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Adjuvant effect of green propolis on humoral immune response of bovines immunized with bovine herpesvirus type 5

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Abstract

Despite recent technological advances in vaccine production, most vaccines depend on the association with adjuvant substances. In this study, propolis, which has been attracting the attention of researchers due to its bioactive properties, was evaluated as an immunological adjuvant. The association of 40 mg/dose of an ethanolic extract of green propolis with an inactivated oil vaccine against bovine herpesvirus type 5 (BoHV-5), resulted in a significant increase ($P < 0.01$) in the neutralizing antibody levels, comparing to the bovines that received the same vaccine without propolis. Besides, propolis increased the percentage of animals with high antibody titers (above 32). Phenolic compounds such as artepillin C (3,5-diprenyl-4-hydroxycinnamic acid) and the derivatives of cinnamic acid besides other flavonoid substances were abundant in the propolis extract used, and they could be the main substances with adjuvant action. The effect of the green propolis extract on the humoral immune response can be exploited in the development of new vaccines.

Keywords: Green propolis; Immunological adjuvant; Antibodies; Bovines

1. Introduction

The recent progress in the development of vaccines has allowed their use not only as a prophylactic product, but also in the treatment of cancer, immunological disorders and chronic infections (Blom and Hilgers, 2004). However, many of them require association with adjuvant substances which, when combined with an antigen, increase its immunogenicity, potentiating the humoral and/or cellular immune responses (Storni et al., 2005; Barr et al., 2006). Besides, immunological adjuvants can be used to extend the duration of the immune response or to stimulate mucosal immunity (Leclerc, 2003).

Even though several substances have been evaluated regarding their adjuvant capabilities, as a general rule,

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production of vaccines is still dependent on the use of aluminum salts (Leclerc, 2003) or oil emulsions, specific for vaccines of veterinary use (Jansen et al., 2006). This way, the development of new vaccines will be highly benefited with the identification of new substances capable of promoting and directing to a proper immune response (Cox and Coulter, 1997; Singh and O'Hagan, 2002).

Oil adjuvants, which are no longer in use for humans due to their adverse effects (Lindblad, 2000), are still largely used in vaccines for veterinary use. These oil emulsions, used specially in association with inactivated antigens (Jansen et al., 2006), potentiate the immune system through the formation of a deposit at the inoculation site, with slow and long release of the antigen (Cox and Coulter, 1997). However, these inactivated vaccines need periodic revaccinations to produce an efficient immunological response (Fenner et al., 1993).

Propolis is a resinous material produced by bees that displays a variety of biological activities against viruses, bacteria, fungi, pathogenic protozoa, and also tumor cells (De Castro, 2001). This natural product also behaves as anti-hyperalgesic and anti-inflammatory agent (De Campos et al., 1998). Despite the demonstration of its immunomodulator activities (Dimov et al., 1992; Orsi et al., 2000), many of the mechanisms of action are still unknown.

Chemical studies revealed the complex composition of propolis, with more than 300 constituents, including several bioactive phenolic compounds such as flavonoids and derivatives of hydroxycinnamic acids (Bankova et al., 2000). These constitutive characteristics can vary according to the bee species and the period of the year (Bankova, 2005). Nevertheless, the botanical origin (Bankova et al., 2000; Bankova, 2005) seems to be the most important factor to be considered when trying to explain the chemical variability among different propolis samples. Green propolis, only found in Brazil, is produced from a plant commonly known as ''Alecrim do Campo'' (*Baccharis dracunculifolia*). This species is not adapted to the natural conditions of other countries (Miyataka et al., 1997), which confers to the green propolis chemical and biological characteristics different from the European propolis, produced predominantly from the exudates of buds of aspen (*Populus* sp., Bankova et al., 2000).

The aim of this work was to evaluate the adjuvant capability of a green propolis extract when associated with an inactivated oil vaccine against bovine herpesvirus type 5, through determination of neutralizing antibody titers, and characterization of the chemical composition of this extract by high performance liquid chromatography (HPLC).

