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Short communication

Immune response of bovines stimulated by synthetic vaccine SBm7462® against *Rhipicephalus* (*Boophilus*) *microplus*

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ABSTRACT

Ten-month-old calves *Bos taurus taurus* were immunized with three doses of SBm7462® with saponin as an adjuvant at 30-day intervals and were evaluated for IgG isotypes, phenotype circulating lymphocytes and changes in the lymph nodes (LN). SBm7462® stimulated the production of predominantly IgG1-isotype IgG antibodies. The lymph nodes exhibited activation at the seventh day after the first immunization, with areas of paracortical and interfollicular hyperplasia and the early formation of germinal centers (GC). Fifteen days after the first immunization, the GC exhibited compartmentalization of cellular populations, a light zone (LZ), a dark zone (DZ) and a mantle. At the same time, hyperplasia of the medullary cords was observed with cells associating with DC cells. Seven days after the first immunization, apoptosis in the DZ and in the paracortical region became evident. By day 15, there was an increase in the medullary cords, which became more numerous at days 35 and 42. PAP-positive cells were found in the paracortical region, medullary cords and GC 7 days after the first immunization. At day 35, there were further strongly PAP-positive cells in the medullary cords. By comparison, none of these changes were observed in the lymph nodes of control groups at any of the days analyzed. The number of $CD21^+$ lymphocytes increased in the immunized groups after the first inoculation, with a maximum number observed at 15 and 10 days after the first and third immunizations, respectively. Compared to pre-immunization counts, the percentage of $WC1^+$ γδ T-lymphocytes displayed more variation, increasing 5 days after the second immunization but decreasing over the following days. According to the results, the synthetic anti *Rhipicephalus microplus* vaccine elicits a complete immune response being T-dependant.

1. Introduction

Ticks are a major concern for public health and commercial animal production due to their ability to vector a number of infectious pathogens. The tick *Rhipicephalus* (*Boophilus*) *microplus* is one of the important arthropods in veterinary medicine because of the economic losses caused in cattle raising in Central and South America and Australia.

The common method of control is the use of chemical products. However, this form of control presents several inconveniences, and the most important of them is the crossed resistance to different chemical bases such as organophosphates (Patarroyo and Costa, 1980) formami-

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dines, cycloamidines, pyrethroids (Lodos et al., 1999) and ivermectins (Martins and Furlong, 2001), in addition to contamination of the environment and food products of animal origin.

In the animal production context, vaccines would be a strategy of control widely accepted because they are safe for the consumer due to absence of residues, for the host and for the environment.

In *R. microplus* the immunity was obtained, in the first instance, after inoculation of "concealed" antigens from the midgut of tick (Opdebeeck et al., 1988) and later on, with the use of a purified glycoprotein (Bm86) (Willadsen et al., 1989), or with its mass production as a recombinant vaccine (Rand et al., 1989).

The recombinant vaccine, already in the market since 1994, vaccination programs have been established; nonetheless, different levels of efficacy to *R. microplus* strains have been experienced, and sequence variations in the target protein among different strains have been found to be associated with variable efficacy (De La Fuente et al., 2000; Sossai et al., 2005).

This has triggered researches for different immunogens, such as the synthetic peptides. The synthetic vaccine SBm7462® containing antigenic determinants of the Bm86 protein which are common to different strains of the tick (Peconick et al., 2008) and added to saponin as adjuvant, was tested in cattle and a efficacy of 81.05% was given (Patarroyo et al., 2002).

The immunity produced by concealed protein antigens, originated from *R. microplus*, in natural, recombinants or synthetics forms, has generated a variable efficacy in a large number of experiments in field and controlled (Rodriguez et al., 1995; De La Fuente et al., 1998; Patarroyo et al., 2002). However, research on this immune protective response has been performed with recombinant proteins, focused on antibodies and their effector properties, although a number or essays characterize the specific antibody isotypes involved (Opdebeeck and Daly, 1990; Valle et al., 2001).

