ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 8, Issue, 08, pp.36248-36253, August, 2016

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

OBTENTION OF TETANUS ANTITOXIN HYPERIMMUNE SERA IN EQUINES

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ARTICLE INFO

Article History:
Received 21st May, 2016
Received in revised form
23rd June, 2016
Accepted 09th July, 2016
Published online 20th August, 2016

Key words:

Tetanus toxoid,
Immune- stimulating oil emulsion,
Equine antitoxin.

ABSTRACT

Tetanus is an illness caused by the neurotoxin produced by the *Clostridium tetani* and its control is a strategy of the vaccination programs developed by the Panamerican Health Organization. For the production of tetanus toxoid are used international standards of antitoxins gotten in equines, however, these antisera are not available for their routine use in Cuba. The purpose of this study was to obtain a potent antitoxin against tetanus toxin. Four groups of mares were immunized. For the immunization, the different amounts of immunogen were emulsified in Immune- stimulating oil emulsion and injected according to the several multisite immunization protocols in low volumes. The second immunizations were carried out in Immune- stimulating oil emulsion. The antibody response was evaluated by Ramón flocculation assay. The antitoxin antibody titers varied significantly from an immunization protocol to another according to the studied parameters. Nevertheless, the same antitoxin titers increased quickly and reached a plateau around the 6 weeks for three groups of animals. The antibody titers were significantly higher in the fourth group of animals, reaching their activity to 2000 LfeqU/mL. We could conclude that the antitoxin antibody concentration increases with the time and the repetition of the immunogenic stimulus, and it is demonstrated that immunization protocols more lingering produce better outputs of antitoxins. This potent antitetanus toxin should be useful as internal reference material in the tetanus toxiod antigen.

Citation: Dr. Rolando Sánchez Artigas, Antonio Miranda Cruz, Oliver Pérez Martín, Liliana Araujo Baptista, Dailín Cobos Valdes and Dr. Onelio Mulet Pupo 2016. "Obtention of tetanus antitoxin hyperimmune sera in equines", *International Journal of Current Research*, 8, (08), 36248-36253.

INTRODUCTION

Tetanus is a serious and often fatal disease caused by the neurotoxin produced by *Clostridium tetani*, widely distributed throughout the world, especially in densely populated regions of hot, humid weather. It is now a rare disease in developed world; however it remains an important cause of death worldwide and is associated with a high case fatality, constituting a health problem particularly in the developing world. The worldwide prevalence is more than one million cases per year and more than 500,000 deaths in the neonatal period. In Cuba, the disease is characterized to be rare or uncommon [4] and neonatal tetanus has been eliminated. In 1889 the organism was isolated by Kitasato and one year later tetanus toxin was identified by Kitasato and Behring for preparations of antitoxin and reported neutralization of toxin by specific antibodies.

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To control tetanus Pan American Health Organization (PAHO) has been launching an Expanded Program on Immunization (EPI) in the countries of the region (Control of diphtheria, pertussis, tetanus, infection with *Hemophilus influenzae* type B and hepatitis B, 2006). It has significantly contributed to reduce vaccine preventable diseases. The vaccine against diphtheria, tetanus and pertussis (DTP) is included in the EPI of the countries of the region (Control of diphtheria, pertussis, tetanus, infection with Hemophilus influenzae type B and hepatitis B, 2006) and it occurs in the Finlay Institute in Cuba. As workplace policy, PAHO requires stringent quality requirements for DPT components, which cannot be characterized by simple chemical or physical procedures, therefore, to establish the activity of these substances or confirm the reliability of the control proceedings International Standards are used as antitoxins, essential to ensure the assurance of the results (Conclusiones de la Reunión de la Red Regional de Laboratorios Nacionales de Control de la Calidad de Vacunas. 2005). However, these standards are not available for routine use in DTP vaccines producing centers, so it is

