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Influence of Immunomodulatory Agents on Bovine Humoral and Cellular Immune Responses to Parenteral Inoculation with Bovine Rotavirus Vaccines

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ABSTRACT

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Sodium diethyldithiocarbamate (DTC), mycobacterium cell wall extract (MCWE, Regressin™), killed *Corynebacterium parvum* (*C. parvum*, Immunoregulin™) and muramyldipeptide (MDP) were each combined with purified, live bovine rotavirus and inoculated into 3 month-old Holstein-Friesian calves in order to examine their ability to potentiate specific humoral and cellular immune responses. The vaccinated calves were boosted twice at 3 and 6 weeks after initial vaccine inoculation. The rotavirus was administered intramuscularly either in an aqueous suspension or in a water-in-oil (WIO) emulsion, prepared with incomplete Freund's adjuvant (IFA). DTC and *C. parvum* were given by the intravenous route, while MCWE and MDP were incorporated directly in the rotavirus suspension. Two groups of calves were also vaccinated either with rotavirus and IFA or with rotavirus emulsified in mineral oil and a mannide oleate compound (MOC, Montanide 888™). A control group of calves was given phosphate-buffered saline (PBS) solution emulsified with IFA. The different vaccine preparations were then compared by studying the kinetics of serum rotavirus-neutralizing antibody production and of proliferative response by blood lymphocytes following in vitro stimulation with bovine rotavirus. The results showed that: (1) the bovine rotavirus should be incorporated in a WIO emulsion in order to induce a cell-mediated immune response as detected by the rotavirus-specific in vitro stimulation test with blood lymphocytes, and to produce higher neutralizing antibody titers in the serum; (2) the vaccines prepared with the mineral oil–MOC complex or IFA both induced comparable levels of humoral and cellular immune responses. The use of mineral oil and MOC as adjuvant may be preferred to IFA, because of the facility of preparing the vaccine and of the low viscosity of the resulting WIO emulsion: (3) the addition of MDP to the WIO emulsion prepared with IFA resulted in a higher cell-mediated immune response as determined by the in vitro blood lymphocyte transformation index specific for bovine rotavirus.

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INTRODUCTION

An adjuvant is defined as any substance able to enhance the immune responses (humoral and/or cellular) to a specific antigen. An increasing number of substances are being identified as being capable of potentiating the specific immune responses. These include the aluminium compounds and complete and incomplete Freund's adjuvants (CFA and IFA), which have been used for many years for the preparation of water-in-oil (WIO) emulsion with antigens (Edelman, 1980; Osebold, 1982). However, these products may induce undesirable side effects such as the formation of granulomas or even abscesses at the injection site (Edelman, 1980; Osebold, 1982). Other immunopotentiating drugs such as sodium diethyldithiocarbamate (DTC) (Renoux and Renoux, 1984), *Corynebacterium parvum (C. parvum)* (Milas and Scott, 1978; Bomford, 1980a, b) and muramyl-dipeptide (MDP) (Chedid, 1985), which represents the minimal structure of peptidoglycan and which can substitute for mycobacteria in CFA, have been shown to enhance the specific immune responses to antigens in several animal species.

Rotavirus is considered to be a major cause of diarrhea in children and newborn animals (Estes et al., 1983). In cattle, passive protection of the calves against rotavirus infection may be achieved by the ingestion of specific antirotaviral antibodies present in the colostrum and milk of the cow, previously vaccinated with rotavirus adjuvanted with IFA (Snodgrass et al., 1980; Saif and Smith, 1983; Saif et al., 1983; Castrucci et al., 1984) or with a combination of aluminium hydroxide and an oil adjuvant (van Opdenbosch et al., 1981). However, the need for finding a better procedure of maternal immunization is relevant owing to the potential clinical complications of these latter adjuvants.

In this article, we compared the ability of several immunomodulatory products to enhance the humoral and cell-mediated immune responses to bovine rotavirus in 3-month-old calves. We have choosen this calf model primarily for economic reasons, owing to the large number of vaccine preparations that required testing.

