Regular Paper ISSN- 0102-9010

INFLUENCE OF A CHOLESTEROL-RICH EMULSION AND BCNU ON THE APOPTOTIC INDICES OF MYELOMA CELLS INDUCED IN BALB/C MICE

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ABSTRACT

Proliferating malignant cells express low-density lipoprotein (LDL) receptors, and a cholesterol-rich microemulsion (LDE) resembling the lipid portion of LDL can be bound to lipophilic drugs such as 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) to increase the drug´s capture and decrease the drug-associated toxicity. In this work, we studied the effect of BCNU and BCNU+LDE on apoptosis in plasmacytoma-related cells obtained from BALB/c mice with myeloma. Four groups of mice (n=4 each) were treated with LDE, BCNU, BCNU in LDE or vehicle solution (control group). Morphological methods (histology and transmission electron microscopy) and immunohistochemistry (TUNEL procedure) were used to evaluate the occurrence of apoptosis. The drugs were injected intraperitoneally in the 14th month after induction of the myeloma and the mice in all groups were sacrificed six hours after this injection. The apoptotic indices of the plasmacytoma mesenteric cells evaluated in the four experimental groups revealed that the LDE emulsion significantly ($p<0.05$) increased the percentage of tumoral cells dying by apoptosis. All the groups with LDE, alone or in combination with BCNU showed significantly higher apoptotic indices than the controls. This enhanced cytotoxicity suggests a potential use for LDE in improving the efficacy of chemotherapeutic agents.

Key words: Apoptosis, cholesterol-rich emulsion, carmustine, BALB/c-induced myeloma cells, immunohistochemistry, transmission electron microscopy

INTRODUCTION

Malignant tumor growth is limited by the incidence of apoptosis, the commonest type of programmed cell death, in tumoral cells [14-16]. The treatment of tumors with chemotherapeutic agents can cause cellular necrosis and apoptosis [8,16], although conflicting results have been reported, probably because of differences in the experimental models and conditions, drug doses, and timing of sample collections [6,10,23,30,34].

The liposoluble drug BCNU (Becenun®, Carmustine (1,3-bis (2-chloroethyl)-1-nitrosourea) has been used alone, or in association with other drugs, to treat various types of tumors, including lymphomas, with reasonable success. However, very little is known about this drug´s mechanism of action. In particular, it is also unclear whether the active ingredient is the drug itself or one or more of its metabolites [28]. BCNU induces tumoral cell death by apoptosis [30] but also produces this same effect in proliferating, non-tumoral cells.

A synthetic, cholesterol-rich emulsion known as LDE (produced by Dr. Raul Maranhão, Atherosclerosis Unit, Heart Institute, Hospital das Clínicas de São Paulo, São Paulo, Brazil.) could be a potential vehicle for drug delivery, thereby allowing more specific drug targeting and a consequent reduction in collateral effects. Proliferating neoplastic cells incorporate LDE, and Maranhão *et al.* [11,21] evaluated the kinetics of this compound in patients with tumors and found that when associated with an antineoplastic agent produces favorable clinical responses with lower side effects than other vehicles. An analysis of the results obtained for patients with multiple myelomas treated with BCNU/LDE [22] suggested that LDE may have several mechanisms of action.

The induction of plasmacytoma in BALB/c mice described by Potter and Boyce [26] is an interesting experimental model, even though it was established in a strain with specific immunological characteris-

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tics. Peritoneal stimulation in a non-sterile environment leads to the production of IgA myelomas from poorly differentiated cells that are disseminated in an aged animal (about 14 months old). As shown by Potter *et al.* [26,27] the development of myelomas in this model depends on more than one factor, and involves lymphocytes, macrophages and interleukin-6 (IL-6) [33]. A relationship between this experimental model and multiple myelomas in humans is possible, but not universally accepted. However, the role of lymphocytes, macrophages and IL-6 in human lymphoma formation is of interest because of their importance in the evolution of this disease, even though "clonal" proliferation consists of B-lymphocytes. Potter's model is also interesting for therapeutic studies and for investigating the action of chemical agents on the cell population involved in lymphomas. According to Potter and Boyce [26], plasma cells, lymphocytes and macrophages (referred to as plasmacytoma-associated cells) participate in the development of plasmacytoma in BALB/c.

In the present study, we used histological analysis, transmission electron microscopy (TEM) and the TUNEL reaction to assess whether LDE enhanced the apoptotic effect of BCNU in mesenteric cells of BALB/c mice treated with mineral oil to induce plasmacytoma.

