Experimental Cell Research **246,** 451–460 (1999)
Article ID excr.1998.4265, available online at http://www.idealibrary.com on IDEAL®

Multimeric α-Lactalbumin from Human Milk Induces Apoptosis through a Direct Effect on Cell Nuclei

Anders Håkansson,†,[1] Jesper Andréasson,† Boris Zhivotovsky,* Diana Karpman,†,‡ Sten Orrenius,* and Catharina Svanborg†

†*Section of Microbiology, Immunology and Glycobiology (MIG), Department of Laboratory Medicine, Lund University, Sölvegatan 23, SE-223 62 Lund, Sweden;* ‡*Department of Pediatrics, Lund University, 22185 Lund, Sweden; and* **Section of Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Box 210, SE-171 77 Stockholm, Sweden*

A fraction from human milk containing spf-multimer α-lactalbumin (MAL) induces apoptosis in tumor cells and immature cells but spares mature cells. The mechanism of apoptosis induction and the molecular basis for the difference in susceptibility between tumor cells and healthy cells have not been defined. In this study we examined the interaction of MAL with different cellular compartments, using confocal microscopy and subcellular fractionation. MAL was shown to accumulate in the nuclei of sensitive cells rather than in the cytosol, the vesicular fraction, or the ER–Golgi complex. Nuclear uptake occurred rapidly in cells that were susceptible to the apoptosis-inducing effect, but not in nuclei of resistant cells. Nuclear uptake was through the nuclear pore complex and was critical for the induction of DNA fragmentation, since inhibition of nuclear uptake with WGA rescued digitonin-permeabilized cells from induction of DNA fragmentation. Ca^{2+} was required for MAL-induced DNA fragmentation but nuclear uptake of MAL was independent of Ca^{2+}. This way MAL differs from most previously described agents in that it crosses the plasma membrane and cytosol, and enters cell nuclei where it induces DNA fragmentation through a direct effect at the nuclear level.

Key Words: **apoptosis; human milk; tumor; nuclear targeting; α-lactalbumin.**

INTRODUCTION

Apoptotic cell death is characterized by loss of cytoplasmic material, nuclear changes with marginalization of chromatin, and the formation of apoptotic bodies [1–3]. The reduction in cell viability is accompanied by stepwise chromatin fragmentation with initial formation of high-molecular-weight (HMW) DNA fragments (50–300 kbp) followed by oligonucleosome-length DNA fragments consisting of oligomers of approximately 200 bp [4–8]. Apoptosis inducing agonists often bind to specific cell surface receptors and activate transmembrane signaling events that trigger a cascade of cytoplasmic and nuclear changes. There are also agents that bypass this sequential activation and instead trigger the apoptosis cascade through targeting of molecules directly in the cytoplasm and/or at the nuclear level. One example is Granzyme B, a serine protease in the cytoplasmic granules of cytolytic T lymphocytes that enters perforin-exposed cells and activates the caspase cascade in the cytoplasm. Intracellular granzyme B is then translocated to the nuclei of target cells and directly cleaves nuclear substrates, such as DNA-PK, poly(ADP-ribose) polymerase, and NuMA, the cleavage of which is a well known event in apoptosis [9, 10].

The transport of macromolecules from the cytoplasm into the nucleus is highly regulated [11–13]. Nuclear pore complexes (NPCs) are the sites of exchange of macromolecules between the cytoplasm and nucleoplasm. The NPCs allow passive diffusion of molecules smaller than 20–40 kDa but larger proteins are delayed. Entry of large molecules or complexes into the nucleus requires active transport to be efficient and is commonly carrier-mediated. The specificity for the carrier may be determined by nuclear targeting or nuclear localization sequences (NLS) that characterize proteins with the ability to enter the nucleus.

We recently described a protein complex in human milk that induces apoptosis in tumor cells and immature cells, but spares other cells [14]. The active complex was initially isolated from casein by ion-exchange chromatography and was shown by N-terminal amino acid sequencing, mass spectrometry, and gel filtration to contain multimers of α-lactalbumin (MAL). The monomeric form of α-lactalbumin, that occurs in human milk whey, did not induce apoptosis. The molecular basis for this difference in activity between MAL and α-lactalbumin (ALA) has not been defined. Furthermore, the difference in sensitivity between tumor cells

[1] To whom correspondence and reprint requests should be addressed. Fax: +46-46-137468. E-mail: anders.hakansson@mmb.lu.se.

0014-4827/99 $30.00

and nonmalignant mature cells has not been explained at the molecular level.

Here we examined the cellular distribution of MAL in sensitive and resistant cells, and compared it to the inactive monomeric form of α-lactalbumin. The results suggested that MAL was transported into cell nuclei of cells that were susceptible to its apoptosis-inducing effect and suggests an alternative mechanism of apoptosis induction where an exogenous molecule that targets cell nuclei can induce the nuclear changes seen in apoptotic cells through a direct effect at the nuclear level.

MATERIALS AND METHODS

Reagents. FITC-conjugated swine anti-rabbit and rabbit anti-human antibodies and FITC-conjugated streptavidin were from Dakopatts a/s (Glostrup, Denmark). The anti-DNA antibodies were kindly provided by the Clinical Immunology Laboratory, Lund University Hospital, Lund, Sweden. DEAE-Trisacryl M was from BioSepra (Villeneuve la Garenne, France). The Biotin Labeling kit was from Boehringer Mannheim GmbH (Germany) and ^{125}I was from Amersham International plc (Amersham, England). Flowcheck and Flowset fluorospheres were from Coulter Inc. (Hialeah, FL). SeaKem GTG agarose and SeaPlaque GTG low-melting-temperature agarose were from SeaKem, FMK Bioproducts (Rockdale, USA). 2-Mercaptoethanol, Dulbeccoś modified Eagleś medium, fetal calf serum (FCS), gentamicin, L-glutamine, nonessential amino acids, penicillin/streptomycin solution, RPMI 1640, and sodium pyruvate were from Gibco/BRL, Life Technology Ltd. (Paisley, Scotland, UK). Dimethylsulfoxide, Hepes, Tween-20, and Tris(hydroxymethyl) aminomethane were from Kebo Lab (Stockholm, Sweden). Heparin, and PD-10 column were from Pharmacia Biotech (Stockholm, Sweden). α-Lactalbumin, antipain, bovine serum albumin, ethylenediaminetetraacetate (EDTA), ethylene glycol-bis(*b*-aminoethyl ether) *N,N,N′,N′*-tetraactetic acid (EGTA), lactoperoxidase, leupeptin, *N*-lauroylsarcosine, nifedipine, phenylmethylsulfonyl fluoride (PMSF), propidium iodide, proteinase K, Triton X-100, trypsin, and verapamil were from Sigma Chemicals Inc. (St. Louis, MO). BAPTA/AM was from Calbiochem (CA, USA). Digitonin and 1,4-ditheotreitol (DTT) were from ICN (CA, USA). Trypan blue was from Chroma Gesellschaft, Schmid & Co (Stuttgart, Germany).

Purification of MAL from human milk. Human milk was thawed, centrifuged to remove fat, and separated into casein and whey by acid precipitation [15]. The casein precipitate was harvested by centrifugation, washed three to five times in distilled water, and lyophilized. The lyophilized casein was dissolved in 1 mM Tris–HCl, pH 8.5, and was fractionated using an ion-exchange column packed with DEAE-Trisacryl M attached to an FPLC system (BioLogic automated system, Bio-Rad Laboratories, Stockholm, Sweden) using a NaCl gradient. The MAL complex eluted with 1 M NaCl. The eluate was desalted by dialysis (membrane cutoff 3.5 kDa) against distilled water for at least 48 h and with four changes of water and lyophilized. MAL, the fraction eluting from the ion-exchange column, contained >75% monomeric α-lactalbumin by weight and less than 20% multimer. We were not able to isolate the active species due to equilibrium between the different molecular species of the protein.

