direct physical effect damage on the mentioned structures in the cerebral cells – mechanical exterior trauma, heat, radiation and other external impact. They break the entity of the neurone, thus provoking oxidation and other biochemical processes in the cell, leading in their turn to hypoxia, cerebral oedema, decreased energy production and malfunction of the emergence and operation of the excitation process.

As a result of the activity or interaction of the described damaging processes occurring in the cerebral cells at their integration with the cerebral cortex and other of its structures, a diffuse hold back of the cortex functions is manifested. It directly causes consciousness disorder under the effect of all types of reasons.

1.2. Classification of unconscious states

The great variety of etiological and clinical characteristics of the unconscious states makes them rather difficult to study and diagnose. Especially complicated is the coma type in critical situations, arisen after mass biological trau-
Etiopathogenetic classification of comatose states (after Al. Monov)

<table>
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<tr>
<th>General type of cerebral disorder</th>
<th>Etiological factors of coma</th>
<th>Type of coma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Coma provoked by endogenous intoxication and metabolite disorders</td>
<td>Diabetes ketoacetosis, hypoglychemic, uremic, liver, thyreotoxic, adrenal, mixedemic, hypopituitary, hyperparathyroid, hypochloremic, hypocaliemic, hypersmollar, lactation etc.</td>
<td></td>
</tr>
<tr>
<td>2. Coma, provoked by exogenous intoxication</td>
<td>Barbiturate, morphine, atropine, alcohol, gasoline, carbon acid, aniline etc.</td>
<td></td>
</tr>
<tr>
<td>3. Coma, provoked by general blood circulation and respiratory disorders and blood damage</td>
<td>Cardiac infarctions, cardiac block, hypoxic, anaemic coma etc.</td>
<td></td>
</tr>
<tr>
<td>4. Coma, provoked by physical agents</td>
<td>Traumatic, thermal, sun, hyperthermal, electrocution etc.</td>
<td></td>
</tr>
<tr>
<td>5. Coma, provoked by infectious and inflammatory processes</td>
<td>Encephalitic, meningitis, malaria, typhus or other comas provoked by infectious diseases</td>
<td></td>
</tr>
<tr>
<td>6. Consciousness disorders, provoked by other neural and psychic diseases</td>
<td>Epileptic, hysteric coma etc.</td>
<td></td>
</tr>
</tbody>
</table>

A. Coma from diffuse damage of the cerebrum

B. Coma mainly from local damage of the cerebrum

Coma by *Insultus cerebri*
The etiopathogenetic classification is based on the general type of the cerebral syndrome, dominating in the clinical picture, and on the etiological factors provoking the different groups of consciousness disorders. Its main positive aspects are:

1. It embraces all the types of coma existing in the clinical medicine in a specific system and underlines some definite interactions among them.
2. The grouping of comatose states is carried out after easily accessible anamnesis data (if such exist) and clinical facts, collected upon patient examination by the physician, whereby conclusions on the essence (and etiology) of the disease are made.

Following this classification, on the basis of the main cerebral syndrome and characteristic manifestations of other systems, established after a thorough examination, the coma type could be determined. In case there are no anamnesis data for the direct etiology of the comatose state, orientation for the coma type can be made based on the cerebral type damage and on differential-diagnostic discussion, contributed by the existing symptoms and quick laboratory tests. Thus could be defined the type of direct or indirect terrorist damage on the organism.

From the above classification can be concluded that the most frequent direct comatose states are of the types: A 2 and 4, while the indirect damage types are: A 1, 3, 6 and B 1 and 2.

1.3. Complex diagnostic system for unconscious states

One of the main activities of the strategy to overcome the consciousness disorders of the patient is a quick and correct diagnostic orientation. In most cases the main reason for mistakes and inadequate treatment of the various coma types is a delayed or incorrect diagnosis. Our investigations show that the diagnostic decision for unconscious state patients can be made quickly and correctly in critical and in biological terrorism situations, on the basis of a complex diagnostic system, contained in the unified doctrine. It includes anamnestic and clinical criteria that can be neurological and somatic in character, as well as laboratory para-clinical information. But there are two main issues that have to be answered: the degree of the unconscious state and the type of the coma.

Anamnestic criteria group (if possible such information can be obtained from patient accompanying persons or relatives). The data from this criteria group important for the diagnosis are the circumstances under which and the fastness with which consciousness disorder has occurred: previous diseases if any and their type; behavior and health status of the victim before the incident; profession and everyday occupations; time and season of incident manifestation; is the pa-
tient a single case or is he part of a group of people affected by a specific disease simultaneously or at short intervals of time.

Clinical data group, received by the basic objective examination methods. Obligatory are the examination, palpation, percussion and auscultation. It includes cerebral neurogenic and somatic syndromes.

The main syndrome of the unconscious states is the cerebral neurogenic. There are four main degrees of quantitative consciousness disorders, established after the following criteria. Obnibilacico – dull, obscured consciousness; somnolencio – unnatural somnolence; soper – unconscious state of which the patient can be briefly drawn out by means of specific irritants; and coma – full loss of consciousness, of which there are no means to draw the patient out.

Very often the milder forms of consciousness disorders with different speed turn into coma. In cases of improvement, the patient gradually comes out of the coma, going through the described degrees of consciousness disorders. The author accepts the following three degrees of comatose states, as best described and studied: superficial coma – complete unconsciousness with suppressed or morbidly changed reflexes and determinable pupil reflexes to light, corneal, swallowing, coughing, tendon and above-bone reflexes; in some specific diseases, pathological reflexes (Babinsky etc.) and muscle hypotonia occur; deep coma – complete unconsciousness with areflexia, severely low muscle tonus or extremely high – in cerebral rigidity; frequent uncontrolled urination and bowel movement; preserved but worsened spontaneous breathing, blood circulation usually with evidence for hypotonia; terminal coma (Coma depasse) – deep coma, complete body areflexia, dilated pupils, hypothermia. This coma type is preconditioned by a strongly increased intracranial pressure, approaching to the arterial, resulting in a severe restriction or termination of the intracranial blood circulation followed by an ischemic infarction of the cerebrum. In addition to the described changes of the consciousness, a number of pathological reflexes, such as neck muscles rigidity, Babinsky, Oppenheim, Gordon symptoms, eye symptoms, convulsions, paretic and paralytic manifestation are included in this group. Following the symptoms and syndromes of this group one can always determine the degree of the cerebral affection and the unconscious state, sometimes even the main disease that has provoked the coma (cerebral insult, inflammatory processes in the cerebrum, etc.).

The next subgroup symptoms and syndromes, mainly of somatic character, are specific for the main disease that has provoked the coma. They are discovered after systematic and thorough examination of the patient. Anamnestic data for sugar diabetes, presence of Kusmual breathing, dry skin and acetone smell around
the patient point to diabetes coma. Anamnestic data for previous renal disease, pale, swallow face, miosis, deep breathing point to renal coma; in the presence of exophtalm, tahicardia, and struma, the attention is drawn to thyrotoxic coma. In establishing an icter, sometimes hepatomegalia one thinks of hepatic coma. Perspiration, tahicardia, low blood sugar values would make us refer to hypoglychemic coma. Observing empty boxes of sleeping pills around the patient, one assumes that it is a barbiturate or similar exotoxic coma provoked by cerebro- and psychic or sedative medication and other agents.

If the patient was found in environment saturated with gasoline or near to a carbon oxide source and evidence of red face and the upper part of the body, the conclusion is for carbon oxide coma. Alcohol evaporations around the patient and snoring point to alcohol coma. If injuries on the head are observed the correct think is to make assessments for a traumatic coma. In high temperatures with or without rigidity of the neck muscles the physicians ought to accept as priority, unless a convincing diagnostic alternative exists, that it is inflammatory process-related coma. Upon establishing strong paleness of or yellowish face of the patient, presence of hepatomegalia and low hemoglobin and erythrocytes values, the physician should think of coma due to severe form of anemia. In hemiplague cases or other local symptoms, high arterial pressure and sudden fall into unconsciousness, the physician should look for insult coma resulting from cerebral hemorrhage. Cases of slow sinking into unconsciousness in adult patients and partial paresis or hemiplague would be evidence for coma resulting from thrombosis of a cerebral artery. A sudden suppression of consciousness, hemiparesis, or hemiplague and valvular disease with arrhythmia would speak for embolism of the cerebral artery.

The above-presented indications offer the possibility for a quick diagnosis of the combined unconscious states and other terrorist inflicted damages. Many of the aggressors of mass biological traumatism and terrorist acts could provoke some of the coma types described here.

Laboratory para-clinical information within the unified diagnosis system of unconscious states included data on the following main indications, which can be quickly obtained by easily-accessible methods: full blood picture; urine – the main indicators are: proteins, sugar, acetone particles, bile pigments, sediment, relative weight; blood for sugar, urea, creatinine, xanthoproteins, bilirubin, transaminase, proteinogram, ionogram, etc.; roentgenography of the head in front and in profile; eye-bottom test; electrocardiogram, etc. When there is not enough evidence for the diagnosis, additional more complicated tests are carried: lumbal
puncture with analysis of the cerebrospinal liquid; chemical blood and urine analysis (at suspicion for poisoning); electroencephalogram, computer tomography (if possible), etc.

In the light of the above mentioned in this chapter, the following model for differentiated diagnosis of unconscious states, according to the unified doctrine of the author, will be presented:

1. From the anamnesis:

1.1. The speed of occurrence of unconsciousness state. Coma occurs quickly in cases of specially prepared cerebrotropic biological substances for terrorist purposes, of cerebral hemorrhage, cerebral embolism, severe cranial-cerebral trauma, epilepsy, Morgani-Adams-Stock outbursts; less quickly it happens after endogenous and exogenous intoxication, infectious and inflammatory diseases, cerebral thrombosis and in some cerebral tumor processes.

1.2. Age. Advanced age speaks in favor of cerebral insult (hemorrhage or thrombosis); young and middle age – for endogenous and exogenous poisonings, cerebral embolism, infectious and inflammatory processes. In extraordinary, extreme situations, age is of no relevance.

1.3. Environment of the incident. Bad working conditions, defecting heating equipment and gas installations, empty packages thrown around the patient suggest exogenous poisonings. When the patient has been found on the street or in his bed, it points to disorder of the general or cerebral blood circulation. For long strong sun exposure – to insolatio, for contact with electrical circuit – to electrocution, etc.

1.4. Established states, preceding the unconscious state. For diabetes, Addison’s or Basedow’s diseases, we think of coma corresponding to the endogenous intoxication group. For infectious diseases – of coma of inflammatory or infectious origin. For high blood pressure, valvular defect, advanced arteriosclerosis – to cerebral insult, for epilepsy – to epileptic coma, etc.

2. From objective examination

The great variety of up-to-date factors which could provoke biological aggression create the possibilities for the emergence of the enumerated changes.

2.1. Smell around the patient: of acetone – points to diabetes coma; of ammoniac – to uremic coma; of raw liver or meat – to liver coma; of alcohol – to alcohol coma.

2.2. Appearance of the patient’s face or skin: pale swollen face speaks
for uremic coma; pale face with cold perspiration – for cardiac infarction and severe blood loss; pale face with abundant perspiration – for hypoglycemic conditions; red face – for apoplectic coma and carbon oxide poisoning; cyanosis on the lips and the extremities – for severe exogenous poisonings (morphine, nitro- and aminobenzenes, etc.); herpes around the mouth or nose – for infectious-inflammatory processes (meningoencephalitis, bronchopneumonia, etc.); icteric skin pigmentation – for hepatalgic and malaria coma; early decubitus – for malaria and barbiturate coma.

2.3. Respiration: deep slowed breathing is found in diabetes coma; stertorous breathing – in cerebral hemorrhages, uremic coma; breathing of the Chains-Stocks type – in heavy exogenous poisonings (by narcotics, alcohol, new terroristic biological poisons, etc.) and in cerebral hemorrhage with rupture in the ventricles; quick breathing – in infectious-inflammatory pathology; very slowed breathing – in morphine coma.

2.4. Pulse: strained pulse accompanies uremic coma; strained slow pulse – cerebral hemorrhage with high blood pressure; mild fast pulse – diabetes coma, some exogenous poisonings, infectious-inflammatory processes; fast irregular pulse – thyreotoxic crisis, general disorder of blood circulation (heart infarction and mitral stenosis); very slow pulse – heart block, big cerebral tumor with hemorrhage, myxedema.

2.5. Blood pressure: high blood pressure is measured in uremic and eclamptic coma, in some exogenous poisonings (by lead complexes), in cerebral hemorrhage; low blood pressure – in some endogenous intoxication (adrenal, hypochlorinaemic coma, cardiovascular type of diabetes coma), in some exogenous poisonings (by barbiturates, narcotic preparations), in some general blood circulation disorders (cardiac infarction).

