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Short communication

Evaluation of cytokines as adjuvants of infectious bovine keratoconjunctivitis vaccines

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ABSTRACT

In this work two cytokines were used in combination with inactivated bacteria (bacterin) to test the bovine conjuctival immune response to the pathogen *Moraxella bovis*, the causative agent of Infectious bovine keratoconjunctivitis (IBK). Treatments using the bacterin vaccine combined with interleukin-2 and interferon-α as adjuvants (Group A), the bacterin vaccine only (Group B), and controls without treatment (Group C), were applied by ocular spraying to evaluate the local immune response in the corneal structure of cattle experimentally infected with *M. bovis*. Six weeks after infection, 14 out of a total of 34 animals presented different corneal lesions; 9 corresponding to the control group C, 4 to the group B and only one to the group A. According to the clinical manifestations, a numeric score was calculated. Control animals presented the highest score value (12 points), followed by group B (7.5 points) and group A (1 point). These results suggest that the addition of cytokines to *M. bovis* treatments can reduce not only eye injuries caused by IBK but also the number of diseased animals.

1. Introduction

Infectious bovine keratoconjunctivitis (IBK) is an important veterinary infection of cattle caused by the bacteria *Moraxella bovis* (Barner, 1952). The infection is spread by direct contact or by the common fly (*Musca* spp.) serving as a vector and is currently the most common ocular disease affecting cattle (Postma et al., 2008). IBK is similar to human "pink eye" and causes severe infection of the conjunctiva, edema, corneal opacity and ulceration. Younger animals are more susceptible but recovery with minimal damage is usual if they are treated early. *M. bovis* uses several different serotyped fimbriae as virulence factors; exploited for the development of new vaccines. In the last decades, different strategies for vaccination against IBK have been developed, however, so far little success have been achieved (Burns and O'Connor, 2008). Variability in the response to vaccines prepared from *M. bovis* can be explained by the isolation of new bacterial species such as *M. bovoculi*, however vaccines prepared with *M. bovoculi* were not effective at all against IBK. Another drawback of *M. bovis* vaccinations is the limitation in the production of recombinant or purified toxins. Numerous tests were performed using adhesins but always with low or no level of protection and with high variability among herds as demonstrated by vaccines that were introduced in countries from the "Southern Common Market" (Mercosur) in 1983. Since 1990 outbreaks in herds routinely vaccinated have been frequently reported, suggesting differences in their genotypic and phenotypic characteristics, including their sensitivity to antibiotics (Rochedo Conceição et al.,

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2004). Antibiotics are used worldwide to treat animals with IBK and to prevent dissemination of bacteria. While *M. bovis* strains grown in vitro showed a quite homogeneous susceptibility to a variety of antibiotics, the effect of them on natural isolates is highly variable (McConnel et al., 2007). Due to these variations in susceptibility, it is advisable to perform antibiogram assays on strains isolated during an outbreak before implementing a massive use of a specific antibiotic. Although IBK has been reported as a contagious disease since the late 1800s, its treatment and control are still difficult (Davidson and Stokka, 2003). Besides from the animal's pain and suffering, there is a significant economic impact due to IBK, for example, in the United States it was estimated that IBK annually affects more than 10 million calves (Snowder et al., 2005). In the Mercosur, animal health authorities from Argentina, Brazil and Uruguay included it among the eight diseases of cattle to be studied by a multinational co-operative project (Rochedo Conceição et al., 2004). Despite the importance of this disease, there are only few descriptive studies in Argentina on the evolution and prevention of the lesions of IBK.

Cytokines are key communication molecules between host cells involved in the defense against a wide variety of pathogens. Bacterial infections induce the expression of multiple chemokines and proinflammatory cytokines, including interferon-α (IFN-α) and interleukin-2 (IL-2) which have been also shown to potentiate the immune response to vaccination in various experimental models (Tovey and Lallemand, 2010). It is now well accepted that type 1 IFNs (IFN-α and IFN-β), in addition to being molecules with powerful antiviral activity, play a critical role in modulating immune responses to foreign antigens (Tompkins, 1999). Moreover, the mechanism of action of several potent adjuvants, such as Freund's adjuvant, has been shown to be due to the induction of cytokines, including type 1 IFNs, IFN-γ, IL-2, and IL-12, that play key roles in the regulation of innate and adaptive immunity (Tovey and Lallemand, 2010).

Considering the poor and variable results obtained with vaccines, in this work, the use of cytokines combined with inactivated *M. bovis*, were evaluated as IBK preventive treatment.

