

# Apoptosis induced by a human milk protein

(epithelial cells/thymocytes/cancer)

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**ABSTRACT** To the breast-fed infant, human milk is more than a source of nutrients; it furnishes a wide array of molecules that restrict microbes, such as antibodies, bactericidins, and inhibitors of bacterial adherence. However, it has rarely been considered that human milk may also contain substances bioactive toward host cells. While investigating the effect of human milk on bacterial adherence to a human lung cancer cell line, we were surprised to discover that the milk killed the cells. Analysis of this effect revealed that a component of milk in a particular physical state—multimeric  $\alpha$ -lactalbumin—is a potent  $\text{Ca}^{2+}$ -elevating and apoptosis-inducing agent with broad, yet selective, cytotoxic activity. Multimeric  $\alpha$ -lactalbumin killed all transformed, embryonic, and lymphoid cells tested but spared mature epithelial elements. These findings raise the possibility that milk contributes to mucosal immunity not only by furnishing antimicrobial molecules but also by policing the function of lymphocytes and epithelium. Finally, analysis of the mechanism by which multimeric  $\alpha$ -lactalbumin induces apoptosis in transformed epithelial cells could lead to the design of antitumor agents.

Through programmed cell death the human body eliminates unwanted cells without evoking an inflammatory response. Cells undergoing programmed cell death show a distinct morphological appearance characterized by cell shrinkage, membrane blebbing, nuclear condensation, and fragmentation with the formation of “apoptotic bodies”; this type of cell death is generally referred to as apoptosis (1, 2).

Human milk provides the breast-fed child not only with nutrients but also with a mucosal immune system. Milk contains a wide array of molecules with antimicrobial activity: antibodies to bacterial, viral, and protozoal antigens (3–5); potentially bactericidal molecules like lysozyme and lactoferrin; fatty acids that lyse bacteria and viral particles; and glycoconjugates that inhibit bacterial adherence to epithelial cells (6–8). These components reach mucosal surfaces in the respiratory and gastrointestinal tracts of the breast-fed child and are thought to interfere with various steps in the pathogenesis of infections at these sites (8, 9). As a result, breast-feeding protects the infant from respiratory and gastrointestinal infections.

During our recent studies of human milk and its antibacterial properties, we observed that transformed cell lines exposed to human milk underwent morphological changes compatible with apoptosis. The active component was identified as multimeric  $\alpha$ -lactalbumin (MAL), which was found to induce apoptosis in a variety of transformed and immature mammalian cells but not in other cells. This report describes these effects and provides preliminary characterization of the cytotoxic mechanism.

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## MATERIALS AND METHODS

**Materials.** Human and bovine  $\alpha$ -lactalbumin, bovine serum albumin (BSA), human and chicken lysozyme, human lactoferrin, and cycloheximide were from Sigma. Thapsigargin was from Gibco.

**Cells.** The A549, NCI H292, A-498, J 82, CaCO-2, HT-29, 5637, GMK, Vero, MDCK, B9, and WEHI 164 cell lines were from the American Type Culture Collection (ATCC). The cell lines were cultured as described (10). Human embryonic lung cells (Hel) and human foreskin fibroblasts (HFF) were kindly provided by the Division of Virology, Department of Medical Microbiology, Lund University, Lund, Sweden. Human peripheral blood granulocytes and lymphocytes were isolated from heparin-treated blood of healthy human volunteers by using a Polymorphprep density gradient (Nycomed, Oslo). Rat thymocytes were harvested as described (11).

**Measurement of Intracellular  $\text{Ca}^{2+}$  Concentration ( $[\text{Ca}^{2+}]_i$ ).** A549 cells ( $4 \times 10^7$  cells) or rat thymocytes ( $5 \times 10^7$  cells) were incubated in Krebs–Henseleit buffer (pH 7.2) with 10 mM Hepes, 15 mM glucose, and 1% BSA. Cells were loaded with 5  $\mu\text{M}$  fura-2 AM for 25 min at 37°C, washed for 30 s at 1500  $\times g$ , and resuspended in Krebs medium (11, 12). After a steady baseline of  $[\text{Ca}^{2+}]_i$  was obtained, inducing agents were added. Control cells had only carrier solvent.