It should be borne in mind that a rational approach to vaccine development for tick control is a long and complicated process and it is necessary to get familiar with some basic immunological mechanisms induced by antigens. The present essay describes, in the "continuum" of the immune response, some induction mechanisms in bovines immunized with the synthetic oligopeptide SBm7462®, such as the kinetics of humoral response (IgG), the predominant isotope, the development of the response in lymph nodes and the phenotypic profile of circulating lymphocytes. The induction of immunity occurs almost exclusively in secondary lymphoid organs (i.e., lymph nodes and spleen). Consequently, examination of lymphoid tissues allowed us to elucidate many of the steps involved in the generation of immunity.

2. Materials and methods

2.1. Experimental animals and immunization protocols

Animals used received treatment in accordance with the animal experimentation rules described in the International Guiding Principles for Biomedical Research Involving Animals. Eight Holstein Frisian, with 10 months age, and intact spleens, were kept individually in an arthropod-free isolation system since birth. They were randomly divided into three groups. One vaccinal group composed of four animals, one control group composed of two animals, and the remaining animals constituted a control group with adjuvant. The animals were inoculated subcutaneously in the neck on days 1, 30 and 60. The formulation was composed of 2 mg of SBm7462® plus 1.5 mg of saponin as adjuvant, resuspended in 4 ml of PBS pH 7.4 (Na_2HPO_4 6.4 mM, KH_2PO_4 10 mM, NaCl 73 mM) per dose. The animals belonging to the control group with adjuvant received 1.5 mg of saponin in 4 ml of PBS per dose, and the animals from the control group received 4 ml of PBS per dose.

2.2. ELISA antibody serology

Serums were collected weekly for 14 weeks after the first inoculation in order to track the antibody response and the isotype of elicited antibody. MaxSorp Nunc® Plates were used and the protocol used was previously described (Patarroyo et al., 2002). Samples were diluted from 1:100 up to 1:2400 (data showed 1:400) in incubation buffer containing Tween 20 and tested in triplicate. Bovine anti-peptide IgG was detected with rabbit anti-bovine IgG (heavy and light specific chains) conjugated with horseradish peroxidase (Sigma® diluted at 1:20,000). Samples were considered positive when showed an optical density (OD) higher than the mean plus two standard deviations of the OD obtained for negative controls.

Specific isotypes were measured, sensitizing the plate overnight at 4 °C with 2 μg/well of peptide diluted in 0.13 M carbonate buffer pH 9.6 serum assayed in duplicate and diluted 1:100; blocked with a solution of gelatine 2% in PBS pH 7.6 during 2 h at room temperature; wells were incubated with purified sheep anti-bovine IgG1 and IgG2 conjugated with horseradish peroxidase (Bethyl Labs.), previously titrated for optimal dilutions (1:30,000).

2.3. Bovine lymph nodes, collection and preparation

Pre-scapular lymph nodes (LNs) from the control were surgically removed 2 days prior to first inoculation, and were used during the research. In each animal from the vaccinated group, the right pre-scapular LN was removed at days 7, 15, 35 and 42 after first immunization. In animals from the saponin group, the right LNs were removed at days 7 and 15 and the left ones at days 35 and 42 after first inoculation. They were sectioned and fixed for 8 h in paraformaldehyde 4% (pH 7.2), and included in Paraplast Plus® (Sigma) resin and stained with H&E.

Apoptosis was observed using the instructions kit "In Situ Death Detection, POD® (Roche USA).

For identifying the presence of the immunogen in presenting cells (APC) of LNs, the peroxidase anti-peroxidase (PAP) technique was used. The primary antibody, anti-SBm7462® rabbit serum was diluted 1:20 and then incubated in moist chamber at 4 °C during 18 h. The secondary antibody goat IgG anti rabbit IgG (specific heavy

and light chains) is diluted 1:10 and incubated at 37 °C during 45 min. The PAP complex (Sigma®) produced in rabbit and diluted according to the manufacturer's instruction was used.

2.4. Phenotype of circulating PBMCs

Bovine PBMC were collected from all groups 1 day prior to immunization, and 15, 35, 63 and 70 days after, put into EDTA tubes (BD Vacutainer, Beckton, Dickinson); the cells were isolated by density gradient centrifugation (Histopaque, 1.077 density, Sigma®), collected from the interphase, washed twice in PBS and preserved in liquid nitrogen until their use and cryopreserved with a solution containing bovine serum albumin 30%, Cryoprotective Solution-HES® 60% (Farmoterápica), 10% of DMSO and 2 UI/ml of sodic heparin.