2. Materials and methods

2.1. Animals and design

The trial was performed at the cattle farm "La Espadaña", Veronica, Buenos Aires, Argentina using 34 rebreed Aberdeen Angus calves, clinically healthy, between 11 and 12 months of age, bred under the same conditions, and with weights between 160 and 180 kg. Before the assay, ocular fluids and blood samples from all animals were analyzed to rule out any pre-existing pathology.

2.2. Bacterial preparations

Three pilliated strains of *M. bovis* were isolated from different IBKs outbreaks which took place in Argentina by the Animal Health Laboratory at the "Pontifical Argentinean Catholic University". The strains were typified according to the following criteria: morphology = diplococcic; gram staining = negative; β-hemolysis in blood agar = positive; oxidase = positive; development in TSI medium = oxidative; catalase = positive; motility = negative; urease = negative; indole = negative; and gelatin = positive. *M. bovis* bacteria were cultured in bovine heart infusion medium (BHI: 375 g L^{-1} heart infusion, 10 g L^{-1} peptone, 5 g L^{-1} NaCl, 5% (v/v) bovine blood) or BHI-agar (15 g L^{-1}) at 37 °C for 48 h. Cells were collected by centrifugation, washed and resuspended in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7) at a final concentration of 10^{10} cells per mL. Vaccine preparations from inactivated *M. bovis* ("bacterin") were performed by bacterial fixation using 0.5% (w/v) formaldehyde during 48 h. After fixation, cells were washed and resuspended in PBS, 0.2% (w/v) formaldehyde at a final concentration of 10^{10} cells per mL. Bacterial and bacterin treatments were applied by ocular spraying (300 μL of the suspension) using the same bacterial strains.

2.3. Animal treatments and sample collection

Calves were subdivided in three experimental groups according to the following: group A, 11 animals treated with 300 μL of bacterin combined with 20 μg mL^{-1} (66,000 UI) of recombinant human IL-2 (Ilcass, Varifarma) and 10 μg mL^{-1} (66,000 UI) of recombinant human IFN-α (Avirostat, Varifarma); group B, 11 animals treated with 300 μL of bacterin only and; group C, 12 animals treated with placebo (300 μL of PBS). Two doses were applied, one at the beginning of the trial and one three weeks later. Forty-five days after the last dose, all animals were experimentally infected by inoculation with *M. bovis*, as described in Section 2.2. Six weeks after infection samples from lachrymal fluid and eyes swabbing were collected, cultured and typified as previously described (Pugh et al., 1966). The doses of cytokines were estimated from a few previous studies available in the literature (Romano et al., 1980; Lin et al., 2005). Diagnostic pH indicator strips (Merck) were used to determine pH values of lachrymal films. Total white blood cell counts were performed using an hemocytometer.

2.4. Scoring of clinical manifestations

Clinical evaluation of infected cattle was performed six weeks after *M. bovis* inoculation and numeric scores were calculated according to the following criteria: normal eye (NE) = 0 pt; conjunctival congestion (CC) = 0.5 pt; corneal clouding (CL) = 1.0 pt; corneal opacity (CO) = 2 pt; corneal ulcer (CU) = 3 pt; keratoconus (KC) = 4 pt (Fiorentino et al., 2001).

2.5. Statistical analyses

Proportions of diseased animals were compared between groups using the chi-square test and post hoc comparisons. The distribution of the obtained scoring data was analyzed using the Kolmogorov–Smirnov test followed

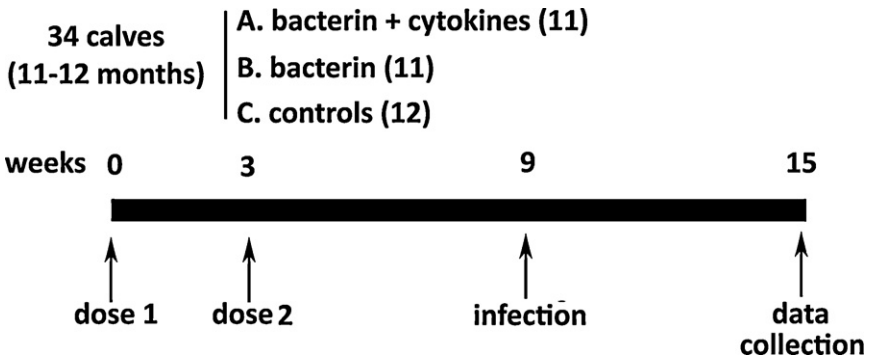

Fig. 1. Schematic representation of the trial. A time-course of the treatments is represented by the black bar, starting from the first dose on week 0 and finishing on week 15 with the data collection. The three experimental groups are also showed and the numbers of calves are indicated between parentheses.

by a Kruskal–Wallis non-parametric comparison. Analyzes were performed using the IBM SPSS Statistics software version 11.5.

