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Review Article

Progress and improvement of the manufacturing process of snake antivenom

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ABSTRACT

Antivenoms have been used successfully for more than a century and up to now constitute the only effective treatment for snakebites .The production of antivenin started long time ago when the calmette was prepared the antivenom in 1894.The method currently used to prepare antivenom by most of the manufacturers are originated from the method of Pope which was develop in 1938. Several new approaches in the production of antivenom have been proposed to produce IgG, F(ab)2, F(ab) antivenin to improve their quality .These improvement include complete or partial modification in the antivenom production regarding animal, immunization protocols , new adjuvants in hyperimmunization of animals , purification processes (caprylic acid ,chromatography , diafiltration and ulterafiltration),enzymatic digestion of IgG (pepsin, papain) and fractionation of venom .When the IgG is digested enzymatically, different fragments are obtained depending on the enzyme used, that is, if papain is used, three fragments are obtained, the crystallizing fragment (Fc) and two antigen-binding fragments F(ab) and, if pepsin is used, one $F(ab')_2$ fragment is obtained, while the crystallizing fragment is digested. Fab and $F(ab)_2$ fragments conserve their capacity to specifically bind to the antigen that gave rise to them.

Keywords: Antivenom, purification, IgG, $F(ab)_2$, F(ab)

INTRODUCTION

The mortality rate from snakebite have been reported to range from 50,000 per year (Swaroop & Grab 1954) to 100,000 per year (Chippaux 1998). However, these largely hospital-based figures are likely to be underestimates, as the majority of snake bite victims seek traditional treatment and may die at home unrecorded. The few attempts to carry out properly designed population-based studies have revealed unexpectedly high rates of mortality, ranging from 2 to 16 per 100,000 per year in Nigeria, Kenya, Senegal and West Bengal (Warrell 1999).

In eighteenth century an effective therapeutic serum against diphtheria and tetanus were developed by (Behring & Shibasaburo Kitasato 1890) for human use. The first successful therapeutic serum treatment of a child suffering from diphtheria occurred in 1891. During the first few years, there was no successful breakthrough for this form of therapy, as the antitoxins were not sufficiently concentrated. Behring and his coworkers prepare the serum in industrial scale by using large animals – first sheep and later horses (Behring & Shibasaburo Kitasato 1890).

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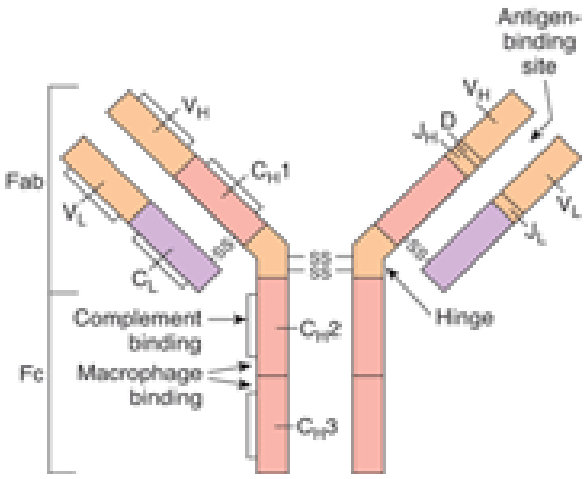

Figure 1. Immunoglobulin G structure.

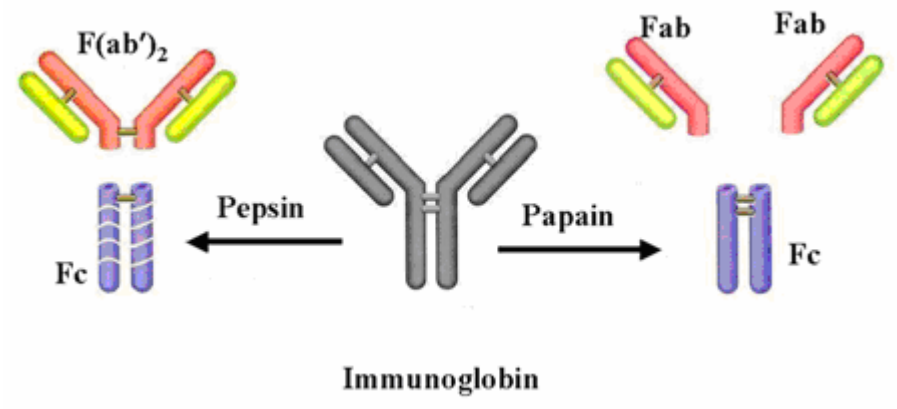

Figure 2. Different fragments of IgG obtained by pepsin and papain digestion.

