Brief Definitive Report

Cytotoxic Differentiation of Mouse Gut Thymodependent and Independent Intraepithelial T Lymphocytes Is Induced Locally. Correlation between Functional Assays, Presence of Perforin and Granzyme Transcripts, and Cytoplasmic Granules

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Summary

Mouse gut intraepithelial lymphocytes (IEL), whether thymodependent ($CD4^+$ or CD8 α/β^+; TCR-α/β^+) or thymoindependent (CD8 α/α^+; TCR-α/β^+ or -γ/δ^+), all display cytotoxic activity in a "redirected lysis assay" using anti-CD3 or anti-TCR β or δ chains secreting hybridomas as targets; this is also observed with IEL of germ-free mice, indicating that this activity, which is absent in peripheral T lymphocytes, does not require stimulation by bacterial antigens. Perforin and granzyme transcripts are detectable in unselected gut IEL, in contrast to normal T lymphocytes of peripheral lymphoid organs. Cytological labeling (with [^{3}H]DFP) of IEL smears reveals labeled granules (i.e., containing serine-esterases, presumably granzymes) in all subsets of gut IEL. This indicates that the gut micro-environment has an inductive role on the cytotoxic differentiation of lymphocytes of various origins when they reach the gut wall to become IEL.

It has recently been shown that gut intraepithelial lymphocytes (IEL) have a dual origin (1, 2). About 45% are the progeny of thymodependent lymphoblasts elicited to proliferate in Peyer's patches under antigenic stimulation and migrate through the thoracic duct (TD) lymph and the blood to seed all along the gut mucosa (3). These thymodependent IEL express TCR-α/β and bear either CD8, or, for a small minority (~10%), CD4 molecules. Their CD8 molecules are made of α (Lyt-2) and β (Lyt-3) chains (CD8 α/β^+ cells), as is also the case for all peripheral CD8 T lymphocytes. About 55% are thymoindependent, arising from bone marrow progenitors migrating directly to the gut, and bear TCR-α/β or -γ/δ. These thymoindependent IEL are easily identifiable since, strikingly, they bear CD8 molecules made only of homodimeric α chains, without β chains (CD8 α/α^+). All subsets of IEL display a peculiar cytological feature: a high percentage of them (20–70%) contain intracytoplasmic granules with sulfated proteoglycans, found in all species studied: rabbits (4), mice (3), rats (5), and humans (6). The function of IEL is incompletely understood, but evidence exists that, whether obtained from normal or nude mice, they can be cytotoxic in vitro, as has been found using "redirected" lysis assays (7–9).

In this study, we compared IEL and their subpopulations with peripheral T lymphocytes from normal mice, from germ-free mice, or from mice with an acute graft-vs.-host reaction (GVHR), a condition leading to a strong in vivo stimulation of grafted T cells. We attempted to correlate the level of cytotoxic activity of these various T lymphocytes (using as targets hybridoma cells secreting anti-CD3 or anti-TCR β or δ mAbs) with that of perforin and granzyme mRNAs transcripts, serine esterase enzymatic activity, and presence of cytoplasmic granules. Perforin and granzymes are indeed thought to be involved in the cytotoxic process (for review, see reference 10).

Materials and Methods

Animals and In Vivo Treatment. C57Bl/6, C_3HeB, and C_3H-DBA/2 mice were raised in our animal house. Germ free (C_3HeB) mice were a kind gift of C. Moreau (INRA CNRZ, Jouy an Josas, France). GVHRs were elicited in lethally irradiated C_3H-DBA/2 mice with C_3H lymphocytes (11).

Lymphocytes Surface Labeling, Selection, and Cytological Procedures. Detection of surface T cell antigens and cell separation were performed as previously described (1). Thymoindependent IEL were obtained by labeling IEL with anti-CD4 (GK1.5), anti-CD8 α/β (H35-17-2), or anti-Thy-1 mAb (TB107), incubation with goat anti-rat Ig–coated steel beads (Dynabeads-Dynal, Oslo, Norway), and sorting of the labeled cells with a magnet (MACS-Miltenyi Biotec Bergisch, Gladbach, FRG). Peripheral T lymphocytes were

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obtained after incubation with goat anti-mouse Ig-coated steel beads. Detection of Di-isopropyl phosphorofluoridate (DFP) binding serine esterases was performed on paraformaldehyde-fixed smears (12) with 10^{-5} M [^{3}H]DFP (Amersham, Burlinghamshire, England) applied for 30 min. This type of labeling can be performed on cells previously stained for surface antigens by fluorescent mAbs, but is not compatible with dye-staining of the granules.

