

A Century of Antivenom

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ABSTRACT

Because it primarily affects the poor in undeveloped parts of the world where medical care is often inadequate and insufficient, envenomation is considered a neglected public health issue, despite the existence of antivenom therapy for more than a century. This article provides an overview of the epidemiological situation for important venomous animals, together with achievements in the production, control, technological progress and safety of antivenoms since their discovery.

Key words: antivenom, epidemiology, production, safety, standardization, stability, review

Introduction

Antivenoms fall into the group of animal immunosera for human use. Depending on their manufacturing process, they can contain whole antibodies (immunoglobulins) or parts of antibodies, Fab/F(ab')₂ fragments, specific for antigens of clinical importance, in this case venoms. Animal immunosera are used, in addition to antivenoms, as antitoxins, antisera against bacterial and viral disease causing agents, and as antilymphocytic immunoglobulins for induced immunosuppression. In addition to antivenoms, the majority of topics addressed by this paper, such as aspects of production, control and safety of use, are also applicable to other medicinal products from this group. The significance of antivenom is its efficacy in what are commonly life-threatening situations that primarily threaten large populations of people in undeveloped areas of the world. In many of these areas, medical assistance is often inaccessible following envenomation. Issues in the production, control and use of antivenoms, such as product purification, potency / activity assessment, standardization, serious side effects due to hypersensitivity to the heterologous components of animal blood, arose soon after their discovery, and many have not yet been fully resolved today. Alongside other factors, including those of an economic nature, such as rising costs of production of antivenom following modernization and quality control standards, logistical issues in the distribution and maintenance of a cold chain,

which are virtually impossible to avoid in the most threatened and impoverished areas where the competent authorities either do not recognize, pay little attention, or are unable to resolve the existing situation as a serious public health problem, production at the global level remains unprofitable. As such, the number of manufacturers of this group of medicines is constantly declining¹, resulting in poor accessibility of this life saving product. There are only a handful of producers, and they are often not able to meet the increasing quality requirements prescribed by pharmacopoeia, guidelines and other regulatory requirements and requirements of good manufacturing practice. Competent authorities, on the other hand, are obliged to monitor and control the implementation of technological progress in the production and improvement of the increasingly strict and demanding quality requirements with regard to maximum drug safety and efficacy. Global initiatives aimed at increasing and improving production and availability of antivenoms where they are most needed have been approved by many, but are primarily under the support and management of the World Health Organization (WHO). This paper provides an overview of the epidemiological situation for the most significant and most common venomous animals, and summarizes the results of studies relating to the production, control, technological progress and safe use of antivenoms, from their discovery to the present day.

History

Back in 1877, Fornara protected a dog after inoculations of toad skin secretions and that was the first official report of successfully performed immunization by repeated injections². Similar experiment was performed in 1887 by Sewall who demonstrated that by applying small but increasing doses of rattlesnake venom, it was possible to expose pigeons to doses of the venom more than seven times higher than the lethal dose, without any obvious effects³. Then in 1892, Kaufmann succeeded in reproducing Sewall's experiment using *Vipera aspis* venom on dogs⁴. This laid the foundation for the procedure of immunizing animals for the purpose of active production of specific antibodies. Not long passed until the practical application of this discovery was implemented, i.e. the application of serum to other, non-immunized animals exposed to venom for therapeutic purposes. Already by 1890, Emil von Behring and Shibasuro Kitasato published a paper on the first antitoxin against tetanus. Behring's continued research on the development of antitoxins against diphtheria brought him the Nobel Prize for Physiology in 1901⁵. These and similar discoveries launched a series of applicable studies in which the immunization of animals was used for the production of specific antibodies for passive immunization or serotherapy. Soon after, in 1984, Phisalix and Bertrand demonstrated antitoxic activity of the blood of animals immunised against *Vipera aspis* venom using heat – detoxified venom⁶. In 1895, Léon Charles Albert Calmette (12 July 1863 – 29 October 1933), a French physician, bacteriologist and immunologist, prepared commercial antivenom against the venom of the Indian cobra (*Naja naja*), obtained through immunization of horses⁷. Another important discovery that contributed to development of immunotherapy was development of toxoids i.e. formaldehyde detoxication method by Gaston Ramon in 1924⁸. Today, more than a century after the discovery of antivenom, the most significant research and discoveries are in the field of pharmacokinetics. These have aided in understanding immunotherapy, and in the procedures of purification, which has largely contributed to the safe use of antivenoms⁶.

