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Evaluation of health status of horses immunized with snake venom and montanide adjuvants, IMS 3012 (nanoparticle), ISA 206 and ISA 35 (emulsion based) during polyvalent snake antivenom production: Hematological and biochemical assessment

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ABSTRACT

Several biochemical and hematological changes in horses are observed during production of snake antivenom. Although conventional adjuvants like Freund's (Complete and Incomplete) are good immunopotentiators, they produce considerable local reactions in animals. Variety of commercial adjuvants, like montanide adjuvants, having high immunopotentiation and showing lesser side effects are available. The prime objective during antivenom production is to strike a balance between safety of immunized horses and efficacy of the product. In our earlier work, efficacy of montanide group of adjuvants in antivenom production has already been established. The aim of the present work was to assess the safety parameters in horses, viz.: biochemical and hematological, during production of snake antivenom. In the present study, 33 new horses were randomly divided into four groups and hyperimmunized using mixture of snake venoms, viz.: Cobra venom, Russell's viper venom, Krait venom and *Echis* venom along with montanide adjuvants, IMS 3012, ISA 206, ISA 35 and Incomplete Freund's adjuvant as a control adjuvant; through subcutaneous route at intervals of two weeks. During the immunization period, biochemical and hematological parameters were monitored at 0th, 14th, 21st, 30th and 42nd weeks. The mean hemoglobin values dropped slightly during initial immunization but subsequently regained to normal levels. The mean serum total protein values and globulin levels showed an increment in all the four groups, compared to day zero, vice-versa a slight drop was observed in albumin levels. No significant changes were observed in serum creatinine, bilirubin, alkaline phosphatase and blood urea nitrogen values. Finally, we conclude that montanide adjuvants

Abbreviations: ALB, albumin; ALP, alkaline phosphatase; BIL, bilirubin; BUN, blood urea nitrogen; CREAT, creatinine; CV, cobra venom; EV, echis venom; GLB, globulin; Hb, hemoglobin; IL1, interleukin-1; IL6, interleukin-6; KV, Krait venom; LD_{50}, median lethal dose; NSS, normal saline solution; PCV, packed cell volume; RV, Russell's viper venom; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEC, total erythrocyte count; TLC, total leukocyte count; TNF-α, tumor necrosis factor-alpha; TP, total protein.

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could be a safer alternative to the conventional adjuvants for primary phase of immunization in antivenom production.

1. Introduction

Snakebites pose a major public health problem in many tropical and subtropical regions in the world including India (Kini and Evans, 1990; Chugh, 1989), approximately 15,000–20,000 snakebite deaths per year being estimated in India. Cobra, Russell's viper, Common Krait and *Echis* are the most important poisonous terrestrial snakes in India (Srimannarayana et al., 2004; Punde, 2005). Venoms of these snakes contain mixtures of enzymatic and non-enzymatic proteins, exhibiting a wide spectrum of biological activities. They possess neurotoxic, myotoxic, cardiotoxic, hemorrhagic and hemostatic edema inducing properties and have direct action on vital organs (Chippaux and Goyffon, 1991). Snake antivenom is the only accepted and specific antidote available against the snake venom action (Thwin et al., 2010; Chippaux and Goyffon, 1998; Theakston et al., 2003; Punde, 2005).

Number of animals like sheep, goats and camels has been tried for production of antivenom depending on their availability in the particular regions. However, horse is the most preferred choice of animal for the commercial production of antivenom worldwide (Pratanaphon et al., 1997; Sjostrom et al., 1994; Christensen, 1979; Theakston et al., 2003). The horses are preferred over other animals because they are easy to handle, thrive in all climates, yield large volume of serum and also the methods of purification of horse antibody are well standardized (Pratanaphon et al., 1997; Sjostrom et al., 1994; Christensen, 1979). A number of reactions have been noted during the immunization of horses with snake venoms and adjuvants, which include local tissue changes like edema abscess, fistula and fibrosis at the site of injection (Angulo et al., 1997). In addition, a slight drop in hemoglobin and hematocrit levels and leukocytosis with increased number of polymorph nuclear leukocytes has also been noted (Angulo et al., 1997; Netto et al., 2004).

In India polyvalent snake antivenom against Cobra venom (CV), Russell's viper venom (RV), Krait venom (KV) and *Echis* venom (EV) is prepared by immunizing horses (Chatterjee et al., 1968; Jadhav and Kapre, 1991). There has been significant increment in number of sera manufacturing firms since last few decades. Earlier, Indian army was main supply of horses for sera production (Waghmare et al., 2009). Since army stopped supply of cast horses to sera production unit, the availability of healthy horses from open market for production purpose has become more acute. Hence the rationale and judicial use of horses has been indispensable and safety of horses is of great concern as they are exposed to venoms and adjuvants during immunization procedures. A number of approaches have been adopted for the preparation of potent and safe antivenom in horses (Theakston et al., 2003). These include use of different adjuvants, use of purified fraction of venom and use of toxin subjected to immunomodulation, making it less toxic but more immunogenic (Theakston et al., 2003; Jadhav and Kapre, 1991; Freitas and Frézard, 1997). Adjuvants are often used along with venom to induce adequate antibody response and to reduce pathological effects (Theakston et al., 2003; Allison and Byars, 1991). Recently at Haffkine Biopharmaceutical Corporation Ltd., India, immunization protocol has been revised by incorporating newer montanide adjuvants (nanoparticles IMS 3012, w/o/w emulsion ISA 206, o/w emulsion ISA 35). The Montanide adjuvants are new generation adjuvants that are ready to use, cost effective, safe and potent. They are biodegradable, compatible, non-toxic, non-carcinogenic, non-teratogenic and non-abortogenic. Formulations based on aqueous phase containing nanoparticles of adjuvant IMS 3012 combined with immunostimulant/s is known to produce significant immune response against *Rhodococcus equi* in horses (Taouji et al., 2004). The adjuvant ISA 206 is composed of derivatives (esters of octadecenoic acid and anhydromannitol) of mannide- monooleate family as a surfactant in mineral oil mix, whereas the adjuvant ISA 35 is based on injectable oils and highly refined emulsifier from mannitol and purified oleic acid from vegetable origin. In addition, they are cost effective for repeated immunization procedures. The presence of refined oil, high grade surfactant and well controlled manufacturing process make these montanide adjuvants less viscous fluid emulsions that are stable and safe (Aucouturier et al., 2002). Water-in-oil-in-water (W/O/W) emulsion ISA 206 and oil-in-water (O/W) emulsion ISA 35 have lower viscosity than that of IFA. An added advantage in the use of these montanide adjuvants is good innocuity for crude antigens, which may exhibit acceptable and minimal local reactions at injection site (Aucouturier et al., 2001, 2002).

In our earlier study these montanide adjuvants have been proved to be excellent in terms of safety and efficacy for antivenom production (Waghmare et al., 2009). However, systemic effect of these adjuvants in horses during immunization has not yet been studied. The objective of present study was to evaluate hematological and biochemical parameters in horses inoculated with venoms (CV, RV, KV and EV) and three different grades of montanide adjuvants (IMS 3012, ISA 206, and ISA 35). Incomplete Freund's adjuvant (IFA) emulsion was used as the control adjuvant in the study.

2. Materials and methods

2.1. Chemicals

Montanide group of adjuvants (IMS 3012, ISA 206 and ISA 35) were obtained from Seppic, Cedex, Paris, France; while Incomplete Freund's adjuvant (IFA) was purchased from Difco, USA; Liver and Kidney function test kits were