Effect of different adjuvants in equines for the production of equine rabies immunoglobulin

SHIVALI ARORA, SOURABH SHARMA, SUNIL K. GOEL, USHA SOREN SINGH

ABSTRACT

Background. Implementation of the recommended post-exposure prophylaxis by vaccination and specific immunoglobulin therapy for rabies is largely hampered by its high cost and inadequate production. Therefore, the development and availability of an economic preparation of rabies immunoglobulin is a high priority for India, where rabies is a major cause of death. We studied the efficacy of four different adjuvants in raising antibodies to rabies antigen in older, discarded equines.

Methods. Eleven equines, 23–26 years old, were divided into 4 groups to receive four different adjuvants in small amounts (1–2 ml)—Freund complete adjuvant with *Mycobacterium tuberculosis*, Freund complete adjuvant with *M. butyricum*, Freund incomplete adjuvant and bentonite—along with purified chick embryo cell vaccine. The immunization schedule was spread over 105 days and the antibody titres were measured on days 56, 91 and 119.

Results. On day 119 (third sampling), Freund complete adjuvant with *M. tuberculosis* provided a geometric mean titre of 654.03 IU/ml in comparison with a titre of 459.19 IU/ml with Freund complete adjuvant with *M. butyricum*, 630.95 IU/ml with Freund incomplete adjuvant and 172.18 IU/ml with bentonite.

Conclusion. Purified chick embryo cell vaccine in combination with Freund complete adjuvant containing *M. tuberculosis* and Freund incomplete adjuvant were better at eliciting an immune response. The low quantity of adjuvants used possibly helped by causing very few side-effects but without compromising the antibody titres.

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INTRODUCTION

Rabies in humans is always fatal. Therefore, effective post-exposure prophylaxis becomes the mainstay in the prevention and control of the disease. Vaccination is a powerful tool to prevent the disease and immunization of humans is widely practised.[1] Human rabies immune globulin (HRIG), a homologous immunoglobulin, is safer but not available in the quantities required. Equine rabies immune globulin (ERIG) is thus the mainstay and should be used much more widely and produced on a much larger scale.

ERIG for human use is produced at the Central Research Institute (CRI), Kasauli by using tissue culture vaccines and gives good results.[2] To increase the immunogenicity of the antigen as well as the volume of ERIG, adjuvants used with vaccines could be useful. In India, antirabies serum (ARS) was first produced in young healthy ponies, and bayol and falba as adjuvants were first used in 1957 and have since then been in constant use at the CRI, Kasauli.

In 1923, Ramon showed that it was possible to artificially increase the level of diphtheria or tetanus antitoxin by adding bread crumbs, agar, tapioca, starch oil, lecithin or saponin to the vaccines.[3] In 1955, Christensen used bentonite-adsorbed Cape cobra venom for the immunization of horses.[4] With the use of adjuvants, a smaller quantity of antigen is required to evoke the immune response, thus reducing production costs.

In 1930, the adjuvant properties of *Mycobacterium tuberculosis* were demonstrated.[5] This observation led Freund in the 1940s to develop the most widely used adjuvant—Freund complete adjuvant (FCA) containing *M. tuberculosis*. Freund incomplete

Central Research Institute, Kasauli 173204, Himachal Pradesh, India
SHIVALI ARORA, SOURABH SHARMA, SUNIL K. GOEL, USHA SOREN SINGH Anti Rabies Vaccine Unit
Correspondence to SOURABH SHARMA

adjuvant (FIA) is prepared in the same manner as the complete adjuvant except that *M. tuberculosis* is not added. Another variant of FCA contains *M. butyricum*.

A vaccine adjuvant can influence the immune response in many ways. The ability of adjuvants to influence several parameters of the immune response complicates the process of finding an effective adjuvant, because our knowledge of how an adjuvant operates at the cellular level is insufficient to support a completely rational approach to match the vaccine antigen with a proper adjuvant. When different adjuvants provide equally good immunogenicity, the choice may depend upon other factors such as cost, availability, reactogenicity and induction of the desired immune response.

We studied the addition of 4 different adjuvants to a common vaccine antigen—the purified chick embryo cell vaccine (PCECV). We aimed to identify the best adjuvant for the production of ERIG.