All attempts to date in discovering, and the discovery of other potential types of medicines for therapy or prophylaxis, such as the use of specific peptides as vaccines or other forms of immunization against animal venom, have not yet been applied⁹. The main reason for the failure in the development of toxin-targeted therapy is the complex composition of venom, which contains hundreds of functionally different proteins which are difficult to purify and characterize. Some groups of authors have also worked on procedures to detoxify venom and apply the toxin as an agent that would cause a reaction of the immune system, thereby providing active protection^{10,11}. According to recent data, thanks to the use of modern bioinformatic technology, promising results have been achieved in the synthesis of multi-epitope DNA immunogens, used to obtain serum specific for the toxin in question and all its isomeric forms.¹² But for the time being,

antivenoms are produced in a similar manner as a century ago, though with much more modern technology. And still, these are the only effective method of specific therapy following a bite or sting from a venomous animal.

Venomous Animals – Epidemiology

Antisera are most commonly used against snake and spider bites and scorpion stings. There are about 600 species of venomous snakes in the world¹³. Though they are found in different parts of the world, the majority inhabit the tropical equatorial regions. Therefore, the incidence of snake bites and the consequential bodily harm or death is tied to those snake species inhabiting, for the most part, undeveloped parts of the world. An accurate number of cases of snake bites and their consequences is not known and is very difficult to determine because of a lack of reliable statistical data¹⁴, as hospitals are not commonly visited and traditional home treatment is often used instead^{15,16}. It can be assumed that about 2.5 million people are envenomed by snakes each year¹⁴, and of these 125,000 will die¹³. Research of J.P. Chippaux shows that although we are not at dispose of precise epidemiological data, many areas of the world, especially Asia and Africa are seriously endangered. For example, this research showed that there is around 1 million snake bites every year in Africa, involving 500,000 envenomations, of which approximately 20,000 ends fatally. Only about 10,000 of mentioned cases are reported by health services¹⁴. Also a good example of the extent of threat to human health and life can be also seen in India, where 250,000 people are bit each year, and of these about 50,000 people die¹³. Most recent and comprehensive research showed that globally, each year, at least 421,000 envenomings occur resulting with at least 20,000 deaths, but this figures could be as high as 1,841,000 envenomings and 94,000 deaths¹⁷. Of the fourteen species of snake found in Croatia, only two are venomous: the nose horned viper (*Vipera ammodytes*) and the adder (*Vipera berus*)¹⁸. According to data from Split Clinical Hospital, a total of 389 victims of bites from the nose-horned viper were admitted for treatment over a sixteen year period (1980–1996)¹⁹.

Even if the snake bite does not result in death, it can still have serious consequences, primarily due to the lack of treatment or treatment received too late. Serious invalidity (dysfunction or loss of limbs) or diseases such as kidney failure, osteomyelitis with malignant transformations, intracranial bleeding, thromboses and the like can result¹³.

Other significant animal species are scorpions. Of the some 1500 species of scorpion, about 30 species are harmful to humans, as they produce venom in sufficient quantities to cause systematic organ failure or death. There is a significant difference in incidence and severity of scorpion envenomation in different areas of the world, depending on species prevalence. The most endangered areas in which we can find the most venomous species are some parts of Africa, Near and Middle east, South India,

Mexico and South Latin America. There are also difficulties in estimation of incidents due to same reason as in the case of snake bites, but mortality data are better known. Some attempts for better control mechanism, including improvement of case recording, first resulted with the »increase« of incidence showing that existing epidemiological data for scorpionism only partially reflect real status and that further study is needed for more realistic data^{20–22}. In Mexico for example, scorpion stings are among the leading public health issues, with more than 250,000 stings recorded *per year* and hundreds of deaths ensuing²³. At the global level, the annual number of scorpion stings exceeds 1.2 million leading to more than 3250 deaths²⁰. The global data indicate that despite of geographical limitation, scorpionism is public health concern.

There are about 200 species of spider worldwide that produce venom that can cause serious harm to humans after a bite. Epidemiologically, it is difficult to pinpoint the number of cases due to difficulties in obtaining a diagnosis as it is not uncommon for the patient to be unaware of the spider bite²⁴. The most important venomous species are the widow spiders (*Latrodectus spp.*), recluse spiders (*Loxosceles spp.*), funnel-web spiders in Australia (*Atrax* and *Hadronyche spp.*) and the Brazilian wandering spider (*Phoneutria spp.*)²⁵. The black widow *Latrodectus mactans tredecimguttatus* is the most venomous spider in the Croatian coastal region. Since 1948, till the end of 1980s, Maretić studied intensively *Latrodectus spp.* ant their toxin^{26–28} and participated in development of the first european anti-latrodectus serum in 1951. Thanks to health care education, which has increased the ability of the locals to recognize the black widow in its habitat, the incidence of latrodectism in Croatia is relatively low²⁹. In the Zadar region, which is the primary locality of this spider species in Croatia, a total of 30 cases of black widow bites were reported from 1998 to 2006³⁰.