2.6. Temperature: high temperature – in infectious-inflammatory processes (viral infections, pneumonia, typhus, tropic malaria, etc.), in cerebral hemorrhage with rupture in the ventricles; subfebrile temperature – in renal and thyreotoxic coma, in some poisonings (by biological toxins, three-cyclic antidepressants, carbon oxide, etc.).

2.7. Neurological evidence:

2.7.1. Paralyses: Hemiparesis, hemiplague and face asymmetry – in cerebral insult coma; obvious paresis or paralysis – in meningoen-
cephalitis.


2.7.3. Convulsions – in hypoglychemic coma, eclampsia, meningoencephalitis, thermoplegia, insolatio, cerebral hemorrhage (subarachnoid, with rupture in the ventricles), exogenous acute poisonings (by biological phyto- and zootoxins, three cyclic antidepressants, by piperidine phenotiasine drugs – torecan, etc.)


2.8. Changes in the eyes:

2.8.1. The look turns aside – in eclampsia, in epilepsy, in some cerebral hemorrhage; narrow pupils – in uremic and morphine coma; wide pupils – in hypoglychemic coma, in hepatal coma by terrorist poisonings with cholinolytic substances, three cyclic antidepressants, carbon oxide and other poisons; anisocoria – in local cerebral damage; low eyeball tonus – in insult and hypoglychemic coma.

3. From clinical-laboratory and paraclinical tests


3.2. Blood: leucocytosis with polynucleosis – in uremic and diabetes coma, in infectious and inflammatory processes, disturbed the consciousness (pneumonia and bronchopneumonia, meningoencephalitis, sepsis, food toxic infections, etc.); leukopenia with lymphocytosis – in virus meningoencephalitis; anemia – in uremic coma after chronic nephritis, in blood diseases, after severe blood loss; increase of nitrogen containing substances (urea, residual nitrogen, creatinine, etc.) – in uremic coma, in severe cerebral hemorrhage, in acute neurotoxic-effect poisonings (halogenated hydrocarbon, ethyleneglycol etc.), sometimes in hepatic coma, cerebral insult, cardiac infarction; hypoglychemia – in hypoglychemic and adrenal comas; bilirubinaemia – in hepatic coma, exogenous hepato- and hemotoxic noxa poiso-
nings caused by biological terrorism, after hemolysis, etc.

3.3. Cerebrospinal liquid: bloody – in cerebral hemorrhage (subarachnoidal or with ventricle ruptures); xantochrome – in meningoencephalitis and tuberculoses meningitis; with protein cell dissociation – in cerebral tumor.

3.4. Pulmonary roentgenography: inflammatory infiltration of the superior area of the lungs, especially at beginning of unconsciousness – in pneumatic coma; multiple bilateral lung infiltrations – coma by viral infection and bronchopneumonia; milliar tuberculosis – in tuberculosis meningitis.

3.5. Electrocardiogram: evidence for necrotic areas of the myocardium – in myocardial infarction; evidence for severe atrioventricular block – in MAC attacks; data for severe depolarization changes with polytypic chamber extrasystoles – in severe poisonings with biological poisons.

3.6. Electroencephalogram: substitution of the normal alpha-wave activity by high voltage slow wave activity – in initial quantity consciousness disorders; gradual transformation of the cerebral bioelectrical activity into low-amplitude one – in engrossing of the unconscious state; measurements without daily or other cyclic changes – in deep unconscious state with bad prognosis [7]; high voltage polyarrhythmia, interrupted by bioelectrical silence of different length periods – in barbiturate coma, monomorphous sinusoidal 4-7 sec., 30-100 mkw amplitude delta waves – in alcohol coma etc.

3.7. Tomograph computer test of the cerebrum: changed areas are established that suggest tumor, hemorrhage or oedema process.

The data assessment, following the described model, should be carried by juxtaposing and comparing anamnestic, clinical, clinical-laboratory and paraclinical results. Using only one type of data – paraclinical or laboratory would very often lead to incorrect conclusions of hyperdiagnosis.

1.4. Complex treatment program for unconscious states

The similar processes and phenomena that accompany the etiopathogenesis and the clinical picture of different types of unconscious states make it possible to build an unified treatment strategy – a program for unconscious states treatment (Table 7). This program includes several basic groups of treatment procedures and methods, namely:

– Reanimation methods
Cerebroprotective drug combinations
Treatment methods, oriented towards the main disease
Diets
Organization procedures

Reanimation methods

They cover procedures for respiratory, cardiovascular and substitution-corrective reanimation. They are applied at the beginning of the treatment in all types of coma and continue till the patient has been drawn out of the unconscious state. They create the main therapeutic background in the patient’s organism, which prevents the quick lethal outcome, prolongs the life and allows to apply all necessary treatment means and to ensure their effect.

Respiratory reanimation. It helps to prevent the hypoxia, which is the basic cortex and other cerebral structure affection which provokes unconsciousness. In deep and terminal coma (Coma depasse) it is applied by means of intratracheal intubation and assisted breathing; if not available, and in the event of a critical state, “mouth to mouth” breathing is done. It is contraindicated if evidence exists for toxic gas poisoning, infection diseases and other contraindications.

In cases of partial or complete respiratory damage, the patient is subjected to controlled respiration. For the milder cases of consciousness disorders, such as obnubilacio, somnolencio and sopor, respiratory reanimation is applied already at the beginning of the treatment by means of oxygen mixture, introduced in the respiratory system by a nasal catheter or an oxygen apparatus mask. The main indicators for the introduction of respiratory reanimation are disorders in the respiratory system functions, changes in the acid-alkaline state indications which are controlled on the average for 6 out of the 24 hours.

Cardiovascular reanimation. It aims to translate cardiovascular functions into physiological parameters.

Upon decrease in different degrees of arterial pressure – venal drop infusion is switched on containing water-electrolyte and aminoacid dilutions, in doses depending on arterial pressure and diuresis values. In shock states and low arterial pressure values – under 13.3 kPa (100 mmHg), the infusion volume is determined according to the changes of the mentioned hemodynamics indications. Upon indications, vasopressor preparations, such as Effortil, Noradrenalin, beta-stimulators – Isoprenalin, Alupent, etc. are given. In severe shock cases to the laid infusion background, are added anti-shock mixtures, containing beta metason, prednisolon preparations (Urbason) etc. – on the average 120-180 mg, diluted in about 500 ml physiological or Ringer solution. Upon data for heart damage,
Strophanthin or Isolanid are added to the treatment, provided there are no contraindications (poisonings by digitalose drugs, bradiarrhythmic and block symptoms, etc.).

In high arterial pressure cases, resulting directly or as complication from damage caused by the terrorist tool, infusion procedures are not applied or are limited to the liquid volumes that have been released by the organism through urine, vomiting, diarrhea, perspiration, etc.

Besides, hypotensive and spasmolytic combinations, such as Papaverinum hydrochloricum – average dose 0.04-0.06g or Novphyllin – 0.24 g per dose, introduced slowly venally or 0.48 g per dose, intramuscularly, 2 – 3 times in 24 hours till reducing the hypertonia or obtaining endurable values.

**Substitutive-corrective reanimation.** It helps restore the loss of liquids and electrolytes, as well as proteins, and overcome the main balances’ disorder determining the homeostasis: acid-alkaline, water-electrolyte, hemostatic, protein, etc. Water-electrolytic monosaccharides (physiological serum, Ringer solution, glucose serum, etc.) in different concentrations, amino-acid and alkaline solutions, plasma and plasma substitutes, blood are infused in drops venally. Their volume is determined depending on the general state, the hemodynamic parameters and the hematologic indications. Special preparations – hemostatics (different protective cocktails) are also included.

In every type of coma irrespective of its origin the treatment begins by reanimation methods. They determine the therapy strategic direction, since they counteract to the main mechanisms that threaten the patient with a lethal out-

<table>
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<th>Complex treatment program</th>
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<td>Reanimation: respiratory, cardio-vascular, corrective-substitutional</td>
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<td>Cerebroprotective procedures</td>
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<tr>
<td>Treatment methods of the main disease</td>
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<tr>
<td>Diets</td>
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<td>Organization events</td>
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</table>

**Table 9**
come. During the reanimation treatment of comatose states, the present complex treatment program requires that the following hemodynamic indications be traced rhythmically in 24 hours – arterial pressure, central venal pressure, hematocrits, diuresis – every 2-4 hours, acid-alkaline indications in the blood – every 4-6 hours, ionogram – every 12 hours, proteinogram and blood picture – every 12-24 hours. The volume and the type of treatment procedures vary depending on fluctuation of indications.

**Cerebroprotective treatment combination**

They are aimed at overcoming the changes in the cerebrum, which have directly provoked the comatose state. From this point of view, they are applied already at beginning of treatment of every type of comatose state, irrespective of its origin.

**Against hypoxia and metabolic disorders in cerebral cells.** The following basic drug combinations are included here:

a) 20% glucose solution with 10-20 E insulin; for sugar diabetes patients – 100-500 ml levulose solution venally according to diuresis and blood pressure indications – as an energy source for the cerebral cells, if indications and the organism allow it – the cocktail is repeated;

b) Vitamins of the B group (B₁, B₂, B₆) in ampules are administered at generally accepted doses, cocarboxylase (Cothiamin) – average dose 4 times each of 200 mg per day intramuscularly. The mentioned vitamins are precursors of some enzyme systems in the neural cells, which are related also to the oxidation processes and activate their suppressed function;

c) Nootropic preparations – Pyramem ampules of 1.0 g. (Bulgarian Nootropil). Average dosage is 1-3 ampules every 6 hours daily or every 6 hours antihypoxic cocktail (after Al. Monov) – Pyramem 1-3 ampules, Centrophenoxin – 250 mg, vitamin B₆ ampules 50-100 mg, mixed with glucose serum 250 ml applied in drops venally every 4-6 hours, or Nootropil ampules in the period of the coma state and if not contraindicated.

**Against cerebral oedema.** They include dehydrating preparations: Furanthril, Mannitol and their combinations – parenteral application (in preserved renal function), glucocorticoids (Prednisolon’s preparations) – Urbason average dose 3 x 60 mg, Betametason (Celeston) – ampules water solution, 1 ampule 2 – 3 times daily, calcium gluconate ampulla 10 ml and vitamin C ampulla 5 ml, applied together intravenously 3 times daily; “intravasal drainage”, including hypertonic
preparations – Tutofuzin S40 2 x 250 ml, Osmofundin etc., drops venally, human albumin 2 x 250 ml or double treated plasma 250-500 ml, drops venally and 40% levulose hypertonic solution 250 ml, drops venally. These infusion preparations are combined with diuretic drugs, which are applied parenterally twice daily. In cases of severe cerebral oedema and disordered renal function, dialysis methods are applied.

On the background of affected consciousness, convulsion attacks or their equivalents are treated with diazepam or other benzodiazepine preparations or barbiturates in moderate therapeutic doses, combined with B₆ vitamin. It is advisable to include the anticonvulsive preparations at the stage of the intratracheal intubation and respiratory reanimation.

Specific treatment methods, oriented towards the main disease, origin of the consciousness disorder

These are applied at the beginning of treatment, when the disease is known. At this stage of the treatment program, therapeutic preparations are included, such as: insulin – against sugar diabetes, antidotes and other anti-poisons – against severe exogenous poisonings, other hormones – against deficiency of endocrine glands, antibiotics – in inflammatory bacterial processes, antiviral preparations – in general viral aggression, cardiac tonic drugs – in heart diseases and cardiac decompensation, dialysis – in renal deficiency, surgical intervention – in traumatic damage, immunoprotective preparations – in terrorist viral and bacterial aggression.

In lack of data pointing to the original disease, unconscious state treatment is carried out till specification of the diagnosis with the other complex treatment program methods of the present doctrine.

Dietary treatment

It is applied at the initial stage of the treatment parenterally with 20% glucose and levulose solutions in drops venally. In the next 24 hours it is combined with aminoacid and vitamin combination solutions to preserve the nutritive balance. It is necessary at the initial stage to supply the organism with an adequate quantity of hydrocarbon, proteins and calories – 10 – 460 kJ (on the average 2500 calories). In cases of prolonged coma, nutrition is reset to a combined type – parenteral and enteral with a permanently fixed stomach-tube (if not contraindicated).
2. **Convulsion states**

Convulsion states are of the most critical events affecting human organism and endanger the life in their most severe and abrupt manifestations. They represent an important group of acute cerebral insufficiency disorders provoked by mass biological traumas and terrorist incidents. The unified doctrine of A. Monov treating these damages, gives a classification for determining the type of damage and offers adapted clinical criteria for quick orientation and medical behavior in urgent situations. It is presented below (11):

2.1. *Classification and diagnostics of convulsion states*

A. Cerebral types

- Primary (idiopathic) cerebral type convulsions: genuine epilepsy of different types and forms, congenital dystrophic and metabolite cerebral disorders with convulsion manifestations, etc.
- Secondary cerebral type convulsions: caused by traumatic, toxic, vascular, inflammatory, tumour processes in the cerebrum.

