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Specific Inhibition of Lymphocyte Proliferation and Induction of Apoptosis by CLL-I, a β-Galactoside-Binding Lectin[1]

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β-Galactoside-binding lectins or galectins are a family of closely related carbohydrate-binding proteins which functions still remain to be elucidated. Several evidence suggest they could play a role in different biological processes, such as cell growth regulation and immunomodulation. In the present study we report that affinity-purified CLL-I (chicken lactose lectin-I), an acidic 16-kDa galectin exhibits specific growth regulatory properties. Con A-stimulated rat spleen mononuclear cells showed a marked dose-dependent growth inhibition upon incubation with the galectin protein. Cell growth arrest was highly prevented by galectin-specific sugars. In addition, biochemical, cytofluorometrical, and morphological evidence are also provided to show that these inhibitory properties are related to a positive control in the apoptotic threshold of spleen mononuclear cells. Flow cytometric analysis showed a dose- and time-dependent increase of cells with hypodiploid DNA content upon exposure to CLL-I. Moreover, cells treated with CLL-I displayed the typical ultrastructural changes compatible with apoptosis, mainly chromatin condensation and margination along the inner surface of the nuclear envelope. Finally, the highly characteristic "ladder" pattern of DNA fragmentation into oligonucleosome-length fragments of ~180-200 bp could be found within 6 h of cell culture with CLL-I, mainly in the T cell-enriched population. Induction of apoptosis by a β-galactoside-binding protein highlights a potentially novel mechanism for regulating the immune response and points to a rational basis for the postulated immunomodulatory properties of this protein family.

Key words: apoptosis, cellular proliferation, CLL-I, galectins, immunomodulators.

β-Galactoside-binding lectins or galectins constitute a family of closely related carbohydrate-binding proteins widely distributed in the animal kingdom (*1-3*). They show highly conserved primary amino acid sequences, in addition to carbohydrate binding specificity (*4, 5*). Despite the growing progress evidenced in the last few years in the identification, cloning, and sequencing of new galectins (*5*), their functions still remain to be elucidated. They have been involved in different biological processes such as cell adhesion (*6*), cell growth regulation (*7-9*), metastasis (*10*), and immunomodulation (*11, 12*).

In multicellular organisms, homeostasis is maintained through a balance between cell proliferation and cell death (*13, 14*). Cell proliferation is a highly regulated process with numerous checks and balances. While growth factors and proto-oncogenes are positive regulators of the cell cycle, tumor suppressor genes act to oppose uncontrolled proliferation by preventing cell cycle progression (*13, 15*). Physiologic cell death takes place primarily through an evolutionary conserved form of cell suicide termed apoptosis (*16, 17*). The decision of a cell to undergo apoptosis can be influenced by a wide variety of extrinsic and intrinsic regulatory stimuli (*18*). This type of regulation allows the elimination of cells that have been produced in excess, have developed improperly, or have sustained genetic damage (*16, 18*). Although diverse signals can induce apoptosis in a wide variety of cell types, a number of evolutionary conserved genes regulate a final common cell death pathway that is preserved from worms to humans (*19, 20*).

Concerning the immune system, a self destructive process such as apoptosis must be under tight control to avoid unwanted effects, such as potentially autoreactive lymphocytes and excess cells after the completion of an immune response (*21, 22*). Studies performed in animal models have clearly shown the importance of dysregulated apoptosis in the etiology of autoimmune diseases (*23, 24*). Galectin-1, a β-galactoside binding protein expressed by stromal cells in human thymus (*25*) and lymph nodes (*26*) showed prophylactic and therapeutic activity against two

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Abbreviations: CLL-I, chicken lactose lectin-I; Con A, concanavaline A; CRD, carbohydrate recognition domain; IL-2R, interleukin-2 receptor; LPS, lipopolysaccharide; mAb, monoclonal antibody; PI, propidium iodide; SpMs, spleen mononuclear cells.

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experimental autoimmune diseases (*11, 12*). These immunomodulatory properties could result from the deletion of autoreactive lymphocytes, and recent findings suggest that endogenous mammalian galectins could regulate the immune response by engaging the apoptotic program of activated T cells (*27*). Supporting this hypothesis, an interesting study reports the overexpression of the human galectin-1 gene during glucocorticoid-induced cell death (*28*).

Two important chicken galectins have been described: C-16 or CLL-I and C-14 or CLL-II. They have first been investigated by Beyer *et al.* (*29*), and their further characterization, cloning, and sequencing has been reported by Sakakura *et al.* (*30*) and Ohyama *et al.* (*31*), respectively. They show 50-60% amino acid sequence homology with mammalian galectins. In the present study we show that chicken liver C-16 or CLL-I, shows specific lymphocyte growth inhibitory properties. In addition, we provide definitive cytofluorometrical, morphological, and biochemical evidence that these properties are related to a positive control in the apoptotic threshold of rat spleen mononuclear cells, mainly the T cell population.

EXPERIMENTAL PROCEDURES

Animals—Eight- to 12-week-old female Wistar rats (average weight 250 g) were used in this study. Animals were housed and cared for at the Animal Resource Facilities, Departamento de Bioquímica Clínica, Universidad Nacional de Córdoba, in accordance with institutional guidelines.

Galectin Purification and Anti-Galectin Serum Preparation—CLL-I was purified from adult chicken liver by affinity chromatography on a lactosyl-Sepharose matrix as previously described (*29, 32*). Lectin activity was tested by an hemagglutination assay following the procedure described by Nowak *et al.* (*33*), using trypsin-treated glutaraldehyde-fixed rabbit erythrocytes. An anti-lectin serum was obtained in rabbits and used for immunochemical and immunocytochemical studies (*32, 34, 35*).