**Milk Fractionation.** Purification of the active component was according to H.S. (unpublished observation). Briefly, casein was precipitated from  $\approx 5$  liters of milk (13) and fractionated by using an ion-exchange column packed with DEAE-Trisacryl M (BioSeptra, Villeneuve la Garenne, France) attached to an FPLC instrument (Pharmacia-LKB). The lyophilized casein was dissolved in 0.01 M Tris-HCl (pH 8.5) and applied to the column. The run was in 0.01 M Tris-HCl (pH 8.5) with increasing concentrations of NaCl (0–1.0 M). The peaks were monitored at 280 nm. Fractions (3 ml) were pooled (pools I–VI), desalted by dialysis (membrane cutoff, 3.5 kDa) against distilled water for at least 48 h, lyophilized, and diluted in medium to a concentration of 10 mg/ml.

**Analysis of the Milk Fractions.** The active fraction (fraction VI) was analyzed by polyacrylamide gradient gel electrophoresis (PAGE) using 4–20% precast gels (Bio-Rad) on a Bio-Rad Mini Protean II cell. After SDS/PAGE, protein bands were transferred by Western blotting onto poly(vinylidene difluoride) membranes (Pro Blott membranes; Applied Biosystems) and subjected to protein sequencing by Edman degradation in an automated pulse liquid sequencer (model 477A; Applied Biosystems) (14). Purified proteins were analyzed by electrospray ionization mass spectrometry (ESI-MS) (15) and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (16). For ESI-MS a VG Bio-Q

Abbreviations: MAL, multimeric  $\alpha$ -lactalbumin;  $[\text{Ca}^{2+}]_i$ , intracellular  $[\text{Ca}^{2+}]$ ; PAGE, polyacrylamide gradient gel electrophoresis; ESI-MS, electrospray ionization mass spectrometry; MALDI, matrix-assisted laser desorption ionization; FIGE, field-inversion gel electrophoresis.

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ESI mass spectrometer (Fisons/VG, Manchester, U.K.) was used. The electrospray carrier solvent was 1% acetic acid in acetonitrile/water (1:1) and the flow rate was 2–4 ml/min. The molecular mass of sample components was estimated from the *m/z* values of series of ions (15). MALDI-MS was performed on an LDI 1700 time of flight mass spectrometer equipped with a pulsed nitrogen laser (337 nm) (Biomolecular Separations, Reno, NV). Sinapinic acid was used as a matrix and BSA was used as the external standard.

**Influence of Milk Components on Cell Viability.** The cells were grown to confluency in 96-well plates. At time 0, 100  $\mu$ l of medium was replaced by 100  $\mu$ l of experimental solution and incubated at 37°C for 30 min. [<sup>3</sup>H]Thymidine (0.5  $\mu$ Ci; 1 Ci = 37 GBq; Amersham) was added and the cells were incubated for 4 h at 37°C. The supernatant was discarded and the cells were washed twice in phosphate-buffered saline, detached by trypsin treatment, and harvested; the radioactivity was measured in a 1205 Betaplate liquid scintillation counter (Wallac). Cell viability was also assayed by trypan blue exclusion.

**Transmission Electron Microscopy.** Cells were double fixed in glutaraldehyde plus osmium tetroxide, embedded in agar 100, poststained with uranyl acetate and lead, sectioned, and examined by transmission electron microscopy.

**DNA Fragmentation.** Cells ( $2 \times 10^6$  cells) were lysed and centrifuged at 20,000  $\times g$ , and the supernatant was extracted with phenol/chloroform. Precipitated oligonucleosome length DNA fragments were loaded on 1.8% agarose gels, electrophoresed with constant current set at 60 mA, and visualized with ethidium bromide (17).

High molecular weight DNA fragments were detected by field-inversion gel electrophoresis (FIGE) as described (17).

