Cytotoxicity and Apoptotic Effects of Microcystin-LR and Anatoxin-a in Mouse Lymphocytes

(microcystin-LR / anatoxin-a / cytotoxicity / mouse lymphocytes / apoptosis)

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Abstract. There is an increasing amount of knowledge on the cytotoxic properties of cyanotoxins, but relatively little is known regarding their fine specificity and mechanisms of action. In this study, we investigated the influence of microcystin-LR and AnTx-a on mouse B- and T-lymphocyte subpopulations *in vitro*. Cyanotoxins significantly decreased the cell viability after 4 and 24 h, compared to the untreated control. After 24 h exposure to microcystin-LR and anatoxin-a, the viability of splenocytes dropped to 23% and 57%, respectively. Our data demonstrate that microcystin-LR induced apoptosis specifically in mouse B cells, probably via the B-cell antigen receptor and mitochondrial pathway, while the T cells were not affected. AnTx-a showed cytotoxic effects on both lymphocyte subpopulations, but the effects were driven by mechanisms different from apoptosis. These findings demonstrate that the cyanotoxins could cause cytotoxic alterations in a variety of cell types different from the major targets, operating via distinct mechanisms.

Microcystin-LR (MC-LR) and anatoxin-a (AnTx-a) are the most common toxins produced by *Cyanoprokaryota* (blue-green algae) in marine and freshwater supplies around the world. These cyanotoxins may cause serious health problems and ecological risks, even death in both humans and animals (for a review, see Hitzfeld et al., 2000; Lakshmana Rao et al., 2002b). The most often noted producers of toxins are *Microcystis*, *Anabena*, *Aphanizomenon*, *Cylindrospermopsis*, *Oscillatoria* and *Nostoc* (Sivonen, 1996).

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Abbreviations: AnTx-a – anatoxin-a, ConA – concanavalin A, LPS – lipopolysacharide, MC-LR – microcystin-LR, MTT – 3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyltetrazolium bromide, PHA – phytohaemagglutinin.

MC-LR is a cyclic hepatotoxic heptapeptide and its main mechanism of action is well known. The inhibition of serine/threonine protein phosphatases PP1 and PP2A by MC-LR (MacKintosh et al., 1990; Yoshizawa et al., 1990; Dawson, 1998) leads to hyperphosphorylation of the cytosolic and cytoskeletal proteins, which consequently causes hepatocyte deformation and liver toxicity (Eriksson et al., 1992; Falconer and Yeung, 1992; Yoshida et al., 1997). The tissue specificity of MC-LR is due to its difficulty in penetrating into the cells and uptake from the blood requires the action of a multispecific bile acid transport system (Eriksson et al., 1990; Runnegar et al., 1991).

AnTx-a is a low-molecular-weight alkaloid with post-synaptic depolarizing activity. It acts as a stereoselective agonist of the acetylcholine receptors in both neurons and muscle endplates with 8–10-fold higher potency (Carmichael et al., 1975; Spivak et al., 1980).

In addition to the hepatotoxic or neurotoxic mode of action, MC-LR and AnTx-a may affect, in different ways, many other types of cells (non-hepatic and non-neuronal) as well, causing tumour promotion (Nishiwaki-Matsushima et al., 1992; Falconer and Humpage, 1996; Humpage et al., 2000), production of reactive oxygen species (Ding et al., 1998; Lakshmana Rao et al., 2002a; Botha et al., 2004), modulation of the immune system (Nakano et al., 1989; Yea et al., 2001; Lankoff et al., 2004a) and induction of apoptosis via activation of caspases, calpains or Ca^{2+}/calmodulin-dependent protein kinase II (McDermott et al., 1998; Fladmark et al., 1999, 2002; Mankiewicz et al., 2000; Ding et al., 2002; Lakshmana Rao et al., 2002a; Botha et al., 2004; Lankoff et al., 2004a).

Cells can die in either of two ways: programmed cell death (apoptosis) or necrosis. Apoptosis is a normal physiological process that is required for the maintenance of cell homeostasis. The cellular changes of this process are both morphological and biochemical, including disintegration of the cytoskeleton and subsequent cell shrinkage, chromatin condensation and activation of specific proteases, the so-called caspases (Kerr et al., 1972; Wyllie et al., 1980; Nicholson and Thornberry, 1997). At the early stage

of apoptosis, phosphatidylserine, which is normally located on the inner face of the plasma membrane, becomes exposed on the outer surface, and provides a recognition signal for engulfment by phagocytes. Apoptosis can be initiated by a variety of internal and external stimuli, including receptor ligation and toxic insults. Necrosis is another form of cell death, which typically occurs as a result of mechanical or toxic cell injury. Necrotic cells are distinguished from apoptotic cells as they undergo stages such as cell swelling, plasma membrane rupture, organelle breakdown and ultimately lysis, allowing release of the cytoplasmic content and hence induction of an inflammatory response (Wyllie et al., 1980).