MATERIALS AND METHODS

Cell culture and virus production

BSC-1 cells were propagated at 37°C in 150-cm^2 plastic culture flasks (Corning Glass Works, New York, NY), using Eagle's minimum essential medium with L-glutamine (EMEM) (Flow Laboratories, McLean, Virginia), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Laboratories, Chagrin Falls, Ohio), 100 I.U. ml^{-1} of penicillin and 100 $\mu g\ ml^{-1}$ of streptomycin. For virus production, infected cells were maintained in EMEM

supplemented with 5 μg ml^{-1} of bovine crystallized trypsin (Sigma Chemical Company, St-Louis, Missouri) (EMEM-T) and antibiotics.

The rotavirus strain used in this study was plaque-purified neonatal calf diarrhea virus (NCDV) (Archambault et al., 1984). Virus was produced as previously described (Archambault et al., 1984) and was used for animal vaccination, neutralizing antibody determination and for the in vitro rotavirus-specific blood lymphocyte stimulation test (LST). When 80–90% of the cells exhibited a cytopathic effect (CPE), virus was harvested by osmotic shock followed by a trichlorofluoroethane extraction (Fauvel et al., 1978). The virus was then purified by CsCl isopycnic gradient ultracentrifugation (Fauvel et al., 1978). Fractions ranging from 1.34 to 1.39 g ml^{-1} in CsCl were collected and dialyzed overnight against 0.05 M tris-HCl buffer, pH 8.0. The viral titers were determined and calculated in median tissue culture infective dose ($TCID_{50}$) per ml (Archambault et al., 1984).

Animals

Three-month-old male Holstein-Friesian calves were purchased from two farms where all the cattle were free of clinical disease. The calves were kept in an isolated barn with controlled temperature and ventilation. An adaptation period of 3 weeks was allowed before starting the experiment. The animals were divided randomly in groups of 3 calves and were checked for the presence of serum-neutralizing antibodies against bovine rotavirus as well as for their in vitro lymphocyte responses to Concanavalin A (Con A) (Difco Laboratories, Detroit, MI) and bovine rotavirus before vaccination.

Vaccine inoculations

The different vaccine preparations used for inoculation of the calves (groups A–L) are summarized in Table 1. The animals were inoculated intramuscularly in the two semi-tendinosus and semi-membranosus regions of the rear legs with 5 ml (2.5 ml per site) of a mixture containing 2.5 ml of live, purified rotavirus suspension (2×10^8 $TCID_{50}$ ml^{-1}) mixed with equal volume of either phosphate-buffered saline (PBS) or IFA (Gibco Laboratories). The calves were boosted at 21 and 42 days after the first vaccine inoculation.

Each immunostimulant drug was combined with either the aqueous rotavirus vaccine or the WIO emulsion prepared with IFA. DTC (Sigma Chemical Company) was suspended in PBS at a concentration of 50 mg ml^{-1} filtered through a 0.45-μm membrane and inoculated by the intravenous (iv) route in the jugular vein at a dose of 10 mg kg^{-1} body weight (Renoux and Renoux, 1984). A suspension of killed *C. parvum* (Immunoregulin ™, Immunovet, Tampa, FL) was given iv at a dose of 0.8 mg, as recommended by the manufacturer. A suspension containing 1 mg ml^{-1} of mycobacterium cell wall ex-

TABLE 1

Description of the different rotavirus vaccine preparations used to immunize the calves

Groups of calves	Vaccine preparation[a]
A	PBS__IFA (IM)
B	Rotavirus (IM)
C	Rotavirus__IFA (IM)
D	Rotavirus (IM) and DTC (IV)
E	Rotavirus__IFA (IM) and DTC (IV)
F	Rotavirus (IM) and *C. parvum* (IV)
G	Rotavirus__IFA (IM) and *C. parvum* (IV)
H	Rotavirus__MDP (IM)
I	Rotavirus__IFA__MDP (IM)
J	Rotavirus__MCWE (IM)
K	Rotavirus__IFA__MCWE (IM)
L	Rotavirus__mineral oil__MOC (IM)

[a]__, Compounds mixed together; PBS, Phosphate-buffered saline; IFA, Incomplete Freund's adjuvant; DTC, Sodium diethyldithiocarbamate; MDP, Muramyl-dipeptide; MCWE, Mycobacterium cell wall extract; MOC, Mannide oleate compound; IM, Intramuscular inoculation; IV, Intravenous inoculation.

tract (MCWE, Regressin™, Vetrepharm Inc., London, Ont.) (2 ml per dose), and MDP (2 mg per dose) (Wells et al., 1982) (provided by Dr. Louis Chedid, Institut Pasteur, France), were directly incorporated in the rotavirus suspension.