2. Material and methods

2.1. Preparation, botanical and chemical characterization of the propolis ethanolic extract

The green propolis sample was collected in the State of Minas Gerais (South-East region of Brazil) by Nectar Farmacêutica Ltda (sample number SBN-54), and stored at −20 °C. The ethanolic extract was prepared as previoulsy described (Paulino et al., 2002). Briefly, the propolis was ground and macerated with an extract solution containing absolute ethanol, using 10 min daily agitation, for 10 days. Then, the solvent was evaporated and the resulting dried matter was dissolved in phosphate buffer solution (pH 6.2), in a final concentration of 40 mg/ml (4%, w/v) and called GP1. The chemical composition of the green propolis extract was determined by high performance liquid chromatography (HPLC), using a Merck-Hitachi chromatographer (Germany), equiped with the L-7100 pressure pump and the L-7455 diode array detector. Separation was carried out in a Lichrochart 125-4 (Merck, Darmstadt, Germany) column as previously described (Marcucci et al., 2001). The detection of components was monitored at 280 nm and standard compounds were co-chromatographed with the extract. Analysis of the data was carried out using the Merck-Hitachi D-7000 (Chromatography Data Station, DAD Manager).

2.2. Vaccines and inoculations

Evaluation of adjuvant properties of the green propolis extract was made through its association with an inactivated oil vaccine against bovine herpesvirus type 5. This virus, supplied by the Virology and Immunology Laboratory, UFPel (Pelotas, Brazil), was propagated in Madin Darby Bovine Kidney (MDBK, ATCC) cell line. After inactivation with bromoethylamine BEI ($C_2H_7Br_2N$, Merck), in a final concentration of 0.02 M and pH 7.5, the viral suspension with titer of 10^8 $CCID_{50}$/ml (cell culture infections dose 50% ml^{-1}) was emulsified in mineral oil (Marcol 52, Esso Standart Oil Co.), together with the propolis extract.

In this study, 60 randomly selected Hereford cattle, male and female, weighting approximately 150 kg and seronegative for BoHV-5 determined by serum

neutralization (House and Baker, 1971), were used. The animals were kept in pasture rotation together with other cattle of the farm. They were allocated into three groups of 20 animals (control, G1 and G2) and vaccinated intramuscularly (IM) at days 0, 30 and 60. The volume inoculated was adjusted according to the concentration of the propolis solution used per dose, varying from 3 to 5 ml, however the antigen concentration was constant in all the treatments (10^8 $CCID_{50}$/ml/dose). The control group received 3 ml of vaccine without propolis, the group G1 received 5 ml of the same vaccine with 20 mg/dose of propolis, whereas group G2 received 5 ml of this vaccine with 40 mg/dose of propolis. The animals were observed daily until the 10th day after each inoculation in order to observe the occurrence of adverse effects due to vaccination. The experiment was approved by the UFPel Committee of Ethics in Animal Experimentation.

2.3. Serology

For titering neutralizing antibodies against BoHV-5, individual serum samples were collected 30 days after each inoculation and stored at −20 °C. Antibodies were titered by the serum neutralization method (House and Baker, 1971). Briefly, each serum was individually diluted in logarithmic base 2 from 1:2 to 1:256. After distribution (25 μl) in quadruplicate in polystyrene plates (TPP), 25 μl of BoHV-5 virus suspension containing 100 CCID 50% was added. After incubation for 1 h at 37 °C in an environment with 5% CO_2, approximately 30,000 MDBK cells were added per well. The microplates were then returned to the incubator until being read in an inverted microscope when the 100 CCID 50% was observed in the control cells. The absence of cytopathic effect was an indication of the viral neutralization by neutralizing antibodies. The antibody titer was calculated by Behrends and Kärber (Mayr et al., 1982) statistical method, and it was represented by the highest serum dilution capable of neutralizing 100 CCID 50% of the virus.

Table 1
Chemical characterization of green propolis determined by high performance liquid chromatography—HPLC

Component identified in propolis	Dry extract (mg/g)
2,2-Dimethyl-6-carboxyethenyl-2H-1-benzopiran	3.17
2.2-Dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic acid	9.77
3.5-Diprenyl-4-hydroxycinnamic acid (derivative 1-9)	17.75
3.5-Diprenyl-4-hydroxycinnamic acid (artepillin C)	27.77
3-Prenyl-4-hydroxycinnamic acid	5.71
Caffeic acid	2.43
Caffeic acid (derivative 1)	11.35
Cinnamic acid derivative	65.98
Ferulic acid	7.21
Kaempferol (derivative 1)	20.45
p-Coumaric acid	18.03
Pinobanksin	31.48
Total (mg/g of crude resin)	221.10
Total (%)	22.11

2.4. Statistical analysis

Antibody titers were compared using variance analysis (ANOVA) with repeated measurements. The L.S.D. test was used to determine significant differences ($P < 0.05$) among the mean of each treatment using the SAS program.