There are several reports that demonstrate the apoptotic effects of MC-LR on chicken and human peripheral blood lymphocytes (Mankiewicz et al., 2001; Lankoff et al., 2004a, b). Compared to the primary target of MC-LR (hepatocytes), human peripheral blood lymphocytes were less sensitive to cyanotoxins (Mankiewicz et al., 2001). McDermott et al. showed that in other cell types apoptosis can be observed, but required MC-LR concentrations are 100-fold higher (McDermott et al., 1998). It has been reported that MC-LR downregulates lymphocyte functions by decreasing IL-2 mRNA stability or induction of apoptosis (Yea et al., 2001; Lankoff et al., 2004a). Using lipopolysaccharide (LPS)- and concanavalin A (ConA)- or phytohaemagglutinin (PHA)-stimulated lymphocytes the same authors hypothesize that B cells are more sensitive than T cells and more prone to apoptosis.

Although MC-LR was found to inhibit the proliferation of T and B cells and cytokine production, the fine specificity of its action on lymphocytes is still unclear. The goal of this study was to further investigate the above hypothesis using flow cytometry analysis and non-stimulated mouse splenocytes. Here we evaluate the cytotoxic and apoptotic effects of pure MC-LR and AnTx-a as well as their fine specificity and possible mechanisms of action.

Material and Methods

Reagents and media

Anatoxin-a (AnTx-a), 3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin, propidium iodide (PI) and ammonium chloride were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Microcystin-LR (MC-LR) was from BIOMOL GmbH (Hamburg, Germany) and foetal calf serum (FCS) was from PAA Laboratories GmbH (Linz, Austria). FITC-conjugated Annexin-V, PE-conjugated anti-TCR (clone H57-597) and PE-conjugated anti-CD45R/B220 (clone RA3-6B2) antibodies were purchased from BD PharMingen (San Diego, CA). Phosphate-buffered saline (PBS) and Dulbecco's Modified Eagle's Medium (DMEM) were from Gibco™ (Paisley, Scotland, UK).

Cells and treatment with MC-LR and AnTx-a

Spleen cells were isolated aseptically from three male 8-week-old BALB/c mice (20–23 g), which were kept in a climate-controlled and pathogen-free environment and fed standard rodent chow and water *ad libitum*. Briefly, spleens were removed, homogenized, and red blood cells were lysed by 0.84% ammonium chloride solution (pH 7.4). After passage of the cells through a 40 μm cell strainer (FALCON®, Becton Dickinson, Le Pont De Claix, France), cells were collected by centrifugation and washed twice with serum-free DMEM. The cells were resuspended and diluted at a final density of 1 x 10^6 cells/ml in DMEM supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin and transferred in 200 μl aliquots to 96-well plates (Nunc, Roskilde, Denmark).

Mouse splenocytes were exposed to 7.5 μg/ml MC-LR or 0.1 μg/ml AnTx-a in complete DMEM and incubated at 37 °C in a 5% CO_2-humidified incubator for 4 or 24 h prior to analysis of cytotoxicity by the MTT assay or of apoptotic effects by flow cytometry.

MTT cell viability assays

The MTT assay was carried out by the method of Edmondson et al. (1988). This assay is based on the capacity of mitochondrial succinyl dehydrogenase to convert the soluble yellow tetrazolium salt into an insoluble purple-blue formazan product. Briefly, after the desired time of exposure with cyanotoxins (4, 24 or 48 h), 20 μl of a 0.5% (w/v) solution of MTT in PBS were added directly to each well and incubated at 37°C for 3 h. Thereafter, the supernatant was removed and 0.1 ml of 0.04 mol/l HCl in isopropanol was added to each well in order to facilitate solubilization of the formazan product. After 30 min at room temperature the plates were shaken, and absorbance was read at 570 nm in a SPECTRAmax®PLUS microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Flow cytometry

Mouse splenocytes treated with MC-LR and AnTx-a were subjected to two separate staining procedures with different antibodies: (1) FITC-conjugated Annexin-V (staining for apoptosis) and PE-conjugated anti-TCR (staining for T cells) and (2) FITC-conjugated Annexin-V (staining for apoptosis) and PE-conjugated anti-CD45R/B220 (staining for B cells).