MATERIAL AND METHODS

Animals

One-month-old male BALB/c mice (mean weight 30 g) obtained in 1998 from the Vivarium (Biotério) of the Biomedical Institute of the University of São Paulo, were housed 5/cage at room temperature on a 12 h light/dark cycle, with free access to food and water. The experimental protocols described here were approved by an institutional (FMUSP) Committee for Ethics in Animal Experimentation and were done according to the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Plasmacytoma was induced in the mice by a single intraperitoneal injection of 0.5 ml of mineral oil (Nujol), as described by Potter and Boyce [26]. On the 14th month after the induction of plasmacytoma, the mice were divided into four groups (n=4/group) to study the incidence of apoptosis following the injection of BCNU, BCNU in LDE, LDE or saline (vehicle) solution (control group). The mice were killed with ether 6 h after injecting the drugs and the mediastinal lymph nodes, the mesentery and the disease-induced mesenteric polyps [26] were removed and cut into fragments 0.5 cm thick for light microscopy and 0.2-0.5 mm thick for electron microscopy.

Preparation of LDE and of BCNU in LDE

The preparation of LDE and the incorporation of BCNU in the lipid emulsion were done as described by Maranhão *et al.* [21].

Administration of LDE, BCNU and the BCNU/LDE emulsion

All of the drugs were injected intraperitoneally (100 µl/per mouse) based on the mean BCNU dosage given to humans as a single application (100 mg/m^2) [10]. The amount of drug was calculated using the total body surface area [16], which for a 30 g mouse was 0.0076 m^2 and resulted in a dose of 0.76 mg/mouse.

Light miscroscopy and transmission electron microscopy (TEM)

For the light microscopical studies, the tissues were fixed in 4% paraformaldehyde for 24 h at 4ºC and then processed using standard laboratory procedures, with 2-µm-thick sections of paraffin-embedded material being stained with hematoxylin and eosin (HE). The plasmacytoma was characterized according to Potter [25] and Potter and Maccardle [27], based on the consistent presence of plasmacytoma-associated cells, plasma cells, macrophages and lymphocytes in histological sections and in electron micrographs. All of the above cells, which represented most of the cellularity of the mesenteric-associated polyps, were clustered near oil droplets in the vicinity of blood vessels.

For TEM, the material was immediately fixed in 1.5% glutaraldehyde plus 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at room temperature. A second fixation was done with osmium reduced with potassium ferrocyanide for 1.5 h (one volume of aqueous 2% osmium tetroxide and one volume of 3% potassium ferrocyanide), also at room temperature. The rest of the procedure followed standard laboratory routine, with embedding in Epon. Ultrathin, silver-gold sections, contrasted with standard procedures using lead citrate and uranyl acetate, were examined with a JEM 1010 transmission electron microscope. The fragments examined by TEM were previously selected based on the analysis of methylene blue-stained semi-thin sections (~0.25 µm thick) using light microscopy.

In addition to the morphological analysis for the initial evaluation of apoptosis [29], apoptotic cells were also identified by their characteristic nuclear phenotype described by Kerr [13,15]. The incidence of apoptosis was determined by counting 500 cells per mouse in TEM using ultrathin sections of blocks prepared from 1-3 fragments of mesentery and mesenteric polyps per mouse, and the apoptotic index was expressed as a percentage of the total number of cells counted. The number of apoptotic cells was scored for every 100 plasmacytoma-associated cells (plasma cells, lymphocytes and macrophages) that were counted [5,18]. The number of fragments examined

depended on the size of the tumors, which varied among the mice.

TUNEL reaction

In apoptotic cells, activated nucleases promote the appearance of cuts or nicks in double- stranded DNA. At these sites, there is localized and limited synthesis of DNA by the enzyme deoxynucleotidyl transferase (TdT), responsible for the **T**dT-mediated d**U**TP-biotin **n**ick **e**nd **l**abeling reaction, referred to by the acronym TUNEL. This reaction reveals the presence of apoptotic nuclei [3] and, in the protocol used here, a fluorescence reaction results in the apoptotic cells fluorescing in green.

For this assay, apoptotic cells in 2-μm-thick paraffin sections from tissues prefixed in 4% paraformaldehyde for ~24 h were detected using an *in situ* cell death detection kit (POD, Boehringer-Roche, Mannheim, Germany), according to the manufacturer´s instructions. After removal of the paraffin with xylol and rehydration in a graded ethanol series, the specimens were incubated with 20 μg of proteinase K/ml for 30 min, at room temperature, and then incubated in 0.3% hydrogen peroxide in methanol for 30 min, also at room temperature. Subsequently, the sections were incubated in 50 μl of the TUNEL reaction mixture [5 μl of enzyme solution (tube I) + 45 μl of staining solution (tube II)] per sample, in a humid chamber. After this stage, the material was examined with a fluorescence microscope.

