Swiss mice CD1 fed on mussels contaminated by okadaic acid and yessotoxins: effects on thymus and spleen

A. Franchini,[a] E. Marchesini,[a] R. Poletti,[b] E. Ottaviani[a,*]
[a]Department of Animal Biology, University of Modena and Reggio Emilia, Modena;
[b]Centro Ricerche Marine, Cesenatico, Italy

The toxicity of okadaic acid (OA) and yessotoxins (YTXs) was studied in mice orally fed on (i) OA (17.80±2.41 μg/Kg) for 24 h and mouse feed for 24 h; (ii) OA (17.2±2.13 μg/Kg) plus YTXs (1.30±0.12 mg/Kg) for 24 h and mouse feed for 24 h; (iii) OA (18.88±1.86 lg/Kg) plus YTXs (1.45±0.12 mg/Kg) for 24 h. After toxin treatments the thymus and spleen were examined. More severe morpho-functional modifications were found in the thymus, which presented atrophy, a significant depletion in the lymphoid compartment and angiogenesis. In spite of the impairment, a number of inflammatory cells, reactive to anti-cytokine antibodies, were recruited. Moreover, greater expression of matrix metalloproteinase-9, particularly in cells located near new blood vessels, was observed. Thymus injury was still observed after 48 h. Histopathological changes to the spleen were more evident in mice orally treated for 24 h and immediately sacrificed. The organ showed a significant loss of volume and a fibrous component invaded regions involved in immune functions. In white pulp the marginal zones were reduced, lymphoid nodules contained large germinal centres and the periarteriolar lymphoid sheaths showed cellular depletion. An inflammatory cell response was activated by the recruitment of granulocytes, an increased number of active macrophages and increased immunoreactivity to cytokines. Unlike in the thymus, some evidence of recovery was seen in the spleen. The data suggest that low oral doses of OA alone or OA plus YTXs are able to provoke immunostimulation and systemic immunotoxicity, thus also indicative of tumorigenic properties.

Key words: neuropeptides; development; immunohistochemistry; zebrafish

Correspondence: *Prof. Enzo Ottaviani
Department of Animal Biology
via Campi 213/D, 41100 Modena, Italy
Tel: 059.205 5536.
Fax: 059.205 5548.
E-mail: ottaviani.enzo@unimore.it

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The defence system is one of the targets of marine algal toxins. Ito and colleagues (1997) reported that repeated injection in male ICR mice of low doses of the toxin palytoxin provoked damage to the lymphoid tissues. Furthermore, oral administration in male ICR mice of azaspiracid provoked serious pathological changes to the thymus, spleen and Peyer's patches, and both T and B lymphocytes were injured (Ito *et al.,* 2000, 2002a). Recently, we have found that the intra-peritoneal (i.p.) injection of lethal and sublethal doses of yessotoxin (YTX) in male Swiss CD1 mice acts primarily on the immune system (Franchini *et al.*, 2004a). The structural organisation of the thymus microenvironment appeared particularly offended. The epithelial compartment showed modifications in morphology and cytokeratin expression, while the number of apoptotic thymocytes increased significantly. Subsequently, a reduced thymocyte population left the organ to colonise T cell areas of peripheral lymphoid organs resulting in an impaired, cell-mediated immune response. The thymus cytokine response was also modified by i.p. YTX. Careful attention is required in assessing the immunotoxic potential of algal toxins, and account must be taken of the well-established correlation between the suppression of immune functions and the increased incidence and/or severity of neoplastic diseases (Germolec, 2004).

Studies from different laboratories of another algal toxin, okadaic acid (OA), have indicated the small intestine as the target organ (Edebo *et al.*, 1988; Ito and Terao, 1994; Ito et al., 2002b). The capacity of OA to promote tumour formation has been reported (Suganuma *et al.*, 1988). The mode of action of this toxin has been indicated as the inhibition of protein phosphatase 1 and 2A (Bialojan and Takai, 1988; Suganuma *et al.*, 1992). On the other hand, a clearer pattern of YTX action has only emerged recently. *In vitro* studies have demonstrat-

ed the ability to destroy the E-cadherin-catenin system selectively (Ronzitti *et al.*, 2004). In addition to the thymus, YTX acts in mice at different levels, i.e. intestine and brain, with time and dose-dependent effects (Franchini *et al.*, 2004 a, b). In particular the brain, is sensitive only to a lethal i.p. YTX dose (Franchini *et al.*, 2004 b).

The present study reports the results obtained in male Swiss CD1 mice fed on OA and YTXs contaminated mussel digestive gland. In particular, we have focused our attention on the effects of OA alone and OA plus YTXs on the lymphoid tissue of the thymus and spleen.