After the exposure period (4 or 24 h) cells were washed twice (PBS, 1% FCS) and incubated with the antibodies for 20 min at 4 °C in the dark. Cells were then washed twice and analysed by setting a gate on lymphocyte populations using forward and side scatter and plotting data on a log scale using a FACSort (Becton Dickinson, Mountain View, CA) equipped with CellQuest software. The dead cell populations (necrotic cells) were gated out by propidium iodide staining

immediately before analysis. A total of 10,000 events in a live gate were acquired, although all ungated events were saved for later analysis.

Statistics

The results from cytotoxicity tests are reported as mean ± SE from individual determinations with at least four replicates. Data were compared using unpaired (Mann-Whitney U) nonparametric tests. A P value ≤ 0.05 was considered significant. Statistical tests were performed with StatView software (SAS Institute Inc., Cary, NC).

Results

Cell viability of mouse splenocytes following treatment with MC-LR and AnTx-a

Freshly prepared mouse splenocytes were treated with 7.5 µg/ml MC-LR or 0.1 µg/ml AnTx-a for 4 and 24 h. Toxin concentrations were chosen according to literature data. As shown in Fig. 1A, MC-LR significantly decreased the cell viability (as measured by MTT assay) after 4 and 24 h, compared to the untreated control. At 4 h post-treatment, rapid loss of viability was observed as more than 60% of the cells were dead. After 24 h exposure to MC-LR, the viability of splenocytes dropped to 23% in comparison to the control. Treatment of splenocytes with AnTx-a (Fig. 1B) had a lower cytotoxic effect than MC-LR (Fig. 1A). The percentages of cell viability at 4 and 24 h post-treatment were 76% and 57%, respectively. Using one concentration of treatment both cyanotoxins MC-LR and AnTx-a showed a time-dependent decrease in cell viability with higher potency for MC-LR.

The results obtained in these experiments demonstrated that hepatotoxins (MC-LR) and neurotoxins (AnTx-a) could cause cytotoxic alterations in a variety of cell types different from the major targets.

Specificity and apoptotic effect of MC-LR and AnTx-a on mouse lymphocytes

Using flow cytometry, we then investigated which lymphocyte subpopulations (T cells and/or B cells) are affected after MC-LR and AnTx-a treatment, and whether this cytotoxic alteration is due to apoptosis or necrosis. Double staining with Annexin-V FITC (which binds to phosphatidylserine, an early apoptotic marker) and propidium iodide (which binds to nuclear DNA after plasma membrane degradation) allowed distinguishing apoptotic and necrotic cells. In Figure 2 it can be seen that 4 h after treatment with MC-LR the T cells (33% from the splenocytes) were not affected (Fig. 2A, B), while the B cells (54% from the splenocytes) showed 18% of Annexin-V$^+$ (apoptotic) cells (Fig. 2C, D). The frequency of necrotic cells at that time point in both subpopulations was negligible (staining by propidium iodide).

