Histopathological examination of lymphoid organs in cattle and mice experimentally poisoned by *Baccharis coridifolia*: Immunohistochemical characterization of B and T lymphocytes

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Recebido para publicação: 23/04/2007
Aprovado para publicação: 31/01/2008

Abstract

Baccharis coridifolia is one of the most important poisonous plants to cattle in the South of Brazil. The plant provokes necrotic lesions in the lymphoid tissues and in the gastrointestinal tract of cattle. Experimental administration to mice produces most of the lesions seen in the lymphoid tissues of cattle. This study was conducted to search possible differences in the susceptibility of T and B lymphocyte subpopulations. Lymph nodes, spleen, thymus and gut-associated lymphoid tissue (GALT) of cattle and mice experimentally poisoned were evaluated. The results were evaluated based on cell populations affected or remaining in the organs. Immunostaining for B lymphocytes (anti-BLA-36) identified the germinal center of follicles of the lymph node, spleen and GALT in both species. Immunostaining for T lymphocyte (anti-CD3) identified the paracortical area of the germinal centers of the lymph nodes and GALT, the periarteriolar area of the spleen, and the whole thymus both in cattle and mice. Experimentally poisoned cattle and mice shows necrosis of the germinal center of secondary follicles of the lymph nodes, spleen and GALT, where necrotic cells were immunostained for B and less often for T lymphocyte. Necrotic cells in the paracortical region of the lymph node were less often and were immunostained. Necrotic lesions of the thymus were seen only in mice, with positively stained for T lymphocyte. The distribution of the lesions in the lymphoid tissues and the immunostaining in necrotic cells suggested that the active principles of the plant are cytotoxic to B and T cells.

Key words:
Baccharis coridifolia.
Toxic plants.
Lymphoid necrosis.
Cattle.
Mice.

Introduction

The poisonous plant *Baccharis coridifolia* (Compositae), popularly known as "mio-mio", is one of the most important toxic plants in the south of Brazil. Field poisoning is more frequent in cattle, occasionally in sheep[1] and rarely in horses[2]. The toxicosis was experimentally reproduced in cattle[3,4], sheep[5], rabbits[6,7], horses[2] and mice[8].

The isolated active principle was the macrocyclic trichothecenes identified as roridin A and E[9,10], verrucarins A and J[10,11,12], miotoxins A[13] and miotoxins C and F[10].

Spontaneous or experimentally poisoned cattle show necrotic lesions in the gastrointestinal tube and remarkable necrosis of the germinal center of secondary follicles in the lymphoid tissues of the lymph nodes, spleen and gut-associated lymphoid tissue (GALT), and discreet and less often necrosis in the paracortical region of the lymph node. The morphological changes in the lymphoid tissues of mice were similar to those

observed in cattle, although the cattle did not show thymus necrosis. However, necrotic lesions in the gastrointestinal tract were more frequent in cattle[8].

Absence of necrotic lesions in the thymus of cattle, associated with the distribution of the lesions in the lymph node, suggested possible differences in the susceptibility to the active principles of the plant by T and B lymphocyte subpopulations. On the other hand, this phenomenon was not seen in mice. In this species necrotic lesions also occurred in the thymus. Formalin-fixed paraffin-embedded sections of the lymphoid tissues of cattle and mice experimentally poisoned with *B. coridifolia* were submitted to immunohistochemical staining with B and T lymphocytes markers, searching for the affected lymphocyte subpopulations.

Material and Method

Formalin-fixed paraffin-embedded sections from ten calves[4] and nine mice[8] experimentally poisoned by *B. coridifolia* were used. For the examination of the lymphoid system 12 lymph nodes, thymus, spleen and GALT were studied. Lymphoid tissues from two calves and three mice that did not receive the plant were used as controls.

For immunohistochemistry, all serial sections were placed on silanized slides and dewaxed. Antigen retrieval was performed after hydration and endogenous peroxidase blocking with H_2O_2 (code S2001, peroxidase-blocking reagent, Dakopatts) for 15 min. Then the sections were rinsed several times in 0.01M PBS (pH 7.2) and treated with 0.05% bovine serum albumin in PBS for 30 min.

Immunohistochemistry procedures for T cell identification**:** the slides were incubated for 3 hrs at room temperature with the primary antibodies anti-CD3 (code AO452, polyclonal rabbit anti-human CD3; Dakopatts) diluted 1 in 50; then incubated with goat-produced secondary antibodies (code EO432, polyclonal goat anti-rabbit immunoglobulins, Dakopatts), diluted 1 in 300 for 45 min at 4ºC, and incubated with an avidin-biotin peroxidase complex (code K0355, Dakopatts) for 30 min at 4ºC.