SDS-PAGE and Western Blot—SDS-PAGE was performed in a Miniprotean II electrophoresis apparatus (Bio Rad, Richmond, CA) as described by Laemmli (*36*). Briefly, crude soluble extracts and purified CLL-I were diluted in 62 mM Tris, pH 6.8, containing 2% (w/v) SDS, 5% (w/v) 2-ME, and 10% (v/v) glycerol, heated for 5 min at 90°C and resolved in a 12.5% separating polyacrylamide slab gel. Protein bands were detected using Coomassie Brilliant Blue staining. After electrophoresis, the separated proteins were transferred onto nitrocellulose membranes (Bio Rad) and probed with a 1:150 dilution of the anti-galectin serum as previously described (*35*). Blots were incubated with 1 μg/ml horseradish peroxidase-conjugated protein A (Sigma Chemical) and developed with 4-chloro-1-naphthol (Sigma Chemical). Control of specific immunoreaction was performed by incubation of the blots with a rabbit preimmune serum.

Cells—Spleen mononuclear cells (SpMs) were obtained from normal rats. Spleens were removed and collected in sterile conditions using RPMI 1640 medium (Sigma Chemical). Spleen cells were prepared by gentle teasing, debris was then separated and the single cell suspension was collected. Next, SpMs were obtained by Ficoll-Hypaque gradient centrifugation, washed and resuspended in culture medium. Cell viability assessed by means of the trypan blue exclusion test was consistently greater than 95%. The T cell-enriched population was obtained by collecting non adherent cells from SpMs through a nylon wool column (UNISORB, Eldan Tech., Israel), and the B cell-enriched population was recovered from the adherent cell fraction.

Cell Proliferation Assay—To examine cell growth inhibitory activity, cells were cultured in 96-well microtiter plates (Corning, NY), at 5×10^5 cells/well in 200 μl of complete medium: RPMI 1640 plus 10 mM HEPES (Sigma Chemical), 2 mM L-glutamine (Gibco Lab), 50 μM 2-ME, and 100 μg/ml gentamicin, supplemented with 10% heat-inactivated FCS (Gibco Lab), in the absence or in the presence of Con A (2.5 or 5 μg/ml) (Sigma Chemical) (Con A-SpMs). Simultaneously, CLL-I was added to the cultures at concentrations ranging from 0.5 to 8 μg/ml (30-500 nM) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. In another set of experiments, galectin-specific sugars such as lactose and thiodigalactoside, and non-specific sugars such as glucose and galactose were added at different concentrations (5, 10, 30, and 100 mM) to the culture medium in order to test whether growth inhibitory effect of CLL-I is related to lectin properties. After 72 h, the cultures were pulsed with 1 μCi/well [methyl-^{3}H]-thymidine ([^{3}H]TdR sp. act.: 20.00 Ci/mmol) for an additional 18 h. Cells were then harvested and [^{3}H]TdR incorporation was monitored by using a liquid scintillation counter. Results are expressed as mean cpm $\pm$ SD of triplicate determinations from a representative out of seven independent experiments. To rule out a possible toxic effect of CLL-I, cell viability was tested by means of the trypan blue exclusion test, after incubation with CLL-I at different concentrations for different culture periods.

Flow Cytometric Analysis—Nuclear DNA content was analyzed by flow cytometry as described by Nicoletti *et al.* (*37*). Con A-SpMs were cultured in 24-well plates (Corning, NY) in complete medium at a density of 2×10^6 cells per well in the absence or in the presence of CLL-I (2, 4, and 8 μg/ml). After different periods of incubation (3, 6, and 18 h), cells were recovered and subsequently washed: once with PBS containing 300 mM lactose to remove excess of CLL-I and twice, with cold PBS alone. In another set of experiments, SpMs were first stimulated with Con A (5 μg/ml) for 48 h and the 48-h blasts were further incubated with CLL-I for different periods (30 min, 1, 3, and 6 h). Activation was confirmed by staining the cells with a mAb raised against IL-2 receptor α (IL-2Rα) (OX-39, Serotec LTD, England), followed by incubation with an anti-mouse FITC-labeled IgG (Sigma Chemical). Then cells were processed for apoptotic cell detection.

Briefly, cell pellets were gently resuspended in 1 ml hypotonic fluorochrome solution: 50 μg/ml propidium iodide (PI) (Sigma Chemical), diluted in 0.1% sodium citrate plus 0.1% Triton X-100, in 12×75 polystyrene tubes and kept at 4°C for more than 3 h in the dark. The PI fluorescence emission of individual nuclei was filtered through a 585/42 nm band pass filter and measured on a logarithmic scale by a FACScan® cytometer (Becton Dickinson & Co., Mountain View, CA). Cell debris was excluded from analysis by appropriately gating on physical parameters. The number of apoptotic cells was determined by evaluating the percentage of hypodiploid nuclei in the

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<2 N DNA peak and were distinguished from necrotic cells by analyzing the light scatter profile (*38*).

Transmission Electron Microscopy—Ultrastructural features of apoptosis were studied in SpMs and Con A-SpMs recovered from cultures performed in the absence or in the presence of CLL-I (4 μg/ml). After 6 h of incubation, medium was removed and cells were fixed by immersion in 1% glutaraldehyde diluted in 0.1 M cacodylate buffer (pH 7.3). Samples were post-fixed in 1% OsO_4, dehydrated and embedded in Araldite. Thin sections were cut in a Porter-Blum MT2 ultramicrotome and examined in a Zeiss 109 electron microscope. Photographs were taken on a Kodak electron imaging film.