Materials and Methods

Animals and toxin treatment

Male Swiss CD1 mice weighing 20-21 g obtained from Morini (San Polo d'Enza, RE, Italy) were used. The animals were maintained in conformity with guidelines in the European Communities Council Directive (86/609 ECC) concerning animal welfare and fed with standard diet for rodents (M mucedola, srl, MI, Italy). Three groups of 5 animals were orally administered toxin as follows: group A was fed on OA (17.80±2.41 μg/Kg) contaminated boiled mussel digestive gland for 24 h, followed by mouse feed for 24 h; group B was fed on OA (17.2±2.13 μg/Kg) plus YTXs (1.30±0.12 mg/Kg) contaminated boiled mussel digestive gland for 24 h, followed by mouse feed for 24 h; group C was fed on OA (18.88±1.86 μg/Kg) plus YTXs (1.45±0.11 mg/Kg) contaminated boiled mussel digestive gland for 24 h. The toxin concentrations were evaluated by HPLC after mussel boiling. After toxin treatments, the animals were sacrificed and the thymus and spleen dissected for the histological, histochemical and immunocytochemical examination. A further five animals were used as controls.

Evaluation of toxins by HPLC method

The presence of OA and YTXs in the mussel digestive glands fed to the mice was evaluated using the HPLC method with fluorescent detector following the procedure of Lee *et al.* (1987) and Yasumoto and Takizawa (1997), respectively.

Histological and histochemical procedures

Thymus and spleen from treated and animals and controls were fixed in 10% neutral formol and Bouin's mixture, embedded in paraffin wax and cut into 7 μm sections. The following stains were performed (Bancroft and Stevens, 1996): hematoxylin/eosin and trichrome stainings for general morphology; PAS/hematoxylin for carbohydrates, and silver impregnation method for reticular fibres.

Immunocytochemical procedure

For the immunocytochemical procedure, the sections were incubated overnight at 4°C with the following primary antibodies: anti-cytokeratin 1 (CK1) monoclonal antibody (mAb) (1:1000) (Novocastra Laboratories, Newcastle, UK); anti-cytokeratin 8 (CK8) mAb (1:1000) (Novocastra); anti-cytokeratin 18 (CK18) mAb (5 mg/mL) (Roche Molecular Bichemicals, Germany); anti-cytokeratin 1/5/10/14 (CK1/5/10/14) mAb (1:1000) (Novocastra); matrix metalloproteinase (MMP)-9 mAb (1:1000) (Novocastra); anti-IL-1· polyclonal antibody (pAb) (1:500) (R & D Systems, Minneapolis, MN, USA); and anti-IL-6 pAb (1:500) (R & D Systems). The labelling sites were revealed using avidin-biotin-peroxidase complex (ABC). Nuclei were counterstained with hematoxylin. Negative controls were performed by substituting the primary antibodies with non-immune sera. A detailed immunocytochemical protocol is described elsewhere (Ottaviani *et al.*, 1995).

Quantification of thymus and spleen areas

Organ sizes were evaluated by determining the areas of the thymus and spleen sections using the Scion Image for Windows software (Scion corporation, Maryland, USA). The differences between control and treated samples were analysed statistically by ANOVA.

Results

The oral toxicity of OA alone and OA plus YTXs affected both the thymus and spleen, but more severe structural and functional damage was found in the thymus. The organ was considerably reduced in size, decreasing more than four-fold (Figure 1). The most altered thymus area appeared to be the cortex and, in particular, the outer region, where a severe depletion of lymphoid cells, an increase in the non-lymphoid compartment and a fibrous involution were observed (Figure 2a, d). Rare structurally intact lympho-epithelial areas were also seen, as well as extensive angiogenesis, with the formation of new blood vessels (Figure 2d). The

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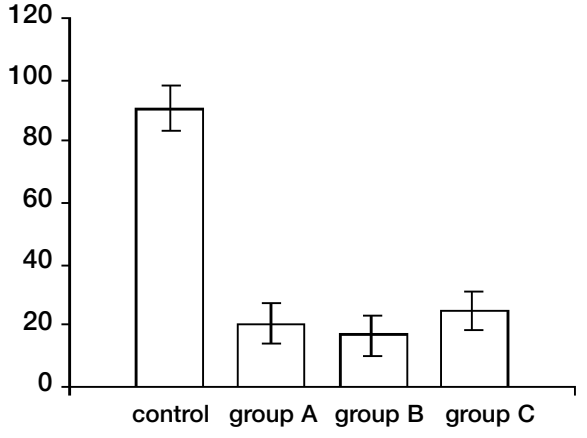

Figure 1. Thymic section areas from control and toxin orally administered mice. Statistical significance is determined by ANOVA (*p<0.05* vs control).