Immunohistochemistry procedures for B cell identification in calves: Dako ARK Kit (code K3955) was used. In a separate container, a mixture was prepared with 40μL of the primary antibody anti-BLA.36 (code MO533-1, monoclonal mouse anti-human B lymphocyte antigen, Dakopatts), diluted in 890.4 μL of 0.01M PBS, pH 7.2, and 29.6 μL of the biotinylated secondary antibody. After 15 minutes, 40μL of a blocking reagent (Dako Ark Kit) of unspecific reactions was added for 5 minutes. The histological sections were incubated with the antibody mixture for 30 minutes in a humid chamber at room temperature and later incubated with the streptoavidin-peroxidase complex for 15 minutes.

Immunohistochemistry procedures for B cell identification in mice: primary anti-IgG antibody (code 16-18-02, KPL) and anti-IgM antibody (code 16-18-03, KPL) were used, diluted at 1:100 in 0.01M PBS, pH 7.2. They were kept in a humid chamber at 4°C for 12 hours, and subsequently incubated with the secondary antibody (HistoMark kit, code 71-00-37, KPL), at room temperature for 30 minutes. Then they were incubated with the streptoavidin-peroxidase complex (HistoMark kit) for 30 minutes at room temperature, in the humid chamber.

Labeling was visualized with 3,3'-diaminobenzidine (code K3466-1, Dakopatts) added with 3% H_2O_2 in phosphate buffered saline (pH 7.6). Sections were counterstained with Harris's hematoxylin diluted at 1:10 and mounted with coverslips. Positive controls consisted of bovine reactive lymph nodes with known immunohistochemical properties. In the negative controls the primary antibody was replaced by 1% bovine serum albumin (BSA) in phosphate buffered saline, pH 7.6.

Immunolabeling was evaluated microscopically.

Results

The main microscopic lesions in lymphoid tissue described in bovines[4] (Figure 1) and in mice[8] (Figures 2 and 3) were reviewed and are presented in table 1. Histopathological examination of the thymus of mice and some lymph nodes of mice and bovine indicated that in some cells the pattern of cell death had an apoptotic morphology with cell shrinkage and nuclear condensation. In both species the control animals did not present lesions.

Immunohistochemistry for B cell identification in mice and calves

In the bovines, immunostaining with anti-BLA.36 antibody predominated in the lymphocytes of the germinal center of the lymph nodes, of the GALT and of the spleen. They were from moderate to discreet in the mantle region, rare in the paracortex and discreet in the medullary region. In four bovines there were immunostaining of fragmented cells in the germinal centers, in a total of 30 lymph nodes (Figure 1) and rare slides immunostained in the GALT. In the thymus immunostained was very rare and random, occurring both in the cortical and in the medullary regions, always in the cells near the blood vessels.

In the mice, anti-IgG and anti-IgM antibody immunostaining predominated in the lymphocytes of the germinal center of the lymph nodes, and it was from discreet to rare in the spleen and GALT. In these, IgG immunostaining was greater than IgM immunostaining. In the mantle, paracortical and medullary regions of the lymph nodes, immunostaining was rare. There was immunostaining of necrotic cells in the germinal centers, totaling eight lymph nodes positively stained for IgG (Figure 4) and two for IgM. One section was immunostaining for IgM in the GALT. In the thymus, positively stained of large lymphocytes for IgG was extremely rare both in the cortical and medullary regions. In the cortical region, IgM immunostaining of large lymphocytes was also extremely rare.

Immunohistochemistry for T cell identification in mice and calves

In all animals, T lymphocyte immunostaining predominated in the paracortical region, including the interfollicular region in the lymph nodes and GALT, in the periarteriolar region of the spleen and along the thymus. In the secondary follicles, despite frequent, there were rare positively stained cells in the regions of the mantle and germinal center of lymph nodes and of the GALT. In the medullary region

Table 1 - Frequence of microscopic findings in the lymphoid tissues of nine mice and ten cattle experimentally fed with *B. coridifolia*. Jaboticabal - SP, 2002

Microscopic findings	Number of affected (mice)	Number of affected (cattle)
Necrosis of lymph node germinal center	6	10
Intense hyperplastic response of the lymph node germinal center	3	1
Discreet necrosis of lymph node paracortical region	2	8
Accentuated necrosis of thymocytes	5	0
Necrosis of spleen germinal center	6	10
Discreet necrosis of spleen periarteriolar region	1	0
Necrosis of germinal center of the gut-associated lymphoid tissue	3	8

immunostaining was from moderate to discreet.