As it can be seen from presented, epidemiological data like incidence and severity of envenomations, are not easy to obtain, but aiming to get more precise data is crucial for identification of adequate needs for antivenom production.

Production of Antivenom

Production procedures

The first step in production is the collection of venom, which represents the antigen for immunization of animals whose serum is then used to produce the antivenom. Snake venom can be collected mechanically by applying pressure to the venom gland, i.e. active milking, by hand or using a specialized apparatus, or by electro-stimulation of the muscles surrounding the gland⁴. Venom is also collected from spiders and scorpions by mechanical or electrical provocation, in which the animal bites/stings the provocation material (e.g. a tube) and injects its venom into it³¹.

The venom is dried in an dessicator using accelerators or by lyophilisation or freeze-drying, which gives crystallized or powdered venom which can be held at refrigerated temperatures for many years^{32,33}.

Considering that the strength of the venom is reduced after the first collection, establishment of the proper collection interval is essential in order to achieve the immunization of the animal with venom of approximately equal strength. This facilitates product standardization, which is an important objective. According to some data, a period of four weeks between collections is sufficient to ensure venom of equivalent strength⁴, however, this needs to be established on a case by case basis, as it is dependent on the type of animal and venom, and on many other factors.

The composition of venom is exceptionally complex and contains many complex molecules such as proteins, enzymes and peptides, as well as many smaller molecules. This demands the use of very sensitive techniques to separate the antigens from the remaining components. These methods must also be adapted to the large variations not only in the composition of various venoms, but also in the size of the venom sample that can be obtained, given the type of animal producing it³⁴.

Variations of venoms are present at many levels: interfamilial, intergenus, interspecies / subspecies, intraspecific, geographical, seasonal, diet / habitat, age and sex variations³⁵.

In compliance with the European pharmacopeia³⁶ and the EMEA guidelines³⁷, immunization antigens must be identified, characterised using chemical and biological methods and must be free extraneous agents, especially virus contaminants. Following this, the animal is immunized using an increasing dose of the venom. The animal of choice is the horse, primarily due to the fact that it can give the largest quantity of serum. A litre of horse serum can give 100 mL of snake antivenom. Horse plasma can be taken once every four weeks, and the animal can be used for this purpose for about six years³⁸. Other animals are also used, such as sheep, goats, donkeys and rabbits. In recent years, research has shown that antibodies obtained from the yolks of eggs of hens immunized with snake venom have successfully neutralized the pharmacological effects of the snake venom^{39,40}, however their safety and efficacy required further study.

After a period in which the immune system produces the specific immunoglobulins in sufficient quantities, the next step is the sterile collection of plasma, primarily through the procedure of plasmapheresis. In this process, the plasma is separated from other blood cell elements, which are then returned to the animal's circulatory system. The plasma is stored at a temperature of –20°C or lower until further use.

After the discovery of the principle of serotherapy at the end of the 19th century, humans were treated with unprocessed and unpurified immune serums. Their use resulted in numerous and, not uncommonly, fatal reactions due to a hypersensitivity to proteins, particularly

albumin. It was considered that these, and not globulin, were the most responsible for the side effects. According to the literature on immunosera from the early 20th century, it could be concluded that these sera were often as dangerous as the venoms themselves⁴¹. This led to a number of important advancements in production, particularly the process of purification, aimed at obtaining an effective, but safe antiserum. Today, the majority of manufacturers use methods that isolate and concentrate immunoglobulins or their active fragments yielded with enzyme digestion on the basis of salt precipitation, procedure published by Pope 1939⁴². If these are medicinal products containing the whole immunoglobulin G, any undesired proteins could be removed through precipitation using caprylic acid (described first time by Chauntin and Curnish in 1960⁴³) and ultrafiltration^{44,45}. In the use of active fragments, fragmentation is achieved through digestion using proteolytic enzymes. With the activity of protease (pepsin, papain) at low pHs and temperatures of 20–25°C, the immunoglobulin molecule is split into Fc (fragment constant) and Fab/F(ab')₂ (fragment antigen binding) fragments^{42,44}.

