Brief Definitive Report

ONTOGENY OF THE Thy-1$^-$, Lyt-2$^+$ MURINE INTESTINAL INTRAEPITHELIAL LYMPHOCYTE

Characterization of a Unique Population of Thymus-independent Cytotoxic Effector Cells in the Intestinal Mucosa

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Intraepithelial lymphocytes (IEL) comprise a large heterogeneous population of immune cells located in the intestinal epithelium of man and rodents. Specific mechanisms of IEL-mediated immunity are not well defined, due in part to difficulties in isolating IEL subsets, and in part to the rather arcane nature of the IEL themselves. Two fundamental characteristics distinguish the IEL from cells of other peripheral immune compartments. First, phenotypic studies of isolated murine IEL show that 80–90% of the cells are Lyt-2$^+$ lymphocytes, of which about half bear Thy-1$^+$ surface antigens (1, 2). The origin and functional role of the Thy-1$^-$, Lyt-2$^+$ IEL are largely unknown (1). It has been proposed (3, 4) that some or all of those cells represent a thymus-independent type of effector IEL, though formal evidence for this has not been demonstrated. Secondly, murine IEL isolates are characterized as having a variety of natural effector activities. These include cytotoxic activities mediated by NK cells (1, 2, 5, 6), spontaneous cytotoxic (SC) cells (6), and natural cytotoxic (NC) cells (2). The relationship of those effector responses to the Thy-1$^-$, Lyt-2$^+$ IEL is uncertain.

The present study has characterized IEL according to the expression of murine lymphocyte antigenic markers, including three surface antigens not previously identified on IEL. As reported here, in both thymus-bearing and athymic nude mice, an unusually high proportion of IEL from unprimed animals expressed an antigen associated with activated cytotoxic cells and a lymphocyte marker present on immature T cells. These findings directly address issues of IEL ontogeny and function, and provide evidence that some cytotoxic IEL may originate by a thymus independent lineage.

Materials and Methods

Mice. Female BALB/cBy mice, 8–10 wk of age, were obtained from The Jackson Laboratories, Bar Harbor, ME. Athymic nude mice (*nu/nu*) of BALB/c background, 8–10 wk of age, were purchased from the Athymic Nude Facility, UCSD.

Monoclonal Antibodies. mAb against murine lymphocyte surface antigens used in these

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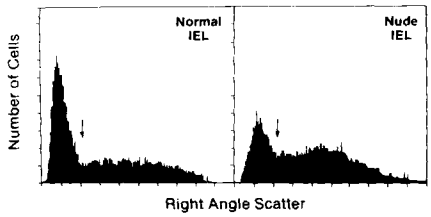

FIGURE 1. Size distribution of IEL from normal and athymic nude mice as determined by flow cytometry analyses. Arrows indicate separation of small (left of arrow) and large (right of arrow) IEL populations.

experiments were as follows: H0.13.4 (7), anti-Thy 1.2; AD4(15) (8), anti-Lyt 2.2; GK 1.5 (9), anti-L3T4; CT-1 (10), anti-CT; and J11d (11), antilymphocyte.

Isolation of IEL. Procedures used to isolate intestinal IEL have been previously described (12). These consist of an EDTA-extraction of the intestinal epithelium and IEL, followed by density gradient separations on Percoll (1). As determined from paraffin-embedded tissue sections, this procedure permits isolation of IEL without penetration into the lamina propria layer.

Flow Cytometry Analysis of Cell Surface Antigens. Analyses of lymphocyte surface markers were done by indirect immunofluorescence, using the primary mAb listed above, followed by FITC-conjugated rabbit anti-mouse or rabbit anti-rat immunoglobulin. Stained cells were analyzed on an Ortho Cytofluorograph, system 50-H (Ortho Diagnostics, Westwood, MA).