Protein labeling. MAL, ALA, BSA, and human IgG were biotinylated according to the manufacturer's instructions. For labeling with ^{125}I, 25 μl of protein (25 μg) dissolved in PBSA-T buffer (30 mM Na_2HPO_4, 10 mM KH_2PO_4, 120 mM NaCl, pH 7.4, with 0.1% Na-Azide, 0.05% Tween-20) was incubated with 2 μl of ^{125}I (0.2 mCi), 2 μl of lactoperoxidase (2.5 mg/ml), and 2 μl of H_2O_2 (1:2000 in PBS; 30 mM Na_2HPO_4, 10 mM KH_2PO_4, and 117 mM NaCl) for 2 min at room temperature. The reaction was stopped by the addition of 500 μl of PBSA-T. The labeled protein was purified on a PD-10 column. Fractions (500 μl) in PBSA-T were eluted and fractions containing radioactivity were stored at −20°C. The labeled proteins eluted in fractions 7 and 8 and had a total activity of approximately 2×10^8 cpm.

Cell lines and primary cultures. The murine lymphoblastoid leukemia cell line L1210 (ATCC CLL 219) was cultured in suspension at 37°C, 5% CO_2, in cell culture flasks (Falcon, Becton Dickinson Labware, New Jersey) in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1:100 dilution of nonessential amino acids, 1 mM sodium pyruvate, 50 μg/ml of gentamicin, and 50 μM 2-mercaptoethanol. At a density of 7×10^5 cells/ml the cells were harvested by centrifugation at 320*g* and the pellet was resuspended in PBS or RPMI depending on the experiment.

The human lung carcinoma cell line A549 (ATCC CLL 185) grew as monolayers attached to the bottom of cell culture flasks (Falcon) under the same condition as described above but in medium without 2-mercaptoethanol. Human renal tubular epithelial cells (HRTEC) were isolated from the kidney of a 3-year-old boy [16]. The cells were cultured in Dulbecco's modified medium with the addition of 15% FCS, 2 mM glutamine, 100 μg/ml of penicillin, 100 U of streptomycin, and 50 U/ml of heparin. Confluent monolayers of A549 and HRTEC cells were detached by addition of versene (140 mM NaCl, 2.4 mM KCl, 8 mM Na_2HPO_4, 1.6 mM KH_2PO_4, and 0.5 mM EDTA, pH 7.2) for 10 min at 37°C and were harvested by centrifugation at 320*g*. The pelleted cells were resuspended in PBS or RPMI depending on experiment.

Cell viability and DNA fragmentation. The cell suspensions (900 μl, 2×10^6 cells/ml) were mixed with MAL (100 μl) in 24-well plates, incubated at 37°C in 5% CO_2, and the cells were harvested by aspiration. Cell aliquots were tested for viability by trypan blue exclusion. Remaining cells were analyzed for DNA fragmentation [14].

HMW DNA fragments were analyzed in cells embedded in low melting point agarose gel treated with proteinase K. Samples were run by electrophoresis at 180 V in 1% agarose gels in 0.5 × TBE (45 mM Tris, 1.25 mM EDTA, 45 mM boric acid, pH 8.0), at 12°C, with the ramping rate changing from 0.8 s to 30 s for 24 h, using a forward to reverse ratio of 3:1. Oligonucleosome-length fragments were analyzed in ethanol-precipitated supernatants from lysed cells run on 1.8% agarose gels overnight applying a 40-V constant voltage. DNA was visualized under ultraviolet light (305 nm) after staining with ethidium bromide and photographed using Polaroid 55 positive–negative film.

Cell surface binding and intracellular distribution of biotinylated protein. L1210, A549, and HRTEC cell suspensions (4×10^6 cells/ml, 95 μl) were incubated at room temperature with 5 μl of biotinylated MAL, ALA, or BSA (5 mg/ml) and then washed in PBS with centrifugation at 320*g* for 10 min to remove unbound protein. To detect surface bound protein, cells were incubated for 30 min at room temperature with FITC-conjugated streptavidin (1:100 in PBS, 100 μl), washed in PBS, suspended in 300 μl of PBS, and analyzed by flow cytometry on a Coulter Epics Profile II flow cytometer (Coulter), equipped with a 488-nm argon laser. Green fluorescence was detected with a 525-nm band pass filter, and the PMT voltage was initially set to 1250 V and adjusted for day to day variation using calibration with Flowcheck and Flowset fluorospheres (Coulter). The binding was calculated as the quotient between the fluorescence intensity of the sample and the fluorescence intensity of the streptavidin control.

To detect intracellular protein, cells exposed to biotinylated protein were permeabilized with saponin to allow entry of FITC-conjugated streptavidin. Cells harvested by centrifugation at 320*g* were fixed by suspension in phosphate-buffered paraformaldehyde (4%) [17] for 5 min at room-temperature, washed in PBS, and permeabilized with 0.1% saponin in PBS. After washing in 0.1% saponin, FITC-conjugated streptavidin (1:100 in 0.1% saponin, 100 μl) was

added and the cells were incubated for 30 min at room temperature. The cells were washed twice in PBS–saponin and once in PBS, mounted on a glass slide, and analyzed in a Bio-Rad 1024 laser scanning confocal equipment (Bio-Rad Laboratories, Hemel-Hempstead, UK) attached to a Nikon Diaphot inverted microscope (Nikon, Japan).

Cell surface binding and intracellular distribution of radiolabeled MAL. L1210, A549, or HRTEC cell suspensions (4×10^6 cells/ml, 100 μl) were incubated with radiolabeled protein (MAL, ALA, or IgG) for 30 min. Unbound protein was removed by washing three times in PBS. Radioactivity in different samples was quantitated in an Epics γ-counter (Coulter).

Subcellular fractionation was according to Graham *et al.* and Trent *et al.* [18, 19]. L1210, A549 or HRTEC cell suspensions (4×10^6 cells/ml, 500 μl) were incubated at 37°C with 1×10^6 cpm of ^{125}I-labeled MAL, ALA, or IgG, washed three times with ice-cold PBS, suspended in 500 μl of cold homogenization buffer (10 mM Tris–HCl, pH 7.8, 5 mM $MgCl_2$, and 2 mM $CaCl_2$ for L1210 cells and 10 mM Tris–HCl, pH 7.5, 2 mM EDTA, for A549 and HRTEC cells, both buffers containing 0.1 mM PMSF, 10 μg/ml of antipain, and 10 μg/ml of leupeptin), and left on ice for 15 min. The cells were disrupted in a Dounce homogenizer (Thomas, Philadelphia, PA) with pestle size 415. Sucrose was added to a final concentration of 250 mM, the homogenate was centrifuged at 1000*g* for 10 min at 4°C, and the pellet was washed once by centrifugation in homogenization buffer. The supernatants from both centrifugations were further centrifuged at 12,000*g* for 30 min at 4°C (pellet 2). The supernatant was centrifuged at 150,000*g* for 1 h at 4°C (pellet 3). The radioactivity of the pellets and remaining supernatant from the differential centrifugation was measured in a γ-counter.