METHODS

Equines

Eleven equines (10 mules and 1 mare), 23–26 years old and in good health were included in the study. These animals were received after being discarded from the Indian Army and the Police Department. On their arrival at our centre, they were subjected to a quarantine of 15 days followed by an injection of tetanus toxoid (10 ml i.m.). They were kept under observation for 1 month before being included in the study. The routine care and management of the equines was done as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).[6]

None of the animals had been immunized against rabies before, nor did they have any rabies-related incident during the course of the study.

Immunizing agents

PCECV (Rabipur, Batch No. 626, date of manufacture April 2000, date of expiry March 2005) manufactured by Chiron Behring Vaccines, Ankleshwar, Gujarat was used as the common vaccine (antigen) in combination with the four different adjuvants. The vaccine was available in lyophilized form with 1 ml diluent per dose and having a potency ≥2.5 IU/dose. The mean potency of this batch of vaccine was 10.5 IU/dose (information provided by manufacturer).

The following adjuvants were used:

1. FCA containing *M. tuberculosis* (H 37 Ra) (Lot No. 139520, Difco Laboratories, USA)
2. FCA containing *M. butyricum* (Lot No. 146825, Difco Laboratories, USA)
3. FIA containing bayol and falba (prepared in-house)
4. Bentonite (2%) adjuvant (aluminium silicate hydrate) (Lot No. 48319, Loba Chemic)

Study design

Eleven equines were divided into 4 groups and immunized as per the schedule given in Table I. The equines were grouped as follows:

Group I: Three equines (numbers 478, 482 and 506) immunized with PCECV and FCA containing *M. tuberculosis*

Group II: Four equines (numbers 484, 486, 511 and 512) immunized with PCECV and FCA containing *M. butyricum*

Group III: Two equines (numbers 494 and 495) immunized with PCECV and FIA

Group IV: Two equines (numbers 496 and 498) immunized with PCECV and 2% bentonite adjuvant.

The schedule of immunization for all the equines was the same, except for the change in adjuvant which was administered in a dose of 1 ml based on the group to which the equine belonged. The FCA and FIA adjuvants were used only with the first (priming) dose owing to their propensity for causing adverse reactions. Two ml of 2% bentonite was used in all the groups on days 21, 27, 35 and 42.

Blood samples were drawn from the equines on days 56, 91 and 119 of the immunization schedule.

Analysis of blood samples

The samples were collected on the days mentioned, sera separated aseptically and subjected to the mouse neutralization test (MNT)[7] for estimation of the antibody titre as recommended by the *Indian Pharmacopoeia, 1996*. The statistical evaluation for determination of antibody titre was done by the Reed and Muench method.[8] The National Reference Standard (Batch no. 1/95) calibrated against the International Standard and having a unitage of 85 IU/ml was received from the Central Drugs Laboratory, CRI, Kasauli and the Challenge Virus Strain (Batch no. 2/2000, titre 10^8/0.03 ml/mouse dose) from the Rabies Research Centre, CRI, Kasauli. The ND_{50} of each sample was calculated along with National Reference Standard Serum, and antibody titres calculated and expressed in international units (IU)/ml.

RESULTS

The sera samples collected from the 11 equines on days 56, 91 and 119 were assayed for estimation of antibody titre against rabies by

TABLE I. Immunization schedule by the subcutaneous route

Day	Dose of PCECV	Adjuvant	Site
0	1 ml	1 ml*	One
21, 27, 35, 42	2 ml	2 ml 2% bentonite	One
56	*First sampling*		
63, 71, 77, 84	4 ml	Nil	Both sides of neck
91	*Second sampling*		
91, 98, 105	4 ml	Nil	Both sides of neck
119	*Third sampling*		

PCECV purified chick embryo cell vaccine * Group I Freund complete adjuvant with *M. tuberculosis*, group II Freund complete adjuvant with *M. butyricum*, group III Freund incomplete adjuvant and group IV 2% bentonite

TABLE II. Antibody titres on days 56, 91 and 119

Group	Equine no.	Antibody titre (IU/ml)			Geometric mean titre (IU/ml)		
		Day 56	Day 91	Day 119	Day 56	Day 91	Day 119
I	478	210	777	819	162.55	457.79	654.03
	482	285	607	612			
	506	72	204	560			
II	484	252	510	591	66.29	310.81	459.19
	486	19	107	117			
	511	68	614	757			
	512	66	314	589			
III	494	84	215	553	91.09	214.78	630.95
	495	99	215	722			
IV	496	19	50	117	38.68	103.51	172.18
	498	79	215	254			

