NONSPECIFIC RECRUITMENT OF CYTOTOXIC EFFECTOR CELLS IN THE INTESTINAL MUCOSA OF ANTIGEN-PRIMED MICE

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The intestinal mucosa is a major host barrier to foreign antigen entry. Yet, little is known about specific cellular immune mechanisms of the gut-associated lymphoid tissues (GALT). Studies using human and murine GALT have demonstrated natural killer (NK) and antibody-dependent, cell-mediated cytotoxic responses and lectin-mediated cytotoxic activities of intestinal lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL) (1–4). Cytotoxic T lymphocyte (CTL) responses to minor histocompatibility antigens and alloantigens have also been demonstrated using mesenteric lymph node and Peyer's patch cells (5). Considerably less is known about antigen-specific cytotoxic responses of the IEL and LPL (6). For this reason, we have examined cytotoxic responses of murine gut IEL after local and systemic priming with alloantigens. Our results detail a series of previously undescribed immunologic events that occur in the intestinal mucosa, but not in the spleen, in response to antigen. Concomitant with the generation of a specific cytotoxic response by gut IEL, additional cytotoxic effector populations are nonspecifically activated locally.

Materials and Methods

Mice.   8–12-wk-old female BALB/cBy mice were obtained from The Jackson Laboratory, Bar Harbor, ME.

Immunization of Mice and Preparation of Effector Cells.   Mice were injected intraperitoneally (i.p.) with 10^7 viable EL-4 thymoma cells. 10 d later, animals were challenged intraperitoneally and intragastrically (i.g.) with 10^7 EL-4 cells. 3 d postchallenge, mice were sacrificed, the small intestine was removed and flushed of fecal material, and Peyer's patches were dissected out. The intestine was opened and cut into 2–4-mm pieces and transferred to a beaker containing 25 ml of phosphate-buffered saline with 1 mM EDTA (PBS-EDTA). Tissues were stirred at 37°C for 20 min. Cells were collected and tissue fragments were stirred in fresh PBS-EDTA for an additional 20 min. Pooled cell suspensions were rapidly filtered through 300 mg of nylon wool in a 10-cc syringe, and recovered cells were washed twice in RPMI 1640 containing fetal calf serum (FCS). This procedure yielded 40–60 × 10^6 lymphoid cells per mouse with >95% viability. Spleen cells were prepared by dissociation of the tissue with forceps and pipetting with a pasteur pipette.

Target Cell Lines and Cytotoxic Assay. EL-4, P815, and YAC-1 cell lines were maintained in RPMI 1640 containing 10% FCS. Spleen cell blasts were prepared by culturing 2–4 × 10^6 nucleated spleen cells for 3 d in 5 ml of RPMI 1640 containing 10% FCS and either 3 μg/ml concanavalin A (Con A) or 50 μg/ml lipopolysaccharide-B (LPS) (*Escherichia coli* 055:B5; Difco Laboratories, Inc., Detroit, MI). Cytotoxic assays were conducted in 96-well microtiter plates. 10^6 IEL, or dilutions, were added to wells in 100 μl of RPMI 1640 + 10% FCS (assay medium). 10^4 ^{51}Cr-labeled target cells (EL-4, P815, YAC-1, or splenic blasts) were added to wells in 100 μl of assay medium and cultures were incubated for 4 h at 37°C in 5% CO_2. The amount of ^{51}Cr present in cell-free supernatants was measured in an automatic gamma counter and the percent specific lysis was calculated as follows: Percent specific lysis = [(cpm release experimental group − cpm spontaneous release)/ (cpm detergent release − cpm spontaneous release)] × 100.

Cold Target Competition Assay. 50 μl of 1 × 10^4 ^{51}Cr-labeled P815 mastocytoma cells were mixed with 50 μl of 3 × 10^4 unlabeled EL-4, P815, YAC-1, or SP2/0 cells. These were added to 1 × 10^6 or 5 × 10^5 gut IEL from EL-4 primed BALB/c mice. After 4 h incubation at 37°C in 5% CO_2, the amount of released ^{51}Cr was determined as described above.

In Vivo Treatment of Mice with Anti-Asialo GM-1 Antibody. BALB/c mice were immunized with 10^7 EL-4 tumor cells by intraperitoneal injection. 10 d later animals were challenged with 10^7 EL-4 i.p. and i.g. and injected intravenously with 100 μl of undiluted anti-asialo GM-1 antibody (Wako Pure Chemical Ind., Dallas, TX). 3 d postchallenge, animals were sacrificed, and IEL and splenic lymphocytes were tested for cytotoxic activities against ^{51}Cr-labeled EL-4, P815, and YAC-1 target cells.