The sections were subsequently stained with peroxidase, which was visualized with diaminobenzidine (DAB). Initially, the sections were incubated with 50 μl of peroxidase converter (converted-POD) in a humid chamber at 37ºC for 60 min, followed by incubation in 50 μl of DAB solution (0.2% DAB + 0.05% hydrogen peroxide in PBS) for 10 min, at room temperature, and counterstained with 0.5% methyl green for 10 min. The progress of this reaction was monitored microscopically. The sections were washed 3-5 times in PBS after each incubation step. Glass coverslips were mounted on the slides with Permount. For the negative control, the TUNEL reaction mixture was omitted from the steps described above, while for the positive control, apoptotic cells from the secretory epithelium of the ventral lobe of the rat prostrate gland, removed four days after castration, were used. In the positive control, apoptotic nuclei and apoptotic bodies derived from atrophying secretory epithelial cells were profuse and reacted strongly in both the fluorescence and the peroxidase steps. No false positive peroxidase reactions occurred in the TUNEL reactions in this study. After assessing the fluorescence, apoptotic cells were identified by a dark brown color covering the cell nucleus [3,5,18].

Apoptosis was quantified with a Kpl 8 X ZEISS compensation ocular lens with a 100- and 25-point grid fitted to an OLYMPUS microscope at magnifications of x40 and x100. Two thousand cells from each preparation of mesentery-associated polyps were examined per mouse. The resulting apoptotic index corresponded to the percentage of apoptotic cells observed in the mesentery and mesenteric polyps [5,18].

Statistical analysis

The results were expressed as the mean ± SEM whenever possible. The Kruskal-Wallis one-way analysis of variance by ranks and the Scheffé multiple-comparisons test were used to compare the apoptotic frequencies of the groups. Since percentages are normally analyzed with binomial statistics, the means were compared after arc sine transformation [19] of the values in percentage. In addition, Student´s *t*-tests with logarithmic transformation of the data used in the Scheffé analyses were used to compare the frequencies of apoptosis of some experimental groups. All statistical comparisons were done using Stata statistical software, release 8.0 (Stata Corporation, College Station, TX, USA).

RESULTS

To facilitate evaluation of the tumor morphology, BALB/c mice were killed 14 months (420 days) after induction of the plasmacytoma with mineral oil. By this time, the tumors were at an advanced stage of development. Of the 33 mice injected with mineral oil, 10 (30%) died before the drugs were tested. Macroscopically, the incidence of plasmacytoma was 100% in the treated and nontreated (control) groups. The disease was recognized by the presence of pedunculated mesenteric polyps 0.5-1.5 mm long and enlarged mediastinal lymph nodes, both found in all of the mice.

Histological analysis

Light microscopy was used to characterize the plasmacytoma. In diseased mice injected with saline, a diffuse lymphoplasmocytic infiltration with discrete vascular proliferation was found in focal areas of the mesentery, as well as in specific areas of this tissue identified macroscopically as mesenteric polyps. This infiltration sometimes had focal areas with aggregations of plasma cells, some of which were binucleated, i.e., a plasmacytoma (Fig. 1A).

In addition to the lymphoplasmocytic infiltration seen in sick mice treated with BCNU, apoptotic cells were present in the mesenteric polyps of most of these animals. In mice injected with BCNU/LDE, the same alterations as seen in mice injected with BCNU were also seen in the mesentery and mesenteric polyps. One mouse in this treatment group showed many atypical plasma cells and apoptosis, as well as an intense lymphoplasmocytic infiltration. Mice injected with the LDE emulsion

showed plasmacytoma and apoptotic cells in the mesentery and mesenteric polyps (Fig. 1B).

The mediastinal lymph nodes of most of the mice had oil granulomas that in histological preparations appeared as transparent areas caused by oil droplets surrounded by an inflammatory process. In some mice, conglomerates of various sized plasma cells, some of them large, complicated the inflammatory process (Fig. 1C). Histological analysis of the mesentery revealed areas with varying degrees of tumoral infiltration in the control and treated groups.

Transmission electron microscopy (*TEM*)

TEM was used to examine the ultrastructural morphology of the mesentery and mesenteric polyps and to assess the presence of apoptosis. The plasmacytomas contained plasma cells that in some cases were anomalous (two nuclei, with signs of immaturity such as a predominance of euchromatin and more than one nucleolus - generally large, and a greatly dilated and much larger rough endoplasmic reticulum), lymphocytes (most of them plasmacytoid), neutrophils, eosinophils and macrophages (Fig. 2A-D).