B. Spinal-cerebral type convulsions: in traumatic, toxic, inflammatory, tumour and other cerebral processes.

C. Somatic type convulsions: in damaged and affected respiratory, cardiovascular, digestive systems, endocrine, bone-marrow structures, stomach-intestine tract, liver, kidney, etc.

D. Psychogenic type convulsions: in hysterical forms, stress conditions, other psychic disorders and diseases.

Convulsion states of the listed above groups can be different in character: generalized, local, tetanic-clonic, tonic-clonic, prolonged convulsion status depending on the etiology of the convulsion state and the localization of the inducing convulsions cause, bringing the changes in the brain.

The classification established by A. Monov opens to the practitioners possibilities for quick orientation on the type of the examined convulsion state and localization of the cause. In most cases this orientation is formed after precisely taken comprehensive anamnesis and thorough clinical examination of the patient.

Biological traumas and terrorist incidents can cause all types of convulsions included in the classification. The condition of patients of group 1.1 – primary cerebral type, in remission, can quickly deteriorate in the mentioned new biological pathology. In the last group – acute convulsion syndrome in the clinical picture – we can include neurogenic damages by a number of phyto- and zootoxins and cerebral and spinal disorders provoked by viral and bacterial neurotropic agents with strong pathogenic activity. Depending on their biochemical nature
these agents, through different mechanisms, cause three main types of disorders in cerebral cells: hypoxia, dysmetabolism, affected neuromediation. These changes determine the occurrence of convulsions and motivate the treatment process.

2.2. Treatment of convulsion states

Intense treatment and reanimation of convulsion states have the following aim:

- Overcome the acute brain changes that have directly caused the convulsion fit (Table 10).
- Overcome the causative agent or the morbid changes in the organism that provoked brain alterations leading to convulsions.

### Table 10

<table>
<thead>
<tr>
<th>Treatment and reanimation of convulsion states in biological aggression</th>
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<tbody>
<tr>
<td>Overcome convulsion fits and their cause</td>
</tr>
</tbody>
</table>

The medications and methods grouped as given below are used to achieve this aim:

**A. Immediate overcome of convulsion attacks and the cause that provoked severe brain changes.**

The following treatment is applied (Table 11):

1. Treatment by antiepileptic sedative effect drugs: antiepileptides, barbiturates (Phenobarbital, Luminal – ampoules, phenitine (Dyphenine etc.), primidone (Primidon etc.), arboxamide (Tegretol etc.), neuroleptics (phenotiazine preparations – in ampoule form, tranquillizers (benzodiazepine preparations etc.)

### Table 11

<table>
<thead>
<tr>
<th>Overcome convulsion fits and their cause</th>
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<tbody>
<tr>
<td>Sedative medications</td>
</tr>
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</table>
The listed drugs are applied one time or at fixed intervals and in forms, depending on the convulsion type and its duration. For convulsion states and unsatisfactory effect of these medications, a short narcosis is required (if not contraindicated) under the direct control of the vital indications.

2. Antihypoxic medicine preparations: pyramem (ampoules) 1 g, aminalon, etc. They are applied fractionated, parenterally, together with the sedative preparations to overcome the hypoxia of cerebral cells.

3. Enzymeprotective medicine preparations: B_1 and B_6 vitamins, enzyme preparations – cocarboxylase (ampoules) 50 mg etc. They activate the affected enzyme groups from the redox-system in the mitochondria. They are prescribed in combination with subgroups 1 and 2.

4. Drugs correcting the intracellular metabolism: monosaccharides (20% glucose and levulose solution etc.); centrophenoxin – in adequate dose for the attack (average values – 150-250 mg parenterally), combined with medication with sedative effect.

5. Dehydrating preparations to overcome the cerebral oedema: 10-20% manitol solution - 250 ml venally in drops; furantril – ampoules 20 mg injected in the muscle or venally, etc. if necessary the two medicines are combined; intravascular dehydrating drainage; high molecular solutions introduced venally in drops (twice enriched plasma 250-500 ml venally in drops, 10% glycerol solution, combined with 5% glucose solution in doses of 0.8 – 1.2 g per kg weight for 24 hours introduced slowly – approximately 100 ml for 20 min) etc.; dehydration therapy applied under control of hemodynamic indications, combined with diuretic preparations and upon indications – venal infusion of water electrolyte, alkalinizing and glucose solutions.

B. Overcoming acute disorders in the organism that have provoked convulsions (Table 12).

<table>
<thead>
<tr>
<th>Table 12</th>
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</thead>
<tbody>
<tr>
<td>Overcome acute disorders of main vital processes</td>
</tr>
<tr>
<td>Respiratory reanimation and oxygenation</td>
</tr>
<tr>
<td>Antishock treatment</td>
</tr>
<tr>
<td>Restauration of water-electrolite balance</td>
</tr>
<tr>
<td>Restauration of alkaline acid balance</td>
</tr>
</tbody>
</table>
In this aim the following treatment is advised:

1. Oxygen therapy and respiratory reanimation. It helps remove respiratory disorders, which have brought changes in the cerebrum, followed by a convulsion state. This procedure is carried out by different methods depending on the degree of respiratory damage (oxygen mixture inhalation by means of nasal catheter or masque; intratracheal intubation to perform assisted or command breathing with respiratory equipment, etc. A barocamera is included after admission of sedative medication upon special indications (severe blood affection by hemotoxic substances etc.); respiratory reanimation is favorably influenced by combinations of nonspecific antihypoxic preparations, administered in fractions during the 24 hours (piramem, centrofenoxin, $B_6$ vitamin in ampoules or in cocktail forms; piramem – ampoules 1 g, centrofenoxin 250 mg, vitamin $B_6$ 50 mg, glucose serum 250 mg, mixed ex tempore and introduced venally in drops; diazepam and piramem in ampoule forms or in cocktail, etc.

a) Hemotransfusion or partial blood infusion – to increase the erythrocytes and haemoglobin quantity in blood circulation in processes having caused heavy blood loss or red blood cells and haemoglobin damage.

b) Enzyme activating and unblocking medication to ensure cell oxidation in CNS which has been blocked by aggressive toxic agents; antidote preparations for different types of intoxication.

2. Overcome severe blood circulation disorders. The following medication is applied in this aim:

a) Antishock preparations: glucocorticoid drugs in adequate to shock state doses (urbason, beta-metason ampoules) applied parenterally; vasopressor preparations – catecholamines, effortil etc., administered in extremely low arterial pressure; water-electrolytic, monosaccharide, aminoacid and plasma-substitute solutions are infused venally in drops in quantities corresponding to the hemodynamic indications to overcome the hypovolemia.

b) Hypotensive preparations: reserpine, chlofasolin, novphyllin, etc. administered in ampoule forms venally or in the muscle in hypertonic attacks.

c) Antiarrhythmic medical treatment: lidocain, procainamid, propranol etc. administered parenterally in ampoule forms controlled by moni-
toring or electrocardiograph checking.

Cardiotonic preparations: cardiac glucosides – isolanid, digitalin in ampoule forms, strophantin (ampoules), administered in adequate doses in cardiac insufficiency.

In abrupt cardiac activity interruption – extrathoracic massage of the heart is done, combined with mouth-to-mouth equipment-controlled breathing. In camera fibrillation - defibrillation is applied or lidocain of 100 to 400 mg is introduced venally according to the generally accepted methods.

3. Severe water-electrolyte disbalance. For its quick overcoming the following medication is applied, specified for its different forms:
   a) For isotonic dehydration: venal infusion in drops of physiological serum, Ringer solution and other water-electrolyte solutions, aminoacid preparations in quantities adequate to the liquid balance of the organism and to the hemodynamic indicators.
   b) For hypertonic dehydration – 5-10-20% glucose solutions are infused venally in drops to overcome the increased sodium concentration in the blood.
   c) For hypotonic dehydration: water-salt solutions with sodium chloride in different percentages are infused to normalize its level in the plasma. The volume corresponds to the ionogram and hemodynamic indications. For hyperkaliaemia: 20% solutions of glucose twice 500 ml each time with 20 UI insulin in every input are introduced venally in drops, or 20-40% levulose solution, twice 500 ml each time introduced venally in drops; for unsuccessful results dialysis is run.

4. Disorders in the acid-alkaline balance. To restore it, venal infusion is applied as follows:
   a) For the worst forms of acidoses (metabolite acidoses) alkaline solutions are administered: 8.4% sodium bicarbonate mixed in equal parts of 20% glucose solution (performs quick buffering and elimination of hydrogen ions and acts as substitute in increased elimination of sodium bicarbonate); the quantity of the infused solution is determined according to the respective formula and acid-alkaline status indications, and the clinical parameters of acidoses; trometamol-buffer solution by organic aminocompound - out of it 30% penetrate the cells and the other 70% are connected to the hydrogen ions and secreted through the kidneys; sodium lactate – 1/6 mol solution in phials of 500 ml.
b) Metabolite alkalose: most often observed with hypokaliaemia after prolonged use of diuretics without adequate hydration. Big doses of vitamin C, potassium chloride solutions and venal infusion by drops of physiological solution are applied. For unsatisfactory results, carboanhydrase inhibitors (diamox, dehydratin, etc) are included.

5. Overcome biochemical and cell composition disorders of the blood. The following medication and methods are applied:
   a) For extreme hyperglychaemia: insulin venally and in muscle according to the level of blood sugar, abrupt decrease is not allowed; insulin therapy is combined with potassium salts.
   b) For hypoglychaemia: 20% glucose solution– from 40 to 60 ml is introduced in flow, or 500 ml venally in drops; for unsatisfactory results urbason is applied venally – 40 to 80 mg, or adrhenalin ½ mg subcutaneously. In prolonged hypoglychaemia and convulsion manifestations – fresh blood of the same group.
   c) For damages erythrocytes: plasma, human albumin and aminoacid solutions in adequate for the clinical and laboratory data doses are introduced venally in drops.

C. Methods for removing exo- and endotoxic substances, that provoked the convulsion state:

1. Forced diuresis: Osmotic forced diuresis is carried out as follows: water-electrolyte and monosaccharide solutions are infused in drops in one of the veins, in a second vein – highly mollecular solutions (aminoacid preparations, plasma and plasma substitutes) in relation of first to the second 4:1 respectively and a total volume for 24 hours of 6000 to 12000 ml). This method helps eliminate through the kindeys the toxic substances, provoking convulsion stimulating disorders of the cerebrum structure.

2. Extracorporal hemodialysis: it is carried out with a dialysator by pumping the blood out from a blood vessel, filtrating the blood through the semipermeable membranes of the apparatus, after which introducing it again through a blood vessel into the organism.

3. Carbohemoperfusion: ready-made spool of specific sorbents granules is used, the latter absorbs toxic substances from the blood. The blood is transferred by a special pump-tube device from the blood vessel through the spool and after purification is returned into the organism through another vessel.
4. Peritoneal dialysis: special hypertonic solutions, introduced after a specific method in the abdominal cavity extract toxic substances and water from the peritoneal blood circulation of the patient. This method can be applied for shock states, but specialized by adequate antishock preparations.

5. Plasmaphoresis and ultrafiltration. Beside the toxic substances, the described dialysis methods applied for the severe convulsion states, extract a big quantity of water (especially so the peritoneal dialysis and ultrafiltration), which has also a dehydrating effect on the cerebrum expressed by an indirect anticonvulsive influence. Dialysis methods as other intensive manipulations are performed under the protection of current medication by sedative drugs against convulsion states.

D. Special medication, neutralizing the effect of convulsion provoking exogene poisons: different types of antidotes.

E. Medical preparations for urgent treatment of acute morbid conditions, provoking convulsion attacks:
   antibiotics, aerosol, injection and ampoule forms for parenteral application of broncholytics in severe respiratory tract damages, glucocorticoids, diuretics, etc.

IV. ACUTE IMMUNE DEFICIENCY

In the available publications, no graphic model for synthesis similar to the unified doctrine for up-to-date forms of this pathology such as the one reflected in Tables 1, 2, 4, 5, 6, 9 was found. Such an approach to the problem is quite useful, since it includes a study of the immune disorders during biological terrorist acts. The introduction of the chemical and biological agents of the terrorist aggression provoke dangerous ecological and immune phenomena presented in unified doctrine (Table 1) (6, 8, 12, 13).