Results and Discussion

BALB/c (H-2^d) mice were immunized with allogeneic EL-4 (H-2^b) thymoma cells as described in Materials and Methods. Both gut and splenic lymphocytes from primed mice lysed EL-4 (antigen-specific target) and YAC-1 (NK-sensitive target) (Fig. 1, *A* and *C*). However, unlike splenic lymphocytes, IEL from primed mice were highly cytotoxic for P815 mastocytoma cells (Fig. 1 *C*). Moreover, the anti-P815 and anti-YAC-1 activities of IEL from primed mice appear to have been generated during the antigen-specific response to EL-4, since normal IEL were not cytotoxic for P815 or YAC-1 (Fig. 1 *D*). Unlike splenic lymphocytes (Fig. 1 *B*), IEL from unprimed mice did not kill YAC-1 NK-sensitive targets (Fig. 1 *D*). It appears that during an antigen-specific response within the intestinal mucosa, additional cytotoxic responses are generated locally to levels that are not usually present in normal GALT.

To define the immunologic specificity of gut IEL, EL-4-primed BALB/c mice were tested for cytotoxic activity against allogeneic and syngeneic target cells. IEL from EL-4-primed BALB/c mice did not lyse syngeneic BALB/c Con A–induced spleen cell blasts, but did lyse EL-4, P815, and YAC-1 cells (Fig. 2*A*). Similarly, syngeneic BALB/c spleen cell blasts induced by LPS were not killed, whereas allogeneic LPS blasts from C57BL/6 mice were lysed (Fig. 2*B*). These experiments demonstrate that the nonspecific activation of cytotoxic effector cells within the gut after antigen priming is an immune event that discriminates between foreign and self antigens.

To further probe the requirements for the nonspecific activation of gut cytotoxic responses, mice were primed with allogeneic spleen cells, i.e., cells bearing alloantigens expressed on non-tumor cells. This was done to rule out the possibility that cytotoxic responses of gut lymphocytes may reflect preferential
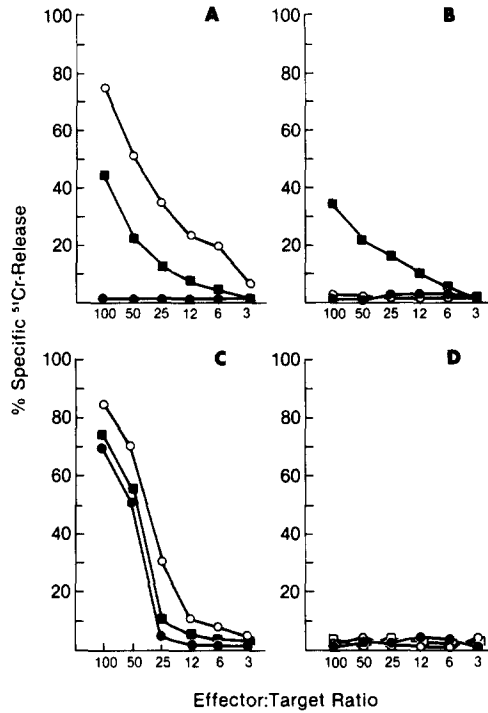
FIGURE 1.   Cytotoxic responses of splenic lymphocytes from EL-4-primed (*A*) and unprimed (*B*) mice, and of gut IEL from EL-4-primed (*C*) and unprimed (*D*) mice when tested against ⁵¹Cr-labeled EL-4 (O), P815 (●), and YAC-1 (■) target cells.
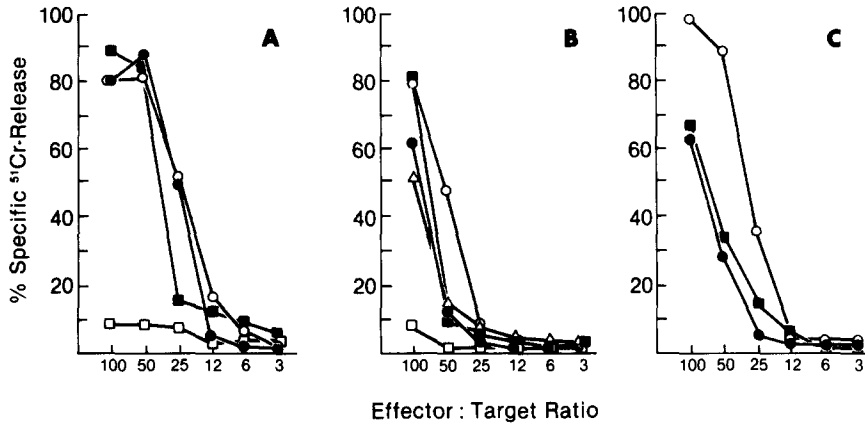