The Fab/F(ab')₂ fragments, which are the bioactive parts of the molecule with specific binding properties for a given antigen, are separated from the inactive Fc part of the molecule. Most manufacturers achieve the separation of these fragments using sedimentation with salts, primarily ammonium and potassium sulphate due to their ability to precipitate globulins, but not albumins. Efficiency of this procedure can be improved by combination of caprylic acid and salt precipitation which was demonstrated in 1990⁴⁶.

This procedure is further followed by heat denaturation at 55°C for ten or more minutes. During this time, the undesired thermolabile Fc proteins coagulate, and the thermostable Fab and F(ab')₂ remain⁴⁷. The denatured proteins can be removed by centrifugation and filtration, while the ammonium sulphate is removed from the sediment by dialysis or ultrafiltration. A small number of manufacturers, use additional, more sophisticated purification methods such as ion-exchange and affinity chromatography⁴⁸, especially since the adaptation of firstly developed methods in 1970s⁴⁹ to an industrial scale⁵⁰.

Chromatography methods can be used alone or in combination with precipitation using caprylic acid.⁵¹ Agglutination with aluminium hydroxide gel is an appropriate method for the removal of lipids. Final product can be in a liquid form or lyophilised. Longer shelf life is attributed to lyophilised antivenom which is a great advantage to this type of pharmaceutical form, considering unfavourable climate conditions and poor distribution organization in endangered areas. Inappropriate lyophilisation and inadequate solubility can result with denaturation and loss of stability and activity. Lyophilisation process also increases cost of production significantly⁴⁸. The majority of commercially available immunoserums are in liquid form and contain preservatives, primarily phenol, cresol or thiomersal^{52,53}. Though with today's advanced

technology and the principles of good manufacturing practice, the use of preservatives in medicines should be avoided wherever possible, their presences in immunosera is still accepted, considering funds that would be necessary to advance the production and secure distribution of antivenoms.

Critical phases of production and their control

Critical phases of production that can be singled out are the procedures of enzyme digestion at low pH values, the procedures of selective heat denaturation and procedures of antivenom purification. The conditions of these procedures must be strictly controlled in order to obtain a product of the desired quality. Proteolytic enzymes of undesirable composition and/or activities, and the differences in quality among batches can be the cause of inadequate breakdown of the immunoglobulin molecule. This can result in an increased incidence of side effects⁴⁸.

During digestion, it is necessary to ensure low pH values. It has been shown that very small differences in pH values, together with the length of time of the digestion itself, can have a significant effect on the immunoglobulin fragments and their biological activity⁵³. The activity of heat in the process of denaturation of undesired proteins, unless controlled appropriately, can result in the loss of active substance (excessive denaturation) or inappropriate product purity (insufficient denaturation). The concentration of salts that precipitate and settle out the globulin protein fragments is also an important factor requiring strict control, as it directly impacts product purity. The purification procedure also removes general impurities such as pyrogenic substances, and undesirable heterologous proteins that cause side effects. It is logical that the use of specific, purified and concentrated antibodies or their parts (production of plasma with high specific activity) reduces the total intake of protein taken with the medication. Therefore manufacturers of these parts of the production process must carefully validate and establish both consistency and standardization, particularly in the sense of activity and purity of the obtained product. This is carried out through quality control according to the established requirements confirmed by the competent authority. The majority of these procedures are important for the safe use of antivenoms.

Pharmacokinetics/Pharmacodynamics

Due to differences in the molecular mass of the active compounds (whole IgG – 150 kDa, F(ab')₂ – 100 kDa; Fab – 50 kDa), different types of antivenom have different pharmacokinetic properties. The Fab fragments arrive first into the extravascular space and have the shortest elimination time through the kidneys. IgG survives for longer in tissues and is excreted extrarenally. The adsorption and elimination of F(ab')₂ fragments is somewhere in between the two previously described types.⁵⁴ The complexes that result between the active compounds of antivenom and the venom are not excreted through the kidney due to their high molecular weight. Com-