Another group of 3 calves were immunized intramuscularly with a WIO emulsion containing the rotavirus and mineral oil (Marcol 52, Esso, Montreal, Que.) with mannide oleate compound (MOC, Montanide 888™, Seppic Company, Paris, France) as emulsifier. Finally, 3 animals were inoculated intramuscularly with PBS emulsified in IFA and served as the control group.

Preparation and characteristics of the WIO emulsion prepared with mineral oil and MOC

A minimal volume of 30 ml of the vaccine was prepared as follows. MOC, used at a concentration of 10% (w/w), was mixed with the mineral oil in a homogenizer (Model 963 C, Yvan Sorvall Inc., Newtown, NJ) at 1500 rpm for 3 min. An equal volume of the virus suspension (2×10^8 $TCID_{50}$ ml^{-1}) was then homogenized with the adjuvant at 3500 rpm for 15 min. During homogenization, the receptacle was kept in a cold water bath to reduce heating.

The following characteristic and stability tests were carried out on the emulsion prepared with mineral oil and MOC. The integrity of the emulsion was tested by placing a few drops into cold water (4°C) for dispersion estimation.

The electrical conductivity test was performed using a power supply (Model IP-17, Heat Company, Benton Harbot, MI) set at 300 V by inserting two brass electrode disks 4.5 cm apart into the emulsion. The current was monitored with a multimeter (Model MX20ZA, Compagnie générale de métrologie, Annecy, France). The relative viscosity of the emulsion was measured with a universal viscosimeter (Saybolt Viscosimeter, Precision Scientific, Chicago, IL). Stability tests were performed by centrifuging 30 ml of the emulsion for 30 min at $2000 \times g$ at room temperature and by prolonged storage at room temperature and at 4°C. The emulsion was then visually checked for alterations.

Virus neutralization test

The level of specific antigen neutralizing antibodies in the animal sera was determined before vaccination (day 0) and at days 14, 28, 49 and 70 after primary immunization (API). For testing, sera were inactivated at 56°C for 30 min. Serial 2-fold dilutions (starting at 1/25) of each serum were made in EMEM-T and the neutralization test was performed by a viral CPE inhibition method (Archambault et al., 1988). The antibody titer of each serum was expressed as the reciprocal of the highest serum dilution neutralizing 1000 $TCID_{50}$ of the rotavirus. Data were then transformed in base 2 logarithmic values ($\log_2 \times 100$) for an analysis of variance and the Duncan multiple-range test (Steel and Torrie, 1980).

Antigen preparation for the in vitro lymphocyte stimulation test

The virus used as specific antigen in the LST was inactivated with binary ethyleneimine (Sigma Chemical Company) and prepared as previously described (Archambault et al., 1988). The resulting inactivated viral suspension was dialyzed for 36 h against three changes of the same culture medium used in the LST without FBS. The viral antigen was checked for any residual infectivity on BSC-1 cells and kept in small aliquots at −70°C. A non-infected cell lysate antigen was similarly prepared from a fraction of 1.34–1.39 g ml^{-1} in CsCl and used as the control.

In vitro lymphocyte stimulation test

Ten ml of blood were collected before vaccination and weekly for up to 70 days by jugular venipuncture and placed into tubes containing 1 ml of 0.15 M sodium citrate as anticoagulant. Lymphocytes were isolated by using Ficoll-diatrizoate gradient centrifugation and the LST specific to bovine rotavirus was performed as previously described (Archambault et al., 1988). Briefly, lymphocytes were resuspended at a concentration of 2.5×10^6 cells ml^{-1} in RPMI 1640 medium (Flow Laboratories), supplemented with 10% of FBS, 2