Cytotoxic Assays and Northern Blot Analysis. Lymphocytes were incubated with 10^4 ^{51}Cr-labeled hybridomas secreting anti-CD3 (13), anti-TCR β (14), or anti-TCR δ (GL3; a kind gift of L. Lefrançois [8]) for 3 h, at varying E/T ratios. Percent lysis was calculated as 100× [(cpm release with effector cells − cpm release alone)/(cpm release by ClH acid − cpm release alone)].

Northern blot analyses, RNA preparations, transfer to Biodyne membrane, and hybridizations were as described (15). For the detection of granzyme mRNA, an oligonucleotide, 5'TTTAGTTCTCTTGGCCTTACTCTC (complementary to a sequence starting on nucleotide 197, 309, and 346, respectively, of the three different granzymes cDNAs described as MCSP-1, -2, and -3 by Kwon et al. [16]), was end labeled with ^{32}P. For the detection of perforin mRNA, a riboprobe containing the EcoRI-Hind III fragment of the mouse perforin cDNA (position 255–766 of the coding region) was used (17) (kind gift of Drs. E. Podack and C. Mueller).

Results and Discussion

In "redirected" cytotoxic assays involving the TCR-CD3 complex, using anti-CD3, anti-TCR β, or anti-TCR δ mAb-secreting hybridomas as targets (Fig. 1), total IEL and their thymoindependent subset (obtained by depletion of CD4$^+$ and CD8 α/β^+ cells or of all Thy-1$^+$ cells) are cytotoxic against the three hybridomas. The cytotoxicity of the thymoindependent population is not altered when it is depleted of its Thy-1$^+$ component, which represents a minority of this population (10–40%; reference 1); this is in contrast to reports that only Thy-1$^+$ IEL are cytotoxic in comparable assays (7, 8). In the thymoindependent population, TCR-α/β^+ and TCR-γ/δ^+ IEL have comparable cytotoxic abilities. Since TCR-γ/δ^+ IEL are found only in the thymoindependent CD8 α/α^+ population (1), thymus-derived IEL should not be cytotoxic for the anti-δ hybridomas cells; this population, however, cannot be adequately separated. When IEL were obtained from mice with acute GVHR induced by the injection of foreign peripheral T lymphocytes, a condition in which all IEL are of donor origin and thus of the thymus-derived variety (1, 11), they indeed displayed cytotoxicity against the anti-CD3 and the anti-TCR β, but not against the anti-TCR δ hybridomas (Fig. 1). In contrast, IEL obtained from germ-free mice, which are in small amounts but predominantly thymoindependent (CD8 α/α^+) and TCR-γ/δ^+ (1) are strongly cytotoxic against anti-δ and slightly against anti-β hybridomas (Fig. 1). Thus, contrarily to prior suggestion (7, 8), IEL do not need an in vivo exogenous stimulation to develop cytotoxic capabilities.

Contrarily to IEL, normal circulating T lymphocytes (thoracic duct lymphocytes or TDL, which contain the precursors of thymus-derived CD8 α/β^+ and CD4$^+$ IEL; references 1 and 3) do not show any cytotoxic activity (Fig. 1). However, when TDL are obtained during a GVHR and thus are, as