The first antivenom was prepared by Calmette on the basis of Behring and Kitasato studies in 1894 (Calmette 1894). The production of the antivenom began by the end of eighteen century. Unrefined heterologue antivenom cause serious reaction and was very dangerous for human use (Russell & Scharfenberg 1962). To overcome these problems, through years a number of improvement in antivenom production have been made by researcher to prepare a high quality antivenom with less unwanted reaction (Russell 1989). The methods nowadays used by all antivenom producers is based on salting out to precipitate specific protein with or without enzyme digestion .The technique used for production of antivenom originated by pope (Pope 1939, Grasset and christensen 1947, Harms 1948, Pope and Stevens 1951) and later modified by several worker (Latifi & Manhouri,1966) for the purification , isolation and concentration of the specific antibody and were later applied to snake antivenom. It is based on the assumption that the albumin, rather than globulin fraction may be responsible for most of the undesirable side effects.

Historical improvement in purification process of antivenoms. There are several kinds of immunoglobulins, known as IgG, IgM, IgD, IgA and IgE, of which IgGs are the most abundant in the blood

circulation. IgGs correspond to a mature immune response and therefore include the vast majority of antibodies that are commercially produced. All the IgGs have the same general structure (Figure 1).

They are composed of four polypeptide chains, two that are heavy (H) and two light (L), which are joined together by disulfide bridges (Consroe P *et al* 1995).The anaphylactic shock and serum sickness reported after administration of equine freeze-dried IgG antivenom (Kitchens CS and Vanmieroplhs 1987). Although the probability of anaphylaxis depends on the patient's sensitivity, the production laboratory can implement various techniques to minimize the occurrence of this adverse reaction. The presence of impurities in antivenoms increases the possibility of anaphylactic shock due to IgE antibodies against these substances (mainly other heterologous animal proteins). Sera not completely purified, or with excessive total protein, can contribute to the development of this reaction. To overcome the side effects, most of the manufacturers introduced an enzymatic digestion step in their antivenom purification procedures to split Fc portion of the IgG molecule that is thought to be responsible for allergic side effects (Morais & Massaldi 2009, Theakston *et al* 2003).

When the IgG is digested enzymatically, different fragments (Fab and $F(ab')_2$) are obtained depending on the enzyme (Figure 2). Fab and $F(ab')_2$ fragments conserve their capability to bind to the antigen that gave rise to them. $F(ab')_2$ fragments also precipitate antigens, while the Fc antibody fraction normally acts as a marker signal.

The Fc fragment comprises the antigenic determinants of the antibody in such a way that when a patient is administered with whole antibodies generated in animal of another species, the patient generates an immune response against these antigenic determinants. This may give rise to varied adverse secondary responses that can even include anaphylactic shock. These problems are significantly reduced when the antibodies are previously digested with papain or pepsin and only the resulting purified Fab or $F(ab')_2$ fragments are administered.

It is proved that F(ab)2 is better than Fab both in its plasma distribution and neutralization. This is explained by the pharmacokinetic differences between the two fragments. However, experimental observations from Brazil showed that IgG protected better than Fab against both Crotalus and Bothrops venoms. IgG was also more efficient in neutralizing the lethal effect, but IgG, F(ab)2 and Fab all had the same efficiency to treat venom-induced haemorrhagic disorders (Ismail *et al* 1998, Ismail & Abd-Elsalam 1998 , Morais *et al* 1994, Leo´n *et al* 1997, Theakston *et al* 2003)**.**

In general, fractionation procedures should not impair the neutralizing activity of antibodies; it should yield a product of acceptable physicochemical characteristics and purity with a low content of protein aggregates, which is non-pyrogenic and which should provide good recovery of antibody activity (WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins 2008).

Purification of IgG antivenoms. In the past, most laboratories have used fractionation protocols based on salting-out procedures employing ammonium sulfate or sodium sulfate (Wüster and Broadley 2007). Two precipitation steps are included using two different salt concentrations in addition to the elimination of "euglobulins" by precipitation in a diluted acidic solution. Such fractionation protocols generally lead to a recovery of antibodies of between 40 and 50% and to the formation of protein aggregates. The final product prepared by this procedure containing a relatively high proportion of contaminating proteins, such as albumin. This compromised the safety of the product, since a high incidence of early adverse reactions has been described in response to such intact IgG antivenoms.