The purity of the fractions was determined by quantitation of specific markers [20]. DNA and RNA were detected with ethidium bromide. Sample (1 vol) was mixed with 1 vol of heparin solution (25 μg/ml) and 1 ml of PBS or with 1 vol of heparin, 1 vol of RNAse A (50 μg/ml), and 1 vol of PBS. The samples were incubated at 37°C for 20 min and 1 vol of ethidium bromide was added (25 μg/ml). Fluorescence was measured after 60 s incubation using an excitation wavelength of 360 nm and an emission wavelength of 580 nm. PBS served as blank, 1 vol of homogenate in 4 vol of PBS served as background correction factor, and the fluorescence was compared to a DNA standard (25 μg/ml of λDNA). Catalase activity was measured by mixing sample (0.5 ml) with H_2O_2 (0.5 ml), for 3 min at 4°C. H_2SO_4 (0.1 ml) was added to stop the reaction, $KMnO_4$ (0.7 ml) was added, and the optical density of the sample was measured at 480 nm within 1 min. Alkaline phosphatase activity was detected by mixing 50 μl of sample with 200 μl of assay mixture (5 ml of 16 mM *p*-nitrophenylphosphate solution, 5 ml of 50 mM sodium borate buffer (pH 9.5), and 20 μl of 1 M $MgCl_2$) and incubated at 37°C for 60 min. The absorbance was measured at 410 nm.

The first pellet (pellet 1 or nuclear fraction) contained nuclei, large mitochondria, and large sheets of plasma membrane. The second pellet contained mitochondria, plasma membrane, and cytoplasmic organelles. The third pellet contained small vesicles, endoplasmic reticulum, and the supernatant contained cytosolic proteins.

Studies of active nuclear uptake. Active nuclear uptake was studied in digitonin-permeabilized cells [21, 22]. Briefly, 5×10^5 cells were incubated in 100 μl of nuclear transport buffer (NTB; 20 mM Hepes, pH 7.3, 110 mM KAc, 5 mM NaAc, 2 mM DTT, 1 mM EGTA), with leupeptin, antipain (1 μg/ml each), and digitonin (40 μg/ml) for 5 min at room temperature. Cells were diluted in NTB and harvested by centrifugation at 320*g* for 10 min.

Biotinylated ALA, MAL, or IgG (5 μl, 5 mg/ml) was added to digitonin-permeabilized cells in a total volume of 100 μl NTB supplemented with 1 mM ATP, 5 mM creatine phosphate, and 20 U/ml creatine phosphokinase, incubated at 30°C, and washed twice in NTB with centrifugation at 320*g* for 10 min. The cells were treated with 0.2% Triton-X 100 in NTB for 6 min, washed, and incubated for 30 min with FITC-conjugated streptavidin (1:100 dilution, 100 μl), washed in NTB and inspected in a Nikon Microphot microscope with epifluorescence equipment or by laser scanning confocal microscopy in a Bio-Rad MRC-1024 instrument. Inhibition of uptake across the nuclear pore was studied in digitonin-permeabilized cells preincubated with 50 μg/ml of wheat-germ agglutinin (WGA). The role of Ca^{2+} was examined after preincubation of the digitonin-permeabilized cells with 0.5 mM EDTA, 0.5 mM BAPTA/AM or 10 μM verapamil for 20 min at room-temperature and exposed to biotinylated protein.

For DNA fragmentation experiments digitonin-permeabilized L1210, A549, or HRTEC cells (2×10^6/ml) were incubated at 37°C with MAL or ALA (0.4 mg/ml) and treated as above. Inhibition of DNA fragmentation was studied by preincubating the nuclei for 20 min at room temperature with 50 μg/ml of WGA before addition of MAL.

Effect of MAL on isolated nuclei. L1210, A549, and HRTEC nuclei were isolated from homogenized cells. Pellet 1 from the homogenization was washed twice in nuclear buffer (25 mM Hepes, pH 7.0, 120 mM KCl, 2 mM K_2HPO_4, 0.3 mM sucrose) for 10 min at 500*g* and suspended in nuclear buffer.

L1210, A549, or HRTEC nuclei in suspension were placed in 24-well plates (900 μl), mixed with MAL, ALA, or BSA (100 μl in nuclear buffer), incubated at 37°C for 1 or 2 h, harvested by aspiration, and analyzed for DNA fragmentation. Inhibition of DNA fragmentation was studied by preincubating the nuclei for 10 min at room temperature with 50 μg/ml of WGA, 500 μM EDTA, 500 μM EGTA, 10 μM BAPTA/AM, or 10 μM verapamil before addition of MAL.

RESULTS

Difference in sensitivity to MAL-induced apoptosis between tumor cell lines and primary cells in culture. Three cell types were selected based on differences in sensitivity to MAL-induced apoptosis (Fig. 1A). The mouse leukemia L1210 cells were highly sensitive with 50% of the cells killed after 6 h exposure to 0.5 mg/ml of MAL. The A549 lung carcinoma cells were intermediately sensitive and required 24 h exposure to 1.25 mg/ml of MAL in order for 50% of the cells to loose their viability. The primary kidney epithelial HRTEC cells remained fully viable after 24 h exposure to 4 mg/ml of MAL. Loss of viability in L1210 and A549 cells was accompanied by DNA fragmentation but DNA fragments were not formed in the HRTEC cells (Fig. 1B). The monomeric form of the protein, ALA (5 mg/ml), had no effect on cell viability and did not induce DNA fragmentation in the L1210, A549, or HRTEC cells.

Interaction of MAL with the surface of sensitive and resistant cells. The surface binding of biotinylated MAL, ALA, or IgG (0.25 mg/ml) to L1210, A549 and HRTEC cells was examined by confocal microscopy (Fig. 2). Both MAL and ALA bound to the three cells types and were distributed in patches separated by unstained areas (Fig. 2). By flow cytometry an increase in surface-bound protein was detected after 10 min and a maximum was reached after 30 min incubation with MAL. There was no quantitative difference in cell surface binding of MAL between the three cell types (binding $\pm$ SD = 10.1 $\pm$ 1.4 (L1210), 11.5 $\pm$ 2.4 (A549), and

8.5 ± 2.8 (HRTEC)). There was no difference in efficiency of cell surface binding between MAL and ALA (binding ± SD = 8.1 ± 1.1 (L1210), 8.8 ± 2.1 (A549), and 6.2 ± 3.0 (HRTEC)).

Cell surface binding of ^{125}I-labeled MAL and ALA increased with the concentration of MAL to reach saturation after 30 min. There was no difference in cell surface binding of ^{125}I-labeled MAL or ALA between the three cell types (Fig. 3). The total amount of cell bound ^{125}I-labeled ALA was, however, lower than for MAL ($P < 0.01$ for all cell types).

These experiments suggested that the differential susceptibility of L1210, A549, and HRTEC cells to MAL-induced apoptosis was not due to differences in MAL surface binding. Furthermore, both the active and the inactive forms of α-lactalbumin bound efficiently to the surface of the three cell types.

Localization of MAL to different intracellular compartments. Cells were examined by confocal microscopy after exposure to biotinylated MAL, ALA, or BSA, fixed, and permeabilized with saponin to allow entry of FITC–streptavidin (Fig. 2). MAL was found in the nuclei of L1210 and A549 cells, but not HRTEC cells.

Nuclear staining was first detected in circa 10% of the L1210 cells after 2 h incubation with 0.25 mg/ml of MAL. After 6 h more than 70% of the nuclei stained brightly. Cytoplasmic staining was not observed in those cells. Nuclear uptake of MAL occurred more slowly in the A549 cells. After 6 h about 15% of A549 cell nuclei stained, and by 24 h about 50% of the A549 nuclei stained brightly. Prior to the detection of nuclear staining, MAL was observed as granular fluorescence evenly distributed throughout the cytoplasm of 549 cells (Fig. 2). Nuclear uptake was not observed in the HRTEC cells exposed to biotinylated MAL.