1. Classification of the immune disorders (after Al. Monov)

The research and investigations that were carried out by the author in recent years, reveal that no matter which immunology component is damaged, and by what etiology factor, the immune system reacts as one of the main regulators and integrators of the human organism, and its whole physiology shows disease deviations. This is due to the interrelations between the different components
of this system. The manifested disease condition in fact represents a general disorder of structures and functions of this system called immunity, due to the various inflicting factors caused by biological traumatism and terrorism.

This classification includes all the immune disease groups established so far, corresponding to the needs of clinical medicine.

1. **Immune deficiency (Immune hypo-activity or immune depression).**
   1.1. Primary (genetic) immune deficiency: T-lymphocyte immune deficiency (inborn hypoplasia of the thymus), B-lymphocyte immune deficiency (hypo- or agammaglobulinemia), combined T- and B-lymphocyte) immune deficiency, C-complementary immune deficiency, (C₃-deficiency), enzyme immune deficiency (chronic granulomatosis etc.) and others. The biological toxin and microbial factors activate the primary (genetic) immune deficiency and the prognosis is very bad.
   1.2. Secondary (acquired) immune deficiency.
      1.2.1. Exogenous type acquired immune deficiency: virus-bacterial type (AIDS), physical-chemical type, medicament type etc.
      1.2.2. Endogenous acquired immune deficiency: endocrine, metabolic, age, etc.

2. **Immune hyper- and para-activity.**
   2.1. Allergic states.
   2.2. Autoimmune aggression.

3. **Stress immunology.**

4. **Malignant immune cytological hyper-proliferation and para-differentiation: tumors, lympho-leucosis, lymphomas, etc. (Table 2).**

On the background of this unified classification of immune disorders and within the frames of its chapters, hereafter will be presented the different types of
ecological and terrorist immune diseases. Their description corresponds to the unified concept of the author on the etiological factors of ecological immune pathology.

2. Main types of immune disorders

Here belong the depressive (hypo-active) and hyperactive forms of immune disorders:

1. Depressive (hypo-active) forms.
   1.1. Acquired immune deficiency.
      1.1.1. Acquired immune deficiency of virus-bacterial type.
      1.1.2. Acquired immune deficiency of physical chemical type.
      1.1.3. Expositional clinically negative immunopathy.

2. Hyperactive (proliferous) forms.
   2.1. Autoimmune aggression.
   2.2. Ecological allergies.
   2.3. Stress immunopathy (Table 2).

The listed representatives of immune pathology are met in risk regions and they tend to increase in frequency in unfavorable ecological conditions. Various ecological and biological terrorist factors participate in their etiology that lead to complicated pathogenic mechanisms and to differentiated forms of the mentioned
clinical forms. In this respect we can talk of clinical groups of immune disorders.

The setting apart of immune disorders in human organism as a current priority pathology, with its own specific characteristics, rises the issue of its etiology.

The etiological factors of this disease are of great interest also due to their relation to the clinical manifestations, their treatment strategy and prophylaxis. This is especially valid for the terrorist ecological immune disease. According to the author’s investigations, its etiology includes the following groups of agents (Table 4).

**Table 3. Etiological factors of immune disorders**

<table>
<thead>
<tr>
<th>Etiological factors of immune disorders</th>
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<tbody>
<tr>
<td>Acquired ecological immune deficiency</td>
</tr>
<tr>
<td>Physical and biological type</td>
</tr>
<tr>
<td>Ecological auto-immune aggression</td>
</tr>
<tr>
<td>Expositional discrete immune therapy</td>
</tr>
<tr>
<td>Stress immunopathy</td>
</tr>
</tbody>
</table>

1. Exogenous agents:
   1.1. Pathogenic microorganisms (bacteria, viruses, etc.)
   1.2. Ecological physical-chemical and biological factors: physical agents, chemical substances, and biological substances.
   1.3. Drugs.
   1.4. Stress influences.

2. Endogenous factors.

**Table 4**

<table>
<thead>
<tr>
<th>Etiology of immune disorders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exogenous agents</td>
</tr>
<tr>
<td>Pathogenic microorganisms</td>
</tr>
<tr>
<td>Physical chemical, biological and other agents</td>
</tr>
<tr>
<td>Drugs</td>
</tr>
<tr>
<td>Endogenous factors</td>
</tr>
<tr>
<td>Cerebral-neurogenous</td>
</tr>
<tr>
<td>Metabolic</td>
</tr>
<tr>
<td>Combined</td>
</tr>
<tr>
<td>Allergic processes</td>
</tr>
<tr>
<td>Endocrine</td>
</tr>
<tr>
<td>Genetic processes</td>
</tr>
</tbody>
</table>

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2.1. Cerebral-neurogenous.
2.2. Endocrine.
2.3. Metabolite.
3. Allergic processes.

The exogenous agents, which are the first group of the presented etiological factors, are direct components of human environment. Most of them are elements of a worsened ecological media, through which it expresses its aggressive impact on human organism. In parallel to the physical, climatic, chemical and other harmful agents, consequence of the “civilized” environmental pollution with the active participation of human beings, to avoid mass drugs intake and misuse, periodic “bacterial and virus explosions” in the air, water and food is unthinkable. We previously included the chemical and biological terrorist agents in the group of exogenous agents.

Investigations of the author and other researchers establish the following data, for the different etiological factor’s subgroups:

Leaders of the pathogenic microorganisms in provoking immune disorders are the virus strains especially represented by the AIDS virus. The other types of bacteria participate mainly in water and food pollution and provoke cholera, stomach-intestinal infections, less often are authors of pulmonary infections.

The most frequent violators of the immune system functions from the group of the ecological physical, chemical and biological factors are the physical agents, namely – the different kinds of radiation: space (ozone layer thinning), secondary (different incidents in factories, nuclear power stations etc.), climate anomalies. Chemical polluters have the widest spectrum of varieties and are the most commonly met violators of the immune units in the human organism. Especially harmful are heavy metals, pesticides, organic diluters and some organic aerosols, created lately, containing carbohydrate molecules. Highly toxic biological poisons of different origins - plant (amanitin – contained in some sorts of mushrooms, the toxic protein of the ricin extract, etc.), animal (snake and scorpion poisons etc.), bacterial (botulin, staphylococcus poisons, etc.) can cause also immune disorders. In the present study the author is presenting medicaments as etiological factors of the disease, mainly in the following two cases: when the methods for application of drugs have been violated (cytostatics, acute and chronic allergic incidents) and in cases of poisoning (heavy acute combined drug intoxication).

An interesting issue is the “stress and immunity” phenomenon and the role of stress as an etiological factor for immune disorders. The molecular and bio-
chemical substrates that appear in cerebral endocrine units after acute and especially during chronic stress state (hypoxic effect, metabolite and mediator disorder, etc.) result in direct disorders of the immune system functioning. The ecological and terrorist character and the consequences of this phenomenon are understandable, having in mind the huge “stress” outbreak in different parts of the world, as a consequence of the difficult and complicated living conditions.

In the etiology of immune disorders, especially their endocrine, metabolite and combined forms, the group of endogenous factors is in many cases a secondary consequence from unfavorable exogenous (ecological and other) impacts on a number of human organs and systems. For example, the etiology of some endocrine diseases with evidence for hypofunction of the internal secretion glands, such as the myxedema syndrome, different forms of hypocorticism, etc., includes in the current conditions, unfavorable exogenous influence (chemical or biological radiation, climatic, etc.). They show immune disorders in their pathogenic manifestations.

The group of allergic processes has multi-dimension relations to the investigated diseases. The allergic process itself is an immune disease. Besides, agents existing in the environment provoke a number of acute and chronic allergies, and they increase in number during ecological incidents. In the third place, severe forms of allergic diseases (the Lyell disease, etc.) result in the occurrence of severe immune deficiency with all the consequences of this condition.

The genetic mechanisms of immune disorders have been known for a long period. Today however, more and more opinions are heard that a number of ecological and terrorist incidents and agents (through their impact on structural and regulatory genes) violate the immune genetic regulation balance.

An important issue for the etiology of immune disorders is the interrelation between the mentioned etiological factors and the human organisms at different periods of time, their interrelation and the impact of the combined etiological complex on the human organism.

Every one of the agents of the first and second groups described in the classification, when taken in significant doses, provokes in a short time different disorders of acute immune character. There are some regions, where the harmful ecological agents are in mildly increased concentrations. The population in these regions, subject to a continuous influence of this “quiet” aggression, slowly and unnoticeably gets immune disorders. By the chemical and biological traumatism and terrorist acts the population in determined regions is namely susceptible to immune disorders.
The influence of several etiological factors simultaneously shows a multiplication effect and results in the growth of new etiological complexes with increased aggressiveness on the human body. The organism exists in a specific reactive and adaptive stagnation, followed by the growth of new nosological units or worsening of the existing diseases by including immune disorders.

In a severely worsened ecological terrorist environment, the influence of the mentioned etiological factors on others of its own components (in the first place microorganisms, and then many other “objects”), very often gives out break to new, dangerous disease agents, with qualitatively different pathogenic effects on the human organism. They create new types of ecological diseases, closely related to immune disorders. Such is the virus, having provoked AIDS in the high risk ecological regions, undergoing continuous draughts, very high temperatures, disastrous rains and other strongly expressed anthropogenic ecological calamities.

Pathogenetic mechanisms of terrorist and ecological immune disorders

The mentioned ecological factors result in the appearance of terrorist ecological immune diseases, by means of the following main pathogenetic mechanisms (Table 5):

1. Eco-immunosuppressive cytological mechanisms. Through these mechanisms, the environmental agents harm the cytological structure of the immune system and its functions. Depending on the type of the damaging factor, the three main groups of cells of this structure and their main sources, are inflicted: B-lymphocytes, T-lymphocytes and different types of macrophages. The bone marrow, the lymph structures, the organs rich in reticule-endothelial formations such as the liver, the lungs, and the cerebrum, etc. are affected. The acting chemical and biological eco- and terrorist agents, depending on their physical and biological characteristics, disturb the leuco- and lymphopoiesis and the activity of the phagocytic cells – the circulating types (monocytes) and the immobile types in the reticule-endothelial system. These disturbances are morphological and cyto-physiological in character and take effect mainly through enzyme inhibition and dysmetabolic processes.

1.1. Terrorist and eco-immune suppressive effects on the B-lymphocytes: decreased production, slowing of the process of their maturation from pre-forms into mature forms and increase of the percentage of pre-forms, production and secretion of incomplete antibodies and decreased number of some of them, significant selective low number of...
Table 5

Pathogenetic mechanisms of ecological and terrorist immunopathies (after A. Monov)

- Immune suppressive cytological mechanisms
  - Immune suppression on T-lymphocytes
  - Immune suppression on B-lymphocytes
  - Immune suppression on phagocytes
  - Eco-allergic mechanisms

- Immune suppressive humoral mechanisms
  - Immuno-globulin disorders
  - Mediator disorders
  - Desorder of the phagocytosis
  - Eco- and terrorist cytological mechanisms
  - Eco- and terrorist genetic mechanisms

- Disorders of the immune response
  - Activation disorders
  - Inter-relations disturbance
  - Effectory reaction disorders
  - Eco- and terrorist immune stimulating malignant effects
the memorizing B-cells, etc.

1.2. Terrorist and eco-immune suppressive effect on T-lymphocytes: decreased production, slowing of the process of their maturation from pre-forms (prothymocytes) into mature forms, selective decrease of some of the mature populations production (especially their helper forms), disorder of their helper functions as concerns B-lymphopoiesis, appearance of malformed representatives of the different populations, especially their membrane receptor completeness against antigens.

1.3. Terrorist and eco-immune suppressive effects on phagocytic cells. The damaging effect on them of the ecological factors goes in three directions: decrease of the monocytopoiesis and the cytolysis of the “quiet” macrophages in the liver, lungs, lymph structures, etc. due to active and accumulating noxes in these organs (heavy metals, biological poisons, aerosols, radiation factors, etc.), disturbance of their immune processes information and cytotoxic regulators.

2. Terrorist and eco-immune suppressive humoral mechanisms. Through these, the offenders of the environment violate the elements of the immune system, the organism’s humoral environment and the its ongoing processes.

2.1. Injury of the immunoglobulins. They are affected in two ways: the immune-competent cells which have been morphologically damaged and deformed suppress their specific production processes in the B-lymphocytes and the T-helpers and provoke damage of the molecule structure through direct impact of the ecofactors or the metabolites of their chemical representatives.

2.2. Damage of the mediator, energy- and metabolite-protecting substances in an inter- and extracellular humoral environment, participating in the immune processes (monocytes, lymphocytes, prostaglandin, enzymes: adenylcylase, guanylcyclase, phosphodiesterase, etc., cyclic adenosine monophosphate, adenosine triphosphate, etc.)

2.3. Disorders of the phagocytosis. It affects mainly the circulating in the blood phagocytes. The effect of heavy metals, radiation agents, some biological poisons provokes phagocyte activity and tissue stationary macrophages disorders (Kupfer cells, microglia cells, peritoneal, splenial, alveolar and other macrophages).