recommended to use internal reference materials (MR), standardized or calibrated against international standards (WHO Expert Committee on Biological Standardization 2007). The anti TT antibodies are traditionally produced in horses which are exceptionally sensitive to oil based adjuvants, so that less potent adjuvants such as aluminum phosphate, aluminum hydroxide can be used, but a high percentage of non-responders horses and resulting antitoxins are often of low potency. The difficulty to raise a response to the TT, allowed to reconsider the use of oil based adjuvants in equine and it proposed that if immunizations were performed in low volumes, the local reaction at each injection site would be limited. Therefore if such immunization could be made with various immunization sites over a wide anatomical area, then more antigen presenting cells would be recruited and antibody production would be enhanced. However, horses are commensurate with the needs of volumes of antibodies and the intentions of using them. Considering also that they are long-lived animals, easy to handle and can bleed for the jugular, taking docile behavior during handling for injection and sampling. Finally, females are preferred as they regularly produce a higher immune response (Guidelines on: antibody production. CCAC. 2002). The production of antitoxins against TT is a problem to be solved because of the intrinsic difficulty of controlling the immune response. The magnitude of the response depends mainly on the dose of the immunogen, the number of stimuli, the spacing between the dose, the frequency of stimulation and the presence of adjuvants. Some of these variables are expensive (immunogen dose, frequency and time of immunization) or dangerous to the health of the animal immunized (adjuvants, boosters). Several studies have focused on obtaining a better immune response with the use of adjuvants. The purpose of the incorporation thereof is to amplify the immune response to the antigen of interest. To obtain these reagents for producing Cuban vaccines containing the TT, the main objective of this study was to produce hyperimmune sera against TT in equine using Immune-stimulating oil emulsion (ISOE) while causing minimal side effects on the species used because of the low volume and multi-site immunization protocols.

MATERIALS AND METHODS

Reagents

Tetanus toxoid was from Finlay Institute and it was obtained from the toxin produced in cultures of *C. tetani*, heat-detoxified with formaldehyde and purified by physical chemical methods. It was kept at 4°C, filtered through a 0.22 µm millipore membrane (Sartorius, Germany). Chemicals were of reactive grade; Span 80, Tween 80 and mineral oil were purchased from Sigma Chemical Company, St. Louis, MO, USA.

Selection of Animals: Quarter Horse mares weighting greater than 370 kg and aged ranging from 6 to 10 years, from the equine breeding farm in the province of Holguin, were used, no previous experience with TT. All animals were maintained and handled in similar circumstances, for this research, a Biosafety Level II (Cuba. Ministerio de Ciencia Tecnología y Medio Ambiente 2003).

Table 1. Immunization protocols

DAYS	GROUPS, TT (LF) X SITES				TOTAL ML/ SITES/ ML X SITE
	1	2	3	4	
1	250	25	250	5	0.5/ 2/ 0.25
14	125	125	500	25	0.5/ 2/ 0.25
21	125	250	500	25	1/ 4/ 0.25
28	250	500	666.7	125	1/ 4/ 0.25
35	625	625	625	250	2/ 4/ 0.5
42				500	4/ 4/ 1
49				500	6/ 6/ 1
56				666.7	6/ 6/ 1
63				625	8/ 8/ 1

Immunization protocols: The mares were divided into four groups of five animals each. Immunization procedures were performed as described in Table 1. All animals were immunized subcutaneously (sc) at several points on each side of the spine with the TT emulsified with Immune-stimulating oil emulsion. The first three protocols were over 35 days and the fourth was over 63 days (Table 1). The immunization protocols, including the TT doses, total volume of injection, number of ejection sites is detailed in Table 1.

Preparation of the Immune stimulating oil emulsion (EOIE): The ISOEs were prepared as fallow. Stable and reproducible emulsions were prepared with 0.39% (w/v) Tween 80 and 2.61% (w/w) Span 80. Tween 80 was added to the aqueous phase and Span 80 was mixed into the oil phase at 60 ºC. The antigen was part of the aqueous phase. The water-to-oil ratio of all emulsions used was 60% (w/w) aqueous phase and 40% (w/w) oil phase. They were types of water-in-oil (W/O) emulsions.

Monitoring animals and local reactions: Local reactions were monitored by measuring the diameter of inflammation at each injection site according to the procedure described by Pratanaphon with modifications relating to the diameter of inflammatory reactions observed. The reactions were graded as zero (smooth surface), medium (1 - 3.0 cm, small swelling), moderate (3.0-6.0 cm, swelled with or without injury), severe (> 6.0 cm, swelling with or without ulceration imminent) depending on the size of the reaction at the injection site. Local reactions were measured for the first injection of 1 mL of ISOE at 2, 4, 6, 8, 10, and 16 weeks after inoculation.