Necrotic cells were immunostaining in the germinal centers and in the region of the lymph node mantle and in the spleen, totaling seven lymph nodes and seven spleens of bovines and six lymph nodes (Figure 2) and one spleen of mice. In one bovine, necrotic cells were positively stained in the paracortical region of two lymph nodes. In two mice immunostaining necrotic thymocytes were observed (Figure3). Necrotic cells in the periarteriolar region of the spleen of mice and in the germinal center of the GALT of bovine and mice were not immunostained.

Discussion and Conclusions

In the immunostaining for B lymphocytes in the lymph nodes, GALT and spleen of bovines, the site most frequently positively stained was the secondary follicle. The immunostaining of fragmented cells in this region suggest that *B. coridifolia* is toxic to B lymphocytes. Mice-produced monoclonal antibody to identify human B lymphocytes was chosen because in a study conducted in paraffin embedded formolized tissue of bovines and equines with B lymphocyte markers, the anti-BLA.36 antibody was the one which best reacted, immunostaining 80-90% of the lymphocytes in the germinal center and mantle zone of lymphoid follicles of lymph nodes, spleen and Peyer's patches[14].

Immunostaining for both IgG and IgM in mice predominated in the large lymphocytes associated with the germinal center, followed by the medullary region. Immunostaining of necrotic cells occurred only in eight lymph nodes for IgG and in

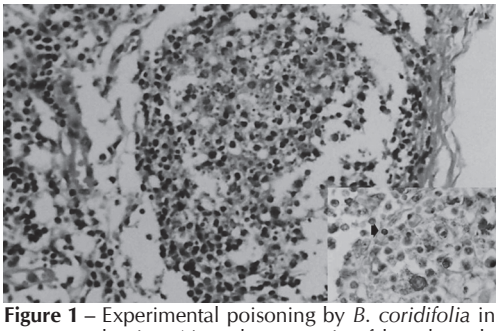

Figure 1 – Experimental poisoning by *B. coridifolia* in bovine. Note the necrosis of lymph node germinal center of the secondary follicle HE. X40. Inset: Immunolabelling of BLA.36 with immunostaining of necrotic cells (arrow) of lymph node germinal center. X100

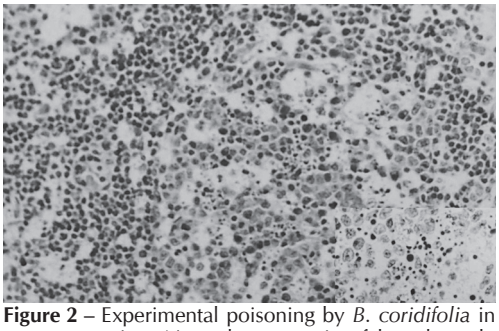

Figure 2 – Experimental poisoning by *B. coridifolia* in mice. Note the necrosis of lymph node germinal center of the secondary follicle HE. X40. Inset: Immunolabelling of CD3 with immunostaining of necrotic cells of lymph node germinal center X100

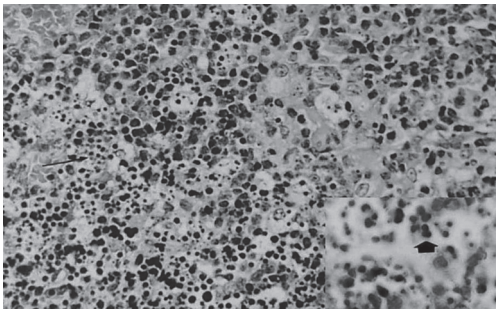

Figure 3 – Microscopic aspect of the thymus of mice poisoned by *B. coridifolia* with necrosis of thymocytes (arrow). HE. X40. Inset: Immunolabelling of CD3 with immunostaining of thymus necrotic cells (arrow). X100

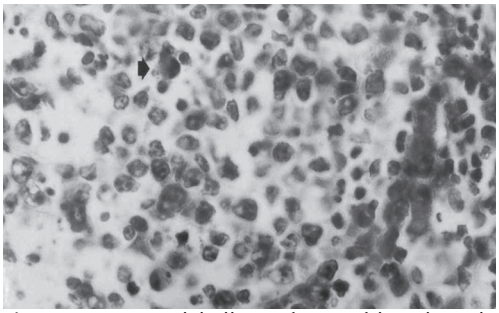

Figure 4 - Immunolabelling of IgG of lymph node germinal center of mice poisoned by *B. coridifolia*. Note immunostaining of necrotic cells (arrow) in the center of the lymph node follicle. X100

two lymph nodes for IgM. The explanation for this may be that most cells were destroyed by necrosis and surface antigens remained only in some of the necrotic cells. Also, the positively stained large lymphocytes might be plasmocytes, expressing IgG and IgM.