## RESULTS

**Human Milk Induces Apoptosis.** Human milk from several donors induced apoptosis in transformed and nontransformed immature cell lines and lymphoid cells but not in mature cells. This effect was first observed in studies with A549 cells, a human lung carcinoma cell line used to investigate bacterial adherence and antimicrobial properties of human milk. Cell viability was reduced by 98% (Table 1), and the cells displayed morphological changes compatible with apoptosis (nuclear condensation, appearance of apoptotic bodies, and cell shrinkage; Fig. 1). Associated with this was the formation of high molecular weight DNA fragments in A549 cells. MDCK cells displayed both high molecular weight DNA fragments and the typical oligonucleosome length DNA fragments (DNA laddering) (data not shown). Human milk produced maximal DNA fragmentation when diluted 1:10. Bovine milk was inactive (data not shown).

**Characterization of the Active Component.** Human milk was fractionated to identify the component(s) triggering apoptosis. The fractions were tested for effects on the viability of four human cell lines (A549, NCI, A-498, and J 82). The results with A549 cells are shown in Table 1. The cytotoxic activity precipitated with the casein fraction of human milk; no activity remained in the whey fraction. After ion-exchange chromatography of casein, the apoptosis-inducing activity was in fraction VI that eluted only after 100% 1 M NaCl (Fig. 2). This fraction contained proteins with molecular masses of 14, 28, and 100 kDa (Fig. 2 *Inset*). The bands of fraction VI showed complete N-terminal sequence homology with human  $\alpha$ -lactalbumin (data not shown). By ESI-MS, the estimated molecular mass of the major component of the active protein fraction (14.088 kDa) was close to the molecular mass of  $\alpha$ -lactalbumin calculated from the amino acid sequence (14.078 kDa). The small differences ruled out most known posttranslational modifications and suggested that the major component did not differ in its covalent structure from  $\alpha$ -lactalbumin. MALDI-MS showed a major peak close to 14 kDa, consistent with mono-

Table 1. Effects of human milk and human milk fractions on viability of human lung carcinoma cell line A549

	Cell viability, %*	
	TBE†	TI‡
Medium control	92	100 (21,750)
Human milk		
Donor 1	2	0
Donor 2	2	1
Donor 3	4	2
Fraction of milk (donor 1)		
Whey	92	62
Casein	0	0
Casein fraction		
I	96	93
II	88	91
III	74	69
IV	89	111
V	79	73
VI§	0	0
Control		
Bovine milk	100	64
$\alpha$ -Lactalbumin, human	92	80
$\alpha$ -Lactalbumin, bovine	93	82
Lysozyme, human	87	69
Lysozyme, chicken	86	75
Lactoferrin, human	98	¶
MAL, human	0	0

\*Equivalent results were obtained with cell lines NCI, A498, and J 82.

†Cell viability was determined by trypan blue exclusion. Mean values from three separate experiments are shown.

‡Cell viability was determined by [<sup>3</sup>H]thymidine incorporation. cpm of the medium control is shown in parentheses. Viability is measured as (cpm sample  $\times$  100)/(cpm control). Mean values from three separate experiments are shown.

§Fractions I–VI were separated by ion-exchange chromatography. Fraction VI was eluted after 1 M NaCl.

¶Not determined.

||Commercial monomeric  $\alpha$ -lactalbumin was multimerized by passage over an ion-exchange column.

meric  $\alpha$ -lactalbumin, but also peaks at 28 and 42 kDa, consistent with the di- and trimeric forms.

Monomeric, commercial human or bovine  $\alpha$ -lactalbumin had no effect on cell viability (Table 1). However, when monomeric  $\alpha$ -lactalbumin was passaged over the ion-exchange column, it multimerized and became cytotoxic. Other human milk proteins (lactoferrin and lysozyme) were found to be inactive at a concentration of 5 mg/ml before and after ion-exchange chromatography (Table 1). We concluded that a multimeric form of  $\alpha$ -lactalbumin in human milk induces cell death.

**Sensitivity of Different Cell Types to MAL.** MAL reduced the viability of human epithelial cell lines (A549, NCI, A-498, J 82, Caco-2, HT 29, and 5637), primate kidney cell lines (GMK, Vero), a canine kidney cell line (MDCK), and two mouse cell lines (WEHI 164 and B9) by >90% (Table 2). Embryonic human cells (Hel, HFF) and human peripheral blood lymphocytes were sensitive, while peripheral blood granulocytes demonstrated intermediate sensitivity to MAL. Human epithelial cells from either the upper respiratory tract or the urinary tract and mouse kidney and bladder epithelial cells were resistant to the cytotoxic effect of MAL (Table 2).