DNA Extraction and Analysis of DNA Fragmentation—To analyze DNA fragmentation, non stimulated and mitogenically-stimulated (Con A 2.5 or 5 μg/ml and LPS 40 μg/ml) total SpMs or cell-enriched populations were cultured in 24-well plates (Corning, NY) at a density of 2×10^6 cells per well in the absence or in the presence of increasing concentrations of purified CLL-I (2, 4, and 8 μg/ml) for the indicated periods (3, 6, and 18 h). Cells were then harvested, washed with cold TNE buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA pH 8) and lysed by addition of 0.5% SDS. Then, cell lysates were incubated at 56°C for 3 h in the presence of 100 μg/ml proteinase K (Sigma Chemical). After digestion, DNA was purified by successive phenol-chloroform extractions and the resultant aqueous phase was mixed with 3 M sodium acetate (pH 5.2) and absolute ethanol. The mixture was incubated at −20°C overnight, and the ethanol-precipitated DNA was washed with 70% (v/v) ethanol. The purified DNA was resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA pH 7.5), and treated with 5 μl of 1 mg/ml DNase-free RNase A (Sigma Chemical) for 1 h. Samples were finally resuspended in loading buffer containing 25% Ficoll 400, 0.25% xylene cyanol, and 0.25% bromophenol blue and resolved on a 1.8% agarose gel containing 0.5 μg/ml ethidium bromide. Electrophoresis was carried out in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA pH 8.4) and DNA visualization was accomplished under UV light.

Statistical Analysis—The mean and standard deviation (SD) were calculated. Comparisons were made among groups and significant differences were determined by one way analysis of variance (ANOVA) and Bonferroni comparisons test. p values less than 0.05 were considered to be statistically significant.

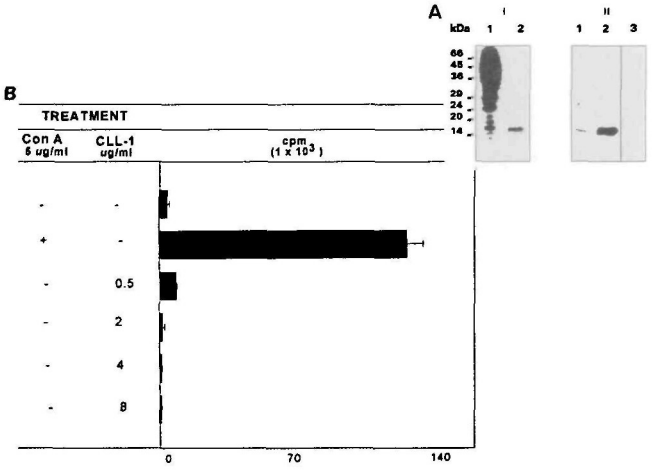

Fig. 1. **Effect of affinity-purified CLL-I on non-stimulated spleen mononuclear cells (SpMs).** A: SDS-PAGE and Western blot analysis showing the electrophoretic and immunoreactive profiles of affinity-purified CLL-I. (I) Protein bands were detected by Coomassie Brilliant Blue staining. (II) Western blot analysis was performed following transference of the electrophoretically separated proteins to nitrocellulose membranes and blots were probed with the rabbit anti-galectin serum (1:150). Lane 1: crude soluble extracts from adult chicken liver (100 μg), lane 2: chicken lactose lectin-I (CLL-I) purified by affinity chromatography on a lactosyl-Sepharose matrix (8 μg), lane 3: affinity-purified CLL-I probed with the same dilution of a preimmune serum as a control of specific immunoreaction. The relative migration of molecular size markers is indicated on the left. B: Spleen mononuclear cells (5×10^5 cells/well) were cultured in 96-well microtiter plates in the presence of increasing concentrations of affinity-purified CLL-I, ranging from 0.5 to 8 μg/ml. Controls included SpMs cultured in medium alone and cells stimulated with Con A (5 μg/ml) in the absence of CLL-I. After 72 h, [^{3}H]TdR was added and uptake was determined for the final 18 h. Data are expressed as mean cpm±SD of triplicate determinations from a representative out of seven independent experiments. $p_{2\mu g/ml}<0.05$ and $p_{4-8\mu g/ml}<0.001$ *versus* non-stimulated SpMs.

RESULTS

Cell Growth Inhibition Induced by Affinity-Purified CLL-I, a β-Galactoside Binding Lectin—To examine whether CLL-I was able to regulate cell growth *in vitro* proliferation, Con A stimulated (2.5 or 5 μg/ml) and non-stimulated rat SpMs were cultured for 72 h in the absence or in the presence of increasing concentrations (0.5, 2, 4, and 8 μg/ml) of affinity-purified CLL-I and monitored by [^{3}H]TdR incorporation.

The purity of CLL-I preparation was analyzed after sequential purification steps (*32*) by SDS-PAGE and Western blot assay (Fig. 1A). The final preparation gave rise to a broad protein band of 16 kDa that strongly

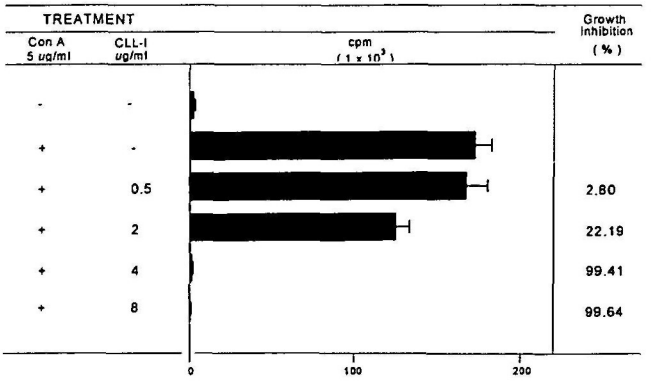

Fig. 2. **CLL-I-induced cell growth inhibition on Con A-stimulated SpMs.** Spleen mononuclear cells (5×10^5 cells/well) were cultured in 96-well microtiter plates in the presence of Con A (5 μg/ml) and increasing concentrations of the affinity-purified CLL-I, ranging from 0.5 to 8 μg/ml. Controls included SpMs cultured in medium alone and cells stimulated with Con A (5 μg/ml) in the absence of CLL-I. After 72 h, [^{3}H]TdR was added and uptake was determined for the final 18 h. Data are expressed as mean cpm±SD of triplicate determinations from a representative out of seven independent experiments. Percentages of growth inhibition were determined in comparison to Con A-treated cells. $p_{2\mu g/ml}<0.05$ and $p_{4-8\mu g/ml}<0.001$ *versus* Con A-SpMs.