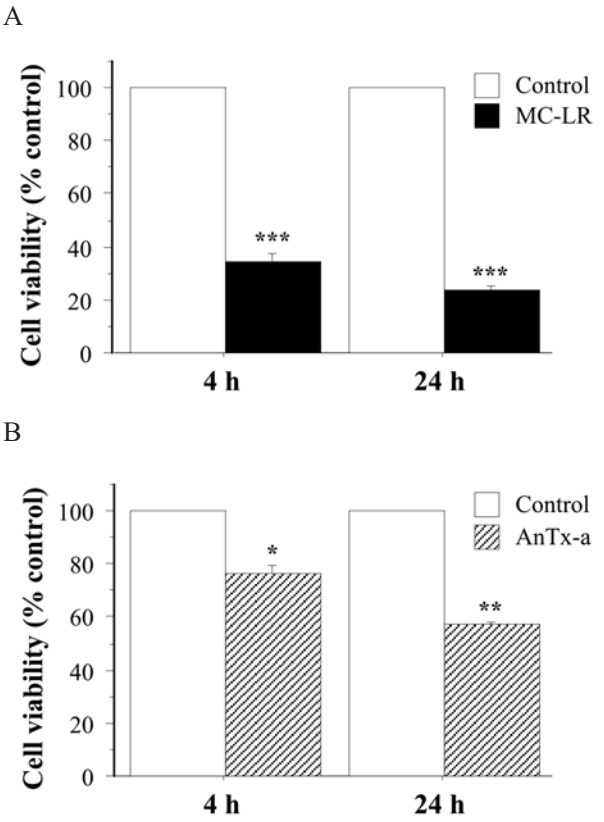

Fig. 1. Effect of MC-LR (**A**) and AnTx-a (**B**) on the viability of freshly prepared mouse splenocytes. The cells were treated with 7.5 µg/ml MC-LR or 0.1 µg/ml AnTx-a for 4 and 24 h, and viability was determined by MTT assay. The values are mean ± SE of four replicates. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

After 24 h exposure of mouse splenocytes to MC-LR, again there was no significant increase in Annexin-V$^+$ T cells (Fig. 3A, B). At the same time point, an increase of approximately 50% in apoptotic B cells was observed (Fig. 3C, D). The percentage of necrotic cells gradually increased with time in both T- and B-cell subpopulations with higher indices for T cells.

These results clearly indicate that MC-LR induces apoptosis only in B cells, probably via B-cell receptor-specific activation of kinase pathways, while the T cells are not affected.

In contrast to MC-LR, exposure of mouse splenocytes to AnTx-a only for 4 h caused significant cell death in both lymphocyte subpopulations as more then 80% of splenocytes were stained with propidium iodide (Fig. 4). This indicates that most cells are in late apoptotic or secondary necrotic phase (Annexin-V$^+$/PI$^+$). No staining of Annexin-V was observed in the viable T- and B-cell subpopulations. Similar results were obtained 24 h after treatment with AnTx-a (data not shown). Therefore, the cytotoxic effects of MC-LR and AnTx-a on lymphocytes operate via different mechanisms.

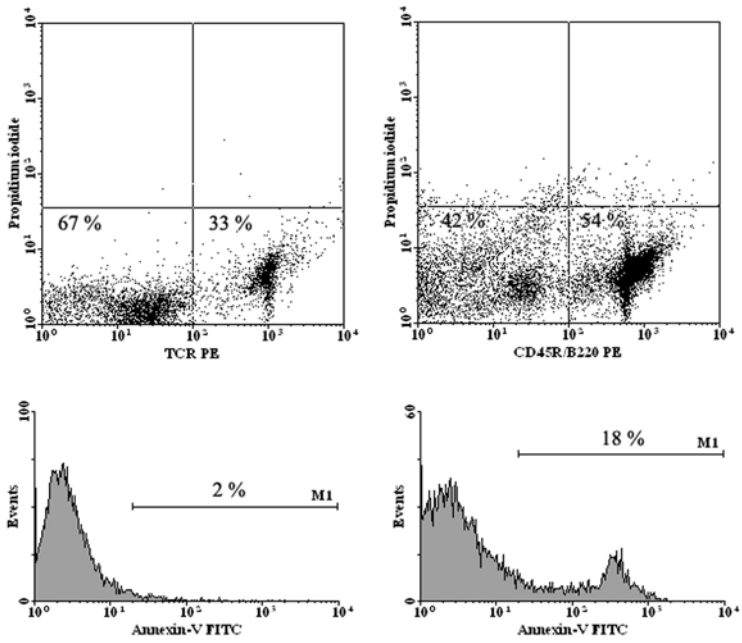

Fig. 2. Flow cytometric analysis of mouse T cells (**A, B**) and B cells (**C, D**) at 4 h post-exposure of the isolated splenocytes to 7.5 μg/ml MC-LR. Cells were stained with anti-TCR PE-conjugated and Annexin-V FITC-conjugated antibodies (for T cells) or CD45R/B220 PE-conjugated and Annexin-V FITC-conjugated antibodies (for B cells) in two separate stainings. Propidium iodide was added immediately before gating. The histograms are gated on TCR^+ or $CD45/B220^+$ cells, respectively, and the region M1 indicates apoptotic cells.