The distribution of ^{125}I-labeled MAL and the kinetics of uptake into the different cellular compartments are shown in Fig. 4. About 80% of the total radioactivity associated with the L1210 cells was recovered in the nuclear fraction after 6 h. The A549 cells had taken about 50% of ^{125}I-labeled MAL into the nuclear fraction after 24 h. In the HRTEC cells only 16% of the total cell associated radioactivity was found in the nuclear fraction after 24 h of incubation. The amount of MAL in the other subcellular fractions did not differ between the cell types (Fig. 4)

There was a marked difference in cellular distribution between MAL and ALA. The monomeric protein was mainly recovered from the cytosol and not from the nuclei (Fig. 5). The cytosolic content of ALA was 80, 75, and 60% of that of MAL in the respective cell type. The nuclear uptake of ^{125}I-labeled ALA did not differ between the cell types.

The cellular localization studies with biotinylated and ^{125}I-labeled MAL suggested that MAL was transported into the nuclei and that the nuclear uptake of MAL into different cells was proportional to their sensitivity to MAL-induced cell death. Nuclear uptake of ALA was much less efficient than that of MAL.

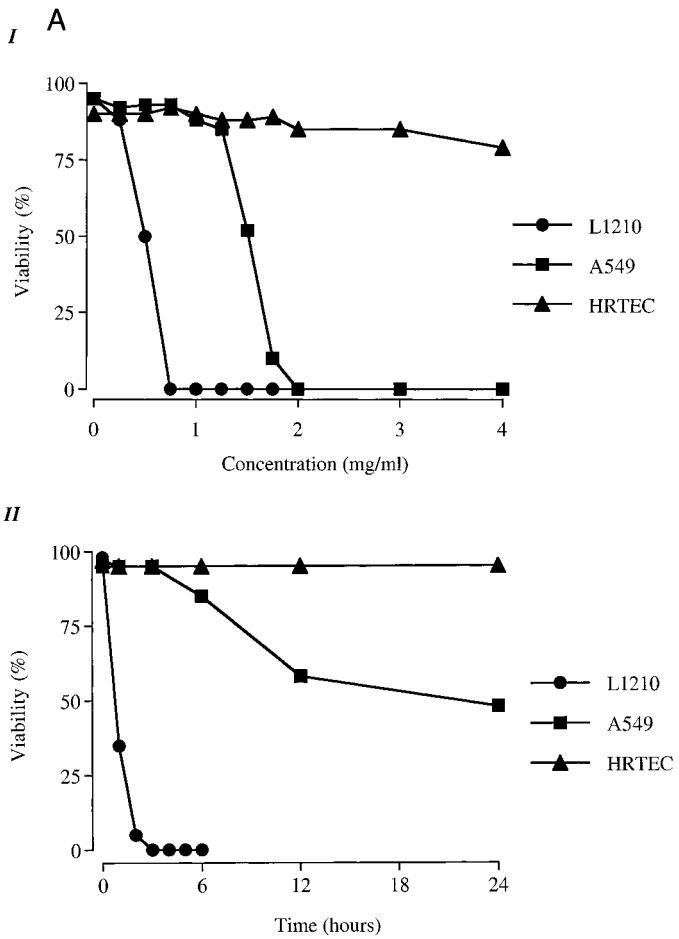


FIG. 1. MAL induces apoptosis in the L1210 and A549 cell lines but not in HRTEC primary cultures. (A) Viability as determined by trypan blue exclusion of cells exposed to MAL in relation to time and concentration. (I) The viability of L1210, A549, and HRTEC cells after exposure to different concentrations of MAL for 6 h (L1210) or 24 h (A549 and HRTEC). The MAL-concentration required to kill 50% of the cells was 0.5 mg/ml for L1210 cells and 1.25 mg/ml for A549 cells. HRTEC cells remained fully viable. (II) The time dependent killing of L1210, A549, and HRTEC cells after exposure to 1.25 mg/ml of MAL. (B) Chromatin cleavage induced by MAL in L1210 and A549 cells was analyzed by field-inverse gel electrophoresis and oligonucleosomal fragmentation was detected by conventional gel electrophoresis. DNA fragmentation was not detected in HRTEC cells. The amount of active multimer in the MAL-fraction was ≤20% by weight.

Interaction of MAL with isolated nuclei. Nuclei were purified from L1210, A549, and HRTEC cells and incubated with different concentrations of MAL (0.2 and 0.4 mg/ml). The formation of HMW and oligonucleosome-length DNA fragments was examined. While some degree of spontaneous DNA fragmentation oc-

