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The use of cyclophosphamide as an enhancer of the vaccine against foot-and-mouth disease

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Abstract. The immunization of biungulate animals with killed foot-and-mouth disease virus (FMDV) requires periodic vaccinations due to a low vaccine immunogenicity. Therefore, FMDV antigens need to be combined with adjuvants such as aluminum hydroxide, saponin or oil emulsions. Animal handling for periodic inoculations, and the repeated doses of vaccines that have to be administered increase the commercialization costs. Moreover, the use of adjuvants may induce adverse effects.
In the present work we show that it is possible to increase the life span of neutralizing antibodies in serum when a single dose of cyclophosphamide (Cy) is administered four days before vaccination with aluminum hydroxide-saponin FMDV vaccine.
Key words. Cyclophosphamide; foot-and-mouth disease; adjuvant; vaccine.

Foot-and-mouth disease is a highly contagious viral disease of cloven-hoofed mammals which causes substantial economic losses[1]. In the countries where FMD has not been eradicated, control of the disease is based upon compulsory vaccination of cattle and occasionally lambs, goats and pigs.
There are seven main serotypes of FMD virus (FMDV) with at least 70 subtypes[2]. Even though the immune response against any one of the serotypes is effective, the antibodies formed in each case do not cross-react with the other serotypes. In addition, post-vaccination (pv) immunity has a short life span (not exceeding 1–2 months in calves and heifers).
These limitations have been partially overcome by the development of vaccines composed of a mixture of the predominant virus serotypes incorporated into different adjuvants. In South America, most of the commercially available vaccines are composed of at least three viral serotypes (A, O and C) incorporated into oil adjuvants. Nevertheless, multiple vaccination at six-month intervals is necessary for the prevention of the disease. Usually, cattle receive two to eight vaccine inoculations before slaughter. In addition to the high costs of repeated vaccination, the oil adjuvant can be harmful. The frequent appearance of subcutaneous abscesses decreases the economic value of the cattle, and this drawback may even cause occasional death[3]. The search for better vaccines to prevent FMD is therefore justified.
Recently, our laboratory has been engaged in research on the immunomodulatory capacity of cyclophosphamide (Cy), a well-known antimitotic and immunosuppressive drug[4]. In a previous study[5] we reported that, used in a very low subimmunosuppressive dose, Cy was able to prevent the development of the characteristic pancreatitis in adult mice experimentally infected with FMDV. This was due to a rapid appearance of FMD neutralizing antibodies (nAb) with high titers. These results prompted us to study the effect of a low dose of Cy in the development of anti-FMDV nAb in cattle vaccinated with commercial FMDV vaccines, in order to find out whether these animals demonstrated a similar behavior.
In this study, we present evidence that Cy is able to increase the titer and duration of serum nAb after the vaccination of cattle with a single dose of FMDV vaccine, although the time of appearance of nAb was not accelerated.

Materials and methods

Drugs. Cyclophosphamide (Endoxan-Asta, Labinca, Argentina).
Animals. Six-month-old Aberdeen Angus heifers free of nAb against FMDV were used for the experiment.
FMD vaccines. Commercial stocks containing a mixture of A_{24}, O_1 Campos and C_3 Resende subtypes in aluminum hydroxide-saponin adjuvant were used.
Virus. Viral stocks of O_1 and C_3 subtypes (MedVet, Argentina) were prepared by infecting BHK_{21} cell monolayers. Viruses were collected from the supernatant and cleared by centrifugation (30,000 *g*). The cytopathic effect of FMDV on BHK_{21} cells 48 h after infection was determined by the $TCID_{50}$ of the Reed and Muench analysis[6]. Viral stocks were maintained at −70 °C.
Neutralizing antibodies assay. Neutralizing antibody titers were determined by a microneutralization test in BHK_{21} cells[7], in which serial dilutions of the sera, in

Table 1. Peripheral blood leukocyte number and differential count of Cy-treated animals.

Animal no.	Cy	Days pv	Cell count ($\times 1000/mm^3$)	N	M	L	E	B
				(percentage of cells ± SD)				
1–8	+	−4	10	28 ± 1	7 ± 1	64 ± 1	4 ± 0	0
		0	6	16 ± 2	6 ± 0	75 ± 3	3 ± 1	0
		7	8	21 ± 2	5 ± 1	70 ± 3	4 ± 0	0
		14	11	29 ± 1	8 ± 2	60 ± 3	3 ± 0	0
		28	10	28 ± 1	6 ± 1	61 ± 1	5 ± 1	0
9–12	−	−4	11	28 ± 1	6 ± 0	61 ± 1	5 ± 1	0
		0	10	29 ± 2	6 ± 1	61 ± 1	4 ± 0	0
		7	9	27 ± 1	8 ± 1	60 ± 2	4 ± 1	1
		14	11	29 ± 1	7 ± 1	59 ± 1	5 ± 1	0
		28	10	30 ± 1	5 ± 1	62 ± 1	3 ± 0	0

Heifers were inoculated with Cy (5 mg/kg) four days before vaccination. Animals were bled on days −4, 0, 7, 14 and 28 pv. N = neutrophils, M = monocytes, L = lymphocytes, E = eosinophils, B = basophils.

Table 2. Immune response of Cy-treated animals vaccinated with an anti-FMDV vaccine.