Several new developments have been proposed for further purification including complete or partial modification in antivenom purification such as ammonium sulfate and caprylic acid precipitation to improve the quality of the antivenom (WHO

Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins 2008).

The caprylic acid precipitation is further purification step which can be improved the purity and quality of the antivenom. The use of caprylic acid (octanoic acid) as an agent for precipitating proteins from animal plasma has been described in the literature (Steinbuch Audran 1969).

Several procedures for the purification of whole IgG antivenoms with a desirable physicochemical properties and purity by caprylic acid precipitation of non-immunoglobulin proteins have been developed, and are now used for the production of antivenoms (Rojas, Jiménez, Gutiérrez 1994).

Caprylic acid is added to plasma, and precipitated proteins are removed and discarded. The filtrate containing the immunoglobulins is concentrated with ulterafilter. The immunoglobulin solution is formulated.Variations of this procedure have been introduced by various manufacturers, including dilution of plasma, changes in caprylic acid concentration, pH, and temperature, etc. (Raweeith & Ratanabanagkoon 2003).

Caprylic acid fractionation allows the production of antivenoms of relatively high purity and with a low aggregate protein. The yield may reach up to 60–75% of the activity in the starting plasma, depending upon the details of the procedure and/or the equipment used. The Caprylic acid fractionation of horse hyperimmune plasma is a simple, convenient and economical protocol for the manufacture of high quality whole IgG antivenoms. It constitutes a potentially valuable technology for the alleviation of the critical shortage of antivenom in Africa (Gutiérrez *et al* 2005, Raweeith & Ratanabanagkoon 2003). The desirable efficacy and safety profiles of caprylic acid fractionated antivenom immunoglobulins have been demonstrated in clinical trials. (WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins 2008).

Isolation and Purification of Fab antivenoms. Production of monovalent Fab fragments is performed by some manufacturers (Wüster W, and McCarthy CJ. 1996), currently using hyperimmunized sheep plasma. Papain is used to carry out the enzymatic digestion, and the process of preparation of the fragment may use ammonium sulfate, sodium sulfate or caprylic acid. Immunoglobulins are precipitated from plasma by salting-out tecnique. The precipitate separated, dissolved and digested by papain. The solution is then applied to a diafiltration system to remove salts and low-molecular-mass peptides. The preparation is then chromatographed on an anionexchanger (usually in quaternary aminoethyl (QAE)-based or diethylaminoethyl (DEAE)-based media). Fc fragments and other impurities are bound on the column, whereas Fab fragments pass through. After an additional diafiltration/dialysis step, the product is formulated, finally the preparation is sterile-filtered and dispensed into the containers (Leon *et al* 2000).

Optional additional steps used by some manufacturers, the basic methodologies described above for the manufacture of IgG, F(ab')2 and Fab antivenoms allows the production of antivenoms of adequate purity, safety and efficacy (Leon *et al* 2000). Nevertheless, some manufacturers include additional steps to enhance product purity. The methodologies include those described below (WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins 2008, KRIFI *et al* 1999).

Preparation and purification of F (ab')2 antivenoms. The classical protocol for F(ab')2 antivenom production developed by Pope(Pope, CG. 1939b), with a number of modifications. The method of pepsin digestion involves the digestion of horse plasma proteins by pepsin, leading to the degradation of many non-IgG proteins, and to the cleavage of IgG into bivalent F(ab')2 fragments by removal and digestion of the Fc fragment into small peptides (Raweeith & Ratanabanagkoon 2003). A heating step and the purification of F (ab')2 fragments by salting-out are also key elements of this method. Some procedures involve performing the pepsin digestion step on a pre-purified IgG fraction that is obtained by salting-out to

obtain an IgG-enriched precipitate, whereas albumin is not precipitated. Each manufacturer should adjust the pepsin concentration to achieve the required enzymatic activity. The precipitate is eliminated and filtrate is heated. The resulting fraction is separated to remove the precipitate. For further purification second salting-out performed on the resulting solution and the F(ab')2 precipitate is separated ,dissolved and concentrated preferentially by ultrafilteration. (Broadley *etal* 2003). The F(ab')2 solution is then formulated as a injectable dosage form.. Such a process, or similar ones developed by other manufacturers, using pepsin digestion, salting-out and diafiltration is the most often used for the manufacture of F(ab')2 fragments. The yield of this fractionation protocol usually ranges between 30% and 40%. Downstream processing using caprylic acid purification of F(ab')2 ,to be achievable by caprylic acid precipitation of non-F(ab')2 proteins after pepsin digestion, with an improved yield (□60%) (Wolfgang *et a*l 1998). Some manufacturers have introduced additional processing steps such as ion-exchange chromatography or ultrafiltration to eliminate low-molecular-mass contaminants. (WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins 2008, Raweeith & Ratanabanagkoon 2003, KRIFI *et al* 1999).