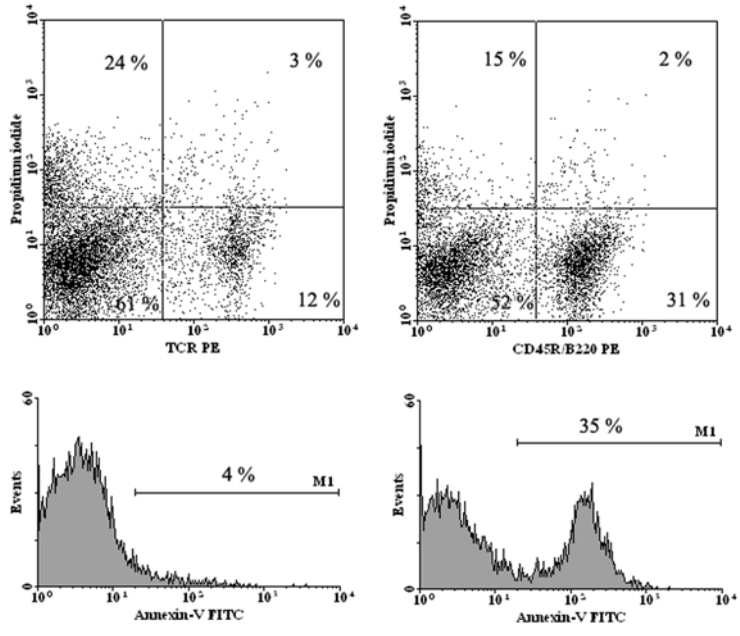

Fig. 3. Flow cytometric analysis of mouse T cells (**A, B**) and B cells (**C, D**) at 24 h post-exposure of the isolated splenocytes to 7.5 μg/ml MC-LR. Cells were stained with anti-TCR PE-conjugated and Annexin-V FITC-conjugated antibodies (for T cells) or CD45R/B220 PE-conjugated and Annexin-V FITC-conjugated antibodies (for B cells) in two separate stainings. Propidium iodide was added immediately before gating. The histograms are gated on TCR^+ or $CD45/B220^+$ cells, respectively, and the region M1 indicates apoptotic cells.

Discussion

The present study was conducted to examine which type of mouse lymphocytes (T and/or B cells) are specifically altered after exposure to MC-LR and AnTx-a. We have clearly demonstrated here that MC-LR specifically induces apoptosis in mouse B cells, while the T cells were not affected. AnTx-a showed cytotoxic effects on both lymphocyte subpopulations.

It has been shown previously that MC-LR and AnTx-a can induce apoptosis in a variety of cell types as well as necrosis when applied at higher concentrations (McDermott et al., 1998; Lakshmana Rao et al., 2002a). Yea et al. (2001) and Lankoff et al. (2004a) reported that MC-LR downregulates lymphocyte functions. Using LPS- and ConA- or PHA-stimulated lymphocytes both groups have shown that MC-LR suppresses the proliferation of LPS-stimulated lymphocytes (B cells). Data about the proliferation of ConA- or PHA-stimulated lymphocytes (T cells) were controversial. Yea et al. (2001) did not found any effect of MC-LR (0–50 μg/ml) on the proliferative response to ConA-stimulated lymphocytes, whereas Lankoff et al. (2004a) showed inhibition of the T-cell proliferation at 25 μg/ml MC-LR as well as decreased production of IL-2 and increased production of IL-6. The authors noticed that B cells were more sensitive to MC-LR exposure than T cells. This assumption was based on the proliferative response of stimulated lymphocytes. Our data from the flow cytometry analysis of MC-LR-treated non-stimulated lymphocytes showed that MC-LR induces apoptosis in the B-cell subpopulation, but not in T cells. To avoid false-positive results due to overstimulation, we have used non-stimulated lymphocytes and measured externalization of phosphatidylserine (by Annexin-V) after short exposure to MC-LR (4 and 24 h). Previous studies showed that MC-LR can induce all the apoptotic changes in hepatocytes in less than 1 h (Ding et al., 2000).

Several pathways of apoptosis have been described during the last years: mitochondrial pathway, death receptor-mediated pathway and perforin-dependent exocytosis pathway. Since MC-LR inhibits protein phosphatases 1 and 2A (Yoshizawa et al., 1990; Carmichael, 1994), it has been suggested that the induction of apoptosis by MC-LR is due to protein hyperphosphorylation (McDermott et al., 1998; Fladmark et al., 2002).

