via vesicular membrane, and degradation and elimination (Fig. 3). Continual degradation of channels ensures that they will not persist after disappearance of the physiological stimulus for their synthesis (aldosterone). It remains unknown whether degradation is enzymatic or accelerated by agents in urine, and whether degraded channels are then broken down intracellularly like other proteins involved in turnover or discarded into urine as suggested by the effect of washing on $I_L$.

Vesicular membrane may be a common vehicle for transferring hydrophobic channels from intracellular sites of synthesis to the plasma membrane. If so, the scheme of Fig. 3 may be relevant to other transport processes that involve vesicular delivery systems (*11*).

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## Human Milk Kills Parasitic Intestinal Protozoa

Abstract. Giardia lamblia, *a common pathogenic intestinal parasite of humans, was rapidly killed by exposure to normal human milk in vitro. The killing did not depend on secretory immunoglobulin A.* Entamoeba histolytica, *the dysentery amoeba, was also killed by normal human milk.* Giardia*-cidal activity cochromatographed with an unusual lipase that is present in the milk of humans but not of lower mammals. Human milk may play a protective role in infants exposed to this parasite.*

*Giardia lamblia* is a major cause of waterborne enteric disease in the United States. Giardiasis may be debilitating, with severe diarrhea and malabsorption. Alternatively, symptoms may be moderate to mild or absent. Infection with this parasitic protozoan is especially prevalent in children and may cause failure to thrive. Some infections are resolved "spontaneously" within a few days, while others cause symptoms for years despite the presence of circulating or secretory antibodies (*1, 2*). Thus, nonimmune factors may influence the incidence, duration, and severity of giardiasis. Such factors are most likely to be present in the site colonized by this flagellate: the upper small intestine. They may be produced by intestinal tissue, secreted into intestinal fluid, or ingested (by breast-fed babies). Normal human milk (NHM) contains a number of antibacterial proteins, some of which are present in other mucosal secretions (*3*). In an animal model, infant mice were protected from *Giardia muris* infection only by milk from previously infected mice (*4*). To our knowledge, however, there has until now been no information concerning antiparasitic factors in *human* milk. We report here that NHM kills *Giardia lamblia* independently of secretory immunoglobulin A (S-IgA).

*Giardia lamblia* trophozoites were rapidly killed by low concentrations of NHM: 50 percent were killed in less than 30 minutes with 3 percent milk, in approximately 60 minutes with 1 percent milk, and in 280 minutes with 0.3 percent milk. At the lowest concentration a lag was followed by rapid killing (Fig. 1A). The lag was not reduced by first incubating the milk in assay medium for 1 or 3 hours. Milk (1 percent) from six other

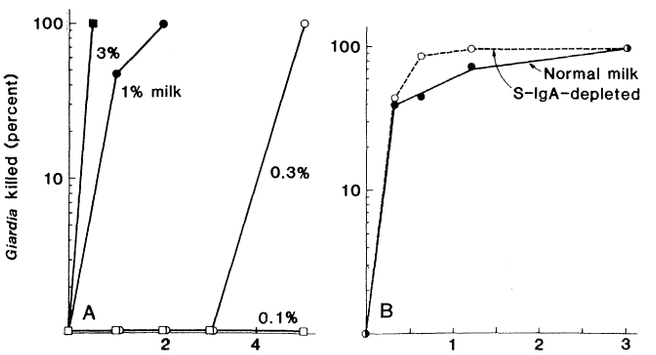


Fig. 1 (A). Kinetics of killing of *Giardia lamblia* trophozoites by NHM. Logarithmic-phase *Giardia lamblia* (ATTC 30888) trophozoites were grown in TP-S-1 growth medium (*6*). Nonattached parasites (including any dead organisms) were removed with the culture medium. Substrate-attached trophozoites were detached by incubation in cold, protein-free maintenance medium (*12*) and washed once in this medium and once in TP-S-1 without serum by centrifugation for 10 minutes at 2000*g* and 10°C. The parasites (5000 per milliliter) were exposed to various concentrations of filter-sterilized milk in serum-free TP-S-1 medium at 35.5° ± 0.5°C for 30 to 300 minutes. Killing was arrested and assayed by chilling the incubation mixtures and diluting them with complete TP-S-1 medium containing serum and melted agarose. This mixture was cooled to solidify it. Individual surviving parasites formed visible colonies after 5 to 6 days' incubation at 35.5°C (*13*). The percentage of parasites killed was calculated as 100 minus the percentage surviving, relative to controls lacking milk. (B) Killing of *G. lamblia* by NHM depleted of S-IgA. Trophozoites were incubated for 3 hours with various concentrations of normal milk or milk depleted (> 99 percent) of S-IgA. The percentage of parasites killed was determined as in (A). No killing was observed with control column buffer.

donors killed > 90 percent of the trophozoites in 180 minutes. None of the milk samples killed at a concentration of 0.1 percent. Unheated cow's milk or goat's milk did not kill *Giardia* during this time (*5–7*).