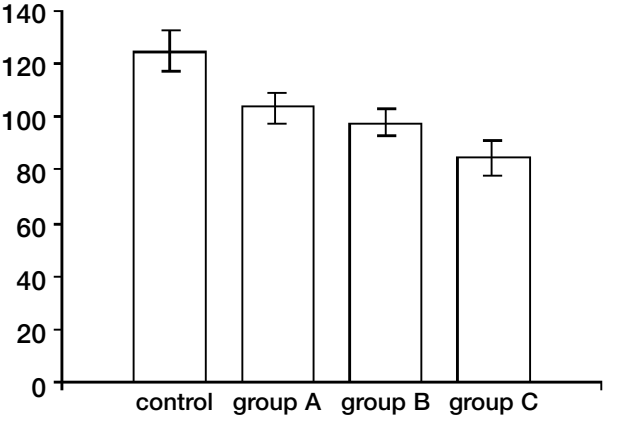

Figure 5. Spleen section areas from control and toxin orally administered mice. Statistical significance is determined by ANOVA (*p <0.05* vs control).

medulla was hardly distinguishable and reduced to small islets, in which small groups of densely packed thymocytes and some apoptotic phenotypes were seen to surround the blood vessels (Figure 2e, f). The development of heterogeneous, Hassall's corpuscle-like bodies was also evident. The immunocytochemical reactions demonstrated changes in the expression of cytokeratin intermediate filament protein in the epithelial thymus sub-populations. In comparison with controls, the thymus from treated animals showed a more extensive network of both low MW cytokeratin (CK8, CK18) and high MW cytokeratin (CK1, CK1/5/10/14) reactive epithelial cells in the cortex (Figure 3a, b). In medullary areas, the epithelial cells were sometimes structurally modified into round cells and showed stronger immunoreactivity to high MW cytokeratins (Figure 3c and d). The cytokine response, i.e. IL-1α and IL-6, revealed a higher number of positive cells, which were mainly inflammatory cells, such as granulocytes and macrophages (Figure 3e, f). In control thymus, rare isolated cells in the cortex and a higher number in the cortico-medullary junction and medulla were stained with anti-MMP-9 mAb (Figure 4a, b). After oral toxin administration, the number of immunoreactive cells and the reaction intensity increased. Most of these cells showed the morphological characteristics of macrophages. Strongly positive cells were seen in the outer cortex and near the new blood vessels which had reactive, flattened endothelial cells (Figure 4c, d). Moreover, the intercellular spaces of this thymus area were immunoreactive (Figure 4e). There were no significant differences in findings under the three experimental conditions (Figure 4c,f). The structural organization of the spleen of orally toxin-treated animals showed particularly evident modifications in group C. The organ showed a significant loss of volume (Figure 5). In the white pulp, the marginal zone, clearly seen in controls, was reduced, contained more fibroblasts and was partly replaced by thick trabeculae invading the spleen parenchyma from the external capsule (Figure 6a, b). The spleen lymphoid nodules showed large germinal centres containing clusters of large lymphocytes and groups of dead cells. The periarteriolar lymphoid sheaths (PALS), enriched in bundles of reticular fibres, surrounded enlarged arterioles and showed cellular depletion (Figure 6d and e). The fibrous trabeculae also invaded the red pulp to the detriment of the cellular component. Immunocytochemical reactions with anti-cytokine pAbs demonstrated groups of intensely stained cells localised in the PALS (Figure 6i) and red pulp spleen cords (Figure 7b). These were mainly inflammatory cells, such as granulocytes and macrophages in active phase (Figure 7c). In control spleen, a few, isolated immunoreactive cells were seen (Figure 6h and 7a). In spleen from group A and group B samples, more limited structural alterations, e.g. an increase in the fibrous component (Figure 6g) and a reduction in marginal zones, were evident than group C animals. The development of germinal centres was stimulated (Figure 6c) and cytokine response was similar to group C. A significant loss of volume was not observed (Figure 5).