3. Terrorist and ecological disorders and deformation of the immune
response. This group of mechanisms is manifested simultaneously in the cytological and humoral spheres, both for the primary and the secondary immune response. After disorder of the primary type, the following processes take place:

3.1. The processes of immune response activation are malfunctioning (starting phase). This pathogenic complex disturbs the contacts of the B-lymphocytes and plasmatic cells with the antigen, and its recognition by the competent cells’ specific receptors. Thus the conditions for interrelating are damaged.

3.2. The processes of interrelating are interrupted (central phase). In this case, the terrorist and ecological offenders can break down single or all of the four types of interrelations (between macrophages and T-lymphocytes, between T-lymphocytes and B-lymphocytes, idio-type-anti-idio-type and polyclonal interrelations).

3.3. Disturbance of effector cells functions (effector phase): synthesis suppression and secretion of specific antibodies from the plasmatic cells; production of lymphokines by the sensibilized lymphocytes, created during the central phase.

After damaging effects of the ecological factors on this type of immune processes, the secondary immune response might be strongly suppressed or switched off due to the inhibition of the proliferate and differentiation etiological factors of the memorizing cells. Then, when one and the same antigen meets the organism for a second time, severe immune-determined pathology might occur.

4. Eco-allergic mechanisms. They include allergic type processes in the organism immune system, arisen at contact and upon effect of environmental agents. In this case they act as allergens. A great variety of ecological and terrorist-ecological allergens exists. They can be organic and non-organic chemical substances, also agents of plant and other origin, that the patients have been in contact with for a long time. This kind of immune disorder proceeds in two phases: sensibilization phase – the primary contact of the organism with the ecological allergen during which the processes of sensibilization begin and antiallergens are formed; phase of the allergic incident – when the patient subsequently meets the allergen, upon which mediators are separated and their effect is manifested, most often of the histamine.

Depending on the type of the allergic process and the separating mediators, the allergic reaction can prove to be of the quick type (shock state) or of the slow
type. When the allergic processes take a severe form, other immune units of the patient start malfunctioning – than combined type of several pathogenic mechanisms is created, that come into action simultaneously or consecutively during the flow of the disease. In this case the allergic changes act as etiological factor to a new immune disorder.

5. **Terrorist eco-cytological mechanisms.** Here belong the processes, through which a number of ecological agents injure the various cell groups, proteins and other of the patient’s organism structures. Depending on the nature of the acting violator and of the damaged organ, various, new for the organism substances are created, that act as foreign bodies and provoke adequate reactions from the immune system. This principle is the basis for the rise of destructive immune processes against the source substances of these new products (specialized cell structures and different organs). These interactions between the terrorist ecological agents, the damaged cells, the immune units and macroorganisms can be of acute or slow, prolonged nature. A state of autoimmune aggression (a separate nosological unit in immune pathology) occurs in the organism. It can be of acute and of chronic type.

6. **Terrorist eco-genetic mechanisms.** They include the damaging processes inflicted by ecological agents on the structure and regulatory genes, responsible for immunity genetic regulation. Lately it has been established that each H- and L-polypeptide chain of the different immunoglobulins is coded with several genetic elements. In the primary sex cells DNA they are separated, but in the B-lymphocytes and in the antibody forming cells, they are united and form one gene. In the primary sex cells, other genes are also separated, which during the process of lymphocyte formation combine and create active single genes. Thus, the synthesis of different immunoglobulins with the participation of other genetic mechanisms is provided for. A number of ecological and terrorist-ecological factors, damaging the mentioned genetic structures and their interaction, result in severe disturbance both of the creation of lymphocytes, immunoglobulins and other immune system elements, and of the correct manifestation of the immune response. A genetic background is laid for a whole row of immune disorders and immune deficiency states.

7. **Specialized eco- and toxic immune mechanisms, stimulating malignant formations.** The immune system, through the T-lymphocytes recognizes the tumor antigens, and the human organism goes through pro-
liferation and specification of the immunocompetent lymphocytes, that destroy the tumor cells in the following manner: through anti-cell dependent cell cytotoxicity – it is done by the killer cells through their Fc-receptors and IgG Fc-fragments that have sensibilized the target neoplastic cells; through the natural immune resistance, in which tumoricidal effector NK-cells, Nc-cells, neutrophil and mononuclear phagocytes participate. Experimental research has established that a number of ecological offenders such as radiation factors, organic substances, lymphocytotoxic medication, specialized substances, used for terrorist purposes with retarded reaction, damage the mentioned immunoprotective mechanisms and benefit the appearance of malignant tumor processes, and respectively of their diffusion. Research and investigation for the last years enrich the knowledge of this group of immune pathology pathogenetic mechanisms.

The above described eco- and toxic immune pathogenetic mechanisms, in the complicated interactions between human beings and worsened ecological conditions including terrorist agents, can affect with one single elements of the mechanisms, but can act as a combination – several elements simultaneously. This circumstance preconditions the variety of expressions of the ecological immune pathology and creates serious difficulties to diagnosis, treatment and prophylaxis of these diseases.

3. **Unified diagnostic criteria for current immune disorders**

The unified doctrine including current ecological and eco-terrorist immune pathology supposes the diagnostic decision for its main nosologic units to be subordinated to the main behavior principles that will facilitate the physician in his struggle against them.

The diagnostic criteria are based on three sources with five groups that furnish information on this kind of disease (Table 6).

<table>
<thead>
<tr>
<th>Purposeful anamnesis</th>
<th>Clinical data for ecological impact</th>
<th>Paraclinical examinations</th>
<th>Clinical and chemical laboratory tests</th>
<th>Immuno-logic indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unified diagnostic criteria for current immune pathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 6**
The anamnesis – the following information will give direction to potential or existing ecological immune disorders:

1. Contacts with affected or endangered population contingents, that live in highly aggressive and harmful ecological environment, including drugs (citostimulants etc.), or having spent long periods of time in comparatively less risky ecological regions.
2. Complaints about disorders in the general condition due to unspecified reasons.
4. Continuous or frequent subfebrile or febrile conditions, whose origin has not been identified after investigation by the traditional medical examinations and tests.
5. Subjective complaints or other data for influence of specific ecological polluters on the human organism in a definite region: toxic aerosols, heavy metals, radiation and chemical effects, skin and other allergic manifestations or their equivalents, etc or if there are doubts about harmful substances taken in by food, liquids or air.

The clinical tests – the evidences listed below bind the physician to think and look for terrorist ecological immune disorder:

1. Symptoms and syndromes for occurred poisoning: by noxa, that could damage the immune system; by drugs – combined forms, cytostatics, etc.; by pesticides – phosphoorganic compounds, halogenated carbohydrates etc.; - organic diluters; - heavy metals; - toxic aerosols; - plant poisons – mushroom (amanitin), poisonous herbs and their analogues produced industrially or in laboratory conditions for terrorist aims (alkaloids, saponines etc.); - poisonous animals – insect, fish, snake poisons etc.
2. Symptoms and syndromes for radiation disorders: hematogene and mielogene, hepatogene, cerebral, etc.
3. Different kinds of allergic conditions.
4. Malignant tumors; blood and other symptoms and syndromes.
5. Symptoms and syndromes for local or general inflammatory disease.
6. Symptoms and syndromes for local or generalized processes resistant to traditional treatment.
7. Inflated lymph nodes or splenomegalia.

The paraclinical and laboratory tests – the diagnosis for ecological im-
mune disorders and their terrorist forms, is supported or revealed by the following main subgroups of paraclinical and laboratory analyses:

1. Hematological: leucopenia, anemia and thrombocytopenia, manifested singly or especially in combination, can be provoked by mielogene disorders due to radiation or toxic agents with immune impact.

2. General urinary: positive tests for protein and gall pigments, as well as pathological sediment changes combined with adequate changes as described above, may be caused by beginning ecoimmune disorders.

3. Chemical: positive blood and urine tests for exogenous or terrorist chemical noxa such as heavy metals, pesticides, hemotropic, mielotropic and cytostatic preparations, as well as a high concentration of other drugs require a checking for immune impact.

4. Radiation: increased quantities of radiation noxa or radiation carriers.

5. Positive medical screening tests with differentiated immunological correspondence, including basic generally accessible and feasible examinations for the population of ecologically risky regions, carried periodically every 6-12 months, depending on the risk degree.

6. Positive medical monitoring results by indicators and constellations, exogenous to the environment and endogenous to humans, which control both the dynamics of the direct human environment offenders and the changes in the organism under their influence.

The results of these tests should be periodically compared and assessed.

The clinical technical investigation of certain organs and systems: X-ray examination, electrocardiogram, cerebral examination, scanning (of the abdomen) and others positive results, compared to positive data from the above listed tests, also configure the diagnostic criteria. Polyvisceral examination of the organism is an important new technique that plays an important role due to the promptness and thoroughness the method.

The immunological examination of the patient: these are results for changes in the cytological, humoral and phagocyte indications, obtained according to a specific program (Table 7). One, two or three of the pointed indications are affected, depending on the degree of immune disorder. They are sufficient to receive information for immunity disorders in specific affected groups and to make conclusions for the whole contingent in a region. After the diagnosis has been set conforming to the additional indications thus received, the number and the type of the indications for further investigation and observation can be widened.
Table 7

Basic laboratory indications for terrorist and ecoimmune pathology

<table>
<thead>
<tr>
<th>Cytologic constellation</th>
<th>Humoral constellation</th>
<th>Phagocyte indicators</th>
<th>Other special indicators</th>
</tr>
</thead>
</table>

Manifestations of leucopenia, anemia, subfibrillity without obvious reasons and clinically negative organism indications identified in the process of specialized medical screening of the population in risk regions, requires that additional observation and examination be carried out on the immunologically affected patients. When the results show early disorders in the organism, adequate treatment and prophylaxis should be carried out. During this period, they are most effective.

The following leading syndromes are outlined on the basis of data from the earlier pointed chapters of the clinical picture of a specific patient, as useful criteria for the diagnostic reference to occurred or forthcoming ecoimmune disorder.

Basic syndromes:

1. The Cerebral febrile syndrome, especially of the protracted type and with comatose period. It is an indication mainly for combined medical substances or strong industrial poisonings.
2. Pulmonary febrile syndrome with or without present infection changes. It is valid whenever inhalation of toxic aerosols and gas mixtures or virus or bacterial microorganisms aggression has taken place.
3. Hepatic or hepatic-renal syndrome with or without febrile reaction. It is especially meaningful for toxic, radiation and secondary occurred bacterial infections.
4. Gastrointestinal protracted syndrome. It is important for toxic, radiation and bacterial disorders.
5. Hematogone syndrome in all its variations, with or without febrile reaction. It is observed after radiation, hemotoxic effects and virus infections.
6. Allergic protracted or repeated recidivating syndrome. In addition to the immune disorder, it requires that secondary inflicted immune deficiency be sought for.
7. Febrile syndrome with or without bronchopneumonia and septic manifestation. It points to a virus or a bacterial infection.

8. Malignant tumor syndrome. In all the cases it points to immune disorders.

An adequate integrated table (table 8) has proven to give good results for the carried investigations.

If necessary other specialized immune indicators can be included in the table.

The indicators in the table have been defined in the “Pirogov” Emergency Hospital immunological laboratory and have been applied in investigations carried jointly by the author and the hospital teams.

Table 8. Unified immune indicators for current disorders

<table>
<thead>
<tr>
<th>Name of the indicator</th>
<th>Normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell constellation</td>
<td></td>
</tr>
<tr>
<td>T-lymphocytes</td>
<td></td>
</tr>
<tr>
<td>- early forms</td>
<td>44,24 %</td>
</tr>
<tr>
<td>- late forms</td>
<td>66,88 %</td>
</tr>
<tr>
<td>B-lymphocytes</td>
<td></td>
</tr>
<tr>
<td>Humoral constellation</td>
<td></td>
</tr>
<tr>
<td>1. Immunoglobulin A</td>
<td>170 000*</td>
</tr>
<tr>
<td>2. Immunoglobulin E</td>
<td>190 000*</td>
</tr>
<tr>
<td>3. Immunoglobulin G</td>
<td>150 000*</td>
</tr>
<tr>
<td>4. Immunoglobulins M</td>
<td>950 000*</td>
</tr>
<tr>
<td>5. Alpha 2-macro-globulin</td>
<td>150-250 mg %</td>
</tr>
<tr>
<td>Mioglobulin C3</td>
<td>60-110 mg %</td>
</tr>
<tr>
<td>Mioglobulin C4</td>
<td>20-50 mg %</td>
</tr>
<tr>
<td>Fagocyte activity:</td>
<td></td>
</tr>
<tr>
<td>6. percentage of the fagocytosis</td>
<td>80 - 90 %</td>
</tr>
<tr>
<td>7. fagocyte number</td>
<td>3,5 - 4,0</td>
</tr>
<tr>
<td>8. fagocyte capacity</td>
<td>2500 – 5000</td>
</tr>
<tr>
<td>absolute fagocyte indicator</td>
<td>12,000</td>
</tr>
<tr>
<td>nitro-blau-tetrasol-sulphate index</td>
<td>62</td>
</tr>
</tbody>
</table>

*relative molecular weight
4. Complex treatment program for terrorist and ecological immune disorders

The multifactor, complex disorders of human organism affected by ecological and terrorist immune diseases demand the implementation of an unified strategy for their treatment. According to the elaborated by Al. Monov doctrine, the therapy of this kind of pathology is carried out according to a complex treatment program including the following means and methods:

1. Means and methods disarming the etiological factor and its mechanisms:

1.1. Antidotes and detoxic depurging. One of the main terrorist ecological agents, provoking immune disorders, are the different kinds of exogenous poisons. A successful treatment would include timely counteraction to these toxic substances by two groups of specific means – antidotes and detoxication methods.