Apoptosis was characterized by a decrease in cell size, marginalization of the chromatin, condensation and fragmentation of the nucleus, and condensation of the cell, with preservation of the organelles [13,16,29,35,36]. Other changes that were considered indicative of apoptosis included the presence of swollen mitochondrial profiles with ruptured outer membranes. In these profiles, the mitochondria were partially covered by the external membrane [31], and the swollen mitocondrial matrix that herniated into the cytoplasm through the ruptured outer membrane were covered by the inner membrane.

The apoptosis present in the mesentery and mesenteric polyps of all of the mice was quantified. The mesentery of all mice in the treated groups had a predominance of lymphocytes (mostly plasmocytoid). In addition, macrophages with many inclusions, notably remnants of apoptotic cells (mainly plasma cells) were also seen in the mesenteric polyps (Fig. 3). The plasma cells in the BCNU/LDE group and the macrophages in the LDE group (Fig. 2D) were in early apoptotic stages. The largest proportion of apoptotic macrophages (32.5%) was found in the LDE group. TEM showed that the cytoplasm of most of the apoptotic cells was filled with rough endoplasmic reticulum cisternae, indicating that the dead cells were plasma cells (Fig. 3).

The incidence of apoptosis in the controls was low (2.28%). In the BCNU groups, the apoptotic index was 7.5%, while in the BCNU/LDE group it

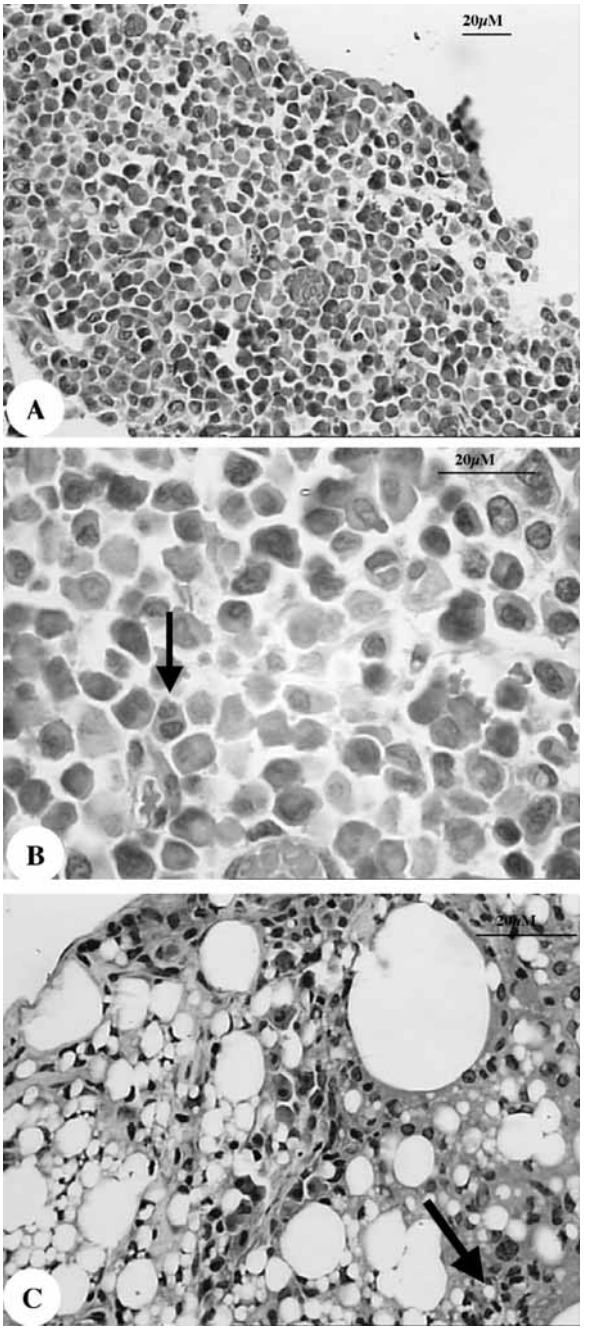

Figure 1. Light micrographs of mesenteric polyps. **(A)** Control group, showing the plasmacytoma. **(B)** LDE group. The arrow indicates an apoptotic nucleus. **(C)** Polyp from an LDE-injected mouse showing the infiltration of inflammatory cells and lipid droplets of various sizes. **Arrow** - apoptotic cells. HE staining. Bars = 10 μm.

was 13.0% and in the LDE group it was 11% (Fig. 4). There were significant differences between the control and the BCNU/LDE (p = 0.001) and LDE (p = 0.004) groups, but no significant differences among the BCNU, BCNU/LDE and LDE groups.