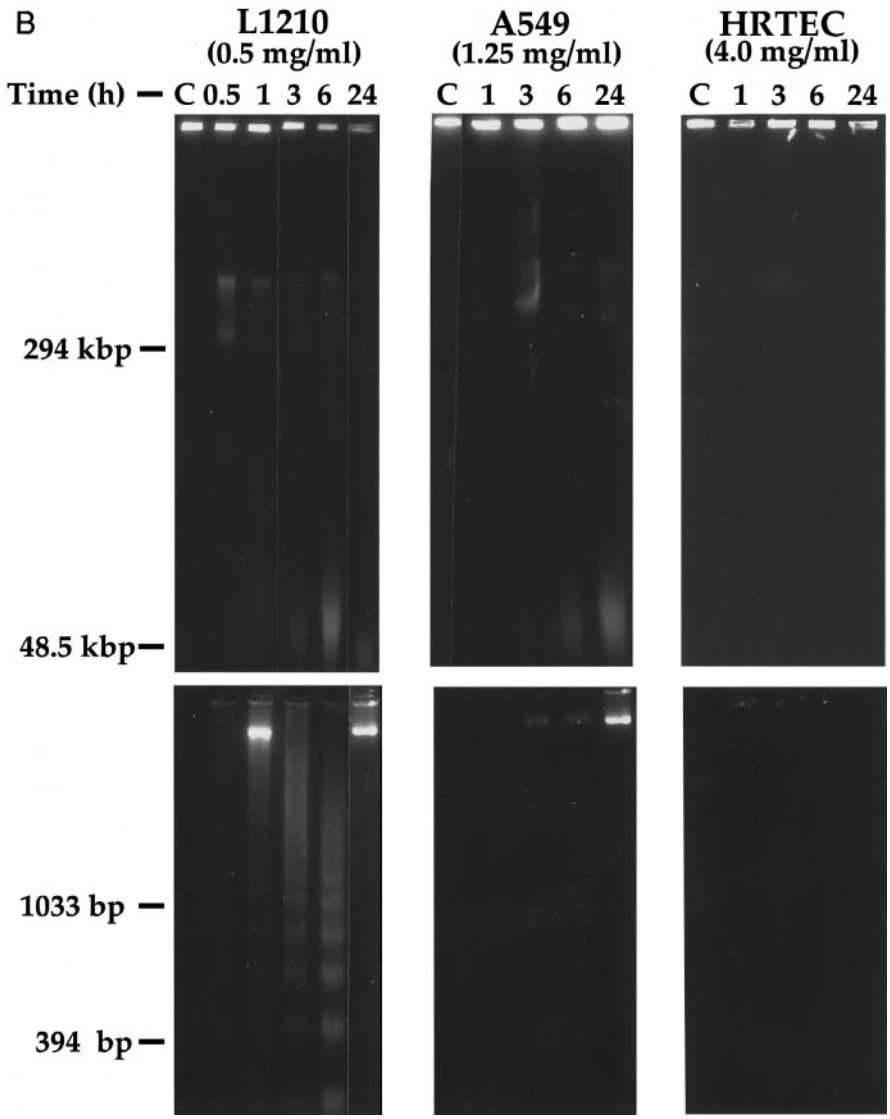


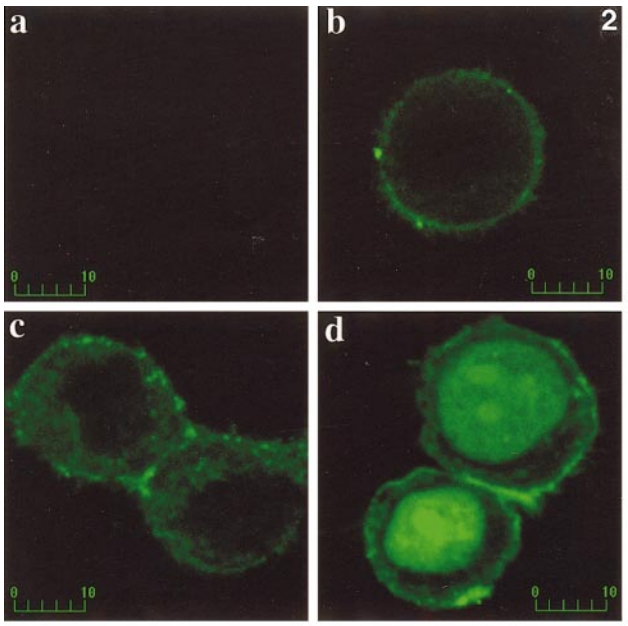
FIG. 1—*Continued*

curred in unstimulated nuclei from the three cell types, MAL (0.4 mg/ml) enhanced the formation of HMW DNA fragments after 1 h and of oligonucleosome length fragments after 2 h of incubation (Fig. 6). MAL was, however, not found to possess endonuclease activity when tested on purified DNA from the sensitive cells (data not shown). ALA did not induce DNA fragmentation in isolated nuclei.

There was no difference in sensitivity to MAL-induced DNA fragmentation between nuclei from the three cell types. These results demonstrated that MAL triggered the induction of DNA fragmentation in the absence of activated cytoplasm and suggested that the differential sensitivity to MAL was determined by the nuclear targeting process rather than by the effect on the nuclei per se.

Uptake of MAL and DNA fragmentation in nuclei of digitonin-permeabilized cells. L1210, A549, and HRTEC cells were permeabilized with digitonin and exposed to biotinylated MAL, ALA, or IgG. MAL was imported directly into the nuclei of digitonin-treated cells with maximum levels reached after 20 min (Fig. 7). HMW DNA fragments were observed after 1 h and oligonucleosomal fragments after 2 h incubation with 0.4 mg/ml of MAL (Fig. 6). ALA entered nuclei of digitonin-permeabilized cells as predicted by its molecular mass (14 kDa) but did not induce DNA fragmentation. There was no nuclear uptake of IgG.

Role of the nuclear pore complex in the uptake of MAL. Active transport over the nuclear pore was examined in digitonin-treated cells, using wheat-germ



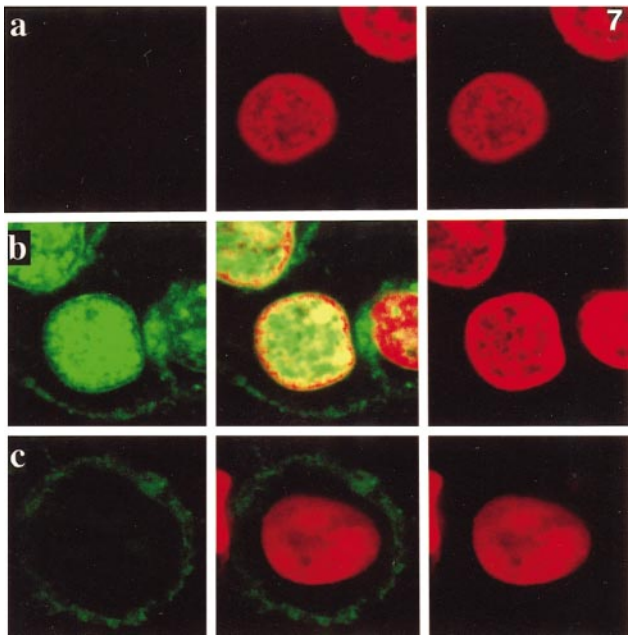


FIG. 2. Interaction of MAL with different cellular compartments in A549 cells. (a) Streptavidin control. (b) Cell surface binding detected after 30 min incubation. The morphology and intensity of MAL cell surface binding was similar for the three cell types and for ALA. (c) Cytoplasmic, granular fluorescence was detected after 3 h incubation with MAL. (d) Nuclear accumulation was maximal after 24 h incubation. The nuclear accumulation was more rapid in the L1210 cells. Nuclear accumulation of MAL was not observed in the HRTEC cells. The scale bar units are in micrometers.

FIG. 7. Nuclear uptake of MAL in digitonin-permeabilized A549 cells, as visualized by confocal microscopy. The cells were incubated with biotinylated protein for 20 min, washed, and counterstained with FITC-conjugated streptavidin (green). There was no nuclear staining in the IgG-treated cells (a), but strong nuclear staining of cells exposed to MAL (b). Nuclear uptake of MAL was inhibited after pretreatment of the cells with WGA (c). Nuclear DNA was counterstained with 25 μg/ml of propidium iodide (red). The integrity of the nuclear membrane of digitonin-permeabilized cells was tested using sera containing anti-nuclear antibodies. After incubation and washing in NTB, the cells were incubated with FITC-conjugated anti-human IgG antibodies (1:100 dilution in NTB) for 30 min at room temperature, washed, and inspected by confocal microscopy. At a concentration of 40 μg/ml of digitonin no nuclear staining was detected. In cells treated with Triton-X 100, which permeabilizes the nuclear membrane, bright nuclear staining was observed after the addition of anti-nuclear antibodies.

agglutinin (WGA) which inhibits the transport of importin–protein complexes through the nuclear pore. Digitonin-permeabilized A549 cells were preincubated with 50 μg/ml of WGA for 20 min, washed, and exposed to biotinylated MAL, IgG, or ALA (Fig. 6). WGA completely blocked the nuclear uptake of MAL, but had no effect on ALA that diffuses freely over the nuclear membrane.

Preincubation of isolated nuclei from A549 cells or digitonin-permeabilized A549 cells with 50 μg/ml of WGA inhibited the MAL-induced DNA fragmentation as compared to control nuclei (Fig. 6).

Role of Ca^{2+} in the nuclear uptake of MAL and in MAL-induced DNA fragmentation. MAL-induced apoptosis was previously shown to require extracellular Ca^{2+} [14]. This suggested that Ca^{2+} might influence the nuclear uptake mechanism and/or the induction of DNA fragmentation once MAL reaches the cell nuclei. L1210, A549, and HRTEC cells were pretreated with an inhibitor of Ca^{2+} uptake (verapamil), with extracellular Ca^{2+}-chelators (EDTA and EGTA) and with an intracellular Ca^{2+}-chelator (BAPTA/AM). ^{125}I-labeled MAL was added to the cells, and the nuclear uptake was examined after 6 h in L1210 cells and after 24 h in A549 and HRTEC cells. No effect on the nuclear uptake of MAL was observed.

The MAL-induced DNA fragmentation was subsequently examined in control nuclei and nuclei incubated with the Ca^{2+} inhibitors. EDTA and BAPTA/AM completely blocked the MAL-induced DNA fragmentation in nuclei isolated from L1210 and A549 cells. These results demonstrate that Ca^{2+} is required for MAL-induced DNA fragmentation in isolated nuclei, but that the nuclear uptake of MAL is Ca^{2+}-independent.