The *Giardia*-cidal activity of human milk is not due to its high concentration of S-IgA (*3*), because full activity was retained after passage over a column of sheep antibodies to S-IgA coupled to Sepharose 4B (Fig. 1B). This treatment decreased the S-IgA content of the milk to below that detectable by an enzyme-linked immunosorbent assay for nonspecific S-IgA (< 0.1 μg/ml). Neither immunoglobulin G nor immunoglobulin M was detected in the milk by radial immunodiffusion (< 5 μg/ml). Thus the *Giardia*-cidal activity of NHM is not due to antibodies.

Human milk appears to have more than one *Giardia*-cidal component, as the killing activity measured at 1 percent was lost after heating at 70°C for 5 minutes but activity stable to heating at 100°C for 20 minutes was detectable with 2 to 5 percent milk. In addition, most but not all of the activity was lost when the milk was reacted with diisopropyl fluorophosphate (DIFP), a specific esterase inhibitor that has been shown to inhibit the bile salt–stimulated lipase (BSL) of human milk (*7, 8*). This enzyme has not been detected in milk of subprimate mammals (*8*). Loss of BSL enzyme activity in heated or DIFP-treated milk is correlated with decreased killing activity (*7*). Thus we suspected that BSL might be one of the *Giardia*-cidal components of NHM.

Human milk was fractionated by molecular sieve chromatography. Both BSL activity and *Giardia*-cidal activity were found in the void volume of a Sephadex G100 column. The two activities also cochromatographed on Sepharose 6B (Fig. 2). Moreover, the parasites were killed by BSL purified to apparent homogeneity (*9*) but not by pure lipoprotein lipase, the other (minor) lipase found in NHM (*10*).

Bile salt–stimulated lipase has been shown to survive *p*H and enzymatic conditions approximating passage through an infant's stomach. In the small intestine bile salts such as sodium cholate and sodium taurocholate protect it from proteolysis (*10*) and stimulate BSL lipolytic activity to a degree dependent on the identity and concentration of the substrate (*11*). Our studies implicate BSL as a novel microbicidal component of human milk. The testing of other milk proteins for *Giardia*-cidal activity requires that they not be contaminated with lipase. To date, we have observed that neither human milk casein, lactalbumin, lactoglobulins, nor egg white lysozyme (Sigma) killed *G. lamblia* (< 5 percent killing in 3 hours at 1 mg/ml).