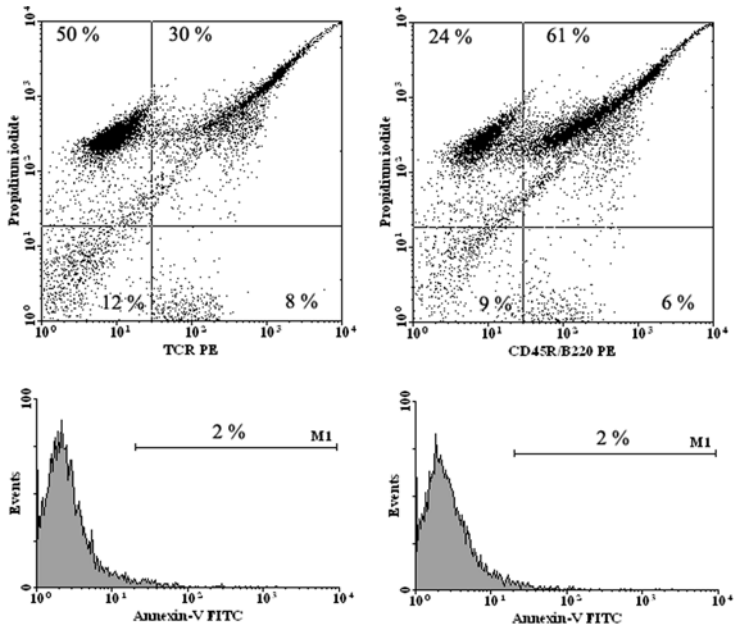

Fig. 4. Analysis of mouse T cells (**A, B**) and B cells (**C, D**) by flow cytometry at 4 h post-exposure of the isolated splenocytes to 0.1 μg/ml AnTx-a. Cells were stained with anti-TCR PE-conjugated and Annexin-V FITC-conjugated antibodies (for T cells) or CD45R/B220 PE-conjugated and Annexin-V FITC-conjugated antibodies (for B cells) in two separate stainings. Propidium iodide was added immediately before gating. The histograms are gated on TCR^+ or $CD45/B220^+$ cells, respectively, and the region M1 indicates apoptotic cells.

Other studies suggested that MC-LR initiates apoptosis by the formation of reactive oxygen species (ROS), which are known to cause damage to cellular membranes and particularly that of mitochondria (Ding et al., 1998, 2000, 2001; Botha et al., 2004). An early event in the mitochondrial pathway is the transfer of cytochrome-c from the outer mitochondrial membrane into the cytosol, where it functions as a cofactor for triggering the effector machinery of apoptosis (Zamzami et al., 1995). The disruption of mitochondrial integrity is not only important for the release of cytochrome-c, but also for the delivery into the cytosol of a fraction of caspase-9 and -3 that is localized in the intermembrane space and has been shown to be involved in apoptosis (Vander Heiden and Thompson, 1999). On the other hand, Ding et al. failed to detect increased activity of caspase-9 and caspase-3 in MC-LR-treated cells (Ding et al., 2002). There are also reports indicating that death receptor-mediated signalling can trigger the activation of the mitochondrial pathway of apoptosis. The protein involved in the triggering is caspase-8, which cleaves Bid (Li et al., 1998; Luo et al., 1998), a member of the Bcl-2 family.

Taking into account all the above evidences and based on the data we have obtained about the functional integrity of mitochondria as determined by the MTT test and externalization of phosphatidylserine as determined by flow cytometry, it seems that MC-LR induces apoptosis in the B lymphocytes via the B-cell antigen receptor (BCR) pathway. Several investigations of BCR-mediated apoptosis point to the mitochondrial/intrinsic pathway with involvement of unique proteases, notably calpain and caspase-2 (Chen et al., 1999; Ruiz-Vela et al., 1999; Ding et al., 2002) or activation of kinase pathways (Graves et al., 1998).

It has been reported that AnTx-a can induce apoptosis in non-neuronal cells mediated by ROS and caspase activation (Lakshmana Rao et al., 2002a). Although the cytotoxic effect of AnTx-a on mouse lymphocytes was not as high as that of MC-LR as measured by MTT (Fig. 1), our flow cytometry data indicated that both subpopulations (T and B cells) are severely affected 4 h after treatment with 0.1 μg/ml AnTx-a (Fig. 4). Therefore, the cytotoxic action of AnTx-a on lymphocytes appears to be non-selective and non-specific. Apoptotic cells from the viable T- and B- cell populations were not detected. Compared to MC-LR, mitochondrial triggering most probably operates by a different mechanism and this mode of action awaits further elucidation.

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