Assay for Cytotoxic Activity. Percoll-passed IEL effector cells and dilutions were added in 100 μl to 96-well microtiter plates. ^{51}Cr-labelled target cells (P815 or YAC-1) were added to effector cells in 100 μl at a final concentration of 10^4 targets cells/well. Cultures were incubated for 4 h at 37°C in 5% CO_2. Complement-mediated depletion of IEL subsets was done by incubating 20–30 × 10^6 IEL with monoclonal anti-CT ($^1/_{100}$ dilution of ascites) or anti-J11d (undiluted tissue culture supernatant) for 45 min at 4°C, followed by rabbit complement ($^1/_{10}$ dilution) at 37°C for 45 min. Treated IEL were passed through Ficoll-Hypaque, and viable cells were assayed for cytotoxic activity. Specific lyses were determined as follows: percent specific lysis = 100 × [cpm release experimental group) − (cpm spontaneous release)]/[(cpm after detergent lysis) − (cpm spontaneous release)].

Results

Expression of T Lymphocyte Antigens on Intestinal IEL. Percoll-fractionated IEL and splenic lymphocytes from unprimed normal and nude mice were studied by flow cytometry analyses for the expression of Thy-1, Lyt-2, L3T4, CT-1, and J11d surface antigens. IEL were analyzed on the cell sorter according to small and large cell populations, (Fig. 1). Small IEL, which were similar in size to resting splenic or lymph node lymphocytes, made up 44% of normal IEL and 38% of nude IEL (Table I). Large IEL were two to four times the size of small IEL, and comprised 56% of normal and 62% of nude IEL. Small IEL from thymus-bearing mice were predominantly Lyt-2^+, L3T4^- lymphocytes, of which about half possessed Thy-1 surface antigens. In contrast, 98% of large IEL, although also Lyt-2^+ and L3T4^-, did not express Thy-1 surface antigens. Thus, nearly 70% of the IEL, overall, were Thy-1^-, Lyt-2^+, L3T4^- cells. Spleen cells from athymic nude mice were essentially void of Thy-1^+ and Lyt-2^+ lymphocytes, whereas IEL from nude mice, which were also Thy-1^-, were Lyt-2^+ for approximately half the cells. The data pertaining to Thy-1 and Lyt-2 expression on IEL correlate with findings of others (1, 2) describing the distribution of those T cell

TABLE I

Cell Surface Antigens on Intestinal and Splenic Lymphocytes from Nude and Thymus-bearing Mice

Mouse	Lympho-cyte source	Cell population	Percent of IEL	Percentage of cells expressing:*				
				Thy-1	L3T4	Lyt-2	CT-1	J11d
Normal	Spleen	Small	—	29 ± 7	ND	ND	3 ± 2	66 ± 9
	IEL	Small	44 ± 3	63 ± 3	6 ± 3	90 ± 6	55 ± 7	17 ± 6
	IEL	Large	56 ± 7	3 ± 2	1 ± 1	82 ± 5	52 ± 4	60 ± 11
Nude	Spleen	Small	—	2 ± 2	1 ± 1	2 ± 2	9 ± 7	91 ± 5
	IEL	Small	38 ± 6	1 ± 2	3 ± 1	56 ± 10	61 ± 5	62 ± 11
	IEL	Large	62 ± 4	2 ± 1	2 ± 2	48 ± 18	78 ± 6	48 ± 8

* As determined by flow cytometry analyses. Data represent mean values ± SEM of 3–5 experiments, each consisting of pooled IEL isolates from four mice.

markers on murine IEL, and are important because they indicate that IEL isolates used in the following experiments represent typical IEL populations.