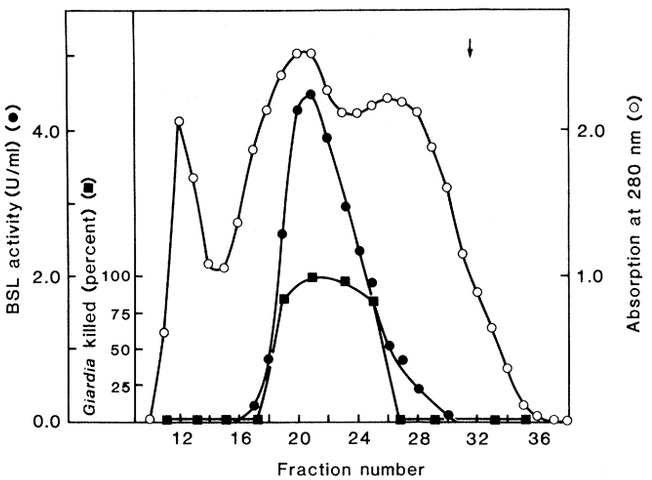


Fig. 2. Cochromatography of BSL and *Giardia*-cidal activity on Sepharose 6B. One hundred milliliters of human milk whey (milk brought to *p*H 4 with HCl to precipitate caseins and then to *p*H 7.4 with NaOH) was fractionated on a column of Sepharose 6B equilibrated with phosphate-buffered saline (*p*H 7.4) at 4°C. The column was eluted with the same buffer until 40 fractions (3 ml) were collected. Protein was estimated by absorption at 280 nm. The position of human milk lysozyme is indicated by an arrow. BSL activity was assayed spectrophotometrically with *p*-nitrophenylacetate as substrate (*11*). To assay killing, washed parasites (ATCC 30957) in TYI-S-33 medium lacking serum and bile (*6*) were incubated with filter-sterilized column fractions for 3 hours. Assays were then terminated by dilution with complete growth medium. Killing was determined by direct microscopic assay based on total disappearance of killed organisms after overnight incubation (*7*). This correlated well with data from the colony assay. There was no killing in controls lacking column fractions or incubated with column buffer alone.

To examine the range of antiparasitic activity, we tested two unrelated mucosal-dwelling pathogenic protozoa, *Entamoeba histolytica* and *Trichomonas vaginalis*, for sensitivity to NHM. The former, an amoeba, colonizes the human colon. The latter lives in the human urogenital tract. Both were killed (> 90 percent) by 1 percent NHM during a 3-hour exposure. *Giardia lamblia* and *E. histolytica* are transmitted by ingestion of cysts. Trophozoites that emerge from cysts in the small intestine of breast-fed children would be exposed to NHM and might be prevented from colonizing. Established infections would be less likely to be interrupted by NHM because trophozoites might not be exposed after they have penetrated the mucus layer.

The *Giardia*-cidal activity of NHM was not dependent on S-IgA, but secretory antibodies to *G. lamblia* might enhance parasite killing. Newborn mice were protected from infection with *G. muris* only if they were suckled by previously infected mothers (*4*). It is not known whether the protection was due to milk antibodies, leukocytes, or both. Natural immunity was not observed in the murine model.

In view of the potent antiparasitic activity of NHM in vitro, it is important to determine whether breast-fed children have a lower incidence of giardiasis or amebiasis than non–breast-fed children and to elucidate the mechanism of parasite killing.


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6. *Giardia* strain PO (ATTC 30888), isolated by E. A. Meyer and given to us by G. Visvesvara and G. Healy, was grown in TP-S-1 medium and used in early experiments [E. A. Meyer, *Exp. Parasitol.* **39**, 101 (1976); G. Visvesvara, *Trans. R. Soc. Trop. Med. Hyg.* **74**, 213 (1980)]. In later experiments, a *Giardia* strain (ATCC 30957) recently isolated by F.D.G. from the duodenal aspirate of a patient with chronic giardiasis (2½

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# Tumor-Derived Growth Factor Increases Bone Resorption in a Tumor Associated with Humoral Hypercalcemia of Malignancy

Abstract. *Evidence is presented that a tumor-derived transforming growth factor is responsible for stimulating bone resorption and causing hypercalcemia in an animal tumor model of the hypercalcemia of malignancy. Both conditioned medium harvested from cultured tumor cells and tumor extracts of the transplantable rat Leydig cell tumor associated with hypercalcemia contained a macromolecular bone resorbing factor with the chemical characteristics of a tumor-derived transforming growth factor.*

The mechanisms underlying tumor-associated hypercalcemia have not been clearly defined. Some tumors cause hypercalcemia without invading bone, presumably by releasing a systemic factor that stimulates bone resorption (*1*, *2*). A family of polypeptide growth factors called "transforming growth factors" (TGF's) are secreted by tumors and display some of the biological properties of epidermal growth factor (EGF) (*3–6*). Some (but not all) TGF's compete with EGF for binding to its receptor (*6*). Since EGF and other growth factors stimulate bone resorption (*7–9*), it is possible that a tumor-derived TGF with properties similar to those of EGF may be one of the factors responsible for increased bone resorption in the humoral hypercalcemia of malignancy. We report that both extracts from an animal tumor associated with hypercalcemia and conditioned medium from cultures of the tumor cells contain a bone resorbing factor, and that purified fractions of this factor contain biological activities associated with TGF's.