**MAL Induces Apoptosis.** Oligonucleosome-length DNA fragments characteristic of apoptosis were observed in thymocytes treated with 0.3, 0.5, and 0.7 mg of MAL per ml for 4 h (Fig. 3) and in MDCK cells treated with 1 mg of MAL per ml for 24 h (Fig. 3). Fragmentation increased with the concentration of MAL. In A549 cells, MAL produced HMW DNA fragments of the kind that is seen early in thymocyte apoptosis

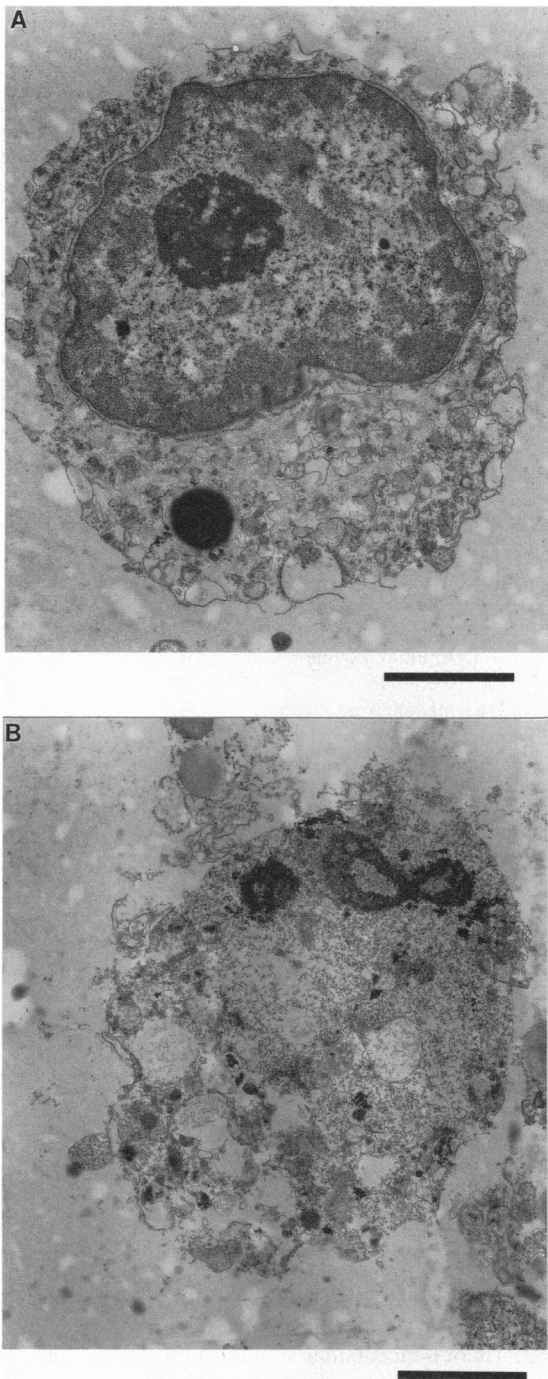


FIG. 1. Apoptosis in MDCK cells exposed to human milk (1:10 dilution; 24 h). (A) Control. (B) Human milk. (Bars = 2.3  $\mu$ m.)

(Fig. 3) (17). DNA ladders were not observed in A549 cells even after higher concentrations of MAL (5 mg/ml) or prolongation of the incubation time (48 h) (data not shown).

In many experimental systems, the induction of apoptosis is accompanied by *de novo* mRNA and protein synthesis and is blocked by protein synthesis inhibitors (2, 18). Cycloheximide effectively prevented MAL-induced DNA laddering in MDCK cells and cell death in A549 and MDCK cells (data not shown).