DISCUSSION

Epidemiological studies have provided evidence that breast-feeding protects against cancer [23–25]. Breast-fed children have a lower incidence, especially of lymphomas, but also of malignancies in general than bottle-fed children, and the frequency decreases with the length of breast-feeding [25]. This association implies

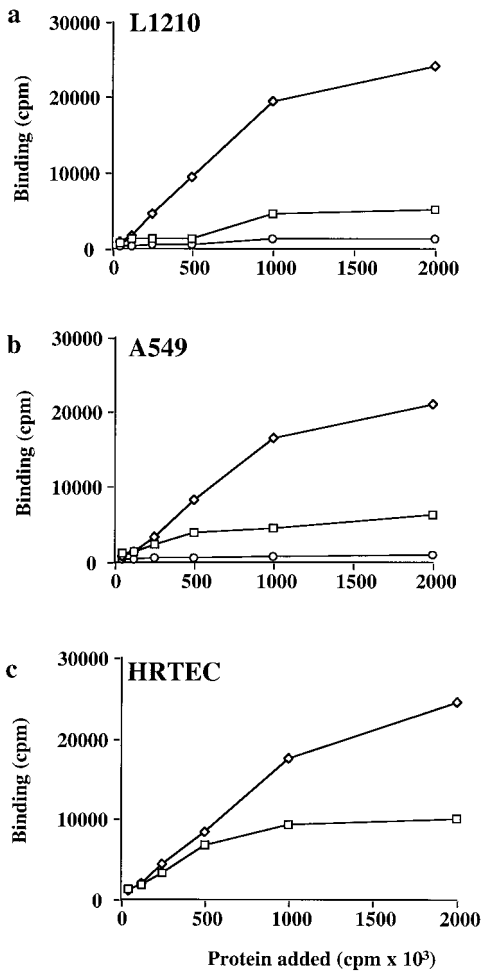


FIG. 3. Cell associated radioactivity in L1210, A549, and HRTEC cells after 30 min exposure to increasing concentrations of radiolabeled MAL (◇), ALA (□), or IgG (○).

that milk contains molecules that influence tissue development.

Tumor cells and mature nonmalignant cells differ in sensitivity to MAL, an α-lactalbumin complex in human milk. The tumor cells undergo apoptosis, while healthy, nonmalignant cells survive and continue to proliferate in the presence of MAL. This study compared the subcellular distribution of MAL in sensitive and resistant cells. By confocal microscopy and subcellular fractionation, MAL was shown to accumulate in nuclei of cells that were sensitive to its apoptosis effect but not in resistant cells. Nuclear uptake occurred before or at the onset of DNA fragmentation and required active transport through the nuclear pore complex. MAL had a direct effect on nuclei; it was able to induce DNA fragmentation in isolated nuclei in the absence of activated cytoplasm. Inhibition of MAL transport through the nuclear pore rescued the nuclei from MAL-mediated DNA fragmentation. The results demonstrate that MAL can target the cell nucleus and that the nuclear targeting mechanisms are more readily available in cells that are sensitive to MAL-induced apoptosis than in resistant cells. MAL differs from most previously described apoptosis-inducing agents in that it induces apoptosis through a direct effect at the nuclear level. Similar intranuclear targeting of apoptosis-inducing agents have been seen only for granzyme B in perforin-treated target cells and for glucocorticoids in thymocytes and certain T-cell lines [9, 10, 26].

α-Lactalbumin is the major protein of human milk whey. In its monomeric form it is a 14-kDa protein with one high affinity Ca^{2+} binding site. We have previously reported that the monomeric, native form of α-lactal-

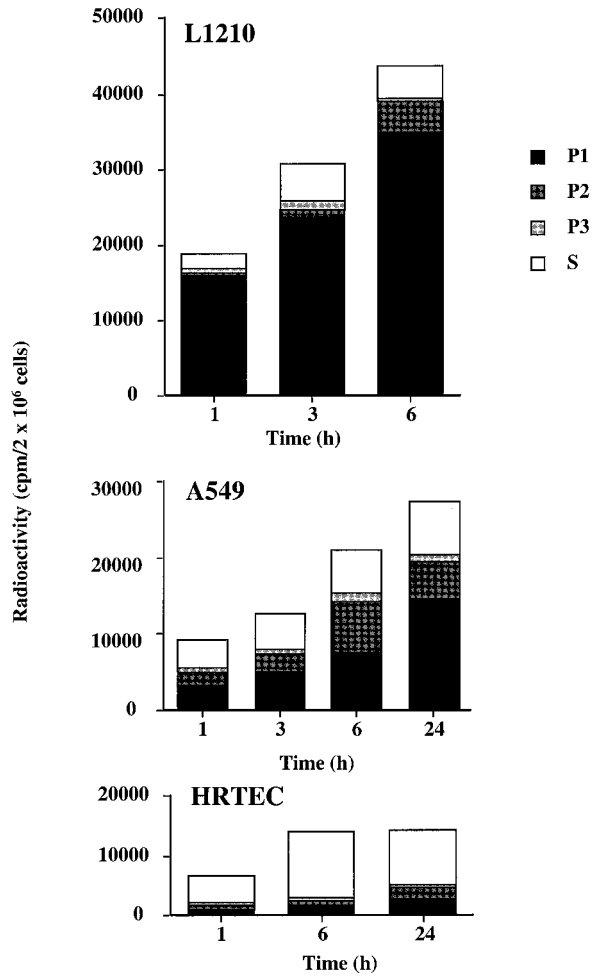


FIG. 4. Localization of ^{125}I-labeled MAL to different subcellular compartments in L1210, A549, and HRTEC cells. Cells were exposed to 10^6 cpm of radiolabeled MAL and homogenized, and radioactivity associated with each subcellular fraction was quantitated in a γ-counter. The nuclear fraction contained mostly nuclei; pellet 2 contained plasma-, Golgi-, and ER-membranes and mitochondria; pellet 3 contained small vesicles; and the supernatant contained the cytosol.

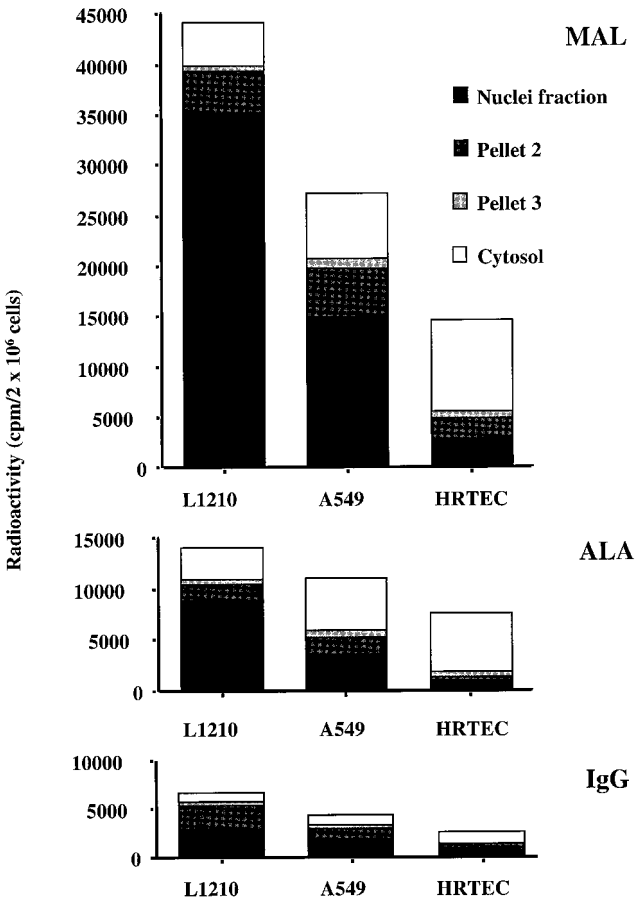


FIG. 5. Difference in subcellular distribution between MAL and ALA. L1210, A549, and HRTEC cells were exposed to 10^6 cpm of radiolabeled proteins for 6, 24, and 24 h, respectively. The cells were homogenized and subjected to differential centrifugation, and the radioactivity of each subcellular fraction was determined. The recovery from the nuclear fraction (pellet 1) was 34,800 cpm (78%), 14,200 cpm (51%), and 2,300 cpm (16%) for the L1210, A549, and HRTEC cells, respectively. The recovery from pellet 2 was 4500 cpm (10%), 5100 cpm (19%), and 2100 cpm (15%). The recovery from pellet 3 was 800 cpm (2%), 1400 cpm (5%), and 1100 cpm (8%) and the recovery from the cytosol was 4500 cpm (10%), 6800 cpm (25%), and 8600 cpm (61%), respectively.