plexes of venom with F(ab')₂ fragments have been observed to be excreted from the body slower than free venom, but faster than the free fragments, which suggests that these complexes are eliminated from the body by phagocytosis⁵⁵. Based on test results, F(ab')₂ fragments have the best pharmacokinetic properties. For this reason, and due to the lower incidence of side effects, which is greatly due to absence of Fc fragments responsible for complement activation, F(ab')₂ fragments are most commonly used⁵⁶. Some authors claim that their efficacy and safety is significantly dependent on the type of animal used in the production of antivenom. Fab fragments obtained from sheep serum have proved to have an equal effect as F(ab')₂ obtained from horses, though with a lower incidence of side effects⁵⁷. However, it has been proven that the neutralization effect of Fab fragments is transient and that repeated application of the antivenom is necessary within a few hours. On the other hand, a single injection of a medication containing F(ab')₂ neutralizes venom for several days⁵⁸. This is due to the shorter elimination half-life ($t_{1/2}$) of the Fab fragments (4.3 hours) in comparison to F(ab')₂ fragments with a $t_{1/2}$ of 18 hours⁵⁹. In line with the newest research on pharmacokinetics of the venoms themselves, which have shown that their kinetic profiles vary greatly, it is clear that the efficacy of the antivenom and the selection of the active compounds depends greatly on the characteristics of the venom⁶⁰. Venoms of snakes from the *Viperidae* family characteristically contain toxins of high molecular weight. These are first quickly absorbed but later switches to a very slow absorption from the site of venom injection. For that reason, clinical symptoms can reoccur, even after the level of antivenom in the blood drops⁶¹. Venoms of snakes from the family *Elapidae* and scorpions contain neurotoxins of low molecular weight which very quickly and easily enter into tissues and have a high volume of distribution. Therefore, it would be ideal for the pharmacokinetic characteristics of the antivenom to appropriately follow the pharmacokinetic profile of the venom⁵⁴.

Safe Use of Antivenom

It is most important to focus on safety considering viral contamination and undesirable effects, i.e. side effects, of these medications. Both these aspects of safety, generally in the production of medicinal products, can best be achieved through product standardization and ensuring consistency in quality and efficacy of batches. Consistency control in the sense of quality implies laboratory characterization of batches using a series of validated procedures for the purpose of proving purity, identity, the content of immunoglobulin, quantity of aggregates, etc.

Safety considering viral contamination

Due to their biological origin, and even more so due to the use of biological materials in production, there is an objective danger that biological medications may contain causative agents of infectious diseases that are danger-

ous for humans. The best known case is the transfer of the SV40 virus in a contaminated poliomyelitis vaccine in the period from 1955 to 1963 from the cells of the monkey from which the vaccine was produced^{62,63}. About half a century later, the virus began to be connected to the etiology of malignant disease⁶⁴. Therefore, the world and European guidelines for the production of biological medications, including immunosera of animal origin^{65,37} stipulate strict quality control testing for viral contamination, beginning from the production animal and all the way to the finished medicinal product. Financial reasons are the main factor for the failure to carry out these procedures, or to carry them out entirely, in the majority of manufacturers. Considering that no cases of viral transfer through immunosera have been recorded⁶⁶, and considering the fact that individual parts of the production procedure have exceptionally strong antiviral effects⁶⁷, it is usually evaluated and concluded that the benefits from their use is significantly higher than the risk of contracting a virus. However, bearing in mind the epizooties that can affect horse breeding, and the zoonosis caused by equine diseases (i.e. Hendra and West Nile virus) that do appear⁶⁸, and in line with the progress in production technologies and medicinal product control, it is necessary to conduct contemporary evaluation of the viral safety of antivenoms and conduct appropriate control accordingly.

Considering that the study of virology of large animals has not been exhausted, and that animals for the production of antivenoms cannot be bred as SPF (specific pathogen free) colonies, it is important to abide by the proper breeding principles with constant monitoring, treatment and prevention where needed, in order to maintain the good health of the animal used for production. In order to ensure that these animals are not vectors of disease causing agents, different methodologies should be used, such as occasional serological testing, pathological examination, and more to continually monitor their health status. Second, but no less important, during production it is necessary to use procedures for the purpose of inactivation and elimination of viruses. In comparison with medicinal products from human plasma, the majority of medications made from animal plasma have not yet undergone procedures for this intent, such as solvent-detergent (S/D), pasteurization or nanofiltration, while their production is largely not validated with regard to viral safety⁴⁸. However, the specific parts of animal immunosera production process disable viruses by inactivation or facilitate their elimination, contributing to the viral safety of these medicinal products.

