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Short Communication

Cytotoxic Potential of Intraepithelial Lymphocytes (IELs)

Presence of TIA-1, the Cytolytic Granule-Associated Protein, in Human IELs in Normal and Diseased Intestine

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Human intestinal intraepithelial lymphocytes (IELs) have phenotypic characteristics of cytotoxic T cells, yet a cytotoxic function has not been demonstrated in redirected lysis assays. A monoclonal antibody that reacts with a cytotoxic granule-associated protein, TIA-1, was used in this study to identify this protein in many, but not all, IELs of normal human proximal small intestine. Furthermore, in active celiac disease, in which the number of IELs is significantly increased, a corresponding increase in the number of TIA-1 cells was found. These results indicate that whereas cytotoxicity of human IELs has been difficult to demonstrate, they contain at least one of the proteins associated with cytotoxicity, and a failure to demonstrate this function may be related to the in vitro assay system used. (Am J Pathol 1993, 143:350–354)

Intestinal intraepithelial lymphocytes (IELs) are located adjacent to the basement membrane and the basolateral aspect of enterocytes. Although the potentially strategic location of these lymphocytes has led to speculation regarding an important role in maintaining an intact mucosal barrier and possibly the pathogenesis of intestinal inflammatory conditions, their functional role has not yet been elucidated. Unlike other primary T cells, freshly isolated murine IELs are constitutively cytolytic.[1] This functional activity has been correlated with the presence of the cytolytic granule markers perforin and granzyme A.[2] The absence of cytolytic activity in the IELs of mice raised in a germ-free environment suggests that IELs are activated *in situ* by exposure to the contents of the intestinal lumen.[3]

The cytolytic capabilities of human IELs have long been the subject of controversy. Like their murine counterparts, human IELs are primarily $CD3^+$, $CD8^+$ T cells.[4–6] Human IELs have membrane-bound granules that resemble those present in cytolytic T cells. These lysosomal granules contain acid phosphatase, betaglucuronidase, and α-naphthyl acetate esterase, but do not contain peroxidase.[7] The presence of proteins associated with cytotoxicity has not previously been described in human IEL granules. Cytolytic T cell granules contain several well-characterized proteins, including the pore-forming protein perforin and

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a family of serine esterases known as granzymes. The granule exocytosis model of cytolytic T lymphocyte (CTL)-mediated lysis[8] postulates that insertion of the pore-forming protein perforin into target cell membranes, and its *in situ* polymerization, result in a striking increase in intracellular calcium concentration. Other granule components initiate a chain of events leading to programmed cell death and the accompanying fragmentation of target cell DNA.

Recently, Anderson et al have identified a 15-kD cytotoxic granule-associated protein, designated TIA-1, whose cytoplasmic expression is restricted to CTLs and natural killer (NK) cells.[9] Functional characterization of TIA-1 suggests that it may be the cytotoxic granule component responsible for inducing programmed death in target cells.[10] Other work has shown that immunohistochemical staining with anti-TIA-1 can be used to identify CTLs in epidermal lesions of human graft versus host disease.[11] We have used this cytolytic granule marker in this report to examine the human intestinal mucosa for the presence of potentially cytolytic lymphocytes.

Materials and Methods

Tissue Samples

Histologically normal human lymphoid tissues were obtained from surgical specimens submitted to the pathology department. Normal adult proximal small intestinal tissue was obtained from three individuals during surgical procurement of tissues for organ transplantation. Proximal small intestinal tissue from five children with active celiac disease was obtained during the initial diagnostic biopsy before treatment with a gluten-free diet. The diagnosis was established on the basis of malabsorption, subtotal villus atrophy, the presence of serum antiendomysial antibodies, and subsequent recovery on a gluten-free diet. Procurement of human tissues and consent forms were reviewed and approved by the Committee for the Protection of Human Subjects from research risks at Children's Hospital and Massachusetts General Hospital.

Monoclonal Antibodies

The monoclonal antibodies (MAbs) used for immunohistochemistry include anti-Leu4 (CD3) from Becton-Dickinson (Mountain View, CA), OKT8 (CD8) from Ortho Pharmaceutical Corp. (Raritan, NJ), and anti-TIA-1 MAb.