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immunoreacted with the anti-galectin serum. Lectin activity associated with this protein band was confirmed by assaying its hemagglutinating properties on rabbit treated erythrocytes (data not shown).

As it is shown in Fig. 1B, when purified CLL-I was added to non-stimulated resting SpMs cultures, we could not detect mitogenic activity at any of the concentrations tested (0.5, 2, 4, and 8 μg/ml). In contrast, [^{3}H]TdR incorporation was partially reduced compared to controls incubated in culture medium alone.

Then, when SpMs were stimulated with 5 μg/ml Con A (Con A-SpMs) and simultaneously incubated with CLL-I, a significant dose-dependent suppression in cell growth was observed, as it is clearly shown by reduced [^{3}H]TdR incorporation in Fig. 2. Dose-response relationship revealed, that cell growth was almost totally inhibited when CLL-I was added at concentrations of 4 and 8 μg/ml (99.41 and 99.64%, respectively) ($p<0.001$). However, when Con A-SpMs were cultured for the same period with a lower concentration of CLL-I (2 μg/ml), cell growth was only partially reduced (22.19%, $p<0.05$), and no significant cell growth inhibition was observed when CLL-I was added at 0.5 μg/ml (2.80%, p=NS). Similar results were obtained when SpMs were stimulated with Con A at a concentration of 2.5 μg/ml. Preserved viability, tested by the exclusion of vital dyes from cells incubated with increasing concentrations of CLL-I, suggested that it did not exert a direct toxic effect on SpMs (data not shown). These results indicate that affinity-purified CLL-I induces a dose-dependent inhibition in Con A-SpMs proliferation, and the critical inhibitory threshold ranges between 2 and 4 μg/ml.

Prevention of CLL-I-Induced Cell Growth Inhibition by Galectin-Specific Sugars—Since the carbohydrate recognition domain (CRD) of β-galactoside-binding lectins share properties among different species beyond similarities on their primary and secondary structures (*2, 5*), we attempted to investigate whether CRD is involved in CLL-I-induced cell growth inhibition.

To answer this question, different specific and non-specific sugars were added at different concentrations (5, 10, 30, and 100 mM) to the culture medium of Con A-SpMs (5 μg/ml) prior to incubation with purified CLL-I (4 and 8 μg/ml) and the proliferative response was monitored by [^{3}H]TdR incorporation. Percentages of growth inhibition are shown in Table I. Results indicate that galectin-specific β-D galactoside sugars such as lactose and thiodigalactoside

TABLE I. **Prevention of CLL-I-induced cell growth inhibition by galectin-specific sugars.** Con A-stimulated SpMs (4×10^5 cells/well) were cultured in 96-well microtiter plates with CLL-I (4 μg/ml) in the presence of galectin-specific and non-specific sugars at concentration ranging from 5 to 100 mM. After 72 h [^{3}H]TdR was added and the uptake was determined for the final 18 h. Data are expressed as percentage of growth inhibition compared to Con A-SpMs cultured in the absence of CLL-I.

Treatment		Growth inhibition (%)				
CLL-I	Sugar concentration	None	Lactose	Thiodigalactoside	Galactose	Glucose
+		97.7	—	—	—	—
+	5 mM		98.6	97.1	Nd	Nd
+	10 mM		98.7	96.4	Nd	Nd
+	30 mM		86.7	63.2	98.0	98.5
+	100 mM		67.5	37.6	97.5	96.4

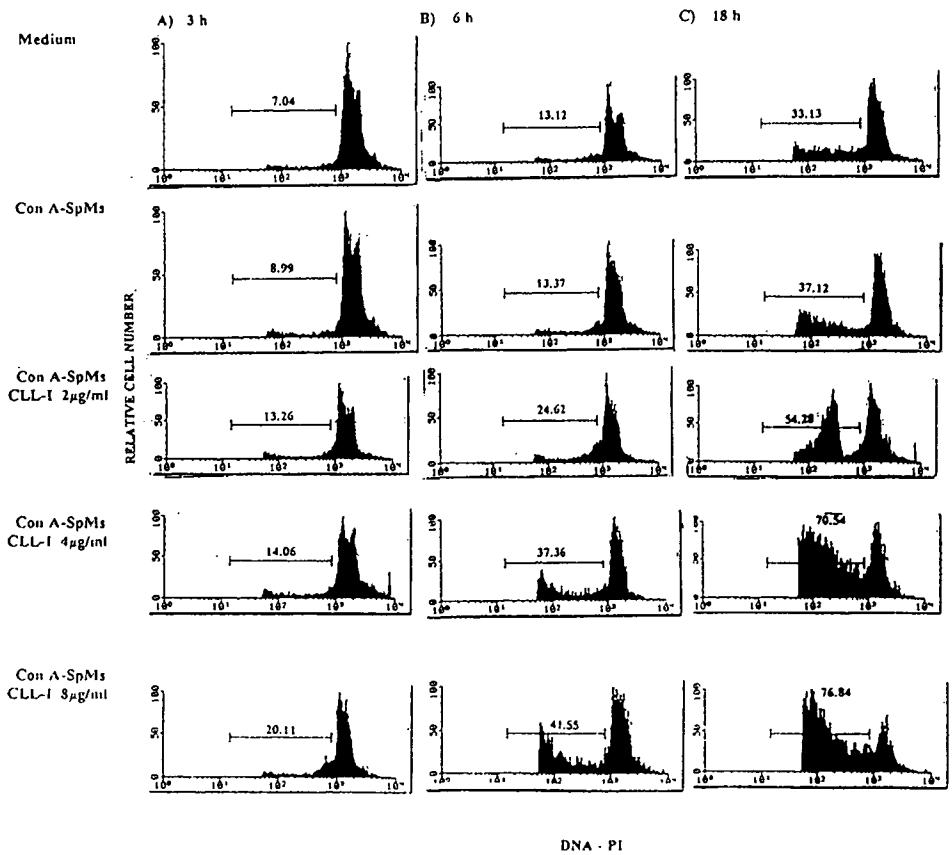

Fig. 3. **Flow cytometric analysis of DNA content of CLL-I-treated Con A stimulated SpMs.** Propidium iodide staining of SpMs cultured in 24-well microtiter plates at a density of 2×10^6 cells/well in medium alone (medium), in medium containing Con A (5 μg/ml) (Con A-SpMs) or in the presence of 5 μg/ml Con A plus CLL-I (2, 4, and 8 μg/ml) for 3 h (A), 6 h (B), and 18 h (C). After the indicated periods, cells were resuspended, washed, stained with the hypotonic fluorochrome solution, and analyzed on a Becton & Dickinson FACScan® cytometer. A representative time course kinetics of the appearance of apoptotic cells is shown, indicating the percentages of cells with hypodiploid DNA content. *x* axis: PI-uptake (FL-3) log scale; and *y* axis: relative cell number.