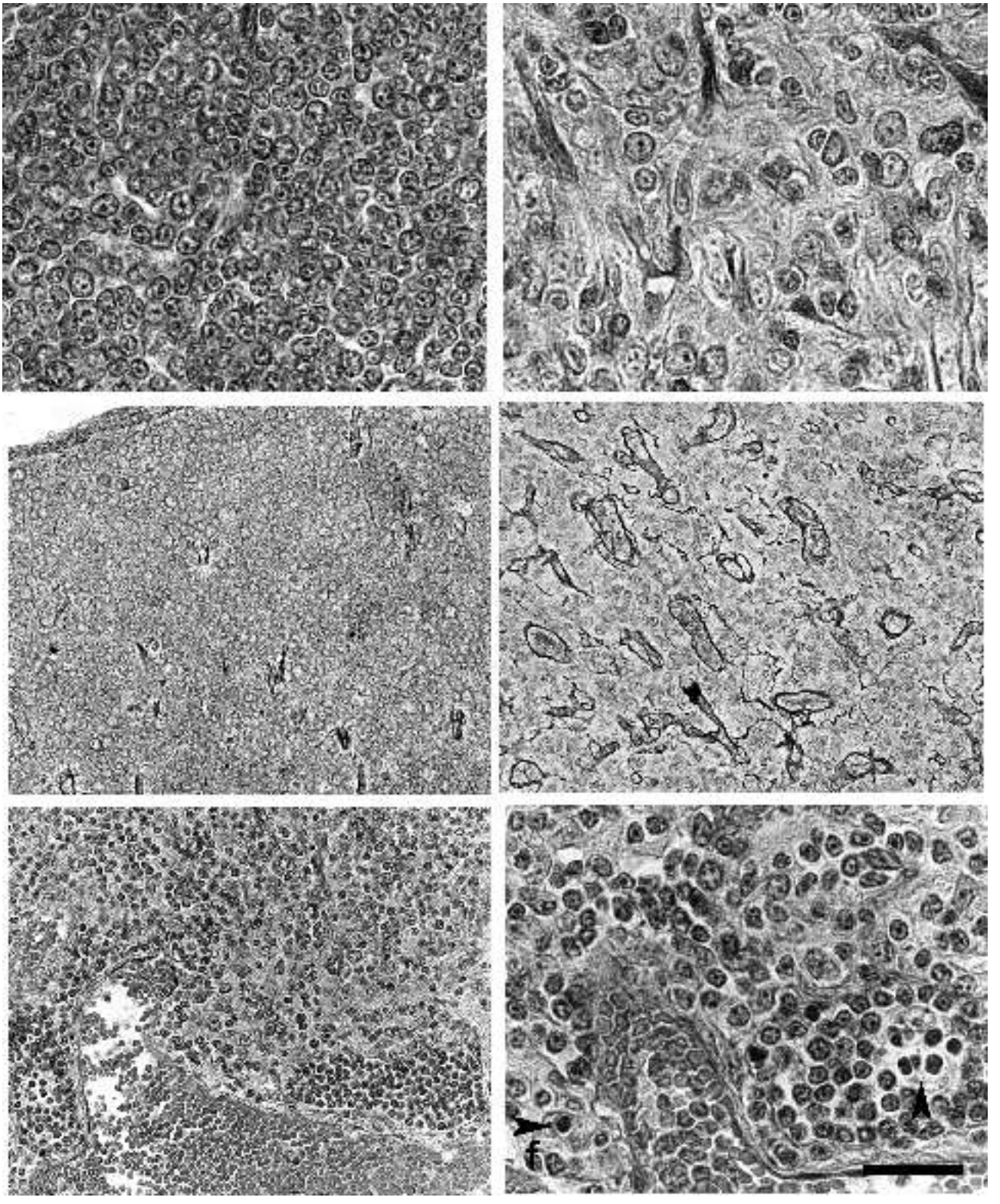

Figure 2. Thymic sections from control (a, c), orally administrated group A (d-f) and group B (b) mice. Trichrome (a, b), silver impregnation (c, d) and hematoxylin/eosin stains (e, f). A severe depletion in thymocyte population (b) and angiogenesis (d) are found in cortical areas after oral treatments. Note densely packed thymocytes surrounding blood vessel in a medullary area (e, f). Cortex (C); blood vessels (arrows), apoptotic cells (arrowheads). Bars = 20 μm.