Immunohistochemistry

Tissue specimens were mounted in optimum cutting temperature compound (Ames Co., Elkhart, IN) and frozen in liquid nitrogen or in a cryostat and stored at −70 C. Four-μ tissue sections were air-dried and acetone-fixed for 10 minutes. Immunoperoxidase staining was done by the indirect avidin-biotin complex method.[6] Briefly, tissue sections were incubated with 2.5% horse serum in phosphate-buffered saline then an optimal dilution of the primary antibody for 1 hour followed by incubation with a 1:240 dilution of biotinylated horse anti-mouse antibody and a 1:120 dilution of avidin-biotinylated peroxidase complex (Vector Laboratories, Inc., Burlingame, CA). Each incubation was followed by three washes in phosphate-buffered saline, and endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in phosphate-buffered saline. The tissue sections were stained with a solution of 3-amino-9-ethyl-carbazole (Aldrich Chemical Co., Inc., Milwaukee, WI) as chromogen, postfixed in 4% paraformaldehyde, counterstained with hematoxylin, and mounted in Glycergel (Dako Corp., Santa Barbara, CA). Nonspecific reactivity of the MAbs of interest was checked by staining several sections in parallel in which the primary antibody was omitted or an irrelevant primary antibody (CD1a, CD4) was used.

Quantitation of Mucosal Lymphocytes

Epithelial cells and stained lymphocytes within the epithelium were counted and expressed as the number of lymphocytes per 100 epithelial cells. At least 1,000 epithelial cells were counted in serial sections for each MAb. Lamina propria lymphocytes were counted with a reticular grid and expressed as the number of lymphocytes per 0.25 square millimeter. Statistical analysis of the data was performed using Student's *t*-test.

Cytotoxicity Assay

Target cell lysis by human IELs was measured by redirected lysis in a standard ^{51}Cr release assay.[12] Human IELs were purified by Percoll gradient fractionation as described.[13] The murine Fc receptor-bearing cell line P815 was used as the target cell for redirected lysis. Target cells were labeled in sodium chromate (^{51}Cr, 1 mCi/ml, New England Nuclear, Boston, MA) for 1 hour at 37 C and then washed in complete Roswell Park Memorial Institute Medium (RPMI 1640 supplemented with 10%

fetal calf serum, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, and 50 μmol/L β mercaptoethanol). They were then precoated with a 1:50 dilution of anti-human CD3 (Leu 4, Becton Dickinson) for 30 minutes at 4 C. The target cells were washed and varying numbers of effector cells (in 100 ml of medium were added to 1×10^4 ^{51}Cr-labeled targets (in 100 μl) and incubated at 37 C for 4 hours. Cells were pelleted by centrifugation, supernatants were assayed for radioactivity, and percentage-specific ^{51}Cr release was calculated from $100 \times (a - b)/(t - b)$ where a is ^{51}Cr release in the presence of effector cells, b is the spontaneous release from labeled target cells in the absence of effector cells (< 10%), and t is the total ^{51}Cr content of the target cells (released by the addition of 1% of NP-40). Phytohemagglutinin-stimulated peripheral blood lymphocytes served as a positive control.

Results

The mucosa of normal small intestine contained many lymphocytes stained with the MAbs against CD3 and CD8. Nearly as many CD8$^+$ lymphocytes (82%) were identified as CD3$^+$ cells within the epithelium, whereas in the lamina propria, the CD8$^+$ lymphocytes represented a smaller proportion of the CD3$^+$ cells (57%) (Table 1). These results are consistent with previously published reports on the distribution of lymphocytes in human intestinal mucosa.[4–6,14] The anti-TIA-1 antibody demonstrated perinuclear and cytoplasmic staining of at least 50% of the number of CD3$^+$ cells in the epithelium and a smaller proportion (28%) within the lamina propria (Figure 1).

Mucosal biopsies from children with active celiac disease contained many more lymphocytes within the epithelium stained by all the monoclonal antibodies. Both anti-CD8 and anti-TIA-1 identified similar numbers of IELs, which represented a majority of the CD3$^+$ IELs. The number of CD8$^+$ and TIA-1$^+$ cells in the lamina propria were also similar and represented a minority of cells compared to CD3$^+$ lymphocytes. Thus, in contrast to normal intestine, the proportion of TIA-1$^+$ cells to CD8$^+$ cells were nearly equal in both the epithelium (100%) and the lamina propria (95%) in celiac disease. Also, the total number of lymphocytes, including TIA-1$^+$ cells was greatly increased in celiac disease.