**Role of  $Ca^{2+}$  in MAL-Induced Apoptosis.** The effect of MAL on  $[Ca^{2+}]_i$  in A549 cells and rat thymocytes was assessed by using fura-2 (12) and with the  $Ca^{2+}$ -ATPase inhibitor thapsigargin as a positive control (19). Thapsigargin caused a sustained increase in  $[Ca^{2+}]_i$  and DNA fragmentation after 4 h in rat thymocytes (20, 21). In A549 cells, thapsigargin (100

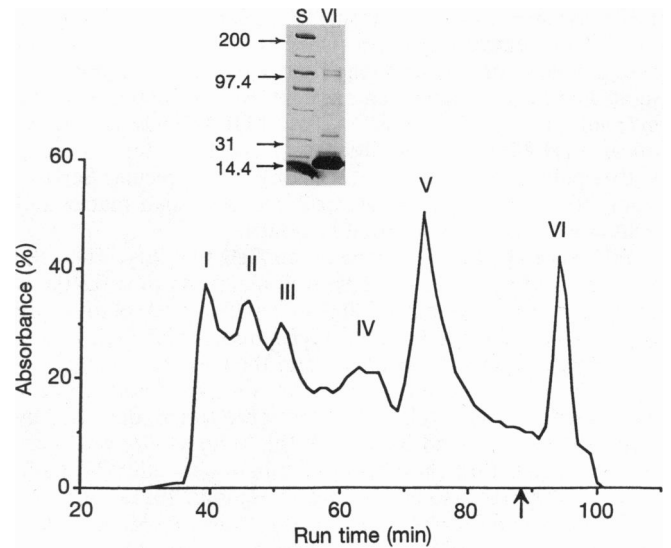


FIG. 2. Casein (100 mg) was fractionated on a DEAE-Trisacryl M ion-exchange column by FPLC. Eluted fractions were pooled as indicated (I–VI). Arrow indicates 100% 1 M NaCl. (Inset) SDS/PAGE of pool VI. Lane S, molecular size markers (kDa) (Bio-Rad).

nM) rapidly induced a sustained increase in  $[Ca^{2+}]_i$  (Fig. 4A), and DNA fragmentation occurred after 24 h (Fig. 4B).

MAL induced a concentration-dependent increase in  $[Ca^{2+}]_i$  in A549 cells and rat thymocytes after a 30-s exposure (Fig. 4). Cell death was prevented under calcium-free condi-

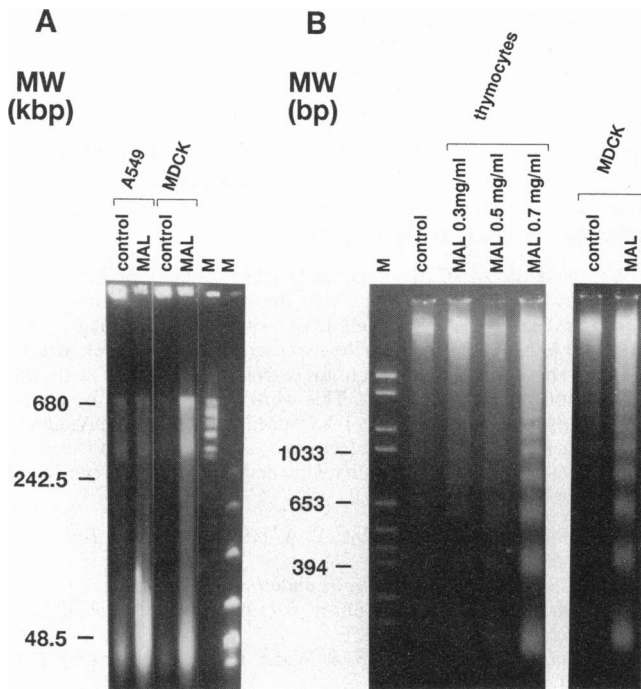
Table 2. Effects of MAL on viability of different cell types