3. Results

3.1. Chemical composition of the propolis extract

As can be observed in Table 1, the HPLC analysis of the green propolis sample utilized in this experiment showed high levels of the phenolic compounds 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C), 2,2-dimethyl-6-carboxyethenyl-2H-1-benzopiran, 3-prenyl-4-hydroxycinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, besides cinnamic acid and the flavonoids pinobanksin and kaempferol. In this sample of green propolis, the flavonoids corresponded to 22.37% of the dried extract.

3.2. Humoral response

In this work, the adjuvant properties of an ethanolic extract of green propolis when associated with an inactivated oil vaccine against BoHV-5 was evaluated. A dose dependent effect was demonstrated. The inclusion of 40 mg/dose of this extract in the experimental vaccine increased the humoral immune response ($P < 0.01$), measured by antibodies titers, compared to the control group (vaccine without propolis) (Fig. 1). Sixty days after the first inoculation, the titer increased from 35 (negative control, without propolis) to 54 in the treatment with 40 mg/dose of propolis. This difference remained the same 90 days after the first inoculation, when the antibody titers were 43 and 67, respectively. However, the addition of 20 mg/dose of the propolis

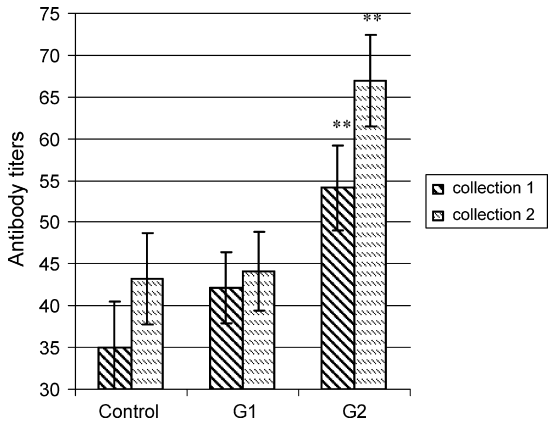

Fig. 1. Mean titer ± S.E.M. of neutralizing antibodies (expressed as reciprocal of the serum dilution) of cattle immunized with an inactivated oil vaccine against BoHV-5 without propolis (control), with 20 mg/dose of an ethanolic extract of green propolis (G1) or 40 mg/dose of propolis (G2). The titer was determined by the serum neutralization test, 30 days after the second inoculation (collection 1) or 30 days after the third inoculation (collection 2). $^{**}P < 0.01$ compared to the control group.

extract did not result in a statistically significant alteration, when compared to the control group. Besides increasing antibody titers of cattle vaccinated with BoHV-5, the use of 40 mg/dose of green propolis solution also increased the percentage of animals with antibody titers equal or higher than 32, especially 30 days after the third vaccination (Table 2).

Adjuvants are used in several types of vaccine aiming at the optimization of the humoral and/or cellular responses. However many substances with adjuvant properties cannot be used in vaccines for human or veterinary use due to their adverse effects (Estrada et al., 2000). In this study, conducted in cattle, no adverse effect was observed due to the association of propolis with the oil vaccine against BoHV-5.

Table 2
Accumulated percent distribution of neutralizing antibodies titers in bovines vaccinated with BoHV-5

Titer[a]	30 days after second dose			30 days after third dose		
	Control[b]	G1[c]	G2[d]	Control	G1	G2
0–7.9	100	100	100	100	100	100
8–15.9	100	100	100	100	93.7	100
16–31.9	72.3	100	100	81.3	77.9	100
32–63.9	44.6	61.2	72.2	43.8	51.6	70.6
≥64	22.3	27.8	33.4	31.3	21.0	58.8

[a] Titers expressed by the reciprocal.
[b] Vaccine without propolis.
[c] Vaccine with 20 mg/dose of ethanolic extract of green propolis.
[d] Vaccine with 40 mg/dose of ethanolic extract of green propolis.