Sample collection: Blood draws were performed by jugular puncture, on the same days of immunization, before proceeding to them. The immune responses of animals in all groups were evaluated to 7 days after the last doses of group 4. Sera were stored at-20ºC prior to the determination of the antitoxin activity.

Determination of antitoxin antibody titers in serum samples: The anti TT antibody response was analyzed using

the Ramon flocculation assay (WHO Expert Committee on Biological Standardization 2007). This assay involves the detection of a complex formed between the antigen and antibody and it is based on visual observation of macroscopic flocculation complexes. The time required for the formation of these complexes depends on the ratio of tetanus antitoxin and specific antitoxin. This time in minutes for the first flocculation occurs is called Kf. Flocculation sequence is defined by the Kf observed for the first, second and third flocculation to occur. The first tube in which flocculation occurs is used to determine the value of Lf flocculation (Limes flocculation is: flocculation units or limit of flocculation) in the sample.

Ethical considerations: All procedures fulfilled with the guidelines established in Canadian Council on Animal Care12, 13 , for handling samples of animal origin in order to decrease biological risk and ensure the protection of workers, the community and the environment.

Statistical Analysis: All statistical analyzes were carry out using GraphPad Prism v4.00 software (GraphPad Software Inc. 2003). Results were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey test to determine differences among groups. P values <0.05 were considered statistically significant.

RESULTS

Kinetics of antibody response to tetanus toxoid immunized mares: The values of anti TT titer in UeqLf/mL of all four groups of animals studied by the test Ramon, during application of the immunization protocols are shown in Figure 1. The anti TT antibody titers obtained in groups 1, 2 and 3 were comparable, in contrast, the same response in group 4 was higher. Antibody responses measured by the Ramon flocculation test did not show a high degree of variation between horses in the same group and between different groups. However, the soluble toxoid mixed with adjuvant induced a strong response in all animals of different groups. Antibody titers increased rapidly and reached a plateau at around 6 weeks for groups 1, 2, 3. In group 4 major titers were achieved after 10 weeks and antibody titers were significantly ($p<0.05$) higher in most animals than those obtained in the other groups. According to the variation of the parameters studied, it appears that the immune response depended mainly on the amount of immunogen and repeating antigenic stimuli (Figure 2 and 3)

Preparation of ISOE. The decision of the used adjuvant was carefully considered and justified. The adjuvant interest was selected, having the current knowledge on the preparation of immunogen / adjuvant, avoiding Freund´s use, which causes