Cells	Cell viability*			
	Medium		MAL	
	%†	cpm‡ (100%)	%†	cpm‡ (%)
<b>Cell lines</b>				
A549, human lung	95	21,750	0	50 (0)
NCI, human lung	91	28,500	0	15 (0)
A-498, human kidney	84	45,400	5	40 (0)
J 82, human bladder	92	7,800	4	20 (0)
Caco-2, human intestine	98	18,700	0	40 (0)
HT-29, human intestine	99	48,400	6	75 (0)
HTB9, human kidney	98	11,200	4	40 (0)
GMK, monkey kidney	72	14,500	0	50 (0)
Vero, monkey kidney	83	45,400	12	400 (1)
MDCK, dog kidney	95	53,700	4	15 (0)
B9, mouse	100	35,400	0	20 (0)
WEHI, mouse	90	14,400	1	20 (0)
<b>Embryonic cells</b>				
Hel, human lung	67	—	8	—
HFF, human foreskin	90	—	8	—
<b>Nontransformed cells</b>				
Nph epithelium, human	43	—	45	—
Urinary epithelium, human	77	—	75	—
Granulocytes, human	100	—	62	—
Lymphocytes, human	100	—	26	—
Kidney, mouse	46	—	62	—
Bladder, mouse	38	—	48	—
Thymocytes, rat	100	—	0	—

\*Cell viability was monitored by  $[^3H]$ thymidine incorporation and trypan blue exclusion. Viability of embryonic and nontransformed cells was assayed only by trypan blue exclusion, since these cell types did not incorporate  $[^3H]$ thymidine.

†% viability as determined by trypan blue exclusion.

‡cpm as determined by  $[^3H]$ thymidine incorporation. Medium control denotes 100%; numbers in parentheses denote (cpm sample  $\times$  100)/(cpm control). Means of three separate experiments are shown.



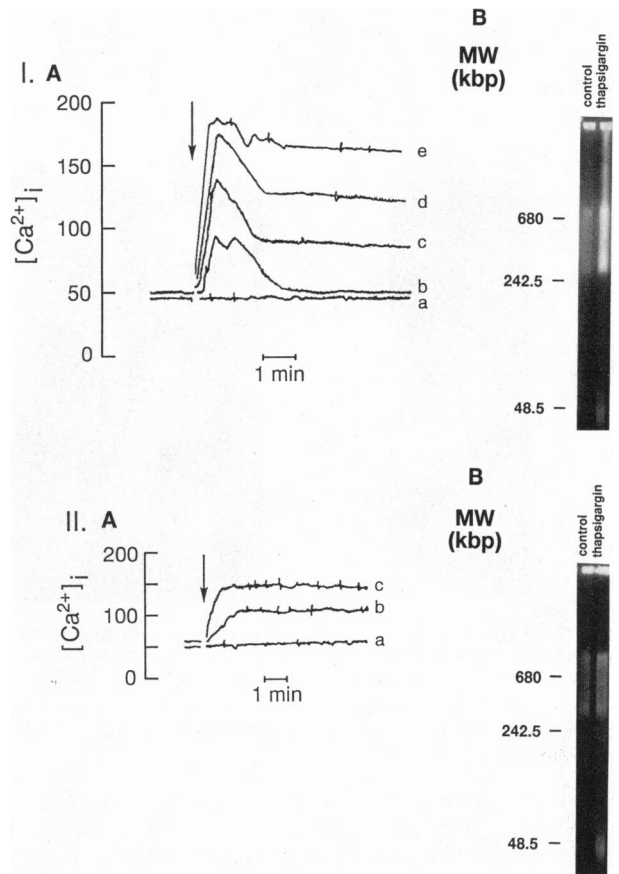
**FIG. 3.** Chromatin cleavage induced by MAL in A549 and MDCK cells and in rat thymocytes. A549 cells and MDCK cells were incubated with 1 mg of MAL per ml for 24 h. Rat thymocytes were incubated with MAL for 4 h. (A) Chromatin cleavage was analyzed by FIGE. DNA size calibration markers were from Sigma; chromosomes were from *Saccharomyces cerevisiae* (225–2200 kbp) (lane 4) and a mixture of DNA *Hind*III fragments, DNA, and DNA concatemers (0.1–200 kbp) (lane 5). (B) Oligonucleosome fragmentation was analyzed by gel electrophoresis. A mixture of fragments from pBR328 DNA cleaved separately with *Bgl* I and *Hinf* I were used as molecular size markers (lanes M) (Boehringer Mannheim).

tions, suggesting  $Ca^{2+}$  to be involved in the apoptotic signal. These results were confirmed by measurement of chromatin cleavage using FIGE. Formation of high molecular weight DNA fragments induced by MAL in MDCK cells was prevented by removal of  $Ca^{2+}$  from the medium (Fig. 5).