Different mono- and poly-medicamentous substances as used as antidotes, to neutralize directly the toxic offender or to correct its specific damaging mechanisms. Different treatment procedures are applied for the different ecological and terrorist poisons, depending on their physical and chemical properties. Antidotes should be included already at the beginning of the treatment of acute forms of ecological or terrorist immune disorders, to prevent further deepening of the damages and stop the process of their transformation into irreversible forms. Detoxic depuration means are applied to throw away the poison from the body and interrupt its contact with the individual. They have three levels of action: entry zone, blood and other humoral structures and cell enzyme level.

Ecological and terrorist poisons penetrate the organism basically from three main areas: the digestive tract, the skin and mucosa and the respiratory system. In the acute forms of poisoning, resulting in immune disorders, these poisons are removed by the following means and methods introduced in modern toxicology: stomach lavage, intestine purgatives, oxygen mixture inhalation (to remove the agents from the respiratory system), toilet of the skin and mucosa.

Methods for renal and out-renal detoxication purging, specialized with other means depending on the acting poison, are applied to remove the agent from the blood and the humoral areas. Detoxic depuration is introduced in acute and chronic forms of ecoimmune pathology. The basic method for renal depuration is the forced diuresis. It is applied already at the beginning of the acute forms of intoxication, provided there are no contraindications. It has a positive effect also in cases of severe endointoxication during different types of immune disorders. The average
volume of prescribed liquids according to Al. Monov should be from 6000 to 12000 ml per 24 hours. The forced diuresis is done in the following manner: through a blood vessel are introduced venously drops water-electrolyte and 5 % glucose solutions, through another – high molecular solutions (aminoacids and plasma-substituters) at the relation low molecular to high molecular solutions 4 to 1 on the average. The hemodynamic indicators are controlled rhythmically throughout the 24 hours: the diuresis, arterial and central venal blood pressure, the hematocrit, etc. Homeostatic disorders of the organism should not be allowed. For the out-renal detoxic depuration, the present complex program includes the following methods: partial blood transfusion (for poisonings with hemotoxic noxa) and dialysis methods – hemodialysis, peritoneal dialysis, carbo-hemo-perfusion, plasma exchange. For mass terrorist ecological poisonings are used the forced diuresis, partial blood transfusion, peritoneal dialysis and carbo-hemo-perfusion.

1.2. Anti-radiation means and depuration methods: They are introduced upon radioactive and radiation aggression on the human organism and upon manifestation of acute and chronic, as well as terrorist ecological incidents, combined with biologically affecting agents. Hepato-medications (pentacin, etc.) are prescribed as multinuclear radiation preparations and iodine preparations for anti-radiation treatment. Detoxic depuration of this type of ecological immune disorders agent is done mainly through blood transfusion and specialized dialysis methods (carbo-hemo-perfusion).

1.3. Antibiotics and other chemical therapeutic preparations. They are applied when the immune damages follow diseases, provoked by the mentioned ecological and terrorist destructive microorganisms: stomach-intestinal infectious diseases (salmonella, cholera, etc.), virus infections or superposed infection from provoked by other agents.
immune disease. The mentioned types of treatment methods should be adequate to the acting microorganism (determined by the bacterial sensitivity and other methods) and protective against medica-mentous disease.

1.4. Antiallergic means. They are applied in acute and chronic forms of the different allergic states. Two main groups of antiallergic preparations are mostly prescribed: antihistamine drugs and glucocorticoids, depending on the allergic process type.

1.5. Anti-stress means: Neurosedative and antidepressive effect medications are applied on the specific individual or population groups manifesting acute stress and chronic reactions and immune disorders in ecological risk regions, affected by severe ecological incidents.

1.6. Treatment methods against endocrine, metabolite and other endogenous diseases in ecological-and terrorist-ecological risk regions. Immune diseases appearing under such conditions could have an etiological connection to this group of endogenous diseases.

1.7. Other treatment methods (surgery, radiology, etc.) used against diseases (tumors, pustule, etc.), that could be etiological factors for immune disorders of ecological origin.


The following types of changes have a cause-effect role for occurrence of immune disorders in the process of the ecological and terrorist aggression on human organism: destructive damage of cells and organs (autoimmune aggressions springs up), local or generalized hypoxia, different forms of cell dismetabolism (carbohydrate, protein, lipid), balance disorders (water-electrolyte, acid-alkaline etc.). The counteractions include:

2.1. Organoprotective medications: hepatoprotective (monosaccharides and amino-acid vitamin solutions, hepatoprotective drugs – Essenciale, lypovitan etc., Legalon, Heparegen, orocetam, vitamin complexes – groups B, C, etc., glycocorticoids, pulmoprotective (inhala- tion of aerosol preparations and combinations – Becotid, broncholitics, acetylcistein, proteolitic enzyme preparations applied locally, etc.); cerebroprotective (nootropic drugs – see below, vitamins of the B group, dehydrating, etc.); hemo- and mieloprotective substances – see below, etc.

2.2. Substitute and corrective preparations: water-electrolyte solutions
(the Ringer solution, physiological serum and alkaline solutions, etc.),
aminoacid solutions, alkalizing solutions, hemostasis combinations
(on hemostasis disorders) etc.

2.3. Anti-hypoxic preparations:
nootropic drugs (pyramem, nootropil),
antihypoxic and antidysmetabolite combinations: pyramem – 1,00 g,
centrophenoxin – 250,0 mg, pyridoxin-vitamin B₆ – 50-100,0 mg,
glucose serum – 250 ml applied venally in drops every 4-6 hours
(AI. Monov) by very critical cerebral insufficiency and polyorganic
hypoxia, equivalent drug preparations; orotous combination
(pyramem ampules or nootropryl, orovit ampules after AI. Monov,
etc.).

3. Immune protective treatment
It is carried out through methods for direct effect on the structures and
functions of the immune system, methods for indirect effect and through substitu-
tive immune procedures. The direct effect is obtained by:

3.1. Cytological immune mechanisms. Through these, the immune cell
structures are influenced to overcome their being damaged and to
enact their reaction against the immune offenders. It is done by means
of:

3.1.1. Cyto-immune stimulating treatment substances: Substances of
biological origin are included here (bestatin, biostim, lentynan,
BCG vaccine – for protracted disorders, etc.) organ physiological
substances (interferon, transfer factor, tymusine fraction V,
etc.), synthetic substances (levamisol, imutiol, tiabendasol, etc.),
that show immune stimulating effect on macrophages, T-cells and
B-cells.

3.1.2. Cyto-immune suppressive treatment substances. Through them
mainly the eco-immune auto-aggression is affected. Alkalizing
medications are used (cyclo- phosphamides, chlorambucil, etc.),
admi metabolites (imuran, metotreksat, hormones, etc.) corticos-
teroids, estrogens and androgen preparations (medroxiprogeste-
rone etc.), enzyme preparations (L-asparigenase and L-glutami-
nase), prostaglandin, imidasol etc., if not contraindicated.

3.2. Molecular immune mechanisms. Immune protection is carried out in
this chapter of the disease treatment by influencing the damaged im-
une system molecular elements (immunoglobulins, lymphokines,
monokines etc.) or by introducing in the organism treatment medica-
tions containing them. Drugs that regulate the deficitous (levamisol, muramildipeptide, indometacin) and the hyperproductive deformations (glycocorticosteroids, steroids, ibuprofen etc.) of the immune response as concerns lymphokines, monokines and other molecular immune elements’ participation are used.

3.3. Regulation of different phases of immune response. The different ecological violators affect this basic manifestation of the immune system in its three phases.

3.3.1. Regulation of the immune response initiation phase. The stimulating effect on enzymes and immune cell processes, ensuring the recognition of the antigen (adenylcyclase, guanidinecyclase etc.) is carried out by means of prostaglandins, imidasol, levamisol, etc.; the inhibiting effect – by means of glycocorticosteroids, methylxantine, calcium preparations etc.

3.3.2. Regulation of the interaction phase between antigen factors and immune cells and of the effector phase (when the immune damage is of terrorist ecological origin) is done by means of stimulating (isoprinosin, levamisol, synthetic polynucleotides etc.) and inhibiting (glycocorticoids, etc.) preparations.

The indirect effect for the immune protective treatment is obtained by including organ and system suitable and adequate medical preparations that have a regulatory effect on the immune processes, affected or not by the ecological aggression. Such medical preparations and procedures are:

3.4. Regulators of the cerebral function and of endocrine units and metabolisms.

3.5. Substitute immune protective treatment. It is carried out mainly in two methods:

3.5.1. Introduction of immune molecular substance preparations: immunoglobulins, interleukines, etc. in the organism.

3.5.2. Introduction of enriched immune plasma protein preparations against different virus and bacterial infections in the organism.


Preparations and methods that have a significant treatment effect on terrorist ecologically provoked immune disorders belong here. They are combined with the whole therapy and with some noteworthy protective measures taken before appearance of this pathology. The wide effect of these preparations and methods is due to their ability to assist the elimination of the ecological and terrorist immune aggres-
sive noxa from the organism (heavy metals, radionuclides etc.), to their properties to strengthen the immune structures and mechanisms and other specialized cells and organs that might be affected by the immune damage (cerebrum, liver, lungs etc.).

According to the investigations carried out by the author, these preparations and methods are presented in two chapters in the present study:

The **pharmacological chapter** of the program includes different pharmaceutical preparations, which manifest enzyme-cell and organoprotective properties and poison-depuration effect. Vitamins of the B group, vitamin C, medications improving cell oxidation and cell-metabolism (pyramem, nootropil, centrophenoxin), aminoacid products, preparations facilitating the elimination of heavy metals from the organism (sodium citrate), helatopreparations (penicilamin) etc. can be used to this effect. The manifestation of some poisons in the organism can be eliminated or prevented by means of medical preparations, for example against phosphate-organic pesticides – atropine and benzodiazepine drugs, against organic cyanide poisons – nitrose preparations etc. Ready combined pharmaceutical preparations in this chapter are orocetam – powerfully strengthening the liver and the cerebrum against terrorist ecological damages. They activate the suppressed oxidizing processes and the substances metabolism of the cells of these organs.

The approximate administration and dosage of the mentioned pharmaceutical preparations, for pills or capsules is:

- vitamin preparations: 20 days, 1-2 pills three times daily, 10 days rest;
- specialized antidotes – drugs (antidotes) in prophylactic doses: 1 pill or ampulla one to two times daily, followed by 10 to 20 days rest.

The mentioned effects influence the processes in two main directions:

- growth of important enzymes that are affected by the heavy metals and other poisons, often combined with biological agents, and disarming the latter (by means of vitamins B₁, B₂, B₆);
- overcoming the disordered oxidizing processes and stabilizing the cell metabolism upset by up-to-date poisons.
- strengthening the overall biochemistry of the cerebrum, liver, renal and myocardial cells and preservation of their structures and functions (by means of vitamin C, vitamin K, monosaccharides, aminoacids etc.).
- enzyme-substituting processes – by introducing in the organism ready enzyme preparations.

**Biological chapter.** This chapter includes a number of preparations and combinations, obtained from plant and animal substances, that associates them
more with the physiologically consistent natural substances. Based on the author’s investigations, the following representatives can be recommended:

- **Deodan** – powder and pills. This is a lysate of Lactobacillus Bulgaricus (Iv. Bogdanov). The biologically active components are: muramil – from the cell wall of Lactobacillus bulgaricus (main component), essential aminoacids, nucleotides, organic polyphosphates etc. – from the cells of the mentioned microorganism. This is a powerful immune stimulator, which modifies the immune answer upon its disturbance. Its biological and immunological properties have been investigated by Iv. Bogdanov, As. Bogdanov and others. A valuable study of the deodan as a stimulator for emitting mediators by immune competent cells has been carried out by G. Gencheva et al. A number of clinicists (Al. Monov and others) have established its clinical effect as an immune protector. All this data proves its properties as an immune preparation with great potential for the therapy and the prophylaxis of a number of immune diseases, including such provoked by chemical and biological agents and of malignant and tumor origin. Administration: on the average one coffee spoon-full or one capsule two to three times daily for a prolonged period following indications; the preparation has no side effects.