TUNEL reaction

Before using peroxidase in the TUNEL reaction, the mesentery and mesenteric polyps were observed by fluorescence microscopy. Fluorescent apoptotic cells were seen in all of the experimental

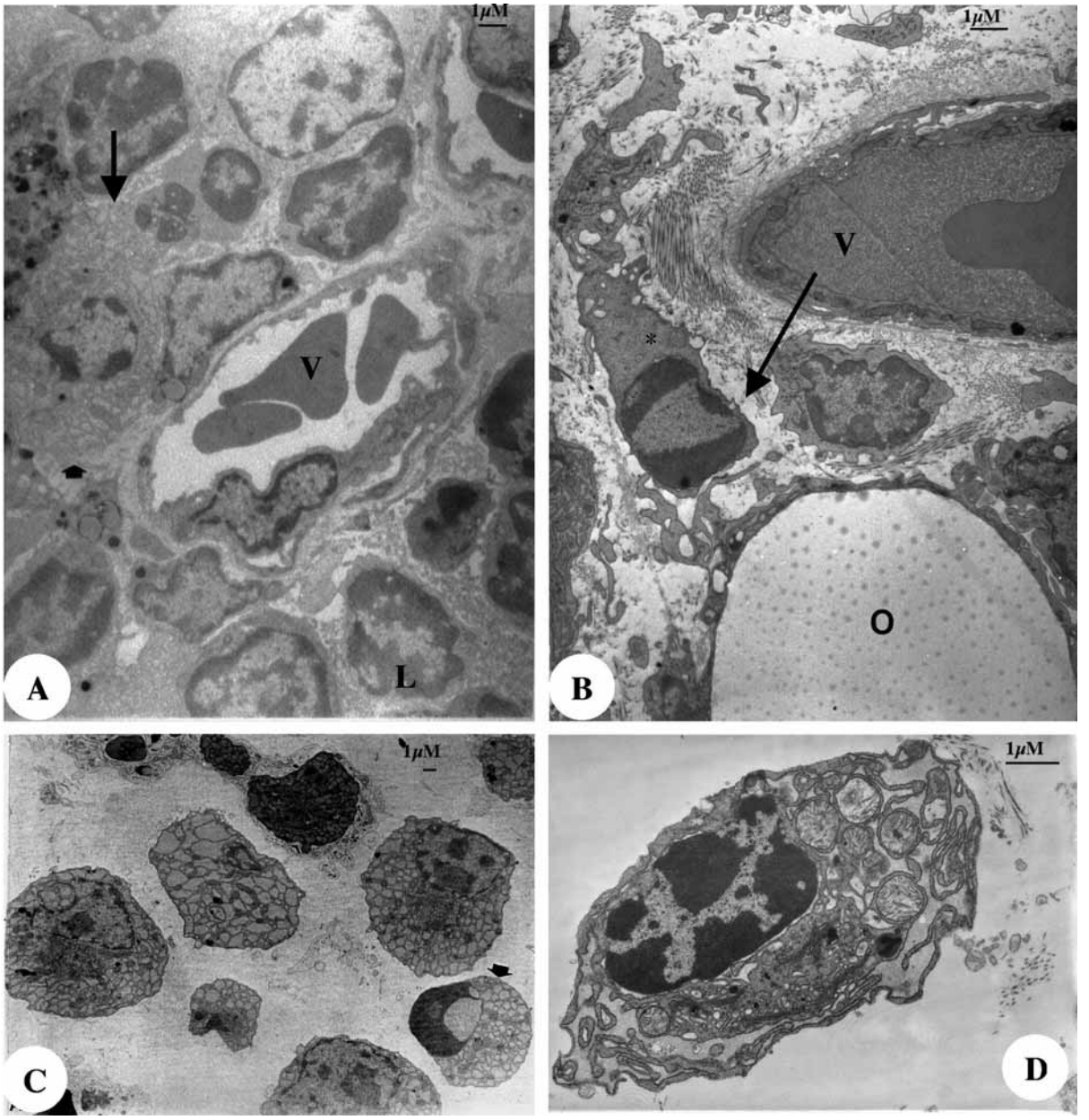

Figure 2. Electronmicrographs of mesenteric polyps. **(A)** Mesenteric polyp of the BCNU group, showing the plasmacytoma, an apoptotic plasma cell **(arrow)**, lymphocytes **(L)** and blood vessel **(V). (B)** Mesentery of the BCNU group showing an apoptotic cell **(arrow)** close to a blood vessel **(V)** and an oil droplet **(O)**. **(C)** Mesenteric region of the BCNU/LDE group showing a predominance of apoptotic plasma cells **(arrow)**. **(D)** Part of a mesenteric polyp in the LDE group showing a macrophage in early apoptosis. Bars = 1 μm.