In particular, this is the activity of the low pH and concentration of pepsin required for the appropriate proteolytic breakdown, and the activity of caprylic acid. It is necessary to bear in mind that these procedures were developed earlier, and validated by manufacturers of human blood and plasma derivatives, and that their antiviral activity was confirmed for these products^{69,70}. In the 1980s, it was proven that exposure to low pH (about 4) greatly contributes to the inactivation and

elimination of viruses from medications containing human immunoglobulins⁷¹, while later this activity was confirmed independently and in combination with other procedures targeted at viral inactivation, i.e. cold ethanol fractionation^{72–74}. It has been proven that caprylic acid, used in the purification of immunosera, in combination with elevated temperatures and low pH values in human immunoglobulins and solutions of human albumin has virucidal properties and the ability to inactivate viruses with a lipid envelope. The inactivation factor was significant, at more than 4 log(10) for the most resistant viruses tested^{75,76}. However, it should be noted that caprylic acid has no effect on viruses without an envelope, and early studies conducted in general for unsaturated fatty acids showed that the inactivation effect is lower in products containing a high amount of endogenous lipids, such as whole plasma and unprocessed plasma fractions, as in the case with the processing of animal antivenoms with caprylic acid⁷⁷. Elevated temperature is another factor that stimulates inactivation through the activity of these mechanisms. Considering that production of animal immunosera to date do not involve temperatures proven to inactivate viruses, such as pasteurization which is used for the production of human immunoglobulins, research was recently carried out on modified production procedures and the processing of horse serum that included exposure to 60°C temperatures over 10 hours. The results confirmed viral inactivation efficacy of pasteurization, low pH and caprylic acid in equine serum, as it was already proven for medicines from human blood and plasma. The same research showed that a combination of the usual procedures with pasteurization ensures protection against viruses that can otherwise survive treatment with low pH and caprylic acid solely. As such, the reduction factor for lipid-enveloped viruses in this procedure was more than 9 log (10), and for those without an envelope was 4 log (10)⁷⁸. External contamination of plasma is prevented during plasma collection using a closed system of plasmaphoresis. At a workshop of the WHO held in London in 2001, it was proposed that manufacturers should carry out a pilot-study that would be considered a standard method and confirmation of validation in the sense of removal and activation of viruses during production process standardization.

Burnouf et al., in an article published in the journal *Biologicals* in 2004, suggested that validation studies should be carried out in which known models of viruses would be used. These would give results that could tell of the speed and degree of inactivation during planned inactivation phases in the production process. The selection of the viral model should be discussed by virologists, and similar studies carried out on human blood and plasma derivatives should be taken into consideration⁶⁶.

Undesired effects/ side effects

The most common side effects following antivenom treatment are immunological reactions to the heterologous (animal) serum proteins. Individual studies have shown that antivenoms produced by the immunization of

camels have a smaller incidence of these types of reactions as their ability to activate the complement is weaker. However, it has also been proven that the antibody titre in human serum after treatment with camel antivenom was lower in comparison with titre achieved after application of antivenom produced from horses and sheep^{79,80}. The results of new studies in the use of human monoclonal antibodies are promising and could in the future become an adequate replacement for heterologous animal serums^{81,82}. Early (direct or delayed) immunological reactions are caused by the IgE antibodies. These results with the degranulation of mast cells and basophils and release of inflammatory factor and histamines, which in turn leads to a disbalance in the circulatory system with vasodilation, vascular permeability, smooth muscle spasms and local inflammation. Consequences can be serious such as urticaria, oedema, drop in blood pressure and asthma. In serious cases when the antigen is present throughout the entire system, anaphylactic shock may ensue⁸³. Further, there may be immunological reactions that arise due to activation of the complement.

The causes of complement activation can be Fc fragments of the heterologous antibodies, proteins/protein aggregates and the formation of immune complexes⁸⁴. Following first two causes, it is important to stress that though a significant difference in the efficacy of medicines containing whole immunoglobulin or their fragments has not been proven⁸⁵, and data on side effects for both types of antivenom are controversial, many authors indicate that purified whole immunoglobulin G cause a greater number of undesirable effects^{86–88}. Excessive proteins in general and the creation of protein aggregates is increased following protein digestion at low pH values⁸⁹, therefore an effective and validated purification procedure is essential.

Later reactions appearing after 1 to 15 days can be seen in local or generalized urticaria, fever and arthralgia. This reaction is often called serum disease. It is an immunological reaction of the body to the accumulations of the antigen/antibody immunocomplex that stimulates an inflammatory response in the body and activates the complement^{90,91}. The use of various diagnostic tests to establish whether the person is sensitive has not proven to be fully applicable, as even following a negative diagnostic test, unexpected side effects have occurred. Emergency medicine physicians at the Faculty of Medicine at the university hospital at Ribeirão Preto, University of São Paulo, published their successful results in the prevention of the appearance of side effects through the use of antihistamines and corticosteroids at the time of application of heterologous animal immunosera⁹². If the diagnosis is made on time and the proper treatment is received, which also implied good medical conditions, the consequences and prognosis for side effects to animal serums are good⁸⁴.