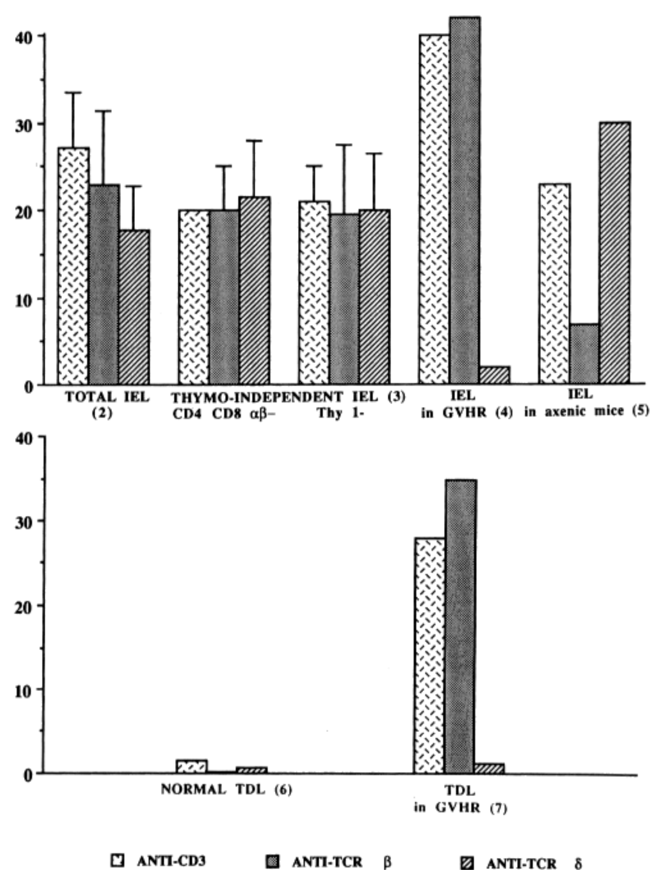

Figure 1. Cytotoxic activity of various populations of gut IEL compared with that of TDL, in redirected lysis assays using secreting hybridomas as targets (1). Results are shown at a 33:1 E/T ratio (see Materials and Methods). Standard deviations were calculated from the results of three to five experiments. The other percentages are the mean of two experiments with close results. For germ-free mice, a single experiment was carried out. (*2*) TCR-γ/δ^+ cells among IEL: 22 ± 7.5%. (*3*) Cytotoxicity against the anti-TCR δ hybridoma does not increase much although there are more TCR-γ/δ^+ in this population (1), but it appears that there is some nonspecific cell loss during the isolation procedure. (*4*) Residual host cells detected with an anti-H2 antibody: <4%. Cells containing TCR-γ/δ^+: 0%. Dye-stained cytoplasmic granules in these experiments: 34% and 49%. (*5*) IEL pooled from eight germ-free mice, with 13% TCR-α/β^+ and 80% TCR-γ/δ^+ cells. (*6*) TDL are deprived of B cells. TCR-γ/δ^+ lymphocytes in the population used: 1.2%. (*7*) Residual host cells: <1%. TCR-γ/δ^+: 0%. Cells containing dye-stained cytoplasmic granules in these experiments: 4.8% and 1%.

the GVHR IEL described above, of donor origin and cytotoxic against the host alloantigens (11), they are cytotoxic against anti-CD3 and anti-β, but not anti-δ hybridomas (Fig. 1).

Since perforin and granzymes are detected in cytotoxic lymphocytes in culture or in immunopathological reactions and are localized in cytoplasmic granules (10), the presence of perforin and of granzyme transcripts in IEL was explored by Northern blot analysis. Perforin and granzyme mRNAs were detectable in gut IEL but not in peripheral lymph node T cells nor in TDL, except in conditions of GVHR; i.e., when stimulated cytotoxic grafted cells represent almost all of the T lymphocytes present in the circulation, the lymphoid organs,

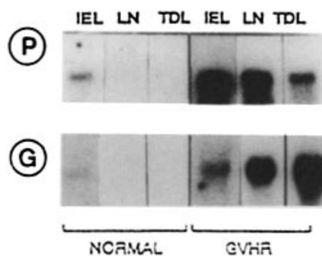

Figure 2. Northern blots of RNA extracts from IEL and TDL using perforin cmRNA (*P*) or granzyme DNA ^{32}P labeled (*G*) probes and showing bands of ~3 kb (perforin) and ~900 bp (granzyme).

and gut IEL (Fig. 2). Since granzymes are serine-esterases; i.e., enzymes characterized by their ability to react with DFP (18), smears of IEL and other T cells were exposed to [^{3}H]DFP. IEL fixed [^{3}H]DFP but TD lymphocytes did not, even the TD blasts that are the precursors of thymus derived IEL (3). The localization and frequency of [^{3}H]DFP silver grains coincided with that of the granules: (*a*) silver grains were distributed in clusters, frequently localized at one pole of the cell, near a nuclear invagination containing the dye-stained granules (Fig. 3); (*b*) the percentages of granule containing IEL and that of [^{3}H]DFP-labeled cells were very close (Table 1). Combination of [^{3}H]DFP binding with determination of the IEL subsets by immunofluorescence showed the presence of silver grains in all subsets: CD4$^+$ and CD8 α/β^+ thymus-derived IEL as well as thymoindependent CD8 α/α^+ IEL, which were the most frequently granulated (81%) and bore especially numerous silver grains. In contrast to normal TDL, [^{3}H]DFP grains were also detected in ~25% of GVHR TD blasts, but they were not concentrated in clusters, and granules are rare or absent in GVHR TD blasts (Fig. 3; Table 1). This observation probably indicates that perforin and granzyme storing granules occur late in differentiation rather than in still rapidly dividing cells.