mmol of L-glutamine and antibiotics. The cell suspension (200 μl) was added to each well of 96 flat-bottomed well microtiter plates (Flow Laboratories). Quadruplicate wells received 25 μl of either the viral antigen (5×10^6 $TCID_{50}$ viral particles before inactivation) or the control cell lysate and the lymphocyte cultures were incubated for 4 days at 37°C in a 5% CO_2 and humidified atmosphere. Stimulation of the lymphocytes by Con A (1 μg per well) was done for each cell sample (1.25×10^6 cells ml^{-1}) to check their immunological activity. All cultures were pulsed with 1 μCi of tritiated thymidine (specific activity of 6.7 Ci $mmol^{-1}$) (New England Nuclear, Boston, MA) 20 h before harvesting on glass-fibre disks with a semi-automated multiple-cell harvester and the bound radioactivity was measured by liquid scintillation. The data were expressed in terms of a stimulation index (SI), which represents the ratio of the mean counts per min (cpm) incorporated by the virus-containing lymphocyte cultures to the mean cpm incorporated by the control cell cultures. Groups of calves were compared by using an analysis of variance and the Duncan multiple range test on the means of the maximal significant SI ($SI \geqslant 2.00$) (Bellanti et al., 1980) obtained for each calf during time intervals of the vaccine inoculations (Days 0–21, 28–42, 49–70).

RESULTS

Characteristics of the WIO emulsion prepared with mineral oil and MOC

Properties of the WIO emulsion prepared with mineral oil and MOC were the following. The emulsion did not disperse when dropped onto the surface of the cold water. The electrical conductivity of the WIO emulsion measured at room temperature (22.7°C) was less than 0.1 μSiemen. The relative viscosity of the emulsion was of 222.7 Seconds Saybolt Universal (SSU) at 37.7°C and of 416.7 SSU at 22.7°C. Stability tests showed that, after storage for 3 months at room temperature and for over 1 year at 4°C, the emulsion sometimes showed a thin clear fluid layer on the top, which could be easily re-incorporated. A similar observation was also recorded after centrifugation of the emulsion at room temperature. Finally, animals inoculated with the WIO emulsion did sporadically develop a slight tumefaction at the injection site, which was generally resorbed within the next 7 days.

Immune responses of vaccinated calves

The kinetics of induction of serum neutralizing antibodies to rotavirus are presented in Fig. 1. Overall, specific antibody titers varied from $\leqslant 25$ ($\leqslant -2$ $\log_2\times100$) to 51 200 (9 $\log_2\times100$) A significant immune response was observed as early as 14 days API for all groups of calves immunized with the rotavirus. The calves vaccinated with the rotavirus WIO emulsion prepared

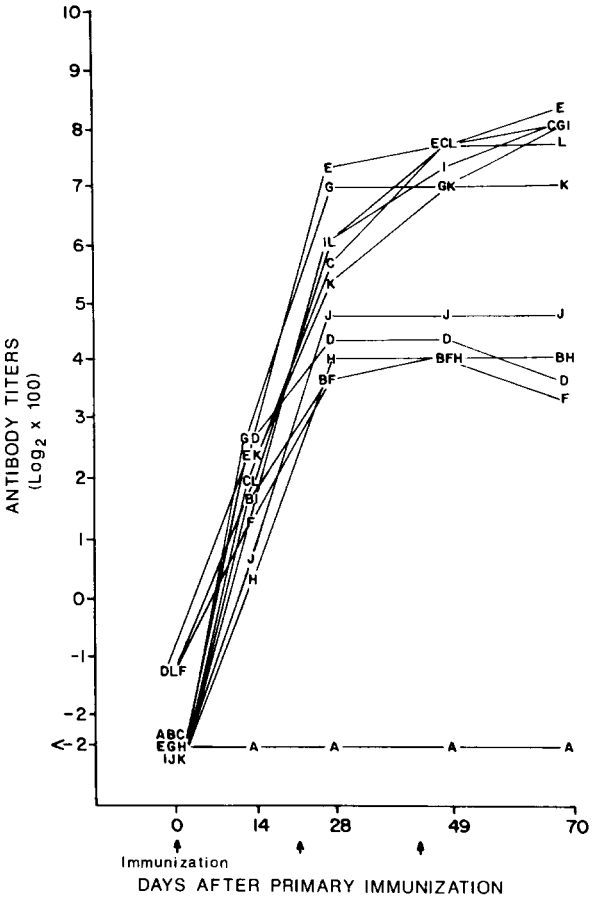

Fig. 1. Kinetics of serum rotavirus neutralizing antibody titers in calves vaccinated with bovine rotavirus. Each point is the mean titer for three calves. Antibody titers were determined in a range of 25 ($-2 \log_2 \times 100$) to 102 400 ($10\ 109_2 \times 100$). The groups of calves are described in Table 1.