Immunostaining of necrotic cells for T lymphocyte occurred in few histological sections. This occurred in the paracortical region of two lymph nodes in one bovine and in the germinal center both of bovines and mice. Studies have demonstrated that there is a great amount of T cells that migrate to the follicle and proliferate during a primary response[15], and *B. coridifolia* might affect those proliferative T cells. In the non-necrotic cells the predominating site of T lymphocyte immunostaining was the paracortical region, both in bovines and in mice. These findings are similar to those described in the literature[14,16]. Studies carried out demonstrated that anti-CD3 antibody reacted to 90% of the lymphocytes in the paracortical region and parafollicular zone of the lymph nodes, spleen and Peyer's patches, to 40-50% of the lymphocytes in the medullary cord of the lymph nodes, and to few cells in the cortical region of the follicle. The anti-CD3 antibody reacted to 95% of the lymphocytes in the red pulp of the spleen, to 50-60% of the cells in the medullary region of the thymus, and to 40-80% of the lymphocytes in the thymus cortex[14].

We believe that, despite the cells being necrotic, some of them still presented surface antigens that allowed immunostaining, since the immunostaining followed the same pattern of distribution as that of the tissue marked in the control animals.

When we compare the results globally, we verify that mice can be used as an experimental model for poisoning by *B. coridifolia* because the necroses in the lymphocyte subpopulations in the tissues studied, with the exception of the thymus, were similar to those described in the bovines. This allows for the study of other aspects of the pathogenesis. We can also suggest that *B. coridifolia* are cytotoxic to B and T lymphocytes, with greater action on B lymphocytes of lymph nodes of both species, and that T lymphocytes of the thymus of mice are also affected by the active principle of the plant, but we do not discard the possibility that another cells populations in the lymphoid organs could be affected by the plant. These hypothesis could be investigated *in vitro* with specific cells population isolated from experimentally poisoned animals with the plant.

Acknowledgements

This paper was funded by Fapesp.

Análise histopatológica de órgãos linfóides de bovinos e camundongos intoxicados experimentalmente por *Baccharis coridifolia*: Caracterização imunoistoquímica de linfócitos B e T

Resumo

Baccharis coridifolia é uma das mais importantes plantas tóxicas para bovinos no Sul do Brasil. A intoxicação pela planta produz lesões necróticas nos tecidos linfóides e no trato gastrointestinal de bovinos. A administração experimental para camundongos produziu a maioria das lesões que ocorrem nos tecidos linfóides de bovinos. Este estudo foi conduzido para detectar as possíveis diferenças na susceptibilidade das populações de linfócitos T e B. Foram utilizados linfonodos, baço, timo e acúmulos linfóides associados ao intestino de bovinos e camundongos experimentalmente intoxicados pela planta. Os

Palavras-chave:
Baccharis coridifolia.
Plantas tóxicas.
Lesões necróticas linfóides.
Bovinos.
Camundongos.

resultados foram avaliados com base na população de células lesadas ou por aquela que permaneceram nos tecidos. Em ambas as espécies, na marcação imunoistoquímica para linfócitos B (anti-BLA-36) predominou a região do centro germinativo dos linfonodos, baço e acúmulos linfóides associados ao intestino. A marcação para linfócitos T (anti-CD3) predominou na região paracortical dos linfonodos, acúmulos linfóides associados ao intestino e região periarteriolar do baço e timo de ambas as espécies. Bovinos e camundongos experimentalmente intoxicados demonstraram acentuada necrose do centro germinativo dos folículos secundários dos linfonodos, baço e intestino, onde as células necrosadas foram marcadas pela imunoistoquímica para linfócitos B e em menor freqüência para linfócitos T. Necrose celular na região paracortical do linfonodo foi discreta e sem marcação imunoistoquímica. Células necróticas no timo foram observadas somente em camundongos, com imunoistoquímica positiva para linfócitos T. A distribuição das lesões nos tecidos linfóides associadas à marcação imunoistoquímica das células necrosadas sugerem que o princípio ativo da planta é citotóxico para células T e B.

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