Ultrafiltration. There are several reports about the use of ultrafiltration or diafiltration in the process of purification of antivenoms. Roges et al reported further purification concentration, preferentially by tangential flow diafiltration and dialysis. Alternatively, the 23% ammunium sulfate (w/v) step is bypassed and, directly after the heating step, the filtrate obtained is subjected to ultrafiltration. They also state that after fractionation by caprilic acid, the additional processing steps may be used, such as ion-exchange chromatography or ultrafiltration, to eliminate low-molecular-mass contaminants (Turner & Amireh 2012). Next after pepsin digestion step, the ultrafiltr used to remove unwanted low molecular weight fragments with a 30-kDa nominal molecular weight cut-off membrane leaving an $F(ab')_2$ solution contaminated only with some pepsin and a small amount of the aggregated low molecular weight fragments (Jones & Landon 2003). Herrera and coworkers showed that the use of ultrafiltration (10 kDa nominal molecular weight limit) in the process of antivenom fractionation by caprilic acid leads to significantly improve in the antivenom quality (María Herrera et al 2009). Therefore it can be concluded that the process of ultrafiltration can be used in the production of antivenom in the different steps to remove low molecular weight proteins and improvement of the product purity.

In the other hand Zolfagharian, H. and Mohammadpour Dounighi, N reported a new method to prepare desirable diphtheria antigen by using gel filtration and ultrafiltration techniques. In this method the diphtheria toxin was purified before detoxification by formaldehyde-stabilizing agent, whereas in the conventional method the crude toxin was detoxified by formaldehyde alone. This report indicates that the purification index and recovery of product prepared by the new method was significantly improved (Zolfagharian & Mohammadpour Dounighi 2004).

Chromatography. The ion-exchange chromatography can be successfully used for antivenom purification based on charge differential with the contaminants. Anion-exchange columns of DEAE or QAE gels or membranes, such as quaternary ammonium cellulose microporous membranes, can be used at neutral pH to adsorb protein contaminants. Alternatively, cation-exchange columns, e.g. carboxymethyl or sulfopropyl gels, have been used for purification of IgG or F(ab')2 fragments . The column is equilibrated at acid pH, e.g. pH 4.5, to bind the antivenom. (KRIFI *et al* 1999, Zhao 1993, Wüster & Broadley 2007).

The affinity chromatography using either immobilized venom or other ligands can be designed to bind immunoglobulins or their fragments. However, columns usually deteriorate rather rapidly, and meticulous care should be taken to wash, sanitize and store them under appropriate conditions. Procedures should be followed to ensure that any substances

leaching from the columns do not affect the quality and safety of the product or else are completely removed during downstream processing; this is especially critical in affinity chromatography using immobilized venom. Affinity processes may affect recovery and high-affinity antibodies may be lost and/or denatured owing to the harsh elution conditions needed to elute them from the chromatographic material (KRIFI *et al* 1999). The use of Fab or $F(ab')_2$ fragments has another advantage that is known as the concept of distribution volume, which is simply the volume of the body in which a determined drug is dissolved. This volume can refer to the circulating blood alone, as is the case of IgG, or can include a larger part of body water in the case of the fragments. For this reason, as Fab and $F(ab')_2$ have a greater corporeal volume they can neutralize toxins lodged in various tissues, not only in the blood. They can even cross the blood/brain barrier in both directions and be used to neutralize or eliminate neurotoxins.The use of $F(ab')_2$ fragments has a particular advantage over the use of Fab fragments in that they are retained far longer in the organism because they have double the molecular weight. Moreover, they conserve their capacity to precipitate the antigen in physiological conditions as well as maintaining a size that allows them access to a distribution volume that is sufficient for treatment purposes. Due to the fact that the $F(ab')_2$ fragments conserve the main characteristics of the antibodies, the applications of the antibodies extend to $F(ab')_2$ fragments, with the additional advantage that because they lack the Fc fragment, recognition as foreign by a patient to whom they are administered is less likely. This provides greater tolerance to application of $F(ab')_2$ fragments and reduces the possibility of secondary reactions, which is particularly useful for prolonged treatments such as those applied in autoimmune diseases (Raw *et al* 1991).