bumin does not induce apoptosis [14]. In this study the monomer was found to bind to the cytoplasmic membrane of sensitive and resistant cells, and to be taken up into the cytoplasm. Like monomeric α-lactalbumin, MAL bound to the surface of sensitive and resistant cells but in addition MAL entered the nuclei of the cells in which it induced apoptosis. This difference in cellular localization may relate to differences in protein folding between the monomeric and the multimeric forms of the protein. Recent studies have shown that MAL consists of α-lactalbumin multimers in the molten globule state complexed with fatty acid. We are currently investigating how changes in protein folding from the native monomer to the molten globule multimer influence the transport over the nuclear membrane, so as to explain the nuclear uptake of the multimer.

For some exogenous apoptosis-inducing molecules, like Fas-ligand or TNF, the binding to their respective cell surface receptors is crucial for apoptosis induction. Ligand–receptor interaction triggers transmembrane and/or intracellular signaling pathways leading to apoptosis [27–29]. Consequently, cells that lack cell surface receptors for these agonists are resistant to their

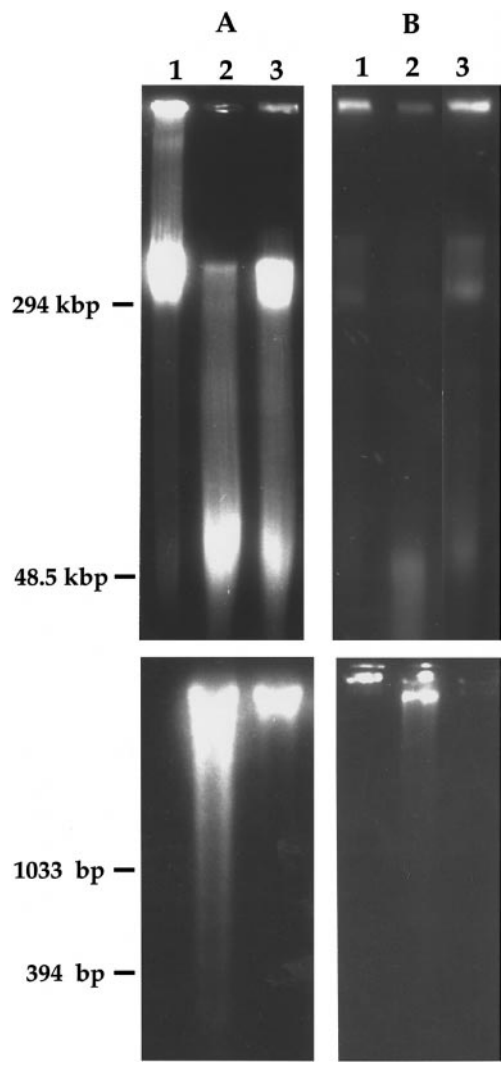


FIG. 6. Chromatin cleavage in isolated nuclei (A) or digitonin-treated A549 cells (B) after 2 h incubation with 0.4 mg/ml of MAL. Transport over the nuclear pore was inhibited by preincubation of the isolated nuclei or the digitonin-treated cells with 50 μg/ml of WGA. Chromatin cleavage was analyzed by field-inverse gel electrophoresis (top) and oligonucleosomal fragmentation was detected by conventional gel electrophoresis (bottom). Lanes A1 and B1 show limited DNA fragmentation in untreated nuclei or digitonin-permeabilized cells. Lanes A2 and B2 show nuclei or digitonin-treated cells exposed to MAL and lanes A3 and B3 show nuclei or digitonin-treated cells exposed to MAL after preincubation with 50 μg/ml of WGA.

apoptosis-inducing effects. In this study there was no difference in cell surface binding of MAL to sensitive and resistant cells, suggesting that receptors for MAL were present on all cells. By confocal microscopy MAL was shown to bind in patches, suggesting either that MAL bound as preformed aggregates or that the bound MAL accumulated in certain areas of the membrane through capping or other mechanisms influencing receptor distribution. Cell surface binding was rapid, saturatable at high MAL concentrations, and it was also specific as defined by competition between labeled and unlabeled MAL (data not shown). There was no difference in cell surface binding between MAL and the monomeric inactive form of the protein. These results suggest that MAL differs from agonists like TNF and Fas-ligand in that cell surface binding does not itself determine if apoptosis will be induced.

There was also no evidence that the cytoplasmic uptake differed between sensitive and resistant cells. Uptake of ^{125}I-labeled MAL into the cytoplasm occurred with similar kinetics in the L1210, A549, and HRTEC cells, and the amount of MAL in the cytoplasm was similar in the three cases. There was, however, a direct link between the susceptibility to MAL-induced apoptosis and the nuclear accumulation of MAL in the three cell types. MAL was detected after 2 h in nuclei of sensitive L1210 cells (20%, 0.5 mg/ml) and the fraction of cells having MAL-containing nuclei increased to about 80% after 6 h. In A549 cells nuclear uptake and DNA fragmentation required almost threefold more protein and still it occurred more slowly. Finally, nuclear uptake was low or absent in HRTEC cells.

Evidence for a direct nuclear effect of MAL was obtained in experiments with isolated nuclei and digitonin-permeabilized cells. MAL entered the nuclei and stimulated the formation of HMW and oligonucleosome-length DNA fragments in isolated nuclei more rapidly than in intact cells. The monomeric form of the protein was able to enter the isolated nuclei and to pass into nuclei of digitonin-permeabilized cells, but did not induce DNA fragmentation. Interestingly, there was no difference in sensitivity to MAL between the nuclear fractions isolated from the three cell types, suggesting that sensitive and resistant cells differ in the transport of MAL into the nucleus and not in the susceptibility to MAL once it reaches the nuclear compartment.

The uptake of MAL into the nucleus was via the nuclear pore complex as shown by inhibition studies using WGA, a lectin that binds to glycosylated regions of the nucleoporins and sterically hinders transport of the importin–protein complex through the nuclear pore. WGA treatment blocked MAL uptake into the nuclei of digitonin-treated cells and inhibited MAL-induced DNA fragmentation. Classical nuclear localization sequences often include clusters of basic amino acids that share little or no sequence homology [11, 30]. When conducting a sequence analysis of the monomeric form of α-lactalbumin we did not find known nuclear targeting motifs and the monomer did not target the cell nucleus. It is likely, therefore that MAL carries structural modifications, not present in ALA, that confer affinity for the nuclear pore and the ability to activate DNA fragmentation in the nucleus.

Lactalbumins are Ca^{2+} binding proteins with one high affinity and one low affinity Ca^{2+}-binding site [31]. MAL was previously shown to increase intracellular Ca^{2+} levels. This Ca^{2+} flux was required for apoptosis to occur; removal of extracellular Ca^{2+} inhibited MAL-induced apoptosis [14]. In this study we examined if the Ca^{2+} dependence reflected an effect on nuclear uptake of MAL and/or the induction of DNA fragmentation. Pretreatment of MAL-sensitive cells with Ca^{2+} chelators had no effect on the nuclear localization of MAL but was found to inhibit MAL-mediated DNA fragmentation in isolated nuclei. These observations suggested that apoptosis occurs as a result of at least two converging mechanisms: a Ca^{2+}-independent transport of MAL into the nucleus and a Ca^{2+} dependent activation step in the nuclear compartment.