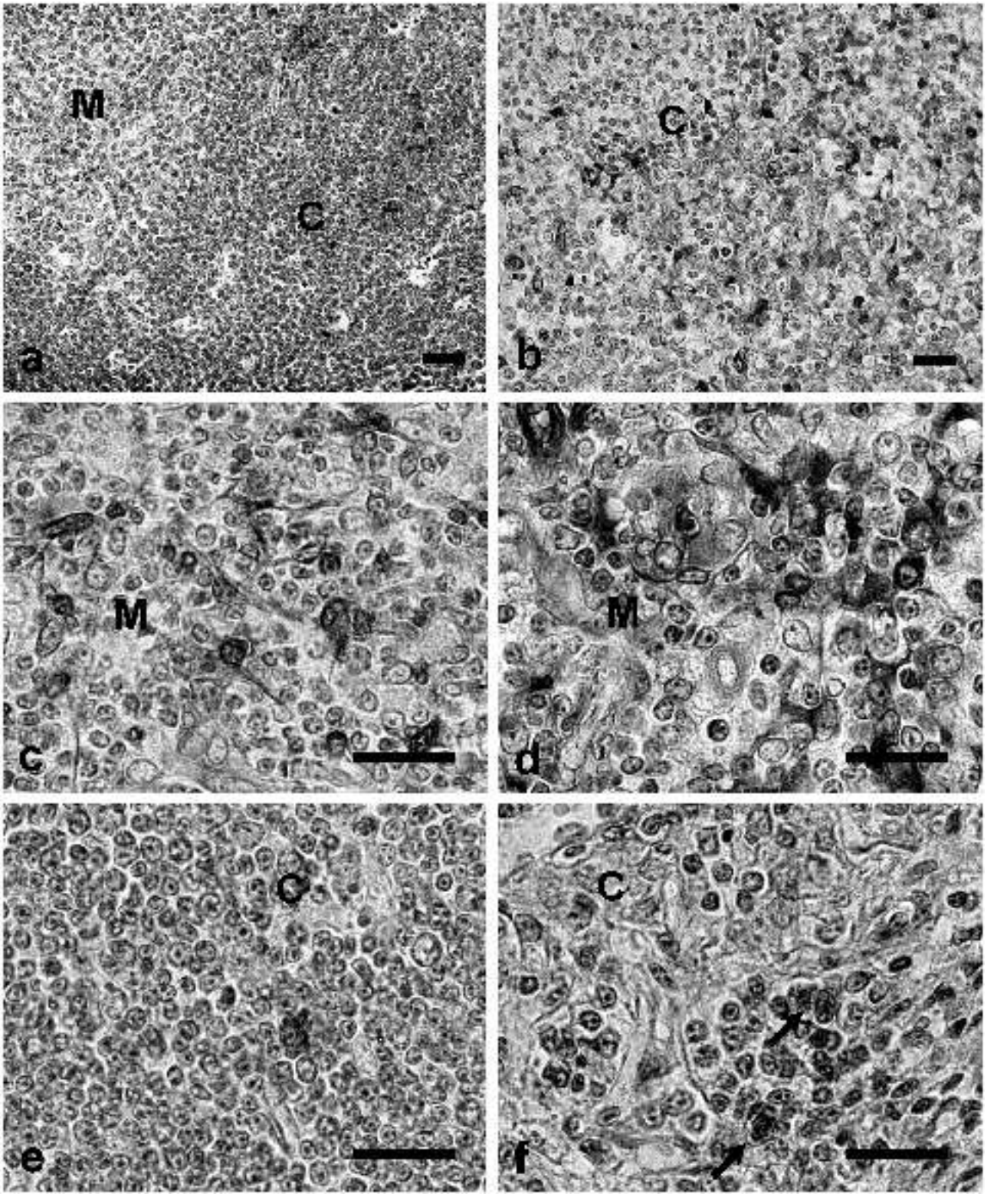

Figure 3. Thymic sections from control (a, c, e), orally administrated group A (b, d) and group B (f) mice, immunostained with anti-CK 1 mAb (a, b); anti-CK 1/5/10/14 mAb (c, d) and anti-IL-1α pAb (e, f). Cortex (C); medulla (M), granulocytes (arrows). Bars = 20 μm.