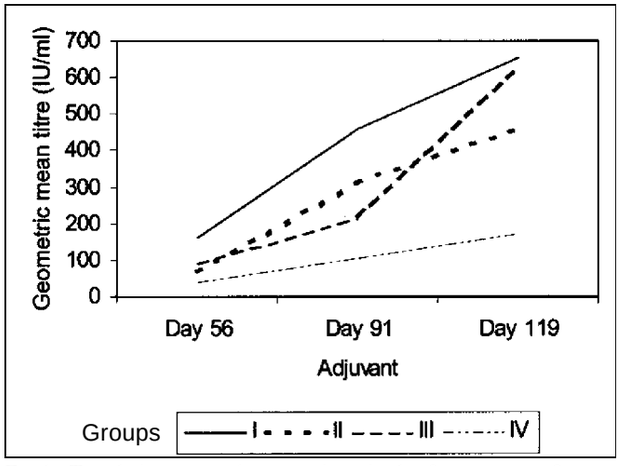

FIG 1. Trends in geometric mean titres in the four groups of equines

TABLE III. Side-effects observed in the equines

Group	Adjuvant	Equine no.	Side-effect
I	Freund complete adjuvant containing *M. tuberculosis*	482	Mild abscess at the site of inoculation 3–4 days after day 42 of immunization
II	Freund complete adjuvant containing *M. butyricum*	484	Abscess at the site of inoculation after day 42 of immunization
		512	Abscess on the right side of neck after day 27 of immunization

No side-effects were seen in the equines in groups III and IV

the MNT. On day 56, the highest geometric mean titres (GMT) were obtained in group I equines (162.55 IU/ml) while the lowest were in group IV (38.68 IU/ml; Table II). This trend in these 2 groups continued over the complete immunization schedule (Fig. 1). However, groups II and III, which showed a low GMT on day 56, had a substantial rise in GMT till day 119. In fact, the GMT in group III almost equalled that in group I, which had the highest GMT on day 119.

Mild side-effects were observed only in equines in groups I and II (Table III).

DISCUSSION

We found that all the adjuvants enhanced the immune response but FCA containing *M. tuberculosis* (group I) led to the development of the highest antibody titre as estimated by the MNT. The GMT of the antibody on day 119 was highest (654.03 IU/ml) in group I equines. In this group of equines the GMT of the antibody on day 56 (162.55 IU/ml) was also the highest. This shows that the use of FCA containing *M. tuberculosis* leads to high antibody titres in a short time. FCA is also known to stimulate an antibody response in low responder animals, probably through activation of T helper cells. On day 56, only equines in group IV had a GMT <38.68 IU/ml but the equines in this group were not withdrawn from the donor population as suggested by Luekrajang *et al*.[9]

FCA containing *M. butyricum* (group II) trailed behind FCA containing *M. tuberculosis* and FIA (group III) in our study with regard to eliciting antibody titres against rabies, although it potentiated the immune response (GMT 459.19 IU/ml on day 119). Similar findings have been reported with regard to the formation of neutralizing antibodies after subcutaneous injections of inactivated rabies vaccines with killed *M. butyricum*.[10] However, some studies have reported that FCA with *M. butyricum* is better than FCA with *M. tuberculosis* especially against *Salmonella typhi*.[11] The main difference between the Wax D of *M. tuberculosis* and Wax D of other mycobacteria is the presence of a peptide moiety in the former, which may be responsible for the enhanced immunogenicity. As the same PCECV was used in all the four groups of equines, the variation in potency, if any, of the different batches was not a factor in the production of antibodies.

FIA is currently used for the production of ERIG at our centre. There was little difference in the antibody titre of FIA compared with FCA with *M. tuberculosis* (GMT on day 119 of 630.95 IU/ml *v*. 654.03 IU/ml). However, FIA lags behind FCA with *M. tuberculosis* in providing an early rise in antibody titres (GMT on day 56 of 91.09 IU/ml *v*. 162.55 IU/ml).

Bentonite (group IV) had the lowest GMT titres and on day 119 this was 172.18 IU/ml. Though bentonite is non-ulcerogenic, it is not as efficacious as either FCA or FIA.