We were unable to detect cytotoxicity by human IELs in several experiments using the NK cell target K562 or in redirected lysis assays in which an antibody to CD3 was bound to the Fc receptor-bearing murine target cell line P815 (data not shown). As a positive control, phytohemagglutinin-stimulated human peripheral blood lymphocytes lysed 50 to 60% of the anti-CD3 coated target cells at the highest effector target ratio tested (40:1).

Table 1. *Distribution of TIA-1$^+$ Cells in Normal Intestine and Celiac Disease*

	Normal small intestine (n = 3)		Celiac disease (n = 5)	
MAb	IEL	LPL	IEL	LPL
CD3	7.6 ± 5.2	229 ± 67	37.4 ± 7.3	148 ± 29
CD8	6.2 ± 3.7	130 ± 53	28.4 ± 4.4	61 ± 17
TIA-1	3.9 ± 2.8	48 ± 30	28.2 ± 7	58 ± 17

IEL = number of Stained lymphocytes/100 epithelial cells (with SD), LPL = number of Stained lymphocytes/0.25 mm^2 (with SD) in lamina propria.

Discussion

Two new findings have emerged from these studies. First, many but not all CD8$^+$ IELs contained TIA-1. In the lamina propria, which has many fewer CD8$^+$ T cells, only about 25% of the lymphocytes expressed TIA-1. Second, in active celiac disease, where the number of CD3$^+$, CD8$^+$ IELs is significantly increased,[14] there was a corresponding increase in the population of TIA-1$^+$ cells, especially among the IELs. The numbers of CD8$^+$ and TIA-1$^+$ lymphocytes present in the epithelium and lamina propria were virtually identical. The intestinal injury (subtotal villous atrophy, crypt hyperplasia, and increase in mucosal lymphocytes) in celiac disease occurs secondary to permanent intolerance to dietary gliadin; the injury is reversed by the elimination of gluten from the diet. It is possible that the increase in mucosal lymphocytes with cytotoxic potential may be involved in the mucosal injury of celiac disease.

Murine IELs are known to have NK activity as well as T cell receptor-mediated cytotoxicity in redirected lysis assays.[2] This functional activity has been correlated with the presence of the cytotoxic granule markers perforin and granzyme A. MAbs reactive with perforin have indicated its presence in cytotoxic human TCR Γδ lymphocyte clones as well as lymphokine-activated killer cells.[15] Immunoperoxidase staining of human cardiac allograft rejection tissue has demonstrated granular cytoplasmic staining with an anti-perforin MAb in 1 to 10% of the mononuclear infiltrate. However, no staining with the anti-perforin antibody was observed in normal human intestinal epithelium.[16] Whereas, human IELs