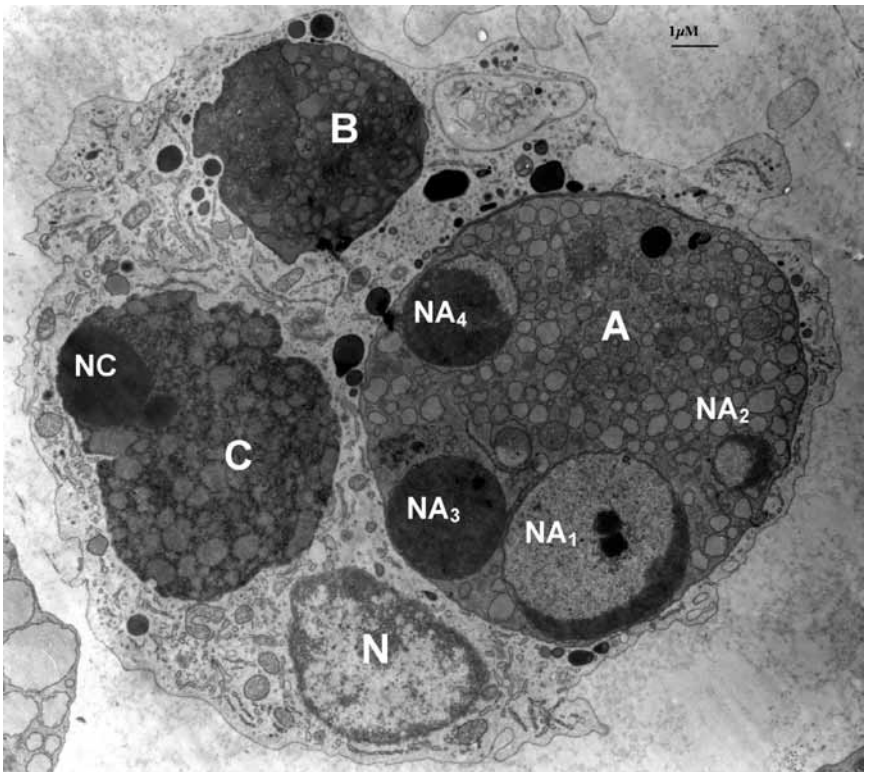

Figure 3. Profiles of macrophage-engulfed apoptotic plasma cells in which the cytoplasm is filled with cross-sections of rough ER cisternae. **NA_1-NA_4** - nuclear fragments in cell profile A, **NC** - apoptotic nucleus in cell profile C, **N** - nucleus of a very large macrophage with a clear cytoplasm that occupies most of the area in the figure. Bar = 1 μm.

groups, including the controls (Fig. 5A-D, apoptotic fluorescent cells indicated by arrowheads), with the greatest number of fluorescent cells occurring in the LDE group (Fig. 5D).

The apoptotic index was 9.6% in the control group, 11.1% in the BCNU group, 13.6% in the BCNU/LDE group and 17.4% in the LDE group. The Sheffé test revealed a significant difference (p=0.038) between the control and LDE groups, with no significant differences in the other inter-group comparisons (Fig. 6). Comparison of the control and BCNU/LDE groups using a Student´s *t*-test indicated no significant difference, even after logarithmic transformation of the data. However, there was a highly significant difference (p=0.0061) in the logarithms of the control and LDE-treated groups.

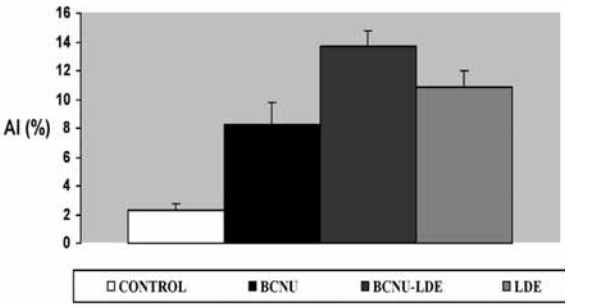

Figure 4. Apoptotic indices determined by TEM in mesentery and mesenteric polyps. The indices in the BCNU/LDE and LDE were significant difference from the control group (p = 0.001 and 0.004, respectively).