In addition to immunological reactions to animal proteins, non-allergic reactions such as pyrogenic shock is possible from insufficiently purified serums containing bacterial endotoxins^{84,52}. Bacterial endotoxins have an

affinity for binding to proteins and it is necessary to apply the appropriate procedure for their removal, particularly since classical methods such as ultrafiltration in the decontamination of water are usually not sufficiently effective for protein solutions⁹³. This fact is important considering that the production of antivenoms includes the procedure of concentrating proteins. This can lead to an increased concentration of endotoxins. It has also been shown that due to the concentration of endotoxins in the precipitate, their concentrations are increased following the procedure of sedimentation of ammonium sulphate in comparison with the activity of caprylic acid⁹⁴. In addition to fever and general symptoms, in higher concentrations they can induce septic shock^{95,96}.

As can be seen from the work cited in this chapter, through the implementation of specific production procedures it is possible to reduce the incidence of side effects to antivenoms. Therefore, it is in the interest of safe use and the reduction of side effects, to improve production in the sense of selecting the most appropriate production procedures, the development of better purification procedures and standardization of the total concentration of proteins.

Standardization

From the regulatory aspect, the standardization of the venom, the antivenom itself, procedure and control procedures is necessary for the estimation and maintenance of quality, efficacy and safety of the antivenom between batches. However, during research in this field, numerous difficulties have arising in meeting the high regulatory criteria.

Research on animal venoms, particularly on snake venoms, is primarily carried out in order to characterize the important components of the venom that are responsible for body impairment and death, for the purpose of finding the most effective treatment strategy. Numerous papers in this field have resulted in the isolation and characterisation of many venoms with regard to their physical and biological properties, and their biological activities^{97–104}. In recent years, proteomic analysis has played an important role and proven to be a useful tool in generating knowledge. In these fields, the name snake venomomics has already emerged for this type of research^{105–107}. Research results have proven, though not explained, the exceptional variability of venoms, including of course their pharmacological properties, even within species, depending on geographical areas, age of the animal and many other factors^{108–111}. This has made attempts to standardize venoms for animal immunization very difficult, though this standardization would contribute to the standardization of production of antivenoms that are not proving to have equal efficacy towards variations of venoms of the same species¹¹². The reasons for difficulties in this type of research are very likely the result of the exceptional composition complexity of the venom which contains numerous complex molecules, such as proteins, enzymes and peptides¹¹³.

The next very important factor is the testing of the potency and biological activity using appropriate methods, by which it is possible to quantitatively express the neutralization activity of the antivenom on various effects of the venom, such as death, necrosis, haemorrhaging¹¹⁴, neurotoxicity¹¹⁵, coagulopathy¹¹⁶ and the like. Considering various effects of the venom, there are various methods that are used, but the most commonly used is one recommended by the WHO (and prescribed in the European Pharmacopoeia) – testing the neutralization of the lethal effect on mice. In order to standardize these types of methods, it is necessary to have local reference preparations of venom and antivenom. However, in 2003, there was only one standard for snake antivenom in the world, the 1st International Standard for antivenom for the *Naja and Hemachatus species*, produced from equine serum and established in 1964⁴⁸. In 2006, Fukuda et al. published a paper on the standardization of the regional referential standard for the *Mamushi* snake (*Gloydius blomhoffi*), which is used in the area of Japan, Korea and China¹¹⁷. In addition to the establishment of reference materials, the problem with testing potency is the use of animals, due to the more active application and stricter demands of the 3R principle. Due to its ethical principles, this is inclined towards the reduction and replacement of the use of laboratory animals in general, including routine drug testing. Therefore, many scientific studies have set out in that direction, setting high standards concerning sensitivity and reproducibility. Good results in the sense of simplicity, speed and economics, as well as good reproducibility, were achieved by Pradhan et al., who developed a method to test the ability of antivenoms to neutralize cobra venom through passive haemagglutination and the inhibition of haemagglutination¹¹⁸. Immunoassays have also proven to have good results in many studies^{119,120}.

In 2001, Sells et al. published a paper on a potency testing method using fertilized chicken eggs in the phase of embryonic insensitivity and they recorded the lethal effect of venom to embryos. A comparison of this method with the conventional ED₅₀ method on rodents gave a strong correlation¹²¹.