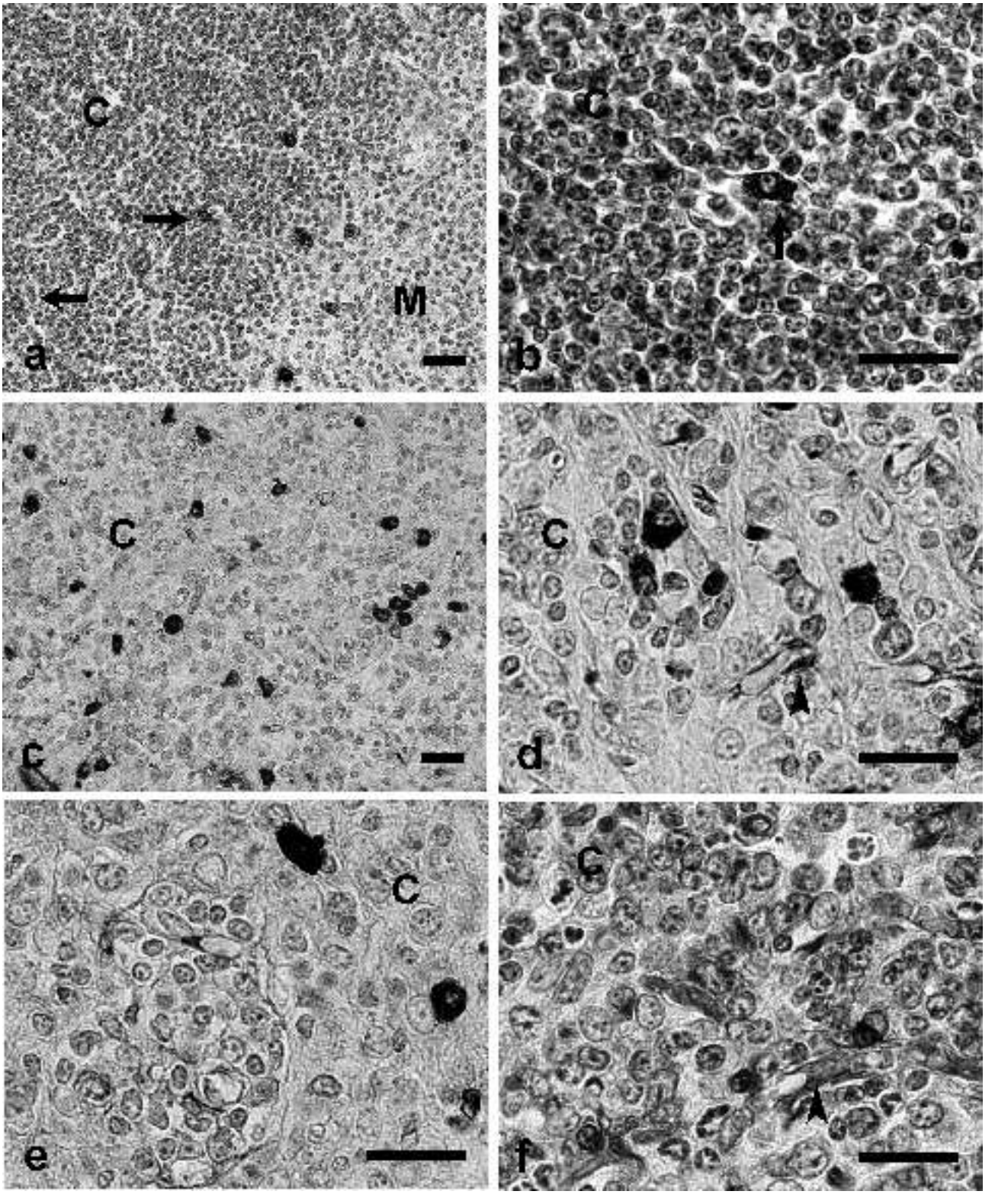

Figure 4. Thymic sections from control (a, b), orally administrated group A (c, d), group B (e) and group C (f) mice immunostained with anti-MMP-9 mAb. In controls, positive cells (arrows) are rare in cortex and more abundant in cortico-medullary junction and medulla. In treated samples, the number of immunoreactive cells and reaction intensity increase. Reactive endothelial cells (arrowheads), cortex (C); medulla (M). Bars = 20 μm.