The cell surface markers were analyzed using FACScalibur BD and monoclonal antibodies (mAb): CD 21 specific for bovine B-cells and CC101 pan-specific for bovine WC1 (Serotec). The PBMCs were thawed at 37 °C, washed by centrifugation (300 × *g*) with PBS (10% bovine foetal serum and 2 UI/ml heparin), during 10 min at 4 °C. An amount of 6×10^5 cells were resuspended in 30 μl of PBS and incubated at room temperature during 20 min with 15 μl of various MoAbs and afterwards were added 2 ml of lysing solution (FACS® Lysing Solution) and centrifuged (300 × *g*) at 18 °C during 7 min. Finally the PBMCs were washed and fixed with a solution (paraformaldehyde 1% in sodium cacodylate buffer 6.63 g/L, pH 7.2).

2.5. Statistic

Data were analyzed by analysis of variance (ANOVA) followed by Tukey–Kramer's multiple comparison tests using the SAEG statistical program 8.X. Version (Federal University of Viçosa) and data with $p < 0.05$ were

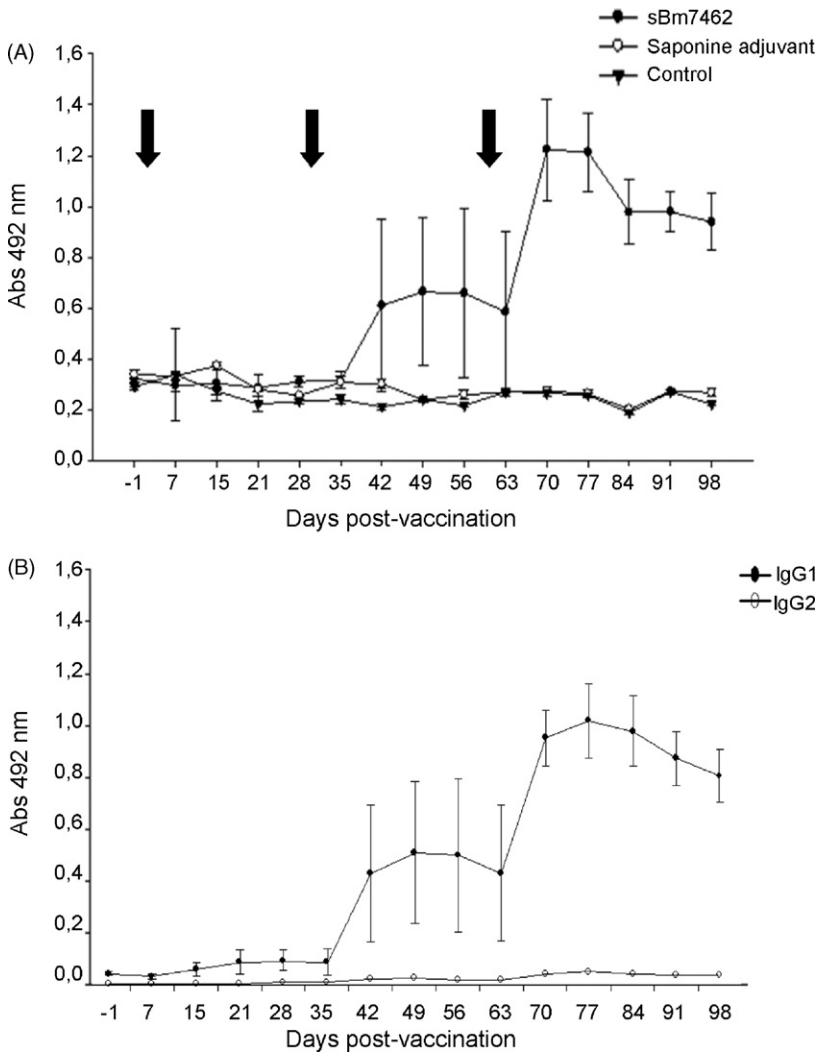

Fig. 1. Kinetics of IgGs (A) and isotypes IgG1 and IgG2 (B) in serum samples from bovine immunized with SBm7462® (sera diluted 1:400). Arrows indicate days of immunization.

considered statistically significant. Mann–Whitney test was used for flow cytometric analyses.