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were able to partially prevent CLL-I–induced growth inhibition at concentrations up to 30 mM, whereas concentrations below this threshold did no longer prevent this effect. In contrast, non specific sugars were not effective to prevent cell growth inhibition at any of the concentrations tested. In addition, controls performed with stimulated and non-stimulated cells cultured with the different sugars in the absence of CLL-I, did not show any change in their proliferative response (data not shown). The results presented herein suggest that cell growth regulatory functions of CLL-I are at least partially related to its carbohydrate-binding properties.

Involvement of Apoptosis in Galectin-Induced Cell Growth Inhibition. Flow Cytometric Analysis of DNA Content—In search for a molecular mechanism responsible for CLL-I–induced cell growth inhibition, DNA content was analyzed by flow cytometric analysis after staining the cells with the DNA-intercalating dye PI. Con A-SpMs cultured in the presence or in the absence of increasing concentrations of CLL-I (2, 4, and 8 μg/ml) for the indicated periods (3, 6, and 18 h), exhibited the DNA content profiles shown in Fig. 3. The kinetic analysis revealed that the proportion of cells with hypodiploid DNA content (shift to the left of G_1 peak) characteristic of apoptosis, increased with dependence of time and galectin concentration.

As shown in Fig. 3A, cells cultured for 3 h with 2, 4, and 8 μg/ml of CLL-I, revealed a slight increase in hypodiploid DNA content (13.26, 14.06, and 20.11%, respectively), in comparison with SpMs cultured in medium alone (7.04%) and Con A-SpMs (8.99%). However, when cells were incubated for 6 h with CLL-I at 2, 4, and 8 μg/ml, the proportion of cells with reduced DNA content increased in a dose-dependent fashion (24.62, 37.36, and 41.55%, respectively). Controls exhibited major peaks representing diploid cells and a minor percentage of cells undergoing apoptosis (13.12% for non-stimulated SpMs and 13.37% for Con A-SpMs) (Fig. 3B). Moreover, as it is shown in Fig. 3C, the proportion of apoptotic cells was greatly enhanced after exposure to CLL-I for 18 h at 2, 4, and 8 μg/ml (54.28, 70.54, and 76.84%, respectively), compared to non-stimulated SpMs (33.13%) and Con A-SpMs (37.12%). Thus, this kinetic analysis indicates that an apoptotic