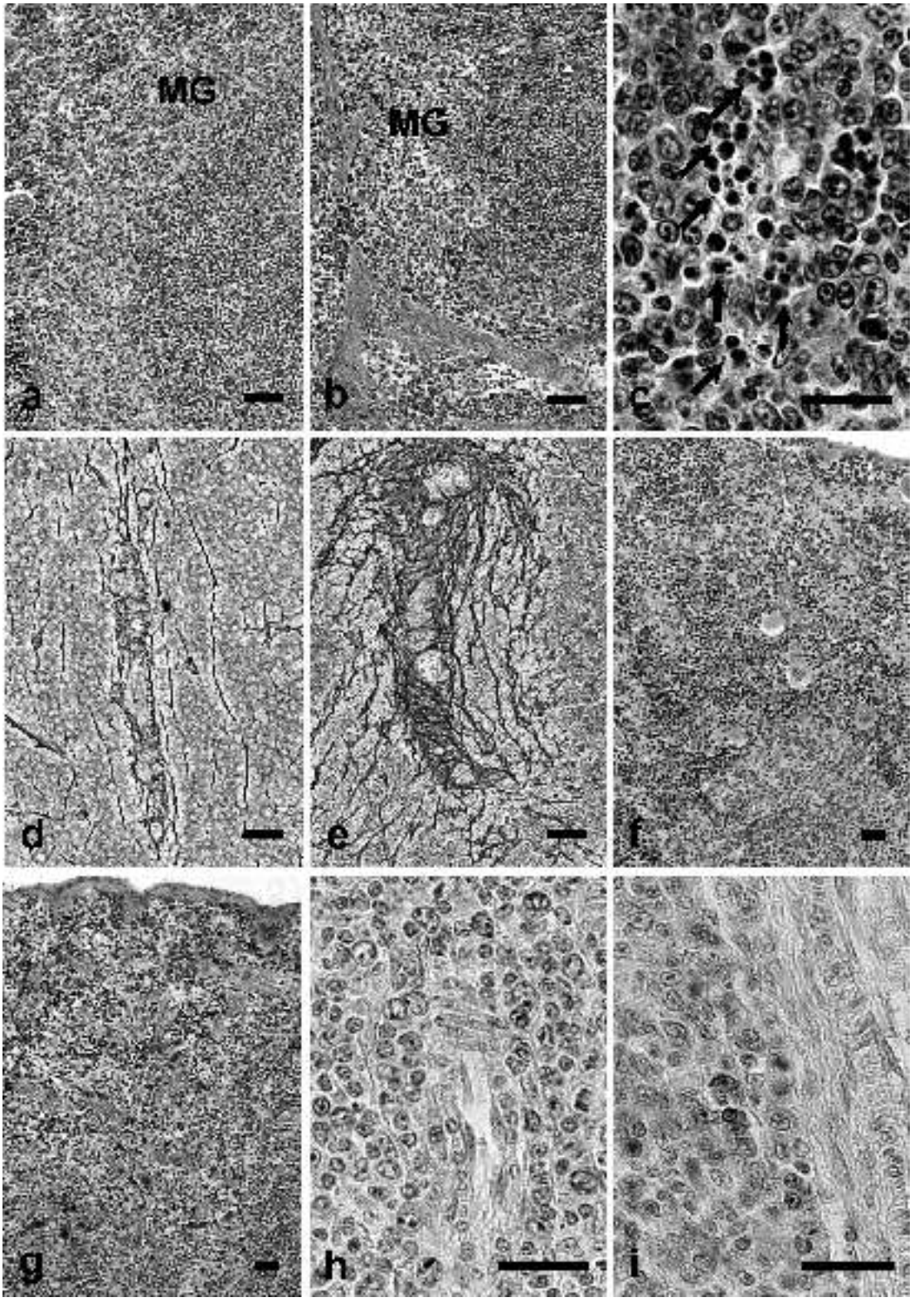

Figure 6. Spleen sections from control (a, d, f, h), orally administrated group A (c), group B (g) and group C (b, e, i) mice. PAS/hematoxylin (a, b, f, g), silver impregnation (d, e) and hematoxylin/eosin stains (c). In treated samples white pulp marginal zone (MG) is reduced (b) and developed germinal centres contain many dead cells (arrows) (c). The periarteriolar lymphoid sheaths (PALS) surround enlarged arterioles and are invaded by reticular fibres (e). Note trabecular invasion of red pulp (g). White pulp PALS immunostained with anti-IL-1· pAb (h, i). Bars = 20 μm.

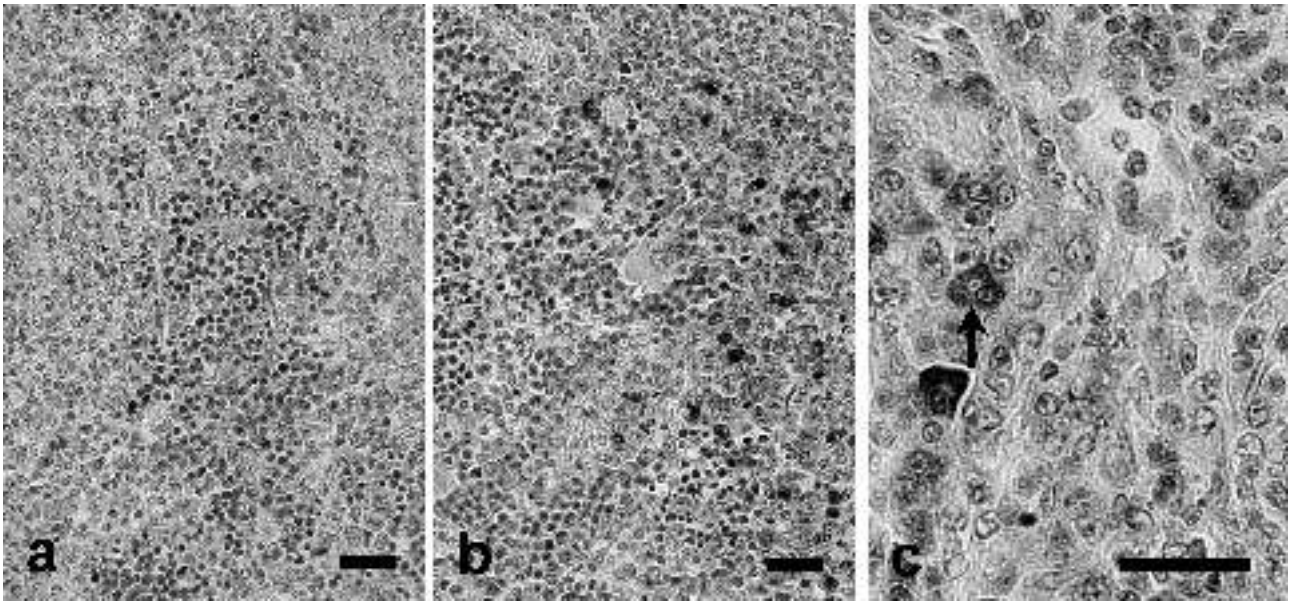

Figure 7. Sections of spleen from control (a) and orally administrated group C (b, c) mice. Red pulp cords immunostained with anti-IL-1α pAb. Note an active macrophage with a cytoplasmic phagocytic vacuola (arrow). Bars = 20 μm