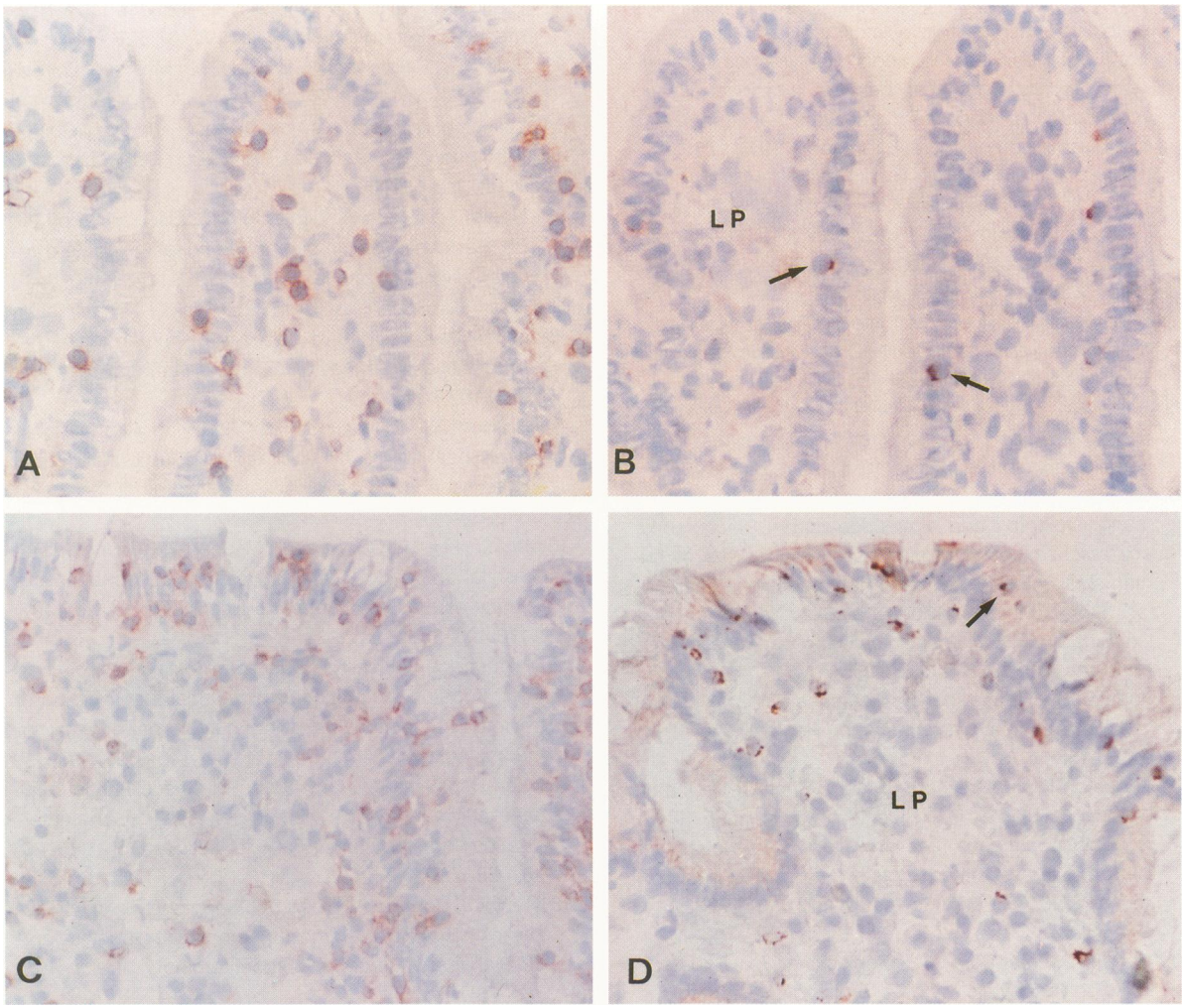

Figure 1. *Immunohistochemical staining of normal proximal small intestine* (A *and* B) *and small intestine of a patient with active celiac disease* (C *and* D), *using MAb anti-Leu 4* (*CD3*) (A *and* C) *and anti-TIA-1* (B *and* D). *Arrows indicate TIA-1$^+$ IEL. Several scattered TIA-1$^+$ lymphocytes are present in the lamina propria* (*LP*) *of the celiac disease tissue. Immunoperoxidase, counterstained with hematoxylin* (*400×*).

contain membrane-bound granules, they rarely express the NK cell markers and have no NK activity as detected by killing K562 target cells.[6,17]

Repeated attempts to demonstrate cytolytic activity by human IELs in conventional ^{51}Cr release assays[12] have been unsuccessful in our laboratories (data not shown). The cytolytic activity of freshly isolated CD3$^+$ IELs was assayed in a redirected lysis assay using a monoclonal antibody to CD3 bound to the Fc receptor-bearing target cell P815 (murine IELs display potent cytolytic activity in this assay). We also failed to detect lysis of the natural killer cell target K562 by human IELs. This observation is in agreement with the work of Taunk et al who reported that jejunal IELs spontaneously lyse a variety of epithelial cell tumor lines but not K562. The spontaneous cytotoxicity detected by Taunk et al, was not mediated by classical NK cells because the cytolytic effector cells were CD3$^+$, CD8$^+$ T cells.[18] Therefore, although a cytolytic function of human IELs has not been clearly defined, it is now apparent that they are capable of cytolytic activity in some assay systems and contain at least one granule protein associated with cytotoxic functions.

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