IEL Express a Cytotoxic Activation Antigen and an Antigen Associated with Immature T Cells. The most surprising findings of these studies pertain to the level of CT and J11d antigen expression on IEL. 50–60% of both large and small IEL from thymus-bearing mice expressed the CT-1 cytotoxic activation antigen. CT-1 antigens were also present on IEL from nude mice to a level equivalent to that found on IEL of thymus-bearing animals. In contrast to the IEL, <3% of spleen cells from thymus-bearing mice were CT-1$^+$, a finding consistent with other studies (10) of the expression of CT antigens in murine spleen cells of normal unprimed mice. Similarly, in thymus-bearing mice, the majority of large and some small IEL were J11d$^+$; in nude mice, roughly half of both small and large IEL were J11d$^+$. Since >95% of the large IEL in thymus-bearing mice were Thy-1$^-$ cells, J11d must be expressed primarily on a Thy-1$^-$, Lyt-2$^+$ IEL population. J11d antigen expression on unfractionated spleen cells was consistent with that reported by others (11), and as expected, nearly all splenic lymphocytes from nude mice were J11d$^+$.

Natural Effector Activities Present in IEL Isolates are Associated with CT$^+$ and J11d$^+$ Subsets. To determine whether CT$^+$ and J11d$^+$ IEL subsets were associated with functional IEL effector populations, fresh IEL isolates were treated with antibody (CT-1 or J11d) plus complement before assay for cytotoxic activity against YAC-1 (NK-sensitive) and P815 (SC-sensitive) target cells. Both NK and SC cytotoxic activities present in IEL isolates were abrogated by treatment with either CT-1 or J11d antibodies (Fig. 2), indicating that both antigens are associated in some way with cytotoxic effector populations of the IEL. Although J11d is specific for nearly all B cells (see Discussion), the finding that J11d was expressed on cytotoxic IEL rules out the possibilities that J11d$^+$ IEL are B cells present in the intestinal epithelium, or that IEL isolates had been contaminated with B cells.

Discussion

The Thy-1$^-$, Lyt-2$^+$ type of IEL constitutes a large proportion (50–70%) of the total IEL, as demonstrated here and as reported by others (1, 2, 14). However, the ontogeny and functional nature of this cell type is a matter of controversy.

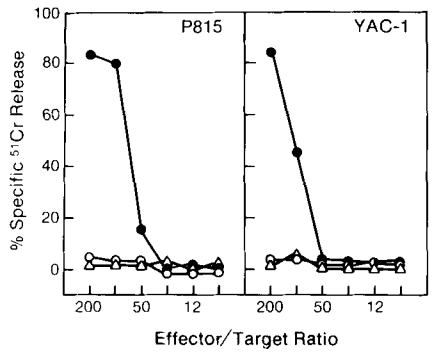

FIGURE 2. Abrogation of cytotoxic activities mediated by SC (P815 target) and NK (YAC-1 target) cell populations following treatment of IEL with CT-1 antibody (○) or J11d antibody (△) plus complement. Complement control without specific antibody (●).

On the one hand, it has been proposed that those IEL represent thymus-independent cells that have acquired lytic activity upon maturation within the intestinal epithelium (3, 4). Alternatively, the Thy-1$^-$, Lyt-2$^+$ IEL may be derived from peripheral CTL that have lost Thy-1 antigen expression.

The finding that more than half of all IEL from unprimed mice express the cytotoxic activation antigen, CT-1, indicates that, as a group, the IEL consist of a population of cytotoxic cells that are highly activated and presumably lytic. Because spleen cells from the same animals used for IEL studies were predominantly CT-1$^-$, similar to what has been reported by others (10) for normal spleen cells, it appears that the intestinal epithelium represents a site uniquely enriched for activated cytotoxic cells. The presence of CT-1 antigens on Thy-1$^-$, Lyt-2$^+$ IEL from thymus-bearing mice (e.g., large IEL) is important in several ways. It shows that CT antigens can be associated with a population of Thy-1$^-$ as well as Thy-1$^+$ cytotoxic cells. It also suggests that some effector IEL may represent a thymus-independent type of IEL. However, the data do not formally rule out the possibility that those IEL originated from Thy-1$^+$ peripheral CTL. More convincing evidence of a thymus-independent origin for the Thy-1$^-$, Lyt-2$^+$ IEL comes from the data pertaining to CT antigen expression on nude IEL, where 60–70% of the IEL were CT-1$^+$ and Thy-1$^-$. The presence of Lyt-2 antigens on IEL but not on splenic lymphocytes from nude mice further implicates the intestinal epithelium as a site of local activation of non–thymus-derived cytotoxic precursors. Moreover, the finding that CT-1 antigen is expressed on nude IEL correlates with observations by others (13) that nude IEL possess lytic activity to NK-sensitive target cells.