Discussion

We have observed a severe alteration in the structural architecture of the thymus following oral administration of OA and OA plus YTXs. The organ exhibits strong atrophy, with significant histological modifications in the outer cortex and medulla, where there is conspicuous depletion of thymus lymphoid elements. Most of the outer cortical region shows disorganization of the lympho-epithelial complexes, an increase in epithelial cytokeratin expression and an invasion of fibrous components and angiogenetic areas.

Physiological thymocyte migration and differentiation are considered the result of multiple cellular interactions between microenvironment components and simultaneous and/or sequential stimuli involving chemokines and extracellular matrix proteins controlling matrix production and degradation (Savino *et al.*, 2003). In this context, key molecules are matrix metalloproteinases (MMPs), good candidates for the intrathymic, extracellular matrix breakdown facilitating T cell migration (Goetzl *et al.*, 1996). Recently, some MMPs have been shown to be constitutively expressed in the human thymus (Kondo *et al.*, 2001) and utilized to disrupt the tissue matrix by mature T cells (Leppert *et al.*, 1995; Xia *et al.*, 1996). On the other hand, MMPs and, in particular, MMP-9 modulate neovascularization, which is known to be necessary for tumour growth and metastasis, by mediating the release of vascular endothelial growth factor from extracellular stores and the angiogenic switch (Bergers *et al.*, 2000). The increased expression of MMP-9 at protein or mRNA level has been correlated to thymus epithelial tumour (Yukiue *et al.*, 2003; Sogawa *et al.*, 2003; Takahashi *et al.*, 2003). Here we demonstrate greater expression of MMP-9, especially in cells located near angiogenetic areas, and modifications in epithelial phenotypes after algal toxin treatment. These observations may support tumorigenic properties. Suganuma *et al.,* (1988) also demonstrated a tumour promoting activity for OA, and our experimental conditions, we have not seen any significant different damages to the thymus of mice fed on OA alone or OA plus YTXs. A dose- and time-dependent immunotoxicity of algal toxins may be surmised as indicated for i.p. injected YTX (Franchini *et al.*, 2004a). Low oral doses of OA provoke more severe thymocyte depletion than higher doses of YTX administered by i.p. injection (Franchini *et al.*, 2004a). Tubaro *et al.* (2004) found that continuous oral exposure to YTX provoked less toxic effects than the same or lower exposure to OA. On the other hand, a sub-lethal i.p. YTX dose was able to provoke more severe damage to mice thymus after 24 h than a lethal dose (death within 2 h) which

resulted in a reduced thymocyte population (Franchini *et al.*, 2004a). This observation may support a similar systemic immunotoxicity of OA and YTXs, but with differing degrees of toxicity over time.

The target organ of OA has been considered the small intestine, and the changes provoked by sublethal, oral doses were observed after 1 h with nearly complete recovery within 48 h (Ito and Terao, 1994; Ito *et al.*, 2002b). The present findings point out the high sensitivity of the thymus to oral toxin treatments, as the organ was still severely injured after 48 h and functionality had not been restored.

With regard to the spleen, histopathological changes were more evident in mice orally treated for 24 h and immediately sacrificed. The thymus atrophy and the consequent reduction in the number of T cells leaving the organ led to a reduction in the number of cells observed in PALS, so altering spleen white pulp function. The spleen size showed significant loss of volume and a fibrous component invaded regions involved in immune functions, i.e. the marginal zones. Mouse spleen architecture is that of a defensive spleen (Haley, 2003) with abundant lymphoid tissue providing immunological defence cells. After oral algal toxin administration, an inflammatory cell response is activated by the recruitment of granulocytes, an increase in the number of active macrophages and immunoreactivity to cytokines. Moreover, the development of the large germinal centres was stimulated, indicating activation of spleen function in response to systemic immunotoxicity. Unlike in the thymus, there was some evidence of recovery in the spleen. Ito *et al.* (2002b) investigated the distribution and excretion of orally administered OA in mice and observed that some toxin becomes inactive, but is still present, after two weeks. Atrophic signs in the spleen and thymus have also been observed after oral administration of high doses of OA (Tubaro *et al.*, 2004) or other algal toxins such as azaspiracid (Ito *et al.*, 2000; 2002a).

In general, the data presented here suggest that low doses of OA alone or OA plus YTXs are able to provoke both immunostimulation and systemic immunotoxicity.

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