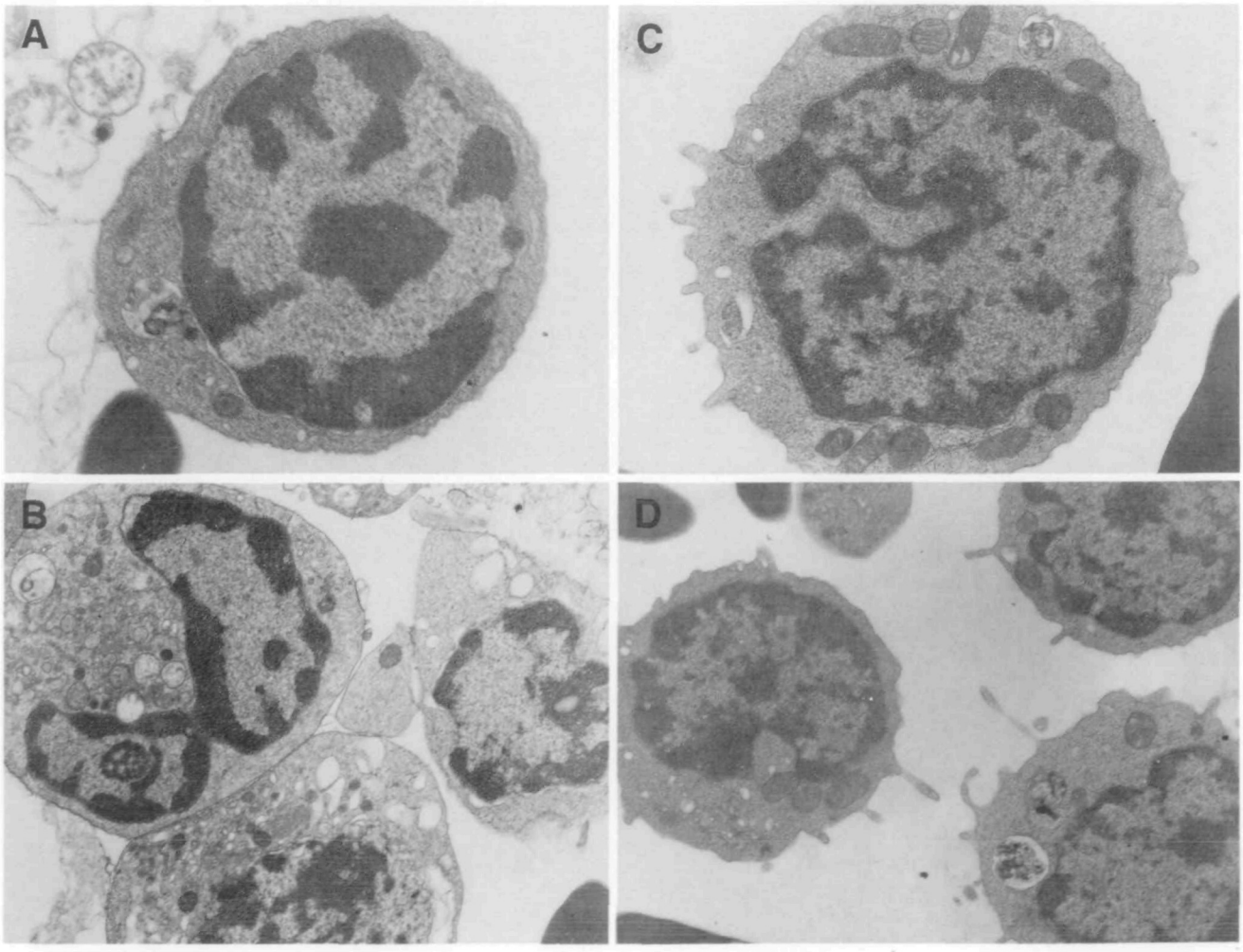

Fig. 4. **Transmission electron microscopical examination of ultrastructural changes induced by affinity-purified CLL-I.** Spleen mononuclear cells were cultured in 24-well microtiter plates, at a density of 2×10^6 cells/well for 6 h in medium containing 5 μg/ml Con A plus 4 μg/ml CLL-I (panels A and B) and in medium alone (panels C and D). After 6 h, cells were harvested, washed and processed for transmission electron microscopical analysis. Cells treated with the galectin displayed the typical ultrastructural features of apoptosis. Magnifications: panels A and C: ×19,000; and panels B and D: ×12,000.

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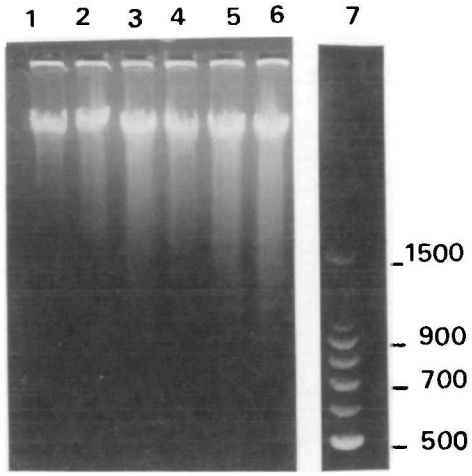

Fig. 5. **Electrophoretic analysis of internucleosomal DNA fragmentation of total SpMs induced by affinity-purified CLL-I.** Spleen mononuclear cells were cultured in 24-well microtiter plates at a density of 2×10^6 cells/well for 6 h, in medium alone (lane 1), in medium containing Con A 2.5 and 5 μg/ml (lanes 2 and 3, respectively), and in medium containing 2.5 μg/ml Con A plus the addition of CLL-I at increasing concentrations: 2, 4, and 8 μg/ml (lanes 4, 5, and 6, respectively). Cells were then harvested, and genomic DNA was extracted as described in "MATERIALS AND METHODS." Samples were diluted in loading buffer and resolved on a 1.8% agarose gel. The relative mobility of oligonucleosome-length DNA fragments of CLL-I-treated cells reflects integer multiples of 180-200 bp respect to controls. Molecular size markers (100 bp DNA ladder) are shown in lane 7.

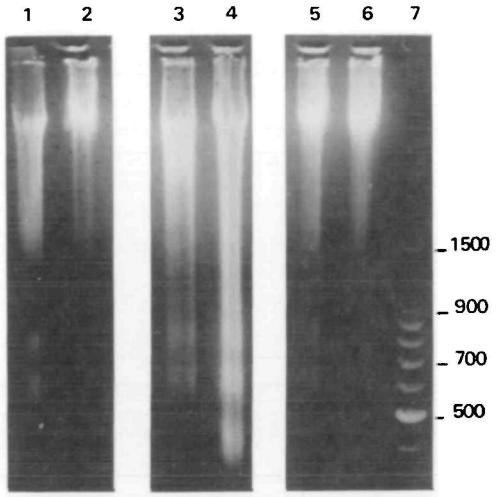

Fig. 6. **Electrophoretic analysis of internucleosomal DNA fragmentation of T and B cell-enriched populations induced by affinity-purified CLL-I.** T or B cell-enriched populations were cultured in 24-well plates at a density of 2×10^6 cells/well for 6 h in medium alone (lanes 1 and 2, respectively), in medium containing Con A (2.5 μg/ml) or LPS (40 μg/ml) in the presence of CLL-I (4 μg/ml) (lanes 4 and 6, respectively), and in medium containing CLL-I alone (4 μg/ml) in the absence of the mitogenic stimuli (lanes 3 and 5, respectively). Cells were then harvested, and genomic DNA was extracted as described in "MATERIALS AND METHODS." Samples were diluted in loading buffer and resolved on a 1.8% agarose gel. The relative mobility of oligonucleosome-length DNA fragments of CLL-I-treated cells reflects integer multiples of 180-200 bp respect to controls. Molecular size markers (100 bp DNA ladder) are shown in lane 7.

mechanism of cell death is clearly involved in cell growth inhibition induced by CLL-I.

To investigate whether the apoptotic effect of purified CLL-I depended upon the activation state of the cells, similar experiments were performed on cultures of 48-h Con A-stimulated SpMs. Activation of the cells was confirmed by measuring IL-2Rα expression (44% for Con A-stimulated compared to 7% for non-stimulated SpMs). CLL-I was added to the 48-h blasts at the optimal concentration tested (4 μg/ml) and DNA content was analyzed from cells recovered after incubation with the galectin for the same time periods. The extent of cells with hypodiploid DNA content was not markedly enhanced, compared to cells in which CLL-I was simultaneously incubated with the mitogenic stimulus at the initiation of the cell culture (result not shown).