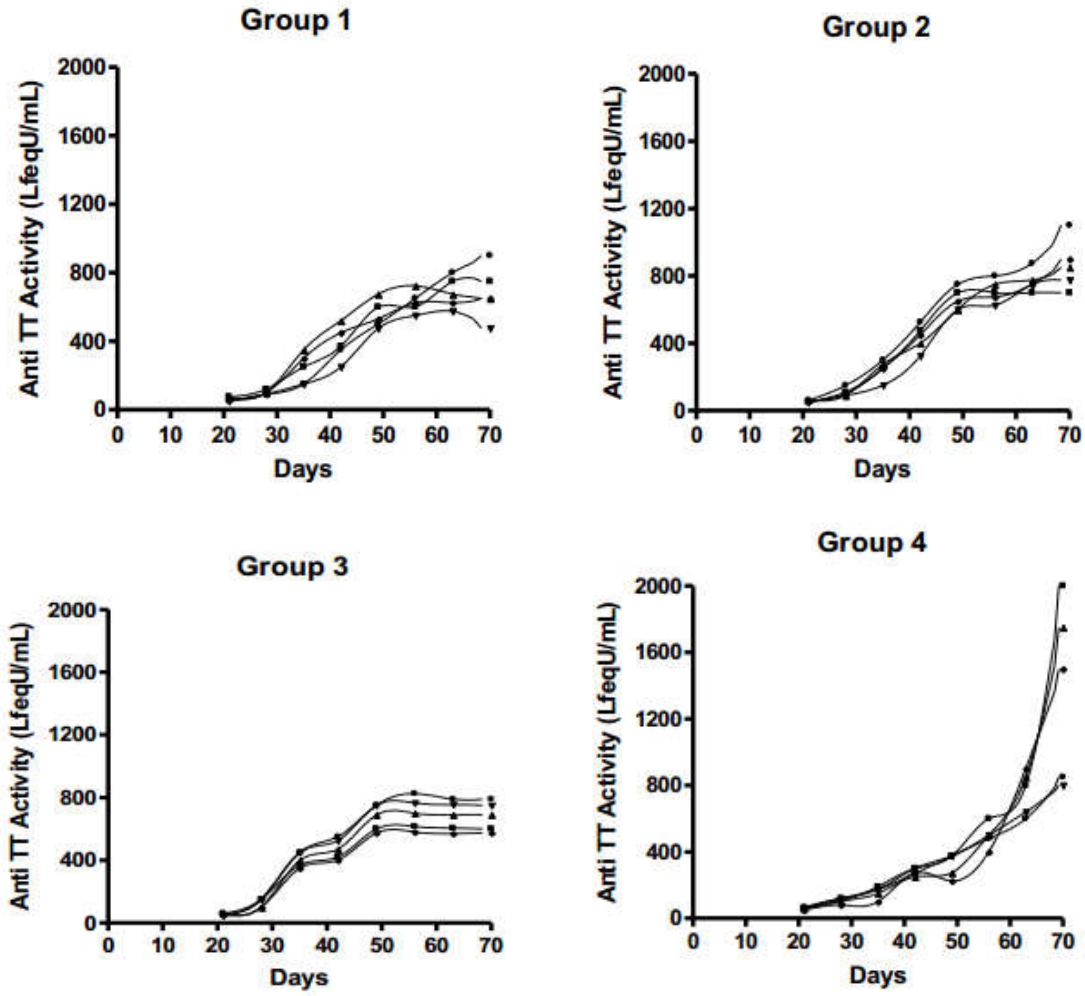

Figure 1. Antibody responses in mares against TT

severe local and systemic reactions and in many countries, such as Canada, is not considered suitable for use in animal experimentation. To minimize local reactions at the injection sites

Animal health. During the course of immunization no weight increase or loss in animals was observed (data not shown). The values of the reactions at EOIEs have been used as incomplete multiple emulsions (Herbert 1968). The immunogen was prepared so as to allow an acceptable response without adverse events in the behavior of animals. The ISOEs were easily injectable in small volumes and the quality of the immunogen preparation denotes the observation of non toxic reactions. They were specially prepared to be sterile administered over a pH within physiological limits. the sites of immunization induced by EOIE/TT were measured and graded after 2, 4, 6, 8, 10 and 16 thereof are shown in Figure 4. These results were similar for all animals and indicate an average reaction during the first 8 weeks of immunization and become normal within 16 weeks.

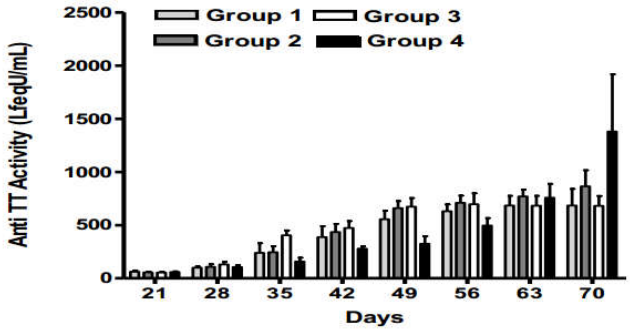

Figure 2. Comparison of tetanus antitoxin titers from the different animal groups according to the immunization protocols

The results are shown as mean of UeqLf/ml±SD.