with IFA (Groups C, E, G, I and K) or with mineral oil and MOC (Group L) showed the greatest immune response with individual antibody titers varying from 12 800 ($7 \log_2 \times 100$) to 51 200 ($9 \log_2 \times 100$) at day 49 or 70 API. Calves vaccinated with the aqueous rotaviral suspension (Groups B, D, F, H and J) demonstrated antibody titers varying from 800 ($3 \log_2 \times 100$) to 3200 ($5 \log_2 \times 100$) at Day 28 or 49 API. Control calves (group A) did not show detectable serum antibodies to rotavirus (titers < 25) throughout the experiment.

Statistical analysis on maximal antibody titers allowed the classification of the 12 groups of calves into three major populations at $P < 0.05$. Calves vaccinated with rotavirus incorporated in WIO emulsions represented the first population with the greatest rotavirus-neutralizing antibody response. The second population with an intermediate humoral response consisted of calves immu-

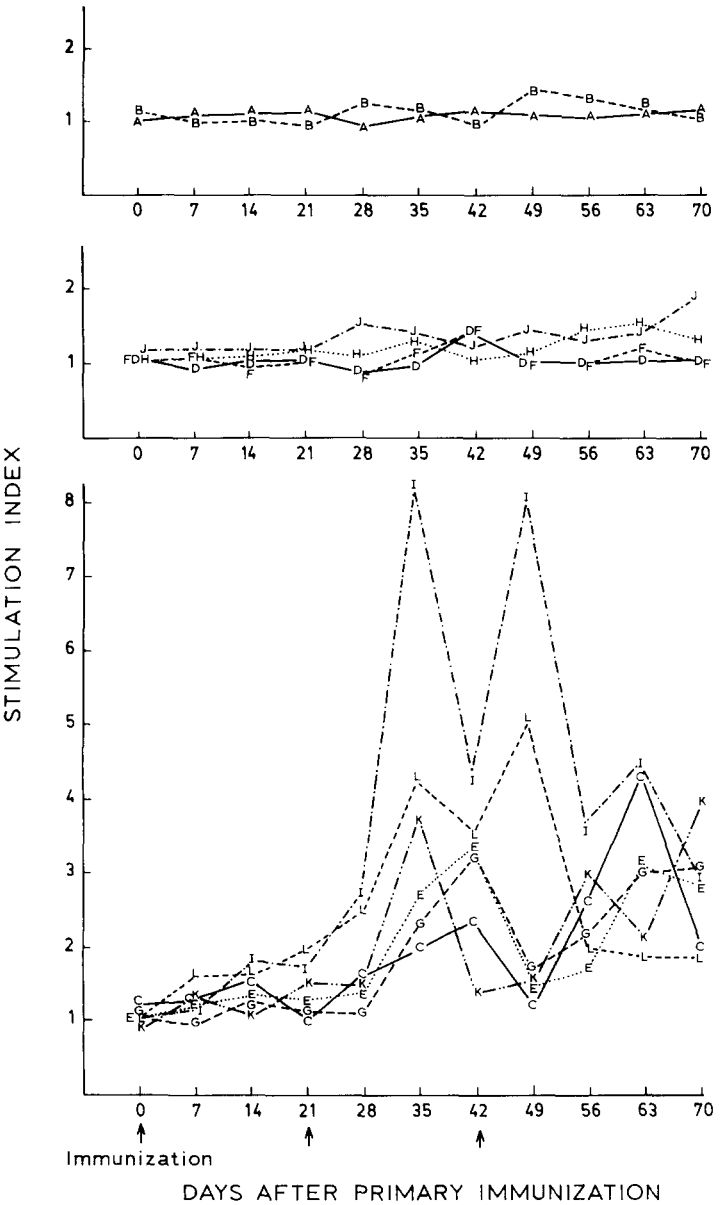

Fig. 2. Kinetics of the in vitro rotavirus-specific blood lymphocyte proliferative responses (as shown by the stimulation index) in calves vaccinated with bovine rotavirus. Each point is the mean stimulation index for three calves. The lymphocyte stimulation test was significant at stimulation index ≥ 2.00. The groups of calves are described in Table 1.

nized with the aqueous rotaviral suspension, while the control calves represented the third population. No potentiation of the antibody responses was observed by using DTC, *C. parvum*, MCWE or MDP.