Ultrastructural Study of Morphological Changes Induced by CLL-I—We examined whether cells incubated with CLL-I displayed the typical ultrastructural changes compatible with apoptosis. Con A-SpMs were cultured for different periods at increasing concentrations of this protein and the cells were processed for transmission electron microscopy. Representative electron micrographs of cells exposed to CLL-I (4 μg/ml) for 6 h are shown in Fig. 4 (panels A and B), in comparison to untreated non-stimulated SpMs (Fig. 4, panels C and D). CLL-I-treated cells showed a significant reduction of their cytoplasmic volume with random assorted but relatively well-maintained organelles, accompanied with condensation and margination of the chromatin along the inner surface of the nuclear envelope, no apparent disruption of the plasma membrane and loss of surface microvilli. In contrast, untreated non-stimulated SpMs exhibited a large nucleus containing regular quantity of diffuse heterochromatin, and as expected, untreated mitogenically stimulated SpMs showed typical morphological hallmark of cells with activated protein synthesis machinery (data not shown). Thus, in broad agreement with biochemical and cytofluorometrical evidence presented in this study, CLL-I-induced cell death occurred with the characteristic ultrastructural features of apoptosis.

Analysis of Genomic DNA Fragmentation Induced by CLL-I—To examine whether the reduction in cellular DNA was associated with internucleosomal breakdown, DNA fragmentation assays were performed in mitogenically stimulated and non-stimulated SpMs cultured in the presence of increasing concentrations of CLL-I. The electrophoretic pattern of DNA extracted from total SpMs cultured for 6 h in the presence of the galectin is shown in Fig. 5. The highly characteristic "ladder" pattern of DNA cleavage into oligonucleosome-sized fragments of ~180-200 bp was intensified in SpMs that were simultaneously stimulated with Con A and exposed to CLL-I at 4 and 8 μg/ml (lanes 5 and 6, respectively). In contrast, ladder-type DNA fragmentation was not observed in cells cultured with medium alone (lane 1). Although it was not extremely evident in our system, it should be noted that cell activation with mitogenic stimuli induces by itself a discrete apoptotic effect in addition to the major stimulatory consequences. In this context, cells stimulated with Con A (2.5 and 5 μg/ml) but untreated with CLL-I exhibited a "very low" pattern of fragmentation (lanes 2 and 3, respectively). In addition,

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CLL-I added to Con A-SpMs at a lower concentration of the inhibitory threshold (2 μg/ml) did not show significant increase of DNA fragmentation (lane 4) in this qualitative assay.

Finally, in order to confirm which target cell was predominantly affected by CLL-I, T and B cell-enriched populations were purified, cultured for 6 h in the presence or absence of the mitogenic stimuli (Con A or LPS) with the optimal concentration of CLL-I (4 μg/ml) and analyzed for DNA fragmentation (Fig. 6). As it is clearly shown, CLL-I induced DNA fragmentation on the Con A-stimulated T cell-enriched population (lane 4), but not on the LPS-stimulated B cell-enriched population (lane 6). In addition, upon incubation with CLL-I alone, fragmentation was observed at a lesser extent, only in the T cell-enriched fraction and was undetectable in genomic DNA extracted from the B cell-enriched population (lanes 3 and 5, respectively). Controls of T and B cell-enriched populations cultured in medium alone did not show fragmentation in this assay (lanes 1 and 2, respectively), and untreated Con A- and LPS-stimulated cells showed the typical slight fragmentation pattern induced by mitogenic signals (data not shown). Hence, endonuclease-mediate cleavage of internucleosomal DNA linker sections would strongly support a particular apoptotic mechanism of cell death (*39*).

DISCUSSION

Conservation of β-galactoside-binding lectins during evolution, their presence in several tissues of diverse species, and their developmental regulation, strongly imply that they could play a role in some relevant physiological processes requiring protein-carbohydrate interaction (*1, 3, 5*).

The rationale behind this study arises from a growing body of evidence suggesting that β-galactoside-binding lectins show specific immunomodulatory properties. Our main findings are: (a) CLL-I, an affinity purified β-galactoside-binding protein induces a dose-dependent growth inhibition in mitogenically stimulated SpMs, (b) the carbohydrate recognition domain appears to be involved in these inhibitory properties, (c) an early induction of the apoptotic program of the cell seems to be at least one of the molecular mechanisms underlying these growth regulatory properties, and (d) the target cell predominantly affected is the T cell-enriched population.

We found that CLL-I shows specific growth inhibitory properties on Con A-stimulated rat spleen cells. Supporting our findings, Wells and Mallucci (*8*) reported that murine β-galactoside-binding lectin functions as a negative growth regulator through an autocrine mechanism by preventing the exit of cells from G_0 and the subsequent progress through the cell cycle. These authors proposed that cell growth regulatory functions were not related to lectin properties, but were rather consistent with CRD independent mechanisms involving ligand-receptor interactions. In our system, however, β-galactoside related sugars were able to partially prevent the inhibitory effects of CLL-I, when added at concentrations up to 30 mM, providing evidence that CRD is involved in this regulatory function. This result is in agreement with a recent study reporting the use of lactose to revert T cell-growth modulatory properties exhibited by human galectin-1 (*27*). To clarify this issue, site-directed mutagenesis studies abolishing the β-galactoside-binding site should be performed.