Animal no.	Cy	Days post-vaccination						Days post-revaccination	
		−4/0	7	14	28	60	90	30	60
1	+	0.00	4.32	4.00	3.86	3.24	2.40	4.27	4.06
2	+	0.00	4.18	3.87	3.45	2.94	2.27	3.92	3.50
3	+	0.00	4.20	3.73	3.54	3.04	2.34	4.10	4.00
4	+	0.00	4.17	3.69	3.48	3.34	2.60	4.55	4.16
5	+	0.00	4.10	3.89	3.37	2.87	2.14	3.87	3.53
6	+	0.00	3.98	3.55	3.15	2.57	1.95	3.95	3.63
7	+	0.00	4.15	3.75	3.51	3.24	2.43	4.01	3.90
8	+	0.00	4.24	4.01	3.65	3.26	2.07	3.66	3.36
9	−	0.00	4.08	3.24	2.50	1.29	0.98	3.15	2.28
10	−	0.00	3.89	2.50	2.00	0.73	0.18	3.86	2.55
11	−	0.00	4.15	2.61	1.27	0.68	0.00	2.20	2.00
12	−	0.00	2.50	1.96	1.43	1.00	0.22	2.56	2.13

Heifers were inoculated with Cy (5 mg/kg) four days before vaccination. Animals were bled on days −4, 0, 7, 14, 28, 60 and 90 pv. On day 180, the animals were revaccinated, and blood samples were obtained 30 and 60 days later. Numbers are expressed as negative logarithms of reciprocal titers against the O_1 Campos subtype of FMDV.

triplicate, were mixed with 1×10^3 $TCID_{50}$ of O_1 or C_3 viral stocks of FMDV. The neutralization titer of the tested sera was obtained according to the Spearman–Kärber method.

Experimental design. Four days before vaccination, the experimental group of animals received Cy (5 mg/kg body weight) dissolved in 5 ml of double-distilled water by an intraperitoneal route. All the heifers were immunized with FMD vaccine by a subcutaneous route. Four days before vaccination, on the day of vaccination, and at 7-, 14-, 28-, 60- and 90-day intervals afterwards blood samples were collected to determine the titer of anti-FMDV nAb. In addition, the peripheral blood leukocyte number and differential count were carried out on samples collected at −4, 7, 14 and 28 days pv.

The same animals received a second dose of the vaccine (without a second challenge of Cy) 180 days after the first immunization. A search for anti-FMDV nAb was carried out 30 and 60 days later.

Statistical analysis. Analysis of the significance of nAb titer differences between treated and non-treated groups, at different times pv, was carried out with the Student *t* test.

Results

Effect of Cy on peripheral blood leukocytes

As can be seen in table 1, Cy induced mild leukopenia lasting for about two weeks. Differential count showed that the decrease in the leukocyte number was mainly due to neutropenia and monocytopenia.

Neutropenia and monocytopenia were transient and significant ($p < 0.005$) but at 14 and 28 days post-vaccination (pv) no significant differences ($p > 0.1$) were found between the Cy-treated and the control group of animals.

Anti-FMDV neutralizing antibodies

At 7 days pv, no significant differences were found in nAb titers against the O_1 serotype between the Cy-treated and non-treated animals. Statistically significant ($p < 0.005$) differences between the two groups were

observed after 14 days pv. At 60 days pv, while in non-treated heifers the nAb titer was reduced almost completely, all the Cy-treated animals showed significantly higher nAb titers ($p < 0.0001$). These differences were more evident at 90 days pv, where an effective level of nAb, as defined by Sutmöller et al.[8], was detected in only one of the four control animals. In contrast, the Cy-treated animals presented nAb titers at a level that can protect more than 65% of vaccinated cattle. Results are summarized in table 2. Serum titration with the C_3 serotype of the FMDV showed similar results (data not shown). The A_{24} serotype of the FMDV was not tested in this work.

In addition, the secondary response was also modified by the Cy-treatment. Sixty days after revaccination the Cy-treated animals presented nAb titers higher than those of the control heifers ($p < 0.0001$).

Discussion

In veterinary medicine, adjuvants are usually used to potentiate the immunogenicity of killed virus vaccines and toxoids. A large variety of compounds have been employed as adjuvants, although in many cases their mode of action is unclear. The simplest adjuvants are those that function by slowing the release of antigens into the body and increasing cell-mediated and humoral immune responses against them[9]. The best adjuvant is one that can induce high antibody levels with a minimal dose of antigen and with a reduced number of inoculations[10]. Due to the non-desirable side effects of traditional adjuvants, new, more efficient products have been studied in the last few years[11–13]. We chose the aluminum hydroxide-saponin supplemented FMDV vaccine because in a short-term analysis no differences could be observed between aluminum hydroxide and oil adjuvants[14]. Moreover, cases of aluminum intoxication are rare[15].

Our results showed that pretreatment with Cy increased the duration of the anti-FMDV nAb response in cattle vaccinated with the commercial aluminum hydroxide-saponin FMDV vaccine. Furthermore, no significant local or systemic side effects were observed in any of the animals. On the other hand, while in non-treated animals nAb titers were significantly reduced at 60 days pv and were not detected at all at 90 days pv, all the Cy-treated animals presented nAb in their sera during that period.

Considering the results, we propose that Cy could be used as a vaccine enhancer. This opinion is based on the minimal adverse effect of the drug in the doses employed for the experiments and the rapid clearance due to its short half-life (2–4 h)[16,17]. Because of its rapid clearance (explaining the reversal of the mild neutropenia and monocytopenia found by days 0–7 pv), it is supposed that no residual Cy is present at the time when cattle are slaughtered.

Our results cannot explain the mode of action of Cy. When the drug is given in large doses, an immunosuppressive effect can be observed[18–20]. At low doses it can act as an immunomodulating drug[21,22], probably by its action on the suppressor cascade[22].

The necessity of drug administration four days before immunization represents a serious handicap because it increases the cost of the vaccination procedure. However, if the drug can lengthen the duration of the immune response, the handicap will be compensated for by the benefit of preventing the necessity of future vaccinations, or at least by increasing the interval between vaccinations.

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