**DISCUSSION**

We report here that human milk induces apoptosis in several mammalian cell lines and immature cells. The apoptosis-inducing activity was in the casein fraction of human milk and was characterized as a multimeric form of human  $\alpha$ -lactalbumin. MAL induced apoptosis in transformed and nontransformed cell lines, both characterized by an immature phenotype. Embryonic cells and peripheral blood lymphocytes were also sensitive, while mature epithelial cells and cells from solid organs were resistant to the apoptotic effect of this milk factor. The induction of apoptosis by MAL required protein synthesis. MAL caused an increase in  $[Ca^{2+}]_i$  and did not trigger apoptosis under calcium-free conditions, suggesting a crucial role for the calcium ion in the process. In addition to the characteristic apoptotic morphology seen in MAL-sensitive cells, MAL-induced apoptosis in MDCK cells was associated with the appearance of both high molecular weight and oligonucleosome-length DNA fragments typical of apoptosis (2, 17), whereas MAL-treated A549 cells exhibited high molecular weight DNA fragments but no DNA ladders (cf. Fig. 3). Similar differences in apoptotic DNA fragmentation have previously been found in other cell types and may reflect differences in protease and/or nuclease activities between cell types.

The purification of MAL from human milk by anion-exchange chromatography was monitored by the biological activity of each fraction. SDS/PAGE profiles of the cyto-



**FIG. 4.** Effects of MAL and thapsigargin on  $[Ca^{2+}]_i$  in A549 cells and rat thymocytes. (IA) A549 cells: control (trace a); with MAL at 0.5 mg/ml (trace b), 1.0 mg/ml (trace c), and 2.0 mg/ml (trace d); or with 100 nM thapsigargin (trace e). (IB) A549 cells were exposed to 100 nM thapsigargin for 24 h and analyzed for high molecular weight DNA fragmentation; (IIA) Rat thymocytes: control (trace a), with MAL at 1 mg/ml (trace b), or with 100 nM thapsigargin (trace c). (IIB) Rat thymocytes were exposed to 100 nM thapsigargin for 24 h and analyzed for high-molecular weight fragmentation. Traces shown are representative of three separate experiments.

toxic fraction showed a major band at 14 kDa and minor bands in the 30- and 100-kDa regions. The N-terminal sequence of each band was identical to the sequence of  $\alpha$ -lactalbumin, but  $\alpha$ -lactalbumin itself lacked cytotoxic activity. However, the presence of additional minor bands on SDS/PAGE suggested that the active component may exist in a different aggregation state. By ESI-MS analysis, the molecular masses of the active component (14.088 kDa) and of  $\alpha$ -lactalbumin (14.061 kDa) were found to be similar, and the small differences ruled out most known posttranslational modifications. Analysis of the active protein by MALDI-MS showed peaks consistent with monomeric (14 kDa), dimeric (28 kDa), and trimeric (42 kDa) forms of  $\alpha$ -lactalbumin, suggesting that the apoptosis-inducing component was possibly a multimeric form of human  $\alpha$ -lactalbumin.

The factors that regulate the aggregation state of  $\alpha$ -lactalbumin in whole human milk are not understood. Both the bioactivity of whole milk and the size fractionation profile of the  $\alpha$ -lactalbumin component indicate that multimers of  $\alpha$ -lactalbumin occur naturally. Moreover, we could induce multimerization of monomeric  $\alpha$ -lactalbumin by anion-exchange chromatography in the presence of high concentrations of NaCl and recover the apoptosis-inducing activity of whole milk. Several peptides and proteins, including cytokines, bacterial toxins, and viral envelope proteins, induce apoptosis in mammalian cells by receptor-mediated signaling or by