The next issue we attempted to elucidate was related to the molecular mechanism underlying CLL-I-induced cell growth inhibition. In this paper we provide definitive cytofluorometrical, morphological, and biochemical evidence to conclude that these inhibitory properties are associated to a positive control in the apoptotic threshold of SpMs. Cytofluorometric analysis of DNA content revealed an increased appearance of cells with hypodiploid DNA content upon incubation with CLL-I. Strikingly, cells with a shift to the left of G_1 peaks appeared in a perfect dose and time-dependent fashion. Moreover, cells incubated with this protein displayed the typical ultrastructural changes compatible with the initiation of an apoptotic cell death program. In addition, SpMs incubated with increasing concentrations of CLL-I revealed the highly characteristic "ladder" pattern of DNA cleavage into oligonucleosome-sized fragments of 180–200 bp, mainly in the T cell population. It is now widely accepted that animal cells have the ability to self-destruct by activation of an intrinsic cell suicide program when they become seriously damaged or are no longer needed (*13, 18*). Not surprisingly, the initiation of apoptosis is carefully regulated. A bewildering diversity of extracellular and intracellular factors have been shown to influence the decision between life and death (*19*). These include lineage information, cellular damage inflicted by ionizing radiation or viral infection, extracellular survival factors, cell interactions, cytokines, oncogenes, and hormones. These diverse signals may act to either suppress or promote the activation of the death program, and the same signal may actually have opposing effects on different cell types (*40–42*). In this context, Lipsik *et al.*'s studies (*40*) performed in nude mice, suggest that chicken galactoside-binding lectins of the 14–16 kDa family have intrinsic mitogenic activity. Furthermore, a recent study performed by Adams *et al.* (*9*) suggests that recombinant galectin-1 has positive or negative growth regulatory properties depending on the experimental conditions.

However, despite considerable progress, it has proven difficult to identify the molecules responsible for mediating apoptosis by conventional biochemical and molecular approaches in mammalian systems. Interestingly, there is increasing evidence that apoptosis occurs by a mechanism that has been at least partially conserved throughout animal evolution (*19, 20*). Therefore, since β-galactoside-binding lectins are among the most highly conserved proteins (*3, 5*), even described in the nematode *Caenorhabditis elegans* (*43*) (one of the most primitive models of apoptosis), it will not be unreasonable to think that they could positively affect the timing with which any individual cell engages its apoptotic program. Supporting the previous assumptions and the present findings, an interesting report describes the overexpression of the human galectin-1 gene during the induction of apoptosis by glucocorticoids (*28*). Considering that these hormones play a crucial role in the induction of cell death and tolerance (*44*), it is possible for galectins to indeed function as linkers between extracellular stimuli and intracellular signaling (*45*), so as to positively regulate the apoptotic program of the cell.

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Interestingly, a recent work reports the expression by human thymic epithelial cells of the endogenous galectin-1, which binds to core 2 *O*-glycans on immature thymocytes (*25*). This striking finding supports the idea that galectin-1 could play a crucial role in the selection of developing thymocytes, a vital process in which apoptosis is extremely relevant (*46, 47*). In broad agreement with our findings, another recent study performed by Perillo *et al.* (*27*) convincingly reveals that human galectin-1 mediates apoptosis of activated T cells. On the other hand, Yang *et al.* (*48*) reported that galectin-3 is able to stimulate proliferation and prevent T cell death. This finding was supported by its sequence similarity to *Bcl-2*, a well-characterized suppressor of apoptosis.

The susceptibility of a given cell to undergo apoptosis after signaling with different exogenous factors seems to depend on at least two parameters: (i) the expression of an appropriate binding site, and (ii) the actual state of activation. Since specific sugars were able to partially prevent CLL-I-induced growth inhibition, it is possible that similar simple structures should be present during this process on the surface of lymphoid cells, as constituents of more complex glycoconjugates. Perillo *et al.* (*27*) concludes that galectin-1-induced apoptosis requires the expression of CD45, a polilactosamine-enriched glycoprotein present in a number of isoforms which are differently glycosylated, and suggestively a recent study highlights a novel role for CD45 in the regulation of lymphocyte death as well as proliferation (*49*). In this context, Schneller *et al.* (*50*) reported the differential binding of chicken galectins to lymphocyte receptors. Regarding the second parameter it seems likely that mitogenic stimulation triggers the initiation of the apoptotic program in a given cell population, producing an irreversible commitment to death upon exposure to CLL-I. Previously published data from *in vivo* and *in vitro* experiments suggest that resting T cells need to be activated before they become susceptible to apoptosis (*51, 52*). In our experiments, the most significant increase of apoptotic cells was detected when CLL-I was simultaneously incubated with the activation signal at the initiation of the cell culture, suggesting a potential effect of CLL-I on early events of activation-driven cell death.

In connection with the possibility that an endogenous galectin could be physiologically released from accessory cells and play a similar role as CLL-I, we have recently characterized and studied the regulation of the expression of a 16 kDa galectin in activated rat macrophages using a policlonal antibody raised against CLL-I (*35*). In a previous study, Kajikawa *et al.* (*53*) reported that the 14 kDa human placenta galectin induced the release of a cytotoxic factor from cultured mouse macrophage-like cell lines and human peripheral blood monocytes, that could eventually interact with lymphoid cells. In our experimental conditions, this possible *trans*-acting effect was ruled out, since purified T but not B cell fractions were selectively induced to undergo apoptosis by the addition of CLL-I in the absence of accessory cells.

Whenever an agent is proposed to cause apoptosis in such a cell type, the question that arises is whether this agent is interacting specifically with the regulation of the death pathway, or simply perturbing normal cellular physiology to an extent sufficient to trip the apoptosis failsafe mechanism (*21*). In our case, this question remains open.

To Conclude—which is the significance and contribution of our findings? At first sight, such filogenetically conserved proteins secreted by multiple variety of cell types could represent a gear of an alternative pathway in the generation of death signals. Second, the studies of Perillo *et al.* (*27*), Yang *et al.* (*48*), and our findings show clear cut evidence supporting potential explanatory mechanisms for the immunomodulatory effects of galectins in experimental autoimmune diseases such as EAMG (Experimental Autoimmune Myasthenia Gravis) (*11*) and EAE (Experimental Allergic Encephalomyelitis) (*12*). Moreover, these findings could also explain the mechanism by which human placenta galectin, may exert its postulated immunosuppressive effects, preventing maternal recognition of paternal antigens expressed on fetal tissues (*54*). Induction of apoptosis by a β-galactoside-binding lectin provides new insights into the mechanisms for regulating the immune response and points to a rational basis for the postulated immunomodulatory properties of this protein family, with its obvious therapeutic potential.

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