3. Results and discussion

3.1. Antibodies

The dynamics of specific anti-peptide IgG production is shown in Fig. 1A. The level of detectable specific antibodies begins its increment from 28 days after first inoculation, without displaying statistic evidence. Fifteen days after second inoculation the antibody levels already show statistic evidence ($^{**}p < 0.01$) when compared to the controls. The highest IgG peak was recorded 15 days after third inoculation, with titers ranging between 400 and 2400 (data not shown).

The kinetics of specific anti-SBm7462® isotopes is shown in Fig. 1B. Since the beginning of antibody production, the predominant isotype was IgG1, and when compared to IgG2 it has always been more numerous and showed significant statistic difference ($^{**}p < 0.01$).

In previous researches, in which different antigens, such as just natural midgut protein, the recombinant Bm86 and its commercial preparation as Gavac® were used; the

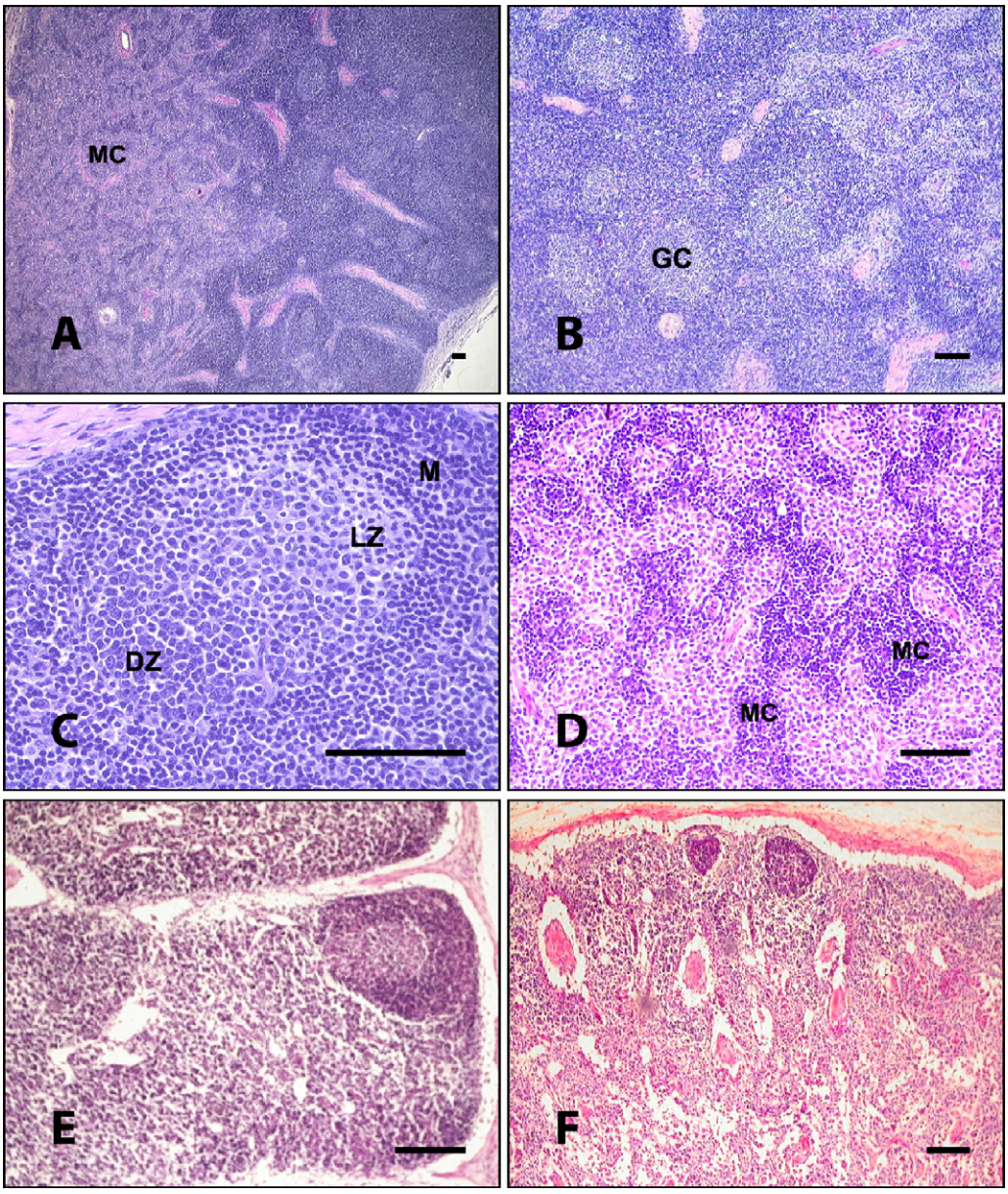

Fig. 2. Microphotography of bovine lymph nodes. (A) 7 days after immunization. (B) 15 days after immunization. (C) Details from CG and medullary cords 15 days after immunization. (D) 35 days after immunization. (E) Saponin control and (F) non-inoculated control. GC: germinal center; DZ: dark zone; LZ: light zone; M: mantle; MC: medullary cords. Scale bar 100 μm.

predominant humoral response was of an IgG1 isotype (Jackson and Opdebeeck, 1990; Rodriguez et al., 2001). As shown in Fig. 1B, the synthetic peptide also elicited an antibody response with the prevalence of IgG1.

3.2. Lymph nodes

The lymph node response was evaluated, by analysis of GC, reactivity of the medullary cord, presence of antigens and apoptosis.

At day 7, the lymph nodes displayed hyperplasia in paracortical (T-dependants) and interfollicular areas as well as a mild degree of hyperplasia of medullary cords and little GC, which was not observed in control lymph nodes (Fig. 2A, E and F).

At day 15, a higher number of follicles with GC formation and evident compartmentation in light zone (LZ), dark zone (DZ) and mantle were observed. At the same period, there was hyperplasia of the medullary cords, with cells that could be histologically characterized as plasmablasts and lymphoblasts (Fig. 2B and C). This hyperplasia of medullary cords was more intense from day 35 after immunization (Fig. 2D). A few secondary follicles were observed in lymph nodes from the control