When defining antivenom potency by determination of its capability to neutralize venom effect, clear difference should be made between lethal and other, specific effects of venom like dermo- and myonecrosis, edema, hemorrhage and inflammation at the site of the bite, because neutralisation of these effect does not mean the neutralisation of lethal effect, which is determined by lethal *in vivo* test in mice.

Stability

Even though appropriate stability testing is regulatory requirement and is an important parameter for the quality of any medicinal product, the issue of stability in antivenoms is imperative. This is not only due to the fact that this is a biological medicine containing complex molecules inclined to change, but also due to the distribution

and application of these medications in undeveloped regions where inadequate medical conditions of keeping and storing these medications and the possibility of breaking the cold chain are not negligible factors. It is known that exposure to elevated temperatures stimulate the formation of protein aggregates. The use of stabilizers such as sorbitol and phenol could reduce this negative effect^{121,122}. Studying the effects of the most commonly used preservatives (phenol and thiomersal) on antivenom properties have proven that their presence increases the formation of aggregates and dimers, and that these preparations can lead to activation of the complements *in vitro*¹²³, weakening of the interaction between leukocytes and endothelial cells, and even the increase or cause of the appearance of certain side effects¹²⁴. In light of this, the safety of these auxiliary compounds in these medications should be re-evaluated.

In comparison to the liquid form, lyophilisation gives a product that is stable for a longer period of time at room temperature or elevated temperatures and has an overall longer expiry period⁴⁸. However, one should not neglect the fact that the procedure of lyophilisation increases production costs, requires the use of additional testing in process controls and quality controls. Decrease in neutralising potency has also been noted in some cases following lyophilisation¹²⁶.

Availability of Antivenom

Despite persistent envenomation problem, as described in chapter Venomous Animals –Epidemiology, the availability and use of antivenom started to decrease in last few decades of 20th century and this trend still exist¹²⁷. For example, although incidence of snake bite didn't decrease in Africa, the number of antivenom commercialised before approximately 25 years dropped from 250,000 doses / year to less than 20,000 vials¹²⁸. The reasons for that are complex. One already mentioned is inconsistency and lack of precise epidemiological data. Increasing costs of improved production technology and rising quality control standards also have negative effect on commercialised manufacture of this type of medicinal product¹²⁹. Finally, poor organization of health services and medicines distribution, deficient, inappropriate and non-standardised treatment protocols are recognised and

not easy solving problems in endangered areas, which contribute additionally to current situation. Possible solutions require collaboration on international level and involvement of many structures including manufacturers, state support, sponsoring, local healthcare providers etc.

Conclusions

By reviewing the current state of production and use of antivenoms, the conclusions of many authors and the World Health Organization are confirmed: despite the fact that envenomation still represents a serious public health threat in many parts of the world, even though it is 21st century, it is a widely neglected problem and improvements in this area are moderate. The most significant problem is the side effects that arise as reactions to the heterologous proteins. A summary of the results of numerous studies can lead to the conclusion that working towards improving the quality of existing preparations is a goal worth striving for, and that quality in relation to the type of origin of the product is the most significant factor in reducing the appearance of undesired effects. Improvement of quality bears a burden of significant cost rise, placing antivenoms in a group of very expensive drugs. Price of a single bottle of antivenom, together with distribution problems, lack of epidemiological data, poor healthcare organization and education are the main reasons that these life saving drugs are not available in proper quantity in countries where they are needed. Worldwide cooperation among manufacturers and regulatory authorities is required in order to ensure accessibility and the safe and effective use of antivenoms, particularly in developing countries. Considering the wide gap between regulatory requirements and the possibilities and profitability of manufacturing processes, one must not forget that the final role of both – the manufacturer and the competent authority is directed at human life protection. Even today, more than one hundred years since the production of the first antivenom, its timely application is still the only effective treatment following envenomation. Antivenoms should be available to all requiring them, independent of the standard of the part of the world in which they live.

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STOLJEĆE ANTIVENOMA

SAŽETAK

Iako specifična terapija u obliku primjene životinjskih antiseruma postoji više od jednog stoljeća, otrovanja koja su posljedica ugriza i uboda otrovnih životinja smatraju se zanemarenim problemom javnog zdravstva, budući se uglavnom događaju u siromašnim i nerazvijenim dijelovima svijeta u kojima je medicinska pomoć i njega neprimjerena i nedostatna. U ovom članku dan je pregled epidemiološke situacije za važnije otrovne životinje, dostignuća u proizvodnji i kontroli, tehnološkom napretku te sigurnosti primjene antivenoma od njihovog otkrića.