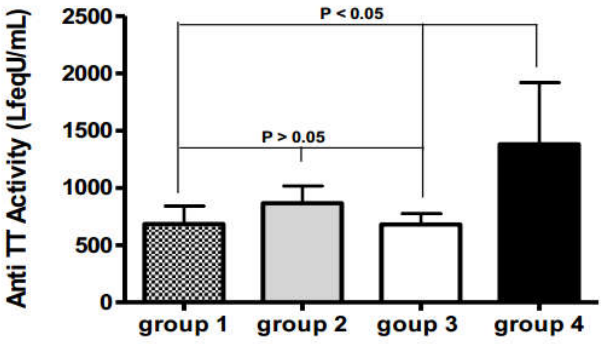

Figure 3. Comparison of tetanus antitoxin titers of the groups of animals according to the immunization protocols on day 70 after immunization

Differences among the means were evaluated by the Tukey test and these indicate significant differences among groups 1, 3 and 4 ($p<0.05$); the same can not among groups 1, 2 and 3.

DISCUSSION

The use of antisera started at the end of the 19th century, following the pioneering work of von Behring, Kitasato, Roux and Calmette (Rabies and envenomings : a neglected public health issue 11 : report of a Consultative Meeting, (WHO, Geneva, 10 January 2007).

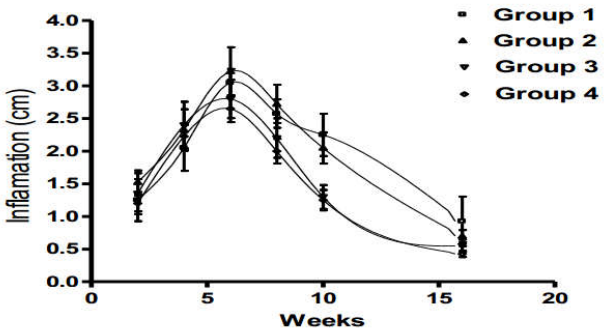

Figure 4. Comparison of the kinetics of local reactions at the injection site after immunizations

At the dawn of immunology, it was observed that animals immunized with specific toxins or venoms developed an antibody response that could be beneficial to the treatment of many different diseases, from tetanus and diphtheria to snake bite envenomings and rabies. The most important factors in the production of antisera with highly specificity and purity are the quality and quantity of the immunogen, the animal model and its immune responses, the immunization protocol, antigen purity poor safety and efficacy of some products and; (iii) deficient or non existent regulation and control of antisera in some countries. This study allowed to compare the anti TT antibody response of the four groups of mares hyperimmunized with different doses of antigen incorporated in ISOE. The antibody titers of antitoxin sera obtained in groups 1, 2, 3 were comparable to each other, it did not happen the same response in group 4 (Figure 1 and 2). The antibody responses measured by Ramon flocculation assay did not show high degree of variation between horses among same group and between groups 1, 2 and 3, however, a significant difference was observed in group 4 with respect to the groups 1 and 3, $P<0.05$ (Figure 3). Not only were the observed high titers of antibody response but every animals responded to the immunization with EOIE, water-in-oil emulsion Miranda (2006).

The oil based adjuvant is capable to form inert depots at the inoculation site, allowing a slow release of immunogens over time to maintain a sustained stimulation of the immune system and activates, directly or indirectly, various types of dendritic cells, macrophages and lymphocytes T CD4+ from reticuloendothelial system, involved in the immune response, but also influence the duration of response in the class and subclasses of immunoglobulins which predominate in the response to toxins and can influence the avidity of the antibodies produced. Many researchers continue the use of Freund as the gold standard because of its well known effectiveness with a wide variety of antigens, although it causes a large chronic inflammatory response (Thommen I, Sartor M, Moleta-Colodel E, Albuquerque D 2011, Mbow ML, Gregorio E, Valiante NM, Rappuoli R 2010, Lambrecht BN, Kool M, Willart MAM, Hammad H 2009), so it must be used only when there is evidence that other builders do not work

(for example, when only small amounts are available soluble immunogenic or when the antibody response is quite weak). The mixture immunogen / adjuvant preparation itself is a major cause of failure of immunizations due to improper emulsification. The EOIE used in experiments, has a low viscosity allowing an easy injection. Anyway, all the desirable features of adjuvants are not found together in any of the 100 or more adjuvants known (Vogel and Powell 1995, Stewart-Tull 2000).