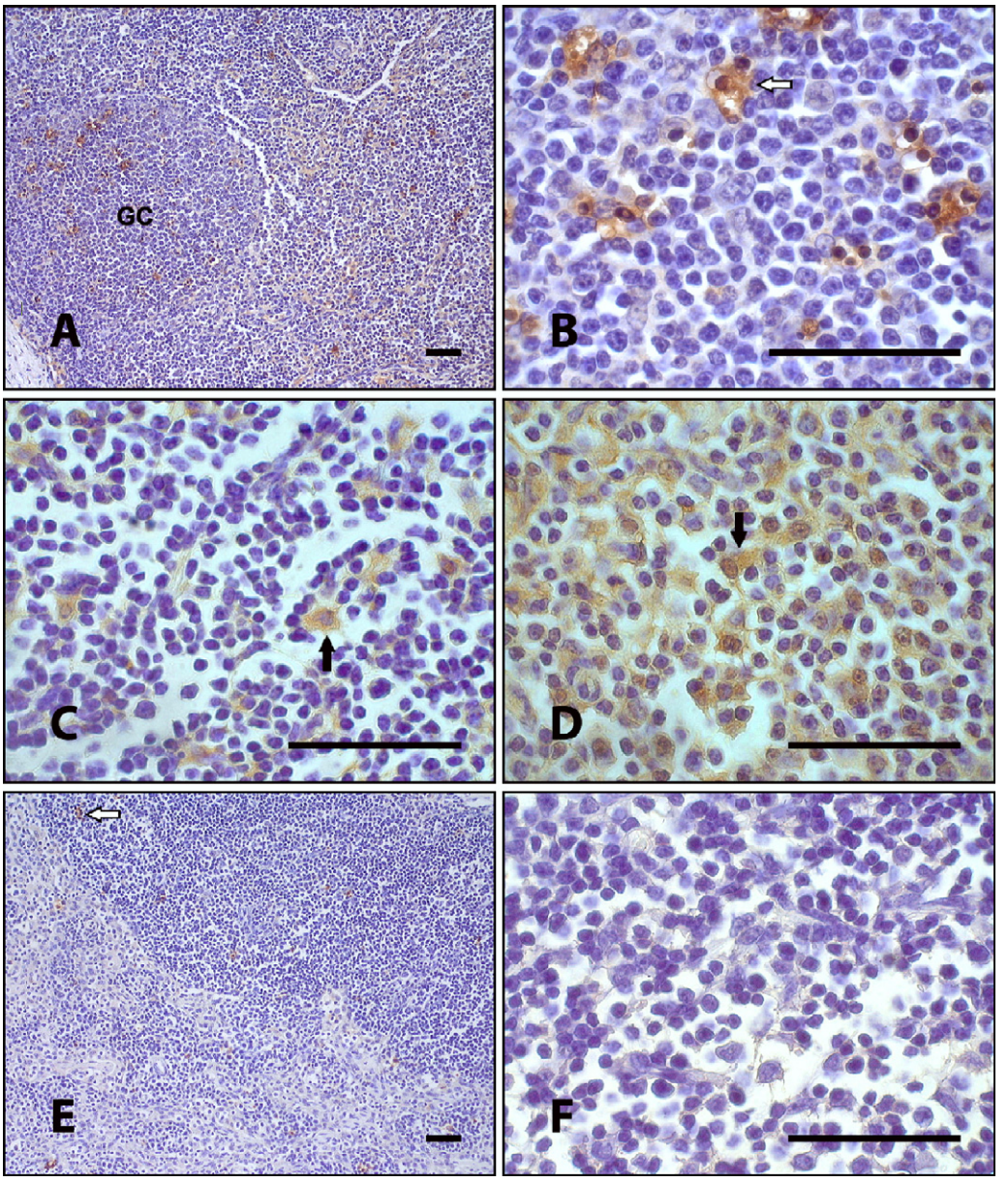

Fig. 3. Microphotography of bovine lymph nodes. (A) TUNEL-positive cells in the dark zone of GCs. (B) Arrow shows details of TUNEL-positive cells 7 days after immunization. (C and D) PAP-positive cells 7 and 35 days after first immunization, respectively. Arrows show the details of DC-like cells. (E and F) controls. Scale bar 50 μm.

groups; however, they did not show the same reactivity that was observed in the immunized animals (Fig. 2E and F).

The characteristic founded, matches with several studies in which T-dependant antigens were used, showing that after *naïve* B-cell activation by the antigen, they migrate to primary lymphoid follicles to start the GC which generally happens between 4 and 8 days after immunization (Liu and Arpin, 1997; Tarlinton and Smith, 2000). At the same period, there was hyperplasia of the medullary cords, with cells that could be histologically characterized as plasmablasts and lymphoblasts (Fig. 2B and C). This hyperplasia of medullary cords was more intense from day 35 after immunization (Fig. 2D).

The hyperplasia of medullary cords, in different times is related to plasmocytic extrafollicular reactions, which occur when memory B-cells are re-stimulated, leading to a rapid differentiation of these cells into plasmocytes; this could also explain why there was a higher specific anti-SBm7462® antibody production.

PAP-positive cells were found in the immunized animals in the paracortical zone, the medullary cords and the germinal centers 7 days after the first immunization (Fig. 3C). At day 35 additional strongly positive cells were found in the medullary cords (Fig. 3D). PAP-positive cells were not observed in the lymph nodes from either the control group or the saponin group at any of the analyzed days (Fig. 3F).

The PAP-positive cells observed at 7 days, indicate that the SBm7462® was captured and retained by resident DCs, transported through the conduit network for initial activation of T-cells, which explains the hyperplasia observed in T-dependant areas. The previously mentioned could be explained according to Sixt et al. (2005) and Allen et al. (2007), who demonstrated that resident DCs keep a specific interaction with some basal membrane components of the reticular fibers, allowing a close contact with conduit contents, consequently there are a efficient capture of substances of small molecular weight. The molecular weight of SBm7462® is 5.5 kDa. Nevertheless, the use of saponin as adjuvant may have helped the peptide capture by DCs, for beginning the immune response (Rosenthal and Zimmerman, 2006). At 35 days the increment of PAP-positive cells explain the increase of the medullary cords and the beginning of production of specific antibodies.