In conclusion, it appears that the gut microenvironment exerts a differentiation influence on lymphocytes becoming located within the epithelium, whatever their origin, their TCR type, or the presence of Thy-1 molecules, leading to synthesis and storage of perforin and granzymes, accompanied by the development of a cytotoxic activity that is not found in peripheral T lymphocytes, except when they are activated.

Table 1. *Granular Differentation in IEL and Circulating Lymphocytes Labeling with [^{3}H]-DFP*

	Normal mice		Mice with GVHR	
	IEL	TDL	IEL	TDL
Percent giemsa-stained granules	69	<0.5	22	<1
Percent [^{3}H]-DFP-labeled cells*				
Clustered labeling	67	0	21	<1
Dispersed labeling	<2	0	4	24

* See Materials and Methods.

What could be the role of IEL with this newly acquired cytotoxic ability? They may insure the defense of the epithelia integrity by rapidly eliminating altered enterocytes; the rapidity of epithelium renewal, which increases in pathological conditions such as GVHR (11), may compensate for cell destruction. Since in normal conditions IEL are not cytotoxic when binding to targets is obtained by PHA (data not shown), recognition of the targets must involve TCR activation. This may occur in a classical MHC-restricted way during viral infections (19); or this may occur in "unclassical" ways, involving other types of specificity, since we have found that IEL (but not unactivated peripheral T cells) are cytotoxic against xenogenic targets bearing enterotoxins with "superantigens" properties (our unpublished observation).

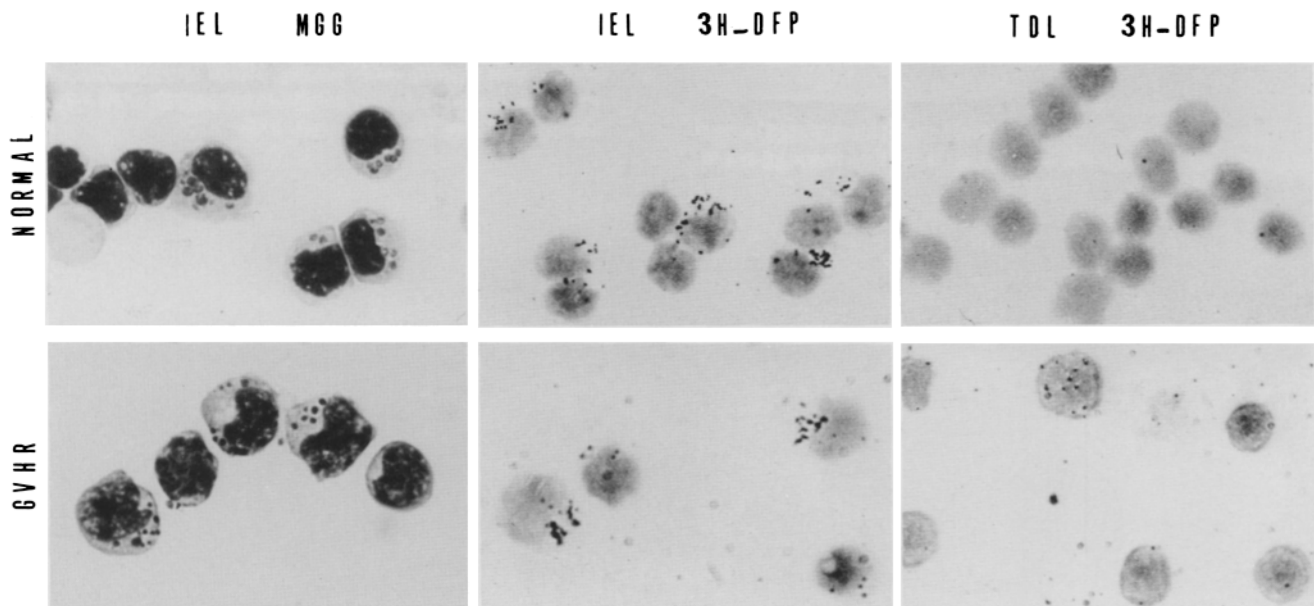

Figure 3. Intracytoplasmic granules and [^{3}H]DFP labeling patterns in IEL and TDL in normal mice and in mice with GVHR.

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