- **Minapel** – a combination of bee honey and apple acid. The doses is two coffee spoon-full three times daily for two – three weeks, after which ten days rest; Upon indications treatment should be repeated several times. The apple acid facilitates the elimination of heavy metals from the organism, while the honey strengthens the liver and neural cells against toxic effect and against the suppression of immune cell groups (Kupffer’s and others cells) functions.

- **Pektavit** – contains pectin and vitamins of the B group. It facilitates the elimination of heavy metals from the organism, respectively from the immune structure depots.

- **Honey-milk immune combination** (after Al. Monov). It is prepared with bee honey and thickened cow sour milk (yogurt). Administration: A mixture of half a cup of the thickened yogurt and one or two spoons of bee honey is taken, prepared just before consummation. The milk protein and the products of the acid ferments membranes activate in the marrow the production of immune system cells, which has been suppressed by ecotoxic, biological, chemical or other harmful agents. The bee honey, by means of its components - monosaccharides, microelements and vitamins, widens this effect
and increases the resistance of the liver, myocardial, renal and neural cells.

- **Briar (Rosa canina) – honey immune combination** (after al. Monov). Bee honey and ground naturally dried briar fruits are used. Preparation and administration: four soup spoon-full of bee honey are mixed homogeneously with one or two coffee spoon-full of briar flour. The mixture is enough for three to four intakes. Effects: the vitamin C in the briar, the monosaccharides and the other biologically active substances in the components of the combination improve the blood protective functions and the immune processes in the medulla, that have been affected by toxic, microbiologic, ecological or terrorist factors. They also have a protective effect against metal and other types of poisons. The effect will improve if a cup of sour milk, yogurt, peanuts or walnuts are eaten after taking the briar-honey mixture.

- **Honey – polyvitamin immune combination.** Geritamin in pills (vitamins A and E), neurobex or another combined preparation of the B vitamins and bee honey are used. Administration and dosage: One pill of geritamin is taken twice daily, together with a spoon full of bee honey; fifteen minutes later one pill of neurobex or similar preparation is taken. Effects: vitamin A increases the resistance of the epithelial tissue and together with vitamin E they improve the chromatin synthesis in the immune competent cells nuclei, that have been damaged by toxic, bacterial, radiation, radionuclide and other agents. The B group vitamins participate in the improvement of the carbon-hydrate metabolism in the cerebral and liver cells and strengthen the vital enzymes of the redox-enzyme system, that participate in the “oxygen explosion” at immune response during phagocytosis. The bee honey, together with the other component of the combination, increases the mentioned effects and manifests immune-active properties by compensating the repeatedly increased energy needs of the T-cells, B-cells and macrophages (after Al. Monov).

The treatment and prophylaxis immune preparations that have been presented, applied together with the medications described in the pharmacological chapter, improve the protective and recovery processes of the immune system after various ecological and terrorist damages.

The treatment of the immune ecological damages of the organism requires that the present complex program be applied comprehensively, with adequate representatives of the different methods. Isolated application of immune protective means or of other methods would be incorrect.
**Recommendation**

The mentioned therapeutic means and methods should be applied only in case of mass traumatism and biological terrorism. The individuality of the patient, the degree of infliction, the phases’ flow of the disease process, the existing contraindication and the main rules for the medical treatment process should be taken into consideration.

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The role of Immunostimulants in Immunotherapy and Immunoprophylaxis of Newly Immerging or Intentionally Spread /Bioterrorism / Infectious Diseases

Bogdan Petrunov, Plamen Nenkov

During the last two decades very intensive investigations are carried out on the preparation, experimental and clinical characteristics of one relatively new category biologically active substances - so called immunostimulants. They are products from natural or synthetic origin with different chemical characteristic and mechanism of action /5,7,13,15,18,19/.

Immunostimulants:
– activate different elements and mechanisms of the immune system of humans and animals;
– they reinforce a body’s natural resistance in order successful to cope with various viral and bacterial infections or to help in the treatment of other pathogenically related with suppressed immune system conditions – cancer / malignant /diseases, AIDS, SARS etc. Immunostimulants created the base of the active and successful development and implementation in the clinical practice of the nonspecific immunotherapy and the nonspecific immunoprevention by stimulating the main factors of the immune system;
– the phagocytosis;
– properdin and complement systems;
– protective secretory IgA antibodies;
– \(\alpha\)- and \(\gamma\)- interferon release;
– T- and B- lymphocytes;
– synthesis of specific antibodies and cytokines;
– synthesis of pulmonary surfactant.

Which are the factors determining the interest of the clinicians to use the immunostimulants in the control of different infectious diseases and the perspective for their large use in the medical practice?

– On the first place that is the increasing multi resistance of the bacteria to antibiotics, which creates in some regions in the world dramatic situation as more than 40% of the circulating bacterial strains are resistant to available antibiotics;
– very serious problem in everyday clinical practice is frequently encountered allergic reactions to antibiotics and chemotherapeutics in patients and in medical personnel what restricts their use;
– it is very important to stress that practically the great part of the antibiotics have well proved immunosuppressive effect. They “kill” bacteria but in the same time diminish the natural resistance of the organism to cope with them;
– one has to have in mind also the lack of activity of the antibiotics in viral infections and finally;
– the lack of specific treatment or vaccines for the greatest part of the viral infections, including HIV/AIDS, SARS and some others newly emerging or intentionally spread bacterial and viral infections.

Undoubtedly all that grew into complex medical problem, placing physicians face to face with the difficult task to carry out the treatment of their patients to successful and safe completion what is particularly observed in the course of acute, chronic and frequently recurring non-specific diseases of respiratory tract, SARS being an typical example in this respect.

The situation is getting worse nowadays because of:
– increasing air pollution
– increasing radiation phone

proved to affect negatively the different segments of the immune system and to increase the risk of sensitization of human organism to different allergens /23/. As a result it is well known that nowadays not less than 10-15% of the human beings are so called immunocompromized persons with damaged immune system and not able to overcome or are easily exposed to common infectious diseases /6/.

All above mentioned considerations are valid in full strength in the case
with SARS epidemics in 2003 for example, as according the information of WHO, CDC and all national health authorities the greatest part of the patients infected by SARS’ Corona virus and developed very severe clinical picture or died are elderly people or suffering from other chronic diseases and we might suggest that they have had suppressed/compromised immune system. Besides it is now proved that SARS’ Corona virus affects CD8 lymphocytes and by this way suppressed the cell mediated immunity.

It is clear nowadays that naturally occurring epidemics / the flu epidemics and the following secondary bacterial infections for instance /as well as newly immerging and mainly – intentionally provoked infection diseases as a form of bioterrorism continually threaten the health of the people of the world. This explains the concerns of the National public health authorities and the international efforts for effective approaches to strengthen the capacities of the health system in order to minimize the risk for the population of deliberately caused disease outbreaks by different bacterial and viral pathogens.

Having all this in mind one can conclude that because of the lack of a specific treatment and a specific vaccine for these infection diseases, the time required for creation and clinical testing of any vaccine or specific drug it is quite reasonable to consider the non-specific immunotherapy and the non-specific immunoprophylaxis based on the use of some immunostimulants reinforcing the natural immune mechanisms as a very promising approach.

In Bulgaria more than 18 years we are working very intensively on the elaboration, experimental study and clinical application of different “targeted” polybacterial immunostimulants for per oral administration intended to stimulate the natural mechanisms of the immune system and to help in recovering and prevention of the infections of respiratory and urine systems, oral cavity and parodonte.

Why did we choose to work with polybacterial immunostimulants composed of Gram-negative and Gram-positive bacteria?

That is because of their well proved mechanism of action in the organism:

– Gram-negative bacteria contain LPS, endotoxins, peptidoglycans, lipoproteins which stimulate macrophages, NK- cells, B- lymphocytes and antibody production and release of interferons and α- and γ- IL-2, IL-6.
– Gram-positive bacteria contain muramildipeptide, lipoteichoic acids, peptidoglicans which stimulate also phagocytosis, T-cell and B-cell function.
– Absorption of these components occurs through gastrointestinal mucosa and on the base of the integral function of the immune system via
GALT / Gut associated lymphoid tissue /is stimulated BALT/ Bronchial associated lymphoid tissue / as a part of the whole MALT / Mucosal associated lymphoid tissue /.

- Bacterial species entering the preparations stimulate the synthesis of homologous specific protective IgG, IgA and IgM antibodies. That means the polybacterial immunostimulants act also like bacterial “vaccines”.

- Finally it is very important that polybacterial immunostimulants are natural products consist of the bacteria which are part of the normal flora of the body.

These per oral polybacterial immunostimulants are composed of freeze-dried lysates and killed bacterial bodies of microbial species with greatest importance for the occurrence of nonspecific respiratory infections /Respivax/ or urogenital /Urostim/ or in oral cavity and parodont /Dentavax/. Their advantage is that they contain not only lysates but the body of the bacteria as in the cell walls are situated the greatest part of the antigens with well proved immunostimulating activity.

Of relevance to conditions with compromised immune system like HIV/AIDS, SARS, malignant diseases etc. would be our polybacterial immunostimulant called RESPIVAX for per oral immunotherapy and immunoprophylaxis of non-specific infections of the respiratory system in adults and children in wide prescription and clinical use now over 15 years in Bulgaria and recently in several other countries. Because of its well proved stimulating capacity on different cells and mechanisms of the immune system Respivax is very convenient to be used as a general immunostimulant in treatment and prophylaxis of newly immerging and intentionally provoked infectious diseases, when we have to rely mainly on the natural resistance of the organism to cope with them in waiting for specific diagnosis and to be developed specific treatment and/or vaccine.

Some of our experimental and clinical data presented here demonstrate clearly the immunostimulating capacity of Respivax.

On tabl. 1 and 2 is seeing the stimulation of antibodies to sheep erythrocytes and to human serum albumin in mice treated with Respivax. This adjuvant effect of the polybacterial immunostimilant is very important as it can be used to provoke better immune response to bacterial or viral vaccines when is administered simultaneously with them. We observed this effect in a study of the immunoresponse to routine revaccination against diphtheria in a group of 87 children. /4/. Paired sera were collected from 47 of them treated with Respivax 30 days before immunization / experimental group / and from 40 children treated
**Table 1.** Hemoagglutination antibodies titre against sheep erythrocytes in mice, treated with Respivax

| Test groups of 8 white mice treated with:                                                                 | Antibody titre after administration of the antigen (sheep erythrocytes) |  |
|----------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|--|---|---|
|                                                                                                                | Primary antibody response | Secondary antibody response |
|                                                                                                                | 7th day | 14th day | 7th day |
| RESPIVAX, perorally, 7 days before and 7 days after Ag                                                        | 1:512   | 1:1024   | 1:2048  |
| RESPIVAX, perorally, 7 days after administration of Ag                                                         | 1:256   | 1:512    | 1:2048  |
| RESPIVAX, s.c., 7 days before and 7 days after Ag                                                              | 1:512   | 1:2048   | 1:4096  |
| RESPIVAX, s.c., 7 days after administration of Ag                                                              | 1:256   | 1:1024   | 1:2048  |
| CONTROL GROUP - injected with Ag only                                                                          | 1:32    | 1:128    | 1:256   |

**Table 2.** Hemoagglutination of antibodies titre against human serum albumin in mice, treated with Respivax

| Test groups of 8 white mice treated with:                                                                 | Antibody titre after administration of the antigen (human serum albumin) |  |
|----------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|--|---|---|
|                                                                                                                | Primary antibody response | Secondary antibody response |
|                                                                                                                | 7-th day | 14-th day | 7-th day |
| RESPIVAX, perorally, 7 days before and 7 days after Ag                                                        | 1:64     | 1:256     | 1:512   |
| RESPIVAX, perorally, 7 days after administration of Ag                                                         | 1:32     | 1:128     | 1:512   |
| RESPIVAX, s.c. 7 days before and 7 days after Ag                                                               | 1:128    | 1:512     | 1:1024  |
| RESPIVAX, s.c. 7 days after administration of Ag                                                               | 1:64     | 1:256     | 1:1024  |
| CONTROL GROUP - injected with Ag only                                                                           | 1:8      | 1:16      | 1:64    |
with placebo /control group/. The serum samples were taken before immunization and 45-50 days after the administration of the vaccine.