Seven days after the first immunization, the presence of TUNEL-positive cells in the dark zone of the GCs and in the paracortical zone became evident appearing as cellular groups (Fig. 3A and B). According to Liu and Arpin (1997), at the beginning of the GC, antigen-specific *naïve* B-cells which migrate to the GCs dark zone in order to maintain GC are subject to selection and therefore they may undergo apoptosis. At day 15, there was an increment of TUNEL-positive cells in the germinal centers and in the medullary cords that became more numerous at days 35 and 42 after first immunization (data not shown). The increment of TUNEL-positive cells at 15 days post-immunization, when the GC reached its maximum extent and when there were a higher number of GCs, is consistent with the process of antigen affinity selection (Camacho et al., 1998). In lymph nodes of the control group TUNEL-positive cells in those areas were scarce or absent; a similar result was observed in the lymph nodes from the saponin group (Fig. 3E). The fact would be considered to be a mechanism of a homeostatic nature that maintains peripheral tolerance of self antigens.

3.3. Circulating PBMCs

As shown in Table 1A, a gradual increase of $CD21^+$ B lymphocytes was observed in the immunized group after first inoculation. The maximum percentages were observed 15 days after the first immunization at 36.84% and 10 days after the third immunization at 39.51%, this change was statistically significant when compared to the average values prior to immunization ($^*p < 0.05$). The increment of $CD21^+$ after the first immunization is specifically related, to other two observations: first, the antigen-specific proliferation observed in lymph nodes during GC development, and second, the presence of antigen-specific antibodies, which increased progressively during secondary and tertiary immune response. Indeed, despite the fact that the specific immunoglobulins progressively increased over the course of the experiment, a concomitant increase of cells was not noted during the secondary and tertiary response; this can be immunologically confirmed by the discreet apoptosis observed in lymph node GCs after the second and third immunizations; this could indicate that the circulating population contains cells with high affinity that left their germinal centres.

In Table 1B, results of $WC1^+$ γδ lymphocytes are shown, which displayed variation along the experiment. When compared to the pre-immunization percentages, this population increased 5 days after the second immunization but decreased over the following days. According to

Table 1
Percentage of lymphocytes (A) B $CD21^+$ and (B) γδ $WC1^+$.

	Days				
	−1	15	35	63	70
(A)					
Immunized	29.85 (±3.10)	36.84* (±3.29)	34.12 (±2.31)	35.73 (±3.36)	39.51* (±6.7)
Control	23.16 (±0.16)	21.67 (±0.80)	22.37 (±1.75)	24.28 (±1.35)	22.92 (±6.36)
(B)					
Immunized	22.23 (±5.10)	20.72 (±5.74)	24.93 (±7.28)	19.99 (±3.9)	15.82 (±0.87)
Control	20.17 (±1.42)	20.81 (±6.19)	20.99 (±7.79)	18.11 (±5.49)	14.57 (±6.44)

* Significant at $p < 0.05$.

Rogers et al. (2005a,b), there are WC1 isoforms and these have a role in the immune response. It is possible that these lymphocytes have a double role of promoting a peptide-adapted immune response, be it by presenting them to $CD4^+$ lymphocytes or directly collaborating with the maturation of DC, enabling them for antigen presentation (Collins et al., 1998; Leslie et al., 2002). Previous reports agree with the results we obtained when, concomitant with the decrease of γδ T-cells in PBL, an efficient antigenic presentation to $CD4^+$ cells started, observed in the hyperplasia of T-dependant areas in lymph nodes and in the presence of PAP-positive cells interacting among T-lymphocytes. The progressive decrease of Tγδ cells in the immune response after the second immunization and the concomitant IgG1 presentation may be corroborated by the studies of Rogers et al. (2005a,b). They hypothesized that the transcription of T-bet and GATA-3 in WC1.1 and WC1.2 isoforms, respectively, could polarize the response.

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