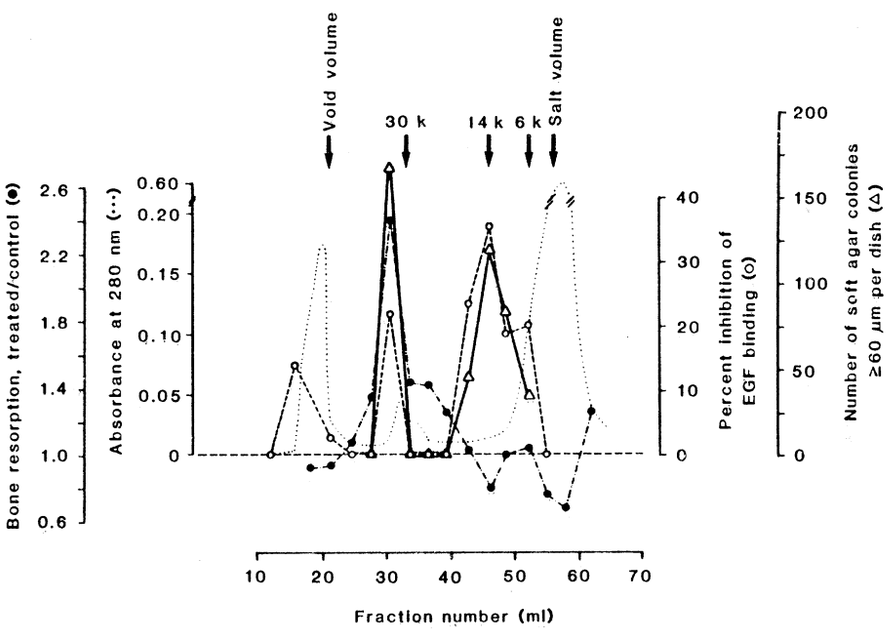


Fig. 1. Biological activities in extracts of Leydig tumors from hypercalcemic rats. Tumor (10 g) was extracted in acid and ethanol and chromatographed on a Bio-Gel P-100 column (1.5 by 40 cm) equilibrated with 1*M* acetic acid. Fractions (1 ml) were pooled in batches of three, divided into portions (2 ml for bone resorbing activity and 0.5 ml each for EGF competition and soft agar growth stimulating activity), and lyophilized. The portions were resuspended in the appropriate medium and tested for bone resorbing activity (bones incubated in control medium alone released 12.3 ± 2.4 percent of total $^{45}Ca$), EGF competing activity, shown as the percentage of [$^{125}I$]EGF specifically bound, and soft agar growth stimulating activity, as described in the legend to Table 1. Values for soft agar colonies were corrected for colonies in control plates (26.6 ± 1.4). Molecular weight markers are blue dextran for the void volume, carbonic anhydrase (29,500), ribonuclease (13,500), insulin (6000), and [$^{3}H$]proline for the salt volume.

The transplantable Leydig cell tumor that occurs in the aged Fischer rat causes hypercalcemia in these animals (*2*). The mechanism of hypercalcemia in this tumor model is increased osteoclastic bone resorption stimulated by a factor produced by the tumor cells. Hypercalcemic rats bearing the tumor have increased osteoclast numbers and activity in bones at sites distant from the tumor, increased urinary hydroxyproline, and reversal of hypercalcemia and the histological changes in bone after treatment with dichloromethylene diphosphonate, a drug that inhibits osteoclastic bone resorption (*10*). Hypercalcemia is due to a systemic mediator because excision of the tumor leads to reversal of the hypercalcemia (*2*, *11*). To identify the mediator of bone resorption responsible for hypercalcemia, we examined tumor extracts and conditioned medium from cultures of tumor cells for bone resorbing activity by using a bioassay for bone resorption that is based on the release of previously incorporated $^{45}Ca$ from fetal rat long bones in organ culture (*12*, *13*).

Pregnant rats in the 18th day of gestation were injected with 0.2 mCi of $^{45}Ca$. The following day the fetuses were removed and the mineralized shafts of the radii were excised and placed in organ culture. Some bones were cultured with tumor products to be tested for bone resorbing activity and other bones were cultured with control medium. Bone resorbing activity was found both in conditioned medium and in tumor extracts (Table 1 and Fig. 1). The activity in the conditioned medium was consistently lost over a fourfold dilution [test/control ratio (mean ± standard error) at one-half dilution, 2.51 ± 0.12; at one-fourth dilution, 1.53 ± 0.11; and at one-eighth dilution, 0.97 ± 0.03).

Freshly excised tumors were extracted with acid and ethanol, precipitated with ethanol and ether, and lyophilized (*5*). The lyophilized samples were chro-