4. Discussion

Due to the high chemical complexity of propolis, it is extremely difficult to identify which substances are responsible for its biological activities. Some researchers state that it is indeed the heterogeneous composition, besides the combination of natural substances that confers to propolis its bioactive properties (Kujumgiev et al., 1999; Ozkul et al., 2005). According to Sforcin et al. (2005) the action of propolis on the immunological system results from the synergism of several substances. The elements detected in the green propolis extract utilized in this experiment, determined by HPLC analysis, show that its botanical origin is the *Bacharis dracunculifolia* plant (Marcucci, 1995; Bankova et al., 2000). In a previous study, multivariate analysis associating ethanol extracts of different samples with the levels of bioactive compounds determined by HPLC allowed the typing of Brazilian propolis (Marcucci et al., 2001). The results were similar to the ones found in this experiment, with predominance of phenolic compounds and cinnamic acid derivatives. In these extracts, the flavonoids corresponded to 22.37% of the dried extract. These substances are known to stimulate humoral as well as cellular immunity (Havsteen, 2002). Although the precise mechanism of action remains unknown, it is possible that the flavonoids stimulate production of cytokines, particularly interleukin 1 (IL-1) and IL-2, which have mitogenic action for B and T lymphocytes (Havsteen, 2002). Artepillin C seems to perform its immunostimulating activity through increase in the number of auxiliar T lymphocytes (Kimoto et al., 1998).

The association of different adjuvant substances aims at combining their properties responsible for the stimulation of the immune system. Complete Freund adjuvant, for example, combines the immunomodulator properties of *Mycobacterium tuberculosis* with the activity of the oil emulsion (Cox and Coulter, 1997). In this experiment, the association of 40 mg/dose of an ethanolic extract of green propolis whith an inactivated oil vaccine against BoHV-5 increased the humoral immune response ($P < 0.01$), measured by neutralizing antibodies titers. It is conceivable that the combination of oil with propolis allowed the formation of a deposit in the inoculation site, resulting in a slow and extended antigen (BoHV-5) and propolis release (Cox and Coulter, 1997), allowing a constant stimulus of the immunological system. According to Jansen et al. (2006), the continuous release of non-replicative antigens results in extended humoral immunity. The propolis extract may have acted as an auxiliary adjuvant

substance, potentiating the humoral response triggered by the antigen associated to the oil. According to Sforcin et al. (2005), propolis ability in modulating antibody synthesis is part of its adjuvant action. The propolis immunostimulating capacity, through an increase in the immunoglobulin levels has already been reported in patients with fibrosing alveolitis (Scheller et al., 1989).

The precise mechanism of action of propolis on cells from the immune system remains unknown (Ansorge et al., 2003). However, it is known that artepillin C, found in large scale in the green propolis sample used, acts on macrophages stimulating the production of IL-12 (Sforcin et al., 2002), which potentiates immunoglobulin production by B cells. Other phenolic compounds, such as cinnamic acid derivatives found in the propolis sample used, also induce production and releasing of cytokines like IL-1, IL-6 and IL-8 by activated macrophages, stimulating antibody production (Orsolic et al., 2005). Such mechanisms can explain the increment in the humoral response observed in the present study. Another hypothesis suggests that propolis can reduce lipid peroxidation and stimulate the immune system by means of direct lymphocyte activation (Kimoto et al., 1998).

Besides increasing humoral immune response of cattle vaccinated with BoHV-5, the use of 40 mg/dose of an ethanolic green propolis extract also increased the percentage of animals with titers higher than 32 (Table 2). According to Lazarowics et al. (1983), bovines with titer equal or higher than 32 resist challenge with field virus. This fact is yet more relevant considering that many farmers do not use vaccines in a prophylactic way, but only after positive diagnosis of the disease in the farm, which implies the presence of the virus in the herd. Even though cellular immunity is more important in the case of BoHV primary infection, humoral immunity is more effective in preventing the resurgence of the disease, once BoHV remains latent in the animal after infection (Babiuk et al., 1996). As comparative parameter, the United States Department of Agriculture considers as immunized a herd which has 80% of vaccinated animals with titer equal to or above 8 (USDA, 2005).

Due to the increase of neutralizing antibody titers, in addition to the percentage of animals with high titers, further studies in the development of this vaccine can take into consideration the possibility of increasing the interval between vaccinations, as well as decreasing the amount of antigen per dose. These approaches, besides lowering the cost of the vaccine, could allow a reduction in the handling of the animals.

5. Conclusions

The data presented in this study showed that an ethanolic extract of green propolis increased the potency of the humoral immune response in cattle when associated with an inactivated oil vaccine against BoHV-5, showing adjuvant action. Besides the increment in neutralizing antibody titers, there was an increase in the percentage of animals with titers higher than 32. Propolis compounds responsible for the immunostimulating activities have not yet been determined, but the flavonoids and other phenolic compounds, like artepillin C, found in large quantity in the sample studied, could have been the main substances with action on the immune system. The use of ethanolic extract of green propolis can contribute for the efficacy of inactivated vaccines, acting as an immunostimulant.

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