DISCUSSION

The treatment of tumors involves cell death, especially apoptotic cell death, and evaluation of the incidence of apoptosis is an important parameter for assessing the therapeutic efficacy of antitumoral drugs. In this work, we evaluated the percentage of apoptotic cells in plasmacytoma cells induced in BALB/c mice that received three combinations of drugs: LDE, BCNU, and BCNU/LDE. The apoptotic index assessed by TEM in the groups injected with BCNU, BCNU/LDE and LDE was significantly higher ($p < 0.05$) than in the control group. In the TUNEL reaction, the apoptotic index of LDE-treated

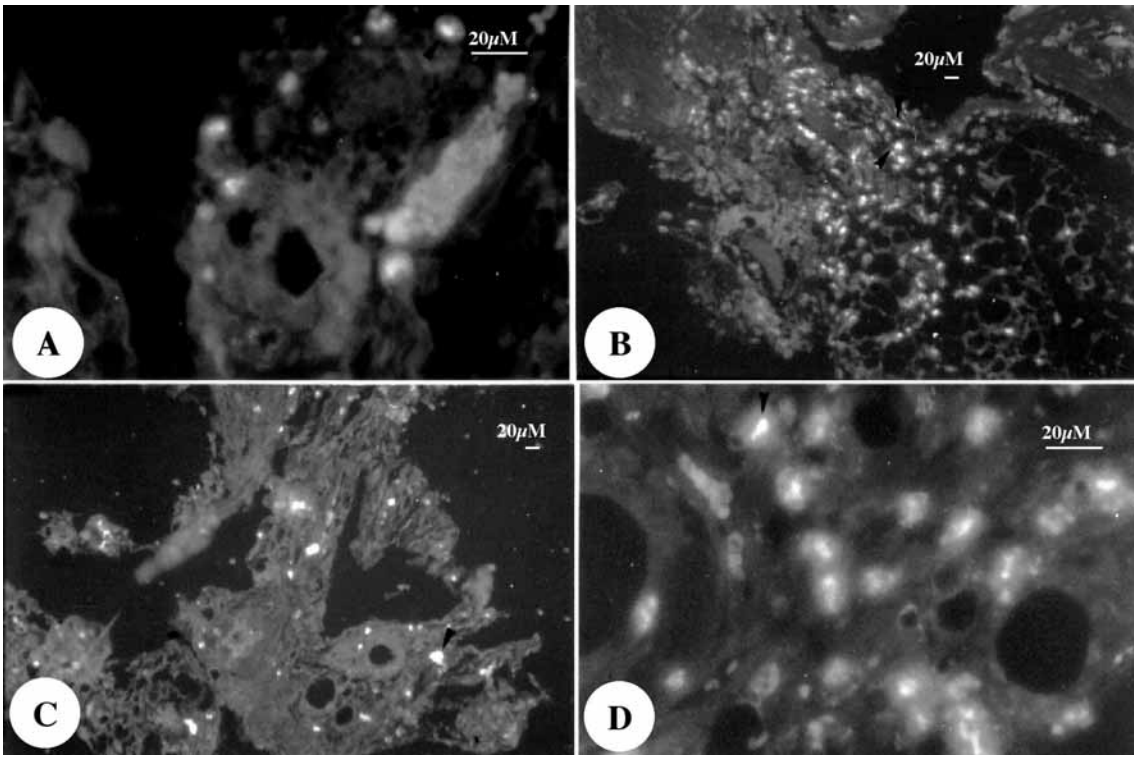

Figure 5. Fluorescence microscopy after the TUNEL reaction. (**A**) Mesenteric polyp of the control group. **(B)** Mesenteric region of the BCNU group. **(C)** Mesenteric polyp of the BCNU/LDE group. **(D)** Mesenteric polyp of the LDE group. Apoptotic cells are indicated by an arrowhead in all of the panels. Bars = 20 μm.

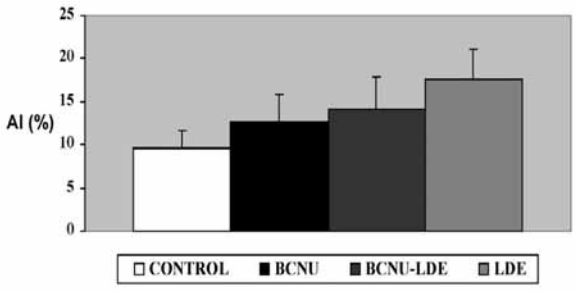

Figure 6. Apoptotic indices in mesentery and mesenteric polyps based on the TUNEL reaction. There was a significant difference in the indices between the LDE and control groups (p=0.038).

mice was greater than that of the controls ($p<0.05$) but did not differ from that of the other treated groups. These results differed from those obtained by TEM for the comparison between BCNU/LDE and the control group. Comparison of the results obtained with these two methods using a *t*-test yielded p=0.1148. There was little improvement in the level of significance (p=0.0891) when the data were log-transformed prior to analysis. This observation suggests that the significance level of p=0.05 was not reached because of two mutually exclusive factors, i.e., the scatter seen in the TUNEL assay results and the relatively small sample size (n=4).