J11d expression on IEL was unexpected, since J11d is a murine lymphocyte marker associated with B cells and is not present on mature or activated T cell populations (11). J11d is, however, present on some immature T cells, notably on cortical thymocytes (11). That J11d on IEL reflects a T cell marker rather than a B cell marker is suggested in the following ways. First, it is generally accepted that IEL are essentially void of B cells; <5% of the IEL bear surface Ig (J. R. Klein, unpublished observation, and ref. 14). Consistent with that fact are the observations in the present study that nearly all IEL were either Lyt-2$^+$ (80–90%) or L3T4$^+$ (6–8%) cells, and as such, J11d must be expressed on one or

both of those IEL subsets. Second, IEL depleted of J11d$^+$ cells lost lytic activity mediated by NK and SC effector populations, indicating that J11d$^+$ IEL were associated with a population of lytically active cells. These findings, which for the first time link J11d antigen expression to functional cytotoxic Lyt-2$^+$ cells, although seemingly incompatible with current knowledge of J11d expression on lymphocytes, may not be incongruent when considering the biology of the IEL. Rather, the data are taken to indicate that the J11d$^+$ cell reflects a thymus-independent type of IEL, i.e., an "immature" T cell that has acquired lytic activity within the intestinal epithelium, and has retained the J11d surface marker. This interpretation applies most notably to the large IEL, which are primarily Thy-1$^-$ cells. However, when all IEL were simultaneously stained for Thy-1 and J11d antigens using a double staining technique, <5% of the IEL were positive for both Thy-1 and J11d antigens (data not shown), suggesting that Thy-1 and J11d are distributed on discrete IEL subsets. Thus, by inference, a substantial proportion of the IEL must be Thy-1$^-$, Lyt-2$^+$, L3T4$^-$, J11d$^+$ cells with lytic activity.

Taken together, the findings reported here suggest that some IEL originate as non–thymus-derived cells that acquire lytic activity presumably within the intestinal epithelium itself. Local activation of effector cell precursors could occur upon exposure to T cell–derived lymphokines such as IL-2. IL-2 has been shown to induce IEL-derived cytotoxic clones to express NK lytic activity (12). Moreover, a mechanism of effector activation such as this would explain recent observations (6) which show that, during an antigen-specific CTL response within the intestinal mucosa, NK and SC cytotoxic activities are simultaneously increased. Finally, it should be noted that the observations described here now can be addressed through molecular studies of T cell receptor gene rearrangements, particularly with respect to the Thy-1$^-$, Lyt-2$^+$, J11d$^+$ cytotoxic IEL.

Summary

Murine intestinal intraepithelial lymphocytes (IEL) from unprimed thymus-bearing and athymic nude mice were characterized according to the expression of murine lymphocyte antigenic markers and cytotoxic activity. The majority of IEL from thymus-bearing mice were Lyt-2$^+$, L3T4$^-$ lymphocytes, over half of which did not express Thy-1 surface antigens. Nude IEL and spleen cells were void of Thy-1$^+$ cells; however, Lyt-2 antigens were expressed on a significant proportion of IEL, but not splenic lymphocytes. Overall, 40–70% of IEL from either thymus-bearing or athymic mice expressed the cytotoxic activation antigen, CT-1, and the J11d lymphocyte marker, both of which were associated with a population of Thy-1$^-$, Lyt-2$^+$ cytotoxic IEL. These data are taken to mean that the intestinal epithelium is a site uniquely enriched for activated cytotoxic cells, a significant proportion of which originate as non–thymus-derived lymphocytes with acquired lytic activity.