The adjuvant effect on EOIE anti TT antibody production can be explained by assuming that the emulsion was a consistent and inert reservoir capable of slowly releasing the antigen over a long period of time, where the stimulus is maintained in contact with effector cells for a prolonged period. This emulsion seems well suited to fulfill this function, provided that the antigen included remain stable for a long period of time when in contact with the components of the emulsion at body temperature. Frequent doses and extreme high, low amounts of antigen of poor immunogenically molecules and without adjuvants can induce immunological tolerance (Lofthouse 2002). Using appropriate adjuvant (Miranda *et al*. 2006), as an efficient antigen release system induces high antibody titers, prolonging immunogenic state of antigen for a sufficient period of time, and applying frequent injectable dose can reach high antibody titers and hyperimmune response without showing immune tolerance (Zinkernagel 2001, Lofthouse 2002). For the production of potent antitoxin and antivenom sera, its has proposed the use of multiple immunizations protocols with small amounts of toxins or venoms to produce a hyperimmune response. Best results are obtained using booster doses at least 4 weeks after the first injection as shown in Figure 1. Similar results were observed by Chotwiwatthanakun *et al*. 2001, Schunk and Macallum 2005. Moreover, the increase of booster frequency not necessarily increases the antibody concentration or improves immune response and its has been shown in some cases opposite effects (Hu 1990), but this does not always happen and other authors reported multiple boosters protocols, but high doses of toxins or toxoids and increasing their frequency (Miranda *et al*. 2006, WHO Expert Committee on Biological Standardization 2007, Wahby *et al*. 2007).

During the course of hyperimmunization, the affinity and quantity of generated antibodies increase. This maturation process is influenced by the immunization protocols used with low dose antigen resulting in high affinity antibodies. In the immunization protocol 4 (Table 1), it was started at doses as low as 10 Lf. The affinity maturation of antibodies has two overlapping strategies for the somatic derivation of new antibody structures. The first involves the hyperimmunization with a selected antigen and a restricted repertoire of antibody genes and the second on changing the primary repertoire. Maturation of the antibody response is evidenced by the change of expression of IgM to predominant IgG isotype in the development of a high-affinity response and the presence of memory T lymphocytes which elevate the speed of response following a broader antigenic challenge. This process usually occurs after, at least, two doses of antigen administration. This agrees with the results of the analyzed immunization protocols where at least 5 doses of TT were administered. However, prolonged antigen exposure induced by a controlled antigen delivery system allows immune maturation and process amplification. In our case is comparable with the immunization protocol 4, it lasted 63 days. The prolonged availability of antigen in optimal amounts, has been shown to be essential for the occurrence of somatic mutation and affinity maturation (Wang *et al*. 2000), when the stimulation is prolonged, there is greater contact with effector cells of the immune response. Severe local reactions, previously reported by other authors, in the injection sites were due to the inoculation of the oil emulsified immunogen in in large volumes (5-10 mL) at each site.

Local reactions at the injection sites are limited If immunizations are performed with small volumes of 0.5-1 mL as in our case (Figure 4). Furthermore, more antigen presenting cells are recruited and antibody production would have more amplification if these immunizations were held at various sites (6 to 10 sites) on an anatomical area (Pratanaphon *et al*. 1997). Not only was a local middle reaction at immunization sites reduced but the resulting mares serum potency was elevated in comparison to that observed in previous studies(Miranda 2006), comparable with other results (WHO Expert Committee on Biological Standardization 2007) and all mares responded to immunogenic stimulus. The low volume, multisite immunization protocols of EOIE / TT (see Table 1) used has proven highly effective in the production of anti TT antibodies. Small volumes of oily immunogen injected at each site resulted only in a middle local reaction. The multi-injection over a wide anatomically area allowed a more effective immune response to take into account in the amplification of the migration and activation of a large number of antigen presenting cells and lymphocytes. The multisite injection strategy may have great importance whether immunization could make direct a large number of antigen presenting cells such as dendritic cells and obtain a more efficient immune response and may be used in immunization / vaccination of both animals and humans. (Chotwiwatthanakun 2001) Shantavasinkul P, Tantawichien T, Jaijaroensup W, Lertjarutorn S, Banjongkasaena A, Wilde H, and Sitprija V. A 4-Site, Single-Visit Intradermal Postexposure Prophylaxis Regimen for Previously Vaccinated Patients: Experiences with 15000 Patients. Clinical Infectious Diseases 2010; 51(9):1070–1072.

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