Rapidly growing tissues need control mechanisms that select for maturity and drive the cells toward differentiation rather than malignancy. This study describes a naturally occurring molecular complex in human milk, that may have a surveillance function in tissue maturation. Such molecules should be explored as cancer therapeutics. They may be predicted to have the advantage over currently available compounds in that they target tumor cells and immature cells rather than healthy cells and in that they kill these cells through apoptosis rather than necrosis, thus minimizing tissue destruction and the side effects that accompany many current treatments.

This work was supported by grants from The Swedish Cancer Foundation (Grants 3807-B97-01XAB and 96-1719), The American Cancer Society (RPG 97-157-01), The Swedish Medical Research Council (Grant 03X-2471), The Royal Physiographic Society, Lund, Sweden, The Swedish Society for Medical Research, Stockholm, Sweden, John and Augusta Perssons Foundation for Scientific Medical Research, Lund, Sweden, Anna-Lisa and Sven-Eric Lundgrens Foundation for Medical Research, Malmö, Sweden, Gunnar, Arvid, and Elisabeth Nilssons Foundation for Fighting Cancer, Helsingborg, Sweden, and Maggie Stephen's Foundation, Lund, Sweden. C.S. gratefully acknowledges the hospitality of the Institute of Molecular Medicine at the John Radcliffe Hospital, Oxford, UK.

REFERENCES

1. Schwartz, L. M., and Osborne, B. A. (1993). Programmed cell death, apoptosis and killer genes. *Immunol. Today* **14,** 582–590.
2. McConkey, D. J., Zhivotovsky, B., and Orrenius, S. (1996). Apoptosis—Molecular mechanisms and biomedical implications. *Mol. Aspects Med.* **17,** 1–115.

3. Kerr, J. F., Winterford, C. M., and Harmon, B. V. (1994). Apoptosis. Its significance in cancer and cancer therapy. *Cancer* **73,** 2013–26.
4. Arends, M. J., Morris, R. G., and Wyllie, A. H. (1990). Apoptosis: The role of endonuclease. *Am. J. Pathol.* **136,** 593–608.
5. Zhivotovsky, B., Gahm, A., Ankarcrona, M., Nicotera, P., and Orrenius, S. (1995). Multiple proteases are involved in thymocyte apoptosis. *Exp. Cell Res.* **221,** 404–412.
6. Kumar, S. (1995). ICE-like proteases in apoptosis. *Trends Biochem. Sci.* **20,** 198–202.
7. Yasuhara, N., Eguchi, Y., Tachibana, T., Imamoto, N., Yoneda, Y., and Tsujimoto, Y. (1997). Essential role of active nuclear transport in apoptosis. *Genes Cells* **2,** 55–64.
8. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998). A caspase-activated DNase that degrades DNA during apoptosis and its inhibitor ICAD. *Nature* **391,** 43–50.
9. Froelich, C. J., Hanna, W. L., Poirier, G. G., Duriez, P. J., D'Amours, D., Salvesen, G. S., Alnemri, E. S., Earnshaw, W. C., and Shah, G. M. (1996). Granzyme B/perforin-mediated apoptosis of Jurkat cells results in cleavage of poly(ADP-ribose) polymerase to the 89-kDa apoptotic fragment and less abundant 64-kDa fragment. *Biochem Biophys Res Commun.* **227,** 658–65.
10. Andrade, F., Roy, S., Nicholson, D., Thornberry, N., Rosen, A., and Casciola Rosen, L. (1998). Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. *Immunity* **8,** 451–60.
11. Jans, D. A., and Hübner, S. (1996). Regulation of protein transport to the nucleus: Central role of phosphorylation. *Physiol. Rev.* **76,** 651–685.
12. Yoneda, Y. (1997). How proteins are transported from the cytoplasm to the nucleus. *J. Biochem.* **121,** 811–817.
13. Görlich, D., and Mattaj, I. W. (1996). Nucleocytoplasmic transport. *Science* **271,** 1513–1518.
14. Håkansson, A., Zhivotovsky, B., Orrenius, S., Sabharwal, H., and Svanborg, C. (1995). Apoptosis induced by a human milk protein. *Proc. Natl. Acad. Sci. USA* **92,** 8064–8068.
15. Melander, O. (1947). *Uppsala Läkarfören. Förhandl.* **3-4,** 107–198.
16. Karpman, D., Håkansson, A., Perez, M.-T. R., Isaksson, C., Carlemalm, E., Caprioli, A., and Svanborg, C. (1998). Apoptosis of renal cortical cells in the hemolytic uremic syndrome: *In vivo* and *in vitro* studies. *Infect. Immunol.* **66,** 636–644.
17. Sander, B., Andersson, J., and Andersson, U. (1991). Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. *Immunol. Rev.* **119,** 65–92.
18. Graham, J. (1992). Isolation of subcellular organelles and membranes. *In* "Centrifugation, a Practical Approach," Ed: Rickwood, D. Oxford IRL Press, Washington, DC pp. 161–182.
19. Trent, D. F., Seither, R. L., and Goldman, D. (1991). Compartmentation of intracellular folates. *Biochem. Pharmacol.* **42,** 1015–1019.
20. Graham, J., and Ford, T. C. (1992). Marker enzymes and chemical assays for the analysis of subcellular fractions. *In* "Centrifugation, a Practical Approach," Ed: Rickwood, D. Oxford IRL Press, Washington, DC pp. 309–333.
21. Adam, S. A., Sterne Marr, R., and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* **111,** 807–816.
22. Shannon Moore, M., and Blobel, G. (1992). The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic fractions. *Cell.* **69,** 939–950.
23. Newcomb, P. A., Storer, B. E., Longnecker, M. P., Mittendorf, R., Greenberg, E. R., Clapp, R. W., Burke, K. P., Willett, W. C., and MacMahon, B. (1994). Lactation and a reduced risk of pre-menopausal breast cancer. *N. Engl. J. Med.* **330,** 81–87.
24. Siskind, V., Schofield, F., Rice, D., and Bain, C. (1989). Breast cancer and breastfeeding: Results from an Australian case-control study. *Am. J. Epidemiol.* **130,** 229–236.
25. Davis, M. K., Savitz, D. A., and Graubard, B. I. (1988). Infant feeding and childhood cancer. *Lancet* **2,** 365–368.
26. Cidlowski, J. A., King, K. L., Evans Storms, R. B., Montague, J. W., Bortner, C. D., and Hughes, F. M., Jr. (1996). The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system. *Recent Prog. Horm. Res.* **51,** 457–90; discussion 490–491.
27. Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R., and Ferguson, T. A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270,** 1189–1192.
28. Tepper, C. G., Jayadev, S., Liu, B., Bielawska, A., Wolff, R., Yonehara, S., Hannun, Y. A., and Seldin, M. F. (1995). Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. *Proc. Natl. Acad. Sci. USA* **92,** 8443–8447.
29. Zheng, L., Fisher, G., Miller, R. E., Peschon, J., Lynch, D. H., and Leonardo, M. J. (1995). Induction of apoptosis in mature T cells by tumor necrosis factor. *Nature* **377,** 348–351.
30. Garcia-Bustos, J., Heitman, J., and Hall, M. N. (1991). Nuclear protein localization. *Biochim. Biophys. Acta* **1071,** 83–101.
31. Ren, J., Stuart, D. I., and Acharya, K. R. (1993). Alpha-lactalbumin possesses a distinct zinc binding site. *J. Biol. Chem.* **268,** 19292–19298.

Received June 9, 1998
Revised version received August 12, 1998