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References

1. Petit, A., P. B. Ernst, A. D. Befus, D. A. Clark, K. L. Rosenthal, T. Ishizaka, and J. Bienenstock. 1985. Murine intestinal intraepithelial lymphocytes. I. Relationship of a novel Thy-1$^-$, Lyt-1$^-$, Lyt-2$^+$ granulated subpopulation of natural killer cells and mast cells. *Eur. J. Immunol.* 15:211.
2. Ernst, P. B., A. D. Befus, and J. Bienenstock. 1985. Leukocytes in the intestinal epithelium: an unusual immunologic compartment. *Immunol. Today.* 6:1.
3. Mayrhofer, G. 1980. Thymus-dependent and thymus-independent subpopulations of intestinal intraepithelial lymphocytes: a granulated subpopulation of probable bone marrow origin and relationship to mucosal mast cells. *Blood.* 55:532.
4. Mayrhofer, G., and R. J. Whately. 1983. Granular intraepithelial lymphocytes of the rat small intestine. I. Isolation, presence in T lymphocyte–deficient rats, and bone marrow origin. *Int. Arch. Allerg. Appl. Immunol.* 71:313.
5. Tagliabue, A., W. Luini, D. Soldateschi, and D. Boraschi. 1981. Natural killer activity of gut mucosal lymphoid cells in mice. *Eur. J. Immunol.* 11:919.
6. Klein, J. R., and M. F. Kagnoff. 1984. Nonspecific recruitment of cytotoxic effector cells in the intestinal mucosa of antigen-primed mice. *J. Exp. Med.* 160:1931.
7. Marshak-Rothstein, A., P. Fink, T. Grindley, D. H. Raulet, M. J. Bevan, and M. L. Gefter. 1979. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J. Immunol.* 122:2491.
8. Gottlieb, P. D., A. Marshak-Rothstein, K. Auditone-Hargreaves, D. B. Berkoben, D. A. August, R. M. Rosche, and J. D. Benedetto. 1980. Construction and properties of new Lyt-congenic strains and anti-Lyt-2.2 and Lyt-3.1 monoclonal antibodies. *Immunogenetics.* 10:545.
9. Dialynas, D. P., F. S. Quan, K. A. Wall, A. Pierres, J. Quintas, M. R. Loken, M. Pierres, and F. W. Fitch. 1983. Characterization of the murine T cell surface molecule designated L3T4 identified by monoclonal antibody GK 1.5: Similarity of L3T4 to the human Leu-3/T4 molecule. *J. Immunol.* 131:2445.
10. Lefrancois, L., and M. J. Bevan. 1985. Functional modifications of cytotoxic T-lymphocyte T200 glycoprotein recognized by monoclonal antibodies. *Nature (Lond.).* 314:449.
11. Bruce, J., F. W. Symington, T. J. McKearn, and J. Sprent. 1981. A monoclonal antibody discriminating between subsets of T and B cells. *J. Immunol.* 127:2496.
12. Klein, J. R., L. Lefrancois, and M. F. Kagnoff. 1985. A murine cytotoxic T lymphocyte clone from the intestinal mucosa that is antigen specific for proliferation and displays broadly reactive inducible cytotoxic activity. *J. Immunol.* 135:3697.
13. Alberti, S., F. Colotta, F. Speafico, D. Delia, E. Pasqualetto, and W. Luini. 1985. Large granular lymphocytes from murine blood and intestinal epithelium: comparison of surface antigens, natural killer activity and morphology. *Clin. Immunol. Immunopathol.* 36:227.
14. Parrot, D. M. V., C. Tait, S. Mackenzie, A. M. Mount, M. D. J. Davies, H. S. Micklem. 1983. Analysis of the effector functions of different populations of mucosal lymphocytes. *Ann. N.Y. Acad. Sci.* 409:307.