Antigen and adjuvant. In order to increase venom or other antigens immunogenicity, use of different adjuvants became routine and more efficient. At present for immunization against snake venom various type of adjuvants, as a solution, suspension or emulsion systems, are used in different centers (Fu *et al* 1999). Above mentioned systems dose not considerable stability and can not protect antigens against environmental factors. In the other hand, their preparation process is time consuming and costly. These adjuvants are metabolized with high rate after injection in animal, so can not have considerable sustained release properties. Therefore, it is necessary to design novel forms of adjuvants. The micro encapsulation has been noticed since 1950, but its application in antigen delivery and pharmacy has been initiated in recent years, and their application for preparation of the sustained and controlled release drug delivery and simultaneously multi-antigen delivery systems is interested. Biodegradable polymers are used as one of the most important vehicles (Pries & Longer 1979, Davis *et al* 1987). The use of microparticles as a antigen delivery system is one of the most reasonable procedures for antigens protection and their sustained release (Jones *et al* 1996, Change & Gupta 1996, Xing *et al* 1996, Yan *et al* 1995 and Esquisabel *et al* 2000). However, antigens are sensitive to organic solvent, drying process, stress pending of miroencapsulation process, acidic medium produced during polymer degradation in body or microencapsulation process. Thus a suitable process should be designed in respect to physicochemical and biological properties of each specific antigen (Fu *et al* 1999). Already, different polymers have been used for this purpose which biodegradable polymers were main among them (Xing *et al* 1996, Yan *et al* 1995). One of the most important polymers for preparation of toxoid microcapsules were polylactides and polyglycols (Change & Gupta 1996, Xing *et al* 1996, Yan *et al* 1995 & Fu *et al* 1999). These polymers are used as a monomer or copolymer (Deng *et al* 1999). On small-scale a double emulsion technique for incorporation of Naja Naja oxiana venom into Poly (lactide-co-glycolide) (PLGA) microspheres were developed by Zolfagharian and Mohmmadpour (Zolfagharian & Mohmmadpour 2009). They have showed the

antigenicity of venom was retained after incorporation into PLGA microspheres. Chitosan, a biodegradable and biocompatible polymer, is a modified natural carbohydrate and the second most abundant polysaccharide in nature (IIIum 1998). It consists of repeating units of glucosamine and N-acetyl-glucosamine, the proportions of which determine the degree of deacetylation of the polymer (Bowman & Leong KW 2006).

It has been reported that chitosan nanoparticles have an excellent capacity for associating proteins (Fernández-Urrusuno *et al* 1999). CS nanoparticles are widely investigated for delivery of polypeptides such as tetanus toxoid, diphtheria toxoid (Vila *et al* 2004, Rezaei Mokaram *et al* 2008). Mohammadpour and his coworkers suggested that the chitosan nanoparticles fabricated may provide a suitable alternative to traditional adjuvant systems for Naja - naja oxiana snake (Mohammadpour *et al* 2010), Hemiscorpius lepturus (Mohammadpour *et al* 2012) and Mesobuthus epueus scorpion venom (Mohammadpour *et al* 2012 **).**

There are some reports regarding venom purification that leads to different purified fractions by ion exchange and gel filtration chromatography (Zolfagharian *et al* 1998, Akbari *et al* 2010).

Antivenom adverse reactions. The side effects of antivenoms are correlated with the purity of the product and the total amount of protein infused. Continuous clinical observation at the bedside is necessary for several hours after treatment to detect acute reactions; late adverse reactions may occur several weeks later. The adverse reaction rates may differ considerably between different antivenoms, but only a small proportion are life-threatening. Studies should aim to detect both early adverse events occurring at the time of, or within 24 hours, of antivenom administration (such as urticaria itching, fever, hypotension or bronchospasm) and late reactions such as serum sickness occurring between 5 and 24 days of antivenom administration (e.g. fever, urticaria, arthralgia, lymphadenopathy, proteinuria, or neuropathy).

As the antigens (Venom,……) used for hyperimmunization have main role in the manufacturing process of immunosera , it is necessary to purify the antigen and use the novel adjuvants (nanoparticles, microparticles,…) to create high level of antibody in the animals. Immunosera consist of foreign proteins (heterologue), adverse effects are an inevitable risk in the therapy. Appropriate manufacturing steps in the purification processes on antisera (salting out, caprylic acid, chromatography, diafiltration and ultrafiltration) can reduce the incidence of adverse reactions.

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