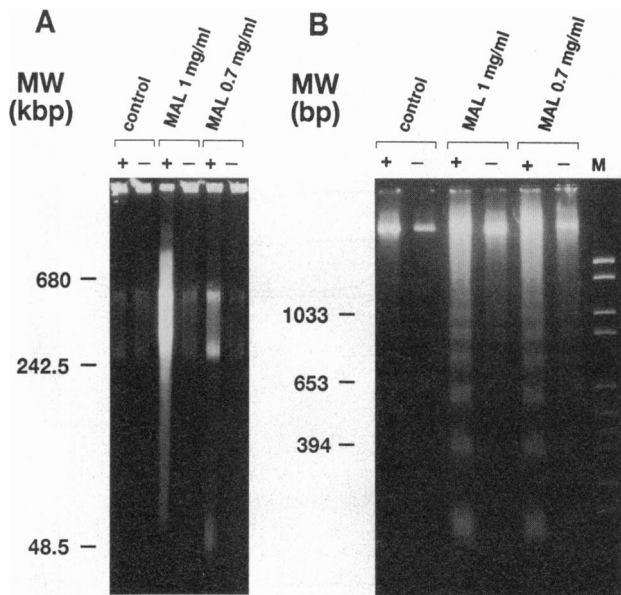


FIG. 5. Effect of removal of extracellular  $\text{Ca}^{2+}$  on formation of high molecular weight (A) and oligonucleosome (B) DNA fragments in MDCK cells. Cells were incubated in the absence or presence of MAL (1 mg/ml) in complete medium (lanes +) or in  $\text{Ca}^{2+}$ -free medium (lanes -) and analyzed for DNA fragmentation. Markers were the same as in Fig. 3.

formation of transmembrane pores (21–23). Whether the induction of apoptosis by MAL reflects cross-linking of receptors to which monomeric  $\alpha$ -lactalbumin normally binds without any cytotoxic consequence remains to be determined. Also, being in an aggregated state, MAL might possibly form transmembrane pores and evoke apoptosis by allowing the influx of  $\text{Ca}^{2+}$  and other solutes.

Calcium signals are crucial for induction of apoptosis in thymocytes by many agents, including antibodies to the T-cell receptor–CD3 receptor complex (18). The  $\text{Ca}^{2+}$ -binding S-100 protein from rat glial cells was recently found to induce apoptosis in PC-12 cells via a sustained increase in  $[\text{Ca}^{2+}]_i$  (24). Like the S-100 protein, MAL induced a rapid increase in  $[\text{Ca}^{2+}]_i$ , and extracellular  $\text{Ca}^{2+}$  was necessary for induction of apoptosis as shown by the protective effect on DNA fragmentation by removal of  $\text{Ca}^{2+}$  from the culture medium. As previously found with thymocytes (19, 20), apoptosis could also be induced in A549 cells by thapsigargin, a  $\text{Ca}^{2+}$ -ATPase inhibitor that produces a sustained increase in  $[\text{Ca}^{2+}]_i$ . Both the S-100 protein and human  $\alpha$ -lactalbumin contain binding sites for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions (25), although the mechanism of  $\text{Ca}^{2+}$  activation by these proteins is still unknown.

Although  $\alpha$ -lactalbumin is one of the most abundant proteins in human milk, its functions are not clear. Very recently, homozygous  $\alpha$ -lactalbumin-deficient mice were generated (26). Their milk was of such high viscosity that it could not be suckled. However, the state of the pups' gastrointestinal mucosa and gut-associated lymphoid systems was not reported. The potency and selectivity of the apoptosis-inducing activity of MAL lead us to suggest that it may act to restrict certain host cell populations. Cytotoxicity toward thymocytes and peripheral blood lymphocytes, which mostly bear  $\alpha\beta$  T-cell receptors, may help explain why the gut mucosa is normally populated by a distinct population of lymphocytes that is largely thymus independent and T-cell receptor  $\gamma\delta$  positive (27). This speculation needs to be tested. Also, it has been reported that human milk contains factors that regulate the proliferation and function of lymphocytes (28).

Unlike normal cells, tumor cells may fail to activate the apoptotic pathway. Indeed, a characteristic of most tumor cells

is the resistance to agents that induce apoptosis in nonmalignant cells. In contrast, MAL induced apoptosis in tumor cells but left other cells intact. Since tumor cells are often resistant to the normal apoptosis-inducing signals, our finding may be important in further elucidation of the pathways that lead to apoptosis in tumor cells. Finally, the ability of MAL to induce apoptosis in transformed, but not in mature, epithelial cells may help direct the neonatal mucosal epithelium toward maturity and away from neoplasia.

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