Regardless of the method of analysis (TUNEL reaction or TEM), there was no additive effect of BCNU on apoptosis when incorporated in LDE, even though the BCNU/LDE group had a greater apoptotic index. Nevertheless, LDE alone enhanced the incidence of apoptosis in macrophages and lymphocytes or plasma cells, and this finding could be useful for the treatment of multiple myelomas in humans.

Although the methods used here did not permit identification of all of the cell types that underwent apoptosis, TEM showed that apoptotis occurred mainly in plasma cells. Nevertheless, we cannot exclude the involvement of some non-plasma cell lineages in advanced stages of apoptosis in which there was extremely condensed chromatin and a cytoplasm devoid of rough endoplasmic reticulum cisternae.

In the TUNEL reaction and TEM, apoptosis is assessed by examining the nuclear material of apoptotic cells. The higher incidence of apoptosis detected with the TUNEL reaction compared to TEM was most likely related to the greater thickness of the sections used for light microscopy (2 μm) than for TEM (0.05-0.07 μm) (thicker sections allow the detection and analysis of more nuclear material). Although the TUNEL reaction allows the rapid identification and quantification of apoptotic cells [3], the technique may give false positive results, even in necrotic cells [37]**.** As suggested by Louagie *et al*. [20], investigations of apoptosis should involve an initial evaluation by TEM. Examination of our preparations by TEM revealed no morphological alterations indicative of necrosis or autophagic programmed cell death [7]**.**

The TUNEL reaction showed that the frequency of apoptosis in the LDE group was greater than in the control, whereas that of the BCNU and BCNU/LDE groups did not differ from the control group. The plasmacytoma of mice consists of undifferentiated B-lymphocytes that are theoretically sensitive to treatment with BCNU, but our results revealed not such sensitivity. On the other hand, in LDE-treated mice the enhanced apoptosis involved cells that form the plasmacytoma, i.e., lymphocytes, plasma cells and macrophages [27]. The level of apoptosis seen in BCNU/LDE mice was similar to that of LDE-treated mice.

TEM allowed analysis of the frequency of apoptosis and of the cell types involved, although the latter was only possible when we examined the initial phase of apoptosis. In LDE-treated mice, the main apoptotic cells were plasma cells and macrophages, all of which were in various stages of apoptosis. There was no increase in the frequency of apoptotic macrophages in the groups that were not treated with LDE. In agreement with this, there are no indications in the literature that BCNU can affect macrophages. Our results agree with the effectiveness reported for BCNU/LDE in humans during therapy for multiple myelomas [22].

LDE may cause apoptosis directly or after oxidation (Maranhão, personal communication, 2001). Potter stated that the stimulation of lymphoid cells and macrophages and the production of IL-6 are fundamental for tumor formation, and that agents cytotoxic to these two types of cells or that prevented IL-6 formation provided effective therapy [2,9].

TEM showed that LDE affected plasma cells, including macrophages and lymphocytes. However, it remains unclear whether when LDE is in combination with BCNU leads to apoptosis in macrophages and lymphocytes, and whether this combination can be used to destroy these cells. A similar phenomenon has been reported for oxy-steroids in human leukemic cells *in vitro* [12] and for other steroids in human myeloma cells [4,17,24,32].

Various types of steroids are effective in preventing and treating tumors, and also affect the immunological system, particularly natural killer cells that are involved in the defense against tumors [1,12]. In agreement with this, the results of the present study show that although LDE is a potentially useful drug carrier, it is not a neutral substance. This aspect needs to borne in mind during therapeutic trials since many cells have LDL receptors and may be able to interact with LDE.

ACKNOWLEDGMENTS

We thank the technicians of the Histology Laboratory (Department of Pathology, Medical School, University of São Paulo - FMUSP) and Dr. Consuelo Junqueira Rodrigues for help with the histological analysis. We also thank Dr. Clovis K. Takiguti and Ivaldo Olímpio da Silva (Department of Preventive Medicine, FMUSP) for their collaboration during the development of this work. The support of CNPq (Projeto Integrado 520359/96-8) and of FAPESP (Proc. 00/06648-2) are acknowledged.

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Received: October 26, 2005
Accepted: January 26, 2006