The main constraint while using adjuvants in vaccine formulations are the side-effects that accompany their use. The Freund adjuvants cause granuloma formation but the intensity of this reaction varies with the quantity of oil used. However, granuloma formation may also contribute to the adjuvant action, as granulomas contain a large number of macrophages which may be important in producing an immune response. Mycobacteria present in FCA stimulate macrophage proliferation at the site of injection, in the lymph nodes and even in the lungs.[12] Contrary to this generally held view, we did not encounter much granuloma formation in our equines. No abscess formation was seen immediately after inoculation of the first dose with FCA. This may be because of the small amount (1 ml) of adjuvant used.

In 1 equine each in the 3 groups receiving Freund adjuvants (Table III), a slight swelling was noticed 3–4 days after inoculation which healed over the next 7–10 days with the local application of BIPP formulation (boric acid, suphanilamide powder, zinc oxide and iodoform). No other side-effect or toxic reaction was observed in the equines.

The antibody response of all equines to the adjuvants is not similar. While some equines may be good responders, others may show a moderate response and yet others may show a poor response. The response may be related to the nutritional status, general health and immunization history of the equine. The equines used in our study were 23–26 years of age but this did not seem to affect their ability to mount an immune response to PCECV. Similar findings have been reported by Chowdhuri and Thomas[13] and Goel *et al*.[2] This can lead to considerable cost savings by using old discarded horses from the army, police and equestary.

The quantity and number of doses of antigen are important as lower doses may not stimulate an appropriate immune response whereas very high doses may cause immune tolerance. The route of inoculation also affects the immune response. A given dose of antigen is usually more effective when injected subcutaneously than when given intravenously. The mode of inoculation also matters as, for example, immunization via ballistic propulsion with a gene gun results in higher primary antibody titres than by the intradermal route.[14]

Therefore, FCA is better in eliciting an immune response to rabies antigen but FIA is also comparable in raising antibody titres

in equines. If antibodies need to be developed in a short time, FCA serves the purpose but over a longer duration FIA provides equally good results. Bentonite is clearly a poor adjuvant. Though it did not elicit a high antibody response with a priming dose, it is safe and non-ulcerogenic and was used till day 42 of the immunization schedule in all the equines.

Conclusion

In India there is a need to produce large quantities of ERIG that is affordable and gives minimal adverse reactions. Our results show that this can be achieved using a small quantity of FCA with *M. tuberculosis* or FIA, and using older equines donated by agencies which do not have any use for them. This will result in substantial savings in the cost of production of ERIG.

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Use of rice bran oil in patients with hyperlipidaemia

REBECCA KURIYAN, N. GOPINATH, MARIO VAZ, ANURA V. KURPAD

ABSTRACT

Background. The quantity and type of dietary fat is known to affect plasma lipid concentration and hence the choice of cooking oil is important to lower the risk of coronary heart disease. Rice bran oil, which was not popular worldwide, is slowly being recognized as a 'healthy' oil in India. We assessed if rice bran oil had hypolipidaemic effects in subjects with elevated lipid levels.

Methods. The study had a cross-over design with subjects (n=14) randomly assigned to consume either rice bran oil or refined sunflower oil in their homes, for a period of 3 months (period 1). After a washout period of 3 weeks, they were crossed over to the other oil (period 2). The serum lipid values were estimated at the beginning, on day 45 and day 90 of each phase. Additional parameters assessed included anthropometry, dietary and physical activity patterns.

Results. The use of rice bran oil significantly reduced plasma total cholesterol and triglyceride levels compared with sunflower oil. The reduction in plasma LDL-cholesterol with rice bran oil was just short of statistical significance (p=0.06). HDL-cholesterol levels were unchanged.

Conclusion. The use of rice bran oil as the main cooking oil significantly reduced serum cholesterol and triglyceride levels. The use of rice bran oil together with dietary and lifestyle modifications may have implications for reducing the risk of cardiovascular disease.

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INTRODUCTION

India is on the threshold of an epidemic of cardiovascular disease, and surveys in urban areas show that coronary risk factors are widespread.[1] Of all the ethnic groups, people of Indian origin have among the highest incidence of cardiovascular and coronary artery disease (CAD),[2,3] often occurring at an early age. Indian immigrants living in various parts of the world have a documented

St John's National Academy of Health Sciences, Bangalore 560034, Karnataka, India

REBECCA KURIYAN, MARIO VAZ, ANURA V. KURPAD Division of Nutrition, Department of Physiology

All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India

N. GOPINATH Emeritus Professor, Department of Cardiothoracic Surgery

Correspondence to REBECCA KURIYAN; *rebecca@iphcr.res.in*