Figure 2. illustrates the kinetics of in vitro blood lymphocyte stimulation with bovine rotavirus. Overall, the mean cpm of the control and rotavirus-containing lymphocyte cultures ranged from 586 to 28 353 and from 594 to 66 837, respectively. Neither the control calves (Group A) nor those vaccinated with the aqueous rotaviral suspension (Groups B, D, F, H and J) showed any specific lymphocyte stimulation. Vaccination of the calves with the rotavirus emulsified in IFA (Groups C, E, G, I and K) or in mineral oil and MOC (Group L) resulted in a specific lymphocyte activity with individual significant SI varying from 2.00 to 16.06. Calves immunized with the virus incorporated in IFA and MDP (Group I) showed a significantly ($P<0.05$) higher lymphocyte activity than the other groups after the second and third vaccine inoculations.

DISCUSSION

Intramuscular inoculation of calves with live rotavirus emulsified in IFA or the mineral oil–MOC complex resulted in higher serum rotavirus-neutralizing antibody titers, when compared with those of calves inoculated with the aqueous rotavirus vaccines. Other authors (Snodgrass et al., 1980; Saif and Smith, 1983; Saif et al., 1983, 1984; Castrucci et al., 1984) also showed the efficacy of IFA to induce a significant humoral immune response following systemic immunization of cows with live or inactivated bovine rotavirus. In our study, live virus was chosen for vaccinating the animals rather than inactivated virus primarily because the antigenicity of some viral proteins may be destroyed by the inactivation process (Saif and Smith, 1983). However, the titer of our virus in the vaccine preparations was not determined.

Vaccination of the calves with the WIO emulsions also induced a specific cell-mediated immune response as detected in vitro by a rotavirus-specific LST. The addition of MDP into the WIO emulsion prepared with IFA resulted in higher in vitro specific lymphocyte transformation, without enhancing the humoral immune response. Enhancement of the in vitro specific blood lymphocyte stimulation, with no potentiation of the antibody response, has also been reported in adult cattle immunized against *Trypanosoma brucei* with a MDP-containing vaccine (Wells et al., 1982). Both their results, as well as ours, suggest a selective effect of MDP on the specific cellular immune response in cattle, comparable to that obtained with CFA in other animal species (Osebold, 1982).

DTC, *C. parvum* and MCWE, either combined with aqueous rotavirus vaccine or with the WIO emulsion prepared with IFA did not potentiate the specific humoral and cellular immune responses. It is well known that immunomodulatory agents may produce variable effects on the immune sys-

tem, from immunostimulation to immunosuppression, or even have no effect at all on the host immune response (Bizzini, 1984). In fact, CFA, MDP, *C. parvum* and DTC demonstrate mostly immunoadjuvant properties (Edelman, 1980; Bomford, 1980a, b; Renoux and Renoux, 1984; Chedid, 1985). However, they may also exert, in certain cases, immunosuppressive activities, which are mainly observed when these products are inoculated before antigen (Scott, 1974; Asherson, 1977; Ferguson et al., 1983; Neveu and Vincendeau, 1983). Finally, a few studies have reported no influence of MDP (Woodard et al., 1980), *C. parvum* (Scott, 1974; Bomford, 1980a) and DTC (Neveu, 1981) on the immune response to different antigens.

MCWE, when incorporated in IFA, failed to potentiate the cell-mediated immune response to bovine rotavirus as detected by LST. Although MCWE is derived from the mycobacterium cell wall and is likely to include MDP, it appears that it is an ineffective adjuvant because the level of MDP is probably inadequate (Maron et al., 1978; Stewart-Tull, 1980).

The use of mineral oil and MOC as an alternative adjuvant to IFA offers many advantages including: the ease of preparing the vaccine in large volumes; the stability as well as the low viscosity of the emulsion (10 times less than that prepared with IFA) (De Lafaire and Brancq, 1984); and the lack of clinical complications at the injection site. Since the immune competence of 3-month-old calves is comparable to that of adult animals (Person et al., 1983), our results obtained by vaccinating calves with the mineral oil–MOC complex will be relevant to undertaking further studies in adult cattle.

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