From results on tabl. 3 is seen that the children treated with Respivax demonstrate a considerably greater increase in GMT / 8-fold / of anti-toxin antibodies and a higher percentage of re-immunized persons having a high level of this protective antibodies /85,1%/.

Ag - antigen - each mouse is injected i.v. with 0.2 ml of human serum albumin. On the 28-th day after the first administration the antigen is injected again in order the secondary antibody response to be followed up. RESPIVAX is administered in a daily dose of 2 mg/0.5 ml of physiological solution perorally or s.c. The antibody titre is determined by passive hemoagglutination with sheep erythrocytes treated with tannin.

### Table 3

<table>
<thead>
<tr>
<th>Studied parameters</th>
<th>Experimental group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; sample</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; sample</td>
</tr>
<tr>
<td>number</td>
<td>%</td>
<td>number</td>
</tr>
<tr>
<td>Studied children</td>
<td>47</td>
<td>100</td>
</tr>
<tr>
<td>Seroconversion</td>
<td>40 persons / 85,1%±10,2% /*</td>
<td>29 persons / 72,5%±13,8% /*</td>
</tr>
<tr>
<td>GMT</td>
<td>0,05</td>
<td>0,4</td>
</tr>
</tbody>
</table>

*=>0,05;  
**<0,05

On tabl. 4 is presented the protective effect of Respivax to a Staphylococcus aureus infection in mice. The results clearly reveal the immunostimulation activity of Respivax as it has favorable influence on non-specific immune mechanisms, which is manifested by the longer survival and lower mortality of the mice treated orally with the product in comparison with the control animals. It is seen that up to the 4-th day of the experiment all control mice / treated with saline instead of Respivax / are dead. The Respivax treated mice demonstrate substantially longer survival time and on day 4 only 14 mice / 50% / are dead. This indicates that the factors of natural resistance in the organism, contributing greatly to overcome the infection, are successfully stimulated by Respivax. One of this factors is obviously the phagocytosis which is stimulated strongly by Respivax as one can see from fig. 1 where is shown that after the administration of Respivax the phagocytic activity significantly is increased in guinea pigs / 24 /. Three days after the end of the administration of Respivax the percentage of phagocytizing cells started to increase and reached a maximum of 50% at the 21 day. Up to 28
day the percentage of phagocytizing cells of the treated animals was 10 times higher than those of controls – 49.5% vs 4.4% / p<0.001 / . These results clearly demonstrate that the oral administration of Respivax stimulates antibacterial functions of phagocytizing cells. This is obviously one of the factors explaining the demonstrative protective effect of Respivax in infected with Staph. aureus Sg 511 mice.

– Each experimental group is of 30 mice/ Suisse strain / with body mass between 15 and 18 gr.
– The mice of Group I are fed for 10 days with Respivax administered by pipette in a dose of 5mg in 0.5ml saline daily.
– The mice of Group II / control / are fed for 10 days with 0.5ml Phisiological sol. daily by pipette.
– After 10 days treatment with Respivax or Phisiological. sol. orally each animal is infected intraperitoneally with 25 million suspension in 0.5ml saline of Staphylococcus aureus Sg 511 strain, coagulase positive, pathogenic for mice.
– The survival of the experimental animals is followed up for 14 day.

In support of the presented data are also the results of the immunomorphological studies, which demonstrate the material substrate of the changes in the immune system under the effect of Respivax / Fig. 2,3, 4 /. It was established that after oral administration of the drug clearly expressed immunomorphological changes were observe, occurring in the lymphoid tissue associated with the intestines, bronchi, spleen and lungs – newly formed lymph nodes localized peribronchially, strongly activated lymphocytes and plasmocytes in mesenteron with well developed endoplasmic reticulum, but without any damaging effect on the intestines. As was mentioned a generalized immune response is unlocked which lays at the basis of stimulated non-specific and specific immune protection of the body.

The large clinical studies / including two placebo controlled studies / of

<table>
<thead>
<tr>
<th>Test groups of mice</th>
<th>Number of dead mice on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>30 mice fed with RESPIVAX 30 days</td>
<td>2</td>
</tr>
<tr>
<td>30 mice fed with Physiological saline 30 days</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 4. Protective Effect of Respivax Administered Orally to White Mice Infected with Staphylococcus aureus Sg 511
Respivax carried out the last 10-12 years clearly demonstrated its very positive effect on the different mechanisms of the immune system in humans and what is most important – on the clinical course of the non-specific respiratory diseases: chronic and recurrent bronchopneumonia, acute and chronic bronchitis and thracheitis, rhinitis /1, 2, 3, 8, 11,12,/. Especially favorable effect is observed during flu epidemics and in winter time when many other respiratory viruses are activated and Respivax is very convenient immunoprophylactic agent strengthening the natural resistance of the organism. From a double-blind controlled study carried out by Yossifov et al. /11/ on 50 children with recurrent acute bronchopneumonia one can see the favorable effect of Respivax with significant reduction of total number of inflammatory episodes, days with antibiotic treatment, days of stay in hospital and increase of the secretory IgA in saliva /tabl. 5 and 6/. The similar results – significant reduction in number and severity of respiratory episodes - are received by Iliev et al. /2/ in children with recurrent viral and bacterial pneumonia and bronchopneumonia treated with Respivax with demonstrative increase of phagocytic activity to the main bacterial strains responsible for development of respiratory diseases /tabl. 7/. 

In a study of 64 adults with chronic non-specific pulmonary diseases /CNPD treated 20 days of each of 4 consecutive months with 50mg Respivax daily/ Kisyova et al. /12/ find substantial reduction of number and severity of inflammatory episodes in comparison with the control group 3 months after the treatment /tabl. 8/. This clinical changes were accompanied with the increase of the titer of specific antibacterial antibodies /to the bacteria entering in Respivax/ of IgG, IgA and IgM classes.

Kojuharova et al. /3/ in placebo control trial find that Respivax is strong interferon inductor. They have studied 56 children and 30 adults divided in two groups: experimental - treated 30 days with 25mg /children/ and 50mg/ adults/ Respivax tablets daily and control- treated with placebo tablets. On the 3rd day after the treatment is assessed the level of the endogenous α-interferon and is observed four-fold increase of its titer in 86% of the treated with Respivax patients. These results demonstrate the capacity of one polybacterial immunostimulant in the treatment of viral diseases and in modulation of the immune reactivity based on interferon production.

The proved immunostimulating effect of Respivax on the cells of the immunocompetent system was the reason to study its action in the complex treatment of patients with HIV/AIDS /22/. Under the name Factor-R tablets we applied this polybacterial immunostimulant in 100 Americans from Texas with HIV/
Fig. 1. Percentage of Phagocytizing PMNC from Guinea Pigs Treated with RESPIVAX
Fig. 2. Small intestine of a mouse 5 times treated per os with 1/10 dose (5mg) of RESPIVAX; on the 7th day after the last treatment. Well preserved brush border. Magnification – 40,000 X.
Fig. 3. Lung of a mouse treated 5 times per os with 1/10 dose (5mg) of RESPIVAX; on the 7th day after the last treatment. New formed lymph nodes localized peribronchially. Staining – H.E. Magnification – 140 X.
Fig.4. Mesenterial lymph node of a rat 5 times treated per os with RESPIVAX (25 mg); on the 5th day after the last dose. Strongly activated lymphoid cells with numerous ribosomes in their cytoplasm and a plasmatic cell with strongly developed endoplasmic reticulum.
Magnification – 16,000 X.
DADS for a period of 6 months. They received every day 60mg Factor-R per orally and were monitored every three months for clinical and laboratory variables of efficacy of Factor-R. On the following fig. 6, 7, 8, 9, 10 are presented the results obtained from this study. As one can see Factor-R has a statistically significant stimulatory effect on different effector cells of host defence reactions. The increased or preserved level of monocytes and neutrophil granulocytes in 50 -to 60% of the subjects contributes to the activation of phagocytosis against infectious agent. This resulted in an increase of non-specific resistance against secondary opportunistic infections, helped by the demonstrative increased of IgA secre-
tory antibodies in 76% of all treated patients. The tendency to maintain and to increase the red blood cells and platelets provides beneficial effect on stabilizing the haemostasis of AIDS sufferers and hence prevents them from haemorrhages. The polybacterial immunostimolant practically has not any substantial effect on CD4 lymphocytes. But its impact on CD8 cells is very demonstrative and impor-

**Table 7. Dynamics of the phagocytic index in the blood serum of the treated with Respivax and the control group children**

<table>
<thead>
<tr>
<th>Treated with Respivax</th>
<th>Before Treatment</th>
<th>10th day</th>
<th>30th day</th>
<th>60th day</th>
<th>90th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Str. pneumoniae</td>
<td>0.720</td>
<td>1.060</td>
<td>1.120</td>
<td>1.100</td>
<td>1.040</td>
</tr>
<tr>
<td>Hem. influenzae</td>
<td>0.760</td>
<td>1.160</td>
<td>1.018</td>
<td>1.080</td>
<td>1.060</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>0.990</td>
<td>1.420</td>
<td>1.560</td>
<td>1.480</td>
<td>1.500</td>
</tr>
<tr>
<td>n = 50</td>
<td>n = 46</td>
<td>n = 44</td>
<td>n = 42</td>
<td>n = 39</td>
<td></td>
</tr>
</tbody>
</table>

p < 0.01

<table>
<thead>
<tr>
<th>Control group</th>
<th>Str. pneumoniae</th>
<th>0.790</th>
<th>0.820</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hem. influenzae</td>
<td>0.770</td>
<td>0.930</td>
<td></td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>0.785</td>
<td>1.070</td>
<td></td>
</tr>
<tr>
<td>n = 20</td>
<td>n = 18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p > 0.05

**Table 8. Clinical Effectiveness of Complex Therapy Including RESPIVAX in CNPD Patients**

<table>
<thead>
<tr>
<th>Endpoints recorded</th>
<th>With Respivax</th>
<th>Without Respivax</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>Shortening of antibacterial treatment duration</td>
<td>51</td>
<td>82.25</td>
</tr>
<tr>
<td>Recurrence within six moths</td>
<td>21</td>
<td>33.8</td>
</tr>
<tr>
<td>Treatment reduction</td>
<td>19</td>
<td>30.6</td>
</tr>
</tbody>
</table>
Fig. 5. The impact of Respivax on α-Interferon synthesis in children and adults

Fig. 6. After 6 months of Polybacterial Immunostimulant IgA

Important as these lymphocytes release a special factor suppressing the replication of HIV in CD4 cells /9, 14/. The substantial increased of CD8 cells of subjects with different duration of HIV positivity gives reason to consider that the treatment with Factor-R by means of its immunostimulating action, on the CD8 cell population, may lead to an extension and improvement of their life as well as contributes to transition of a number of subjects into long-term non-progressors.
Fig. 7. After 6 months of Polybacterial Immunostimulant Red Blood Cells

**Total 75 Subjects**

- Subjects with a decrease in red blood cell level (average % of decrease 20.1%) 13% (10)
- Subject's level of red blood cells not recorded 1% (1)
- Subjects with an increase in red blood cell level or remained the same (<10%) (average % of increase 22.1%) 86% (64)

Fig. 8. After 6 months of Polybacterial Immunostimulant Hemoglobin

**General population, n=53**

- No change (stable)
- Decrease
- Increase

- 13.21%
- 7.54%
- 79.25%
Fig. 9. After 6 months of Polybacterial Immunostimulant Platelets

Total 75 Subjects

Subjects with a decrease in platelet level (average % of decrease 20.7%)
21.3% (16)

Subject's level of platelets not recorded 1.3% (1)

Subjects with an increase in platelet level or remained the same (<10%)
(average % of increase 31.2%)
77.3% (58)

Fig. 10. After 6 months of Polybacterial Immunostimulant Monocytes

Total 75 Subjects

Subjects with a decrease in monocyte level (average % of decrease 48.9%)
46.7% (35)

Subject's levels of monocytes not recorded 1.3% (1)

52% (39)

Subjects with an increase in monocyte level or remained the same (<10%)
(average % of increase 107.2%)
52% (39)
Fig. 11. After 6 months of Polybacterial Immunostimulant CD$_4$ Cells

**After 3 months**
Total NR=100 (54<200; 46>200)

- 41% (41) decreased > 10%
- 59% (59) increased > 10%

**After 6 months**
Total NR: 75 (37<200; 38>200)

- 61.3% (46) increased > 10%
- 38.7% (29) decreased > 10%
Fig. 12. After 6 months of Polybacterial Immunostimulant CD₈ Cells

After 3 months
Total NR=100 (54<200; 46>200)

38% (38)
62% (62)

After 6 months
Total NR: 75 (37<200; 38>200)

32% (24)
68% (51)

↑ = increased > 10 %
↓ = decreased > 10 %
REFERENCES