3. Results and discussion

3.1. Effect of cytokines on animals' infection and clinical manifestations

After treatment and infection, as represented in the diagram in Fig. 1, ocular examination showed that 14 out of a total of 34 animals (41%) presented different corneal lesions; 9 corresponding to the control group C, 4 to the group B and only one to the group A. Chi-square analysis and post hoc comparisons showed significant differences between the proportion of diseased animals from groups A and C (Table 1). Other controls, such as animals treated with cytokines without bacterin, were omitted due to the limitation on the number of animals available. Considering that the comparison between the treatments involving cytokine supplemented bacterin and bacterin control would be much more informative, we chose a single control (in addition to the untreated group).

According to the clinical manifestations, a numeric score was calculated for each group (see Section 2). Control animals presented the highest score value (12 points), followed by group B (7.5 points) and group A (1 point). Control group C showed the most serious clinical manifestations, two cases of bilateral ulcers, a single case of complete corneal opacity, two cases of bilateral corneal clouding, and four cases of mucopurulent conjunctivitis. Group B presented two cases of bilateral ulcers, one case of corneal clouding, and one case of mucopurulent conjunctivitis. Group A included only one case of unilateral corneal clouding (Table 1). Non-parametric statistical analysis showed significant differences between group A and group C (Table 1). These results are consistent with those obtained by comparing the proportions of diseased animals, demonstrating the effect of cytokines as adjuvants of the IBK vaccine. Finally, all animals were tested by eyes swabbing and subsequent bacterial culture and microbiological identification of *M. bovis*. As expected, no infection was found in healthy animals and those animals diagnosed for IBK were also positive for *M. bovis*, according to the criteria described under Section 2, thus confirming the protocol previously done.

3.2. Cattle ocular pH, and white blood cell counts

In order to evaluate the effects of IBK on the lachrymal film pH, diseased and healthy animals were compared. The pH values of all animals analyzed were similar, in the

Table 1
Analysis of animals' clinical signs.

	Group A ($N = 11$)	Group B ($N = 11$)	Group C ($N = 12$)
Animals W/ clinical signs (total)	1	4	9
Animals W/ clinical signs (%)	9	36	75
Normal eye (NE)	10 (0.0)	7 (0.0)	3 (0.0)
Conjunctival congestion (CC)	–	1 (0.5)	4 (2.0)
Corneal clouding (CL)	1 (1.0)	1 (1.0)	2 (2.0)
Corneal opacity (CO)	–	–	1 (2.0)
Corneal ulcer (CU)	–	2 (6.0)	2 (6.0)
Total score	1.0	7.5	12
Normalized score	0.09	0.68	1.00

Chi-square analysis followed by post hoc comparisons of proportions of animals with clinical signs showed significant differences between groups A and C (chi-square = 10.448, $P = 0.005$). Numeric scores were calculated according the following criteria: normal eye (NE) = 0; conjunctival congestion (CC) = 0.5; corneal clouding (CL) = 1.0; corneal opacity (CO) = 2; corneal ulcer (CU) = 3. Clinical scores are indicated in parenthesis. The normalized score refers to the ratios of the total score and the number of animals per group. Since the data were not normally distributed (Kolmogorov–Smirnov $P < 0.05$) total score was compared between groups using the Kruskal–Wallis test. The results show significant differences, $H_2 = 8.47$, $P = 0.014$. Paired comparisons, using Mann–Whitney U-test, indicated that there are differences between group A and group C, $U = 23$, $n_1 = 11$ $n_2 = 12$, $P = 0.03$. Other comparisons were not significant, $P > 0.05$.

normal range (6.0–6.5), even in those individuals who showed severe eye injuries.

A preliminary determination of the immunological response to *M. bovis*, in animals subjected to the different treatments was performed by white cells counting (leukogram) using an hemocytometer. Samples from animals treated with bacterin, with or without cytokines showed statistically significant differences (1.44×10^{10} and 1.46×10^{10} cells per L, respectively) only with the control group (0.96×10^{10} cells per L), but not between them.

Finally, no differences in terms of weight gain were observed between the experimental groups.

This work clearly demonstrated the benefits of the application of cytokines together with bacterin-based vaccines as a preventive IBK treatment since only one treated calf became diseased presenting only slight clinical manifestations. The observed adjuvant properties of IFN-α and IL-2 combined with a bacterin vaccine was probably based on the triggering effect of the IFN-α over the immune response and the leukocytotrophic properties of IL-2 that is instrumental in the body's natural response to bacterial infections. In addition, the use of bacterin prepared using *M. bovis* strains isolated from the endemic area presents advantages in terms of costs and availability compared to recombinant vaccines.

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