FIGURE 2.   Cytotoxic activity of gut IEL from EL-4-primed BALB/c mice tested against EL-4 (O), P815 (●), YAC-1 (■) and BALB/c Con A–induced spleen cell blasts (□) (*A*); and against C57BL/6 (△)and BALB/c (□) LPS-induced spleen cell blasts (*B*). Cytotoxic activity of gut IEL from BALB/c mice primed with 10⁷ C57BL/6 allogeneic spleen cells tested against EL-4 (O), P815 (●), and YAC-1 (■) target cells (*C*).

recognition of tumor antigens shared by EL-4, P815, and YAC-1. BALB/c mice were immunized intraperitoneally with 10^7 freshly isolated C57BL/6 spleen cells suspended in unsupplemented RPMI 1640. Although gut IEL from those mice show a slight preference for killing of EL-4 cells, YAC-1 and P815 cells were also lysed (Fig. 2 C), indicating that the activation of multiple cytotoxic responses within the gut is not limited to antigen presentation via tumor cells.

Since YAC-1 and P815 cells are known (7, 8) to be susceptible to lysis by murine NK and spontaneous cytotoxic (SC) cells, respectively, studies were undertaken to determine whether NK and SC activities account for YAC-1 and P815 killing by gut IEL from primed mice, and if so, whether one or more than one effector population is present. It has been reported (9) that cloned antigen-specific CTL lines, when maintained in medium containing high levels of interleukin 2 (IL-2) can express NK surface antigens and NK-like cytotoxic activities. Other studies have reported the acquisition of multiple cytotoxic specificities in CTL miniclones derived by micromanipulation (10) or by limit dilution (11). Thus, the appearance of multiple cytotoxic activities within the gut of primed mice could reflect a single effector population that is responsible for the killing of YAC-1, P815, and EL-4 cells. Alternatively, several effector populations may be activated locally within the gut of primed mice. Recently (12), it has been reported that memory CTL can be induced to express cytotoxic function when cultured with IL-2 in the absence of antigen.

Two experimental approaches were used to define the type(s) of effector populations activated within the intestinal mucosa. The first approach was based on the awareness that in vivo treatment of mice with anti-asialo GM-1 antibody depletes NK, but not CTL, activity (13). EL-4-primed BALB/c mice were injected intravenously with 100 μl of undiluted anti-asialo GM-1 antibody 3 d before a cytotoxic assay. Antibody treatment completely abrogated YAC-1 killing by both splenic and gut lymphocytes (Fig. 3), indicating that NK cells were responsible for those activities. However, anti-EL-4 and anti-P815 responses of IEL, and anti-EL-4 killing by spleen cells, were retained in antibody-treated mice.

A second group of experiments used cold target competition studies to characterize cytotoxic cells responsible for anti-P815 activity of IEL from primed mice. SC cells are known (8) to lyse P815 and SP2/O cells, another SC-susceptible cell line, but not EL-4 or YAC-1 cells. IEL from BALB/c mice primed with EL-4 were tested for their ability to lyse ^{51}Cr-labeled P815 cells in the presence of unlabeled EL-4, YAC-1, P815, or SP2/O. Neither EL-4 nor YAC-1 blocked P815 killing, whereas both SP2/O and P815 effectively competed for the killing of P815 by IEL (Table I). These data, in conjunction with the above experiment, suggest that discrete cytotoxic effector populations are activated within the intestinal mucosa during an antigen-specific response.

We consider the findings described here to be highly important with respect to immune defense mechanisms within the GALT. In the case of enteric infection, the nonspecific recruitment of unrelated immune effector cells may prevent the establishment of secondary opportunistic infections. A mechanism of immunity such as this would be particularly relevant within the GALT since the intestinal mucosa, unlike the spleen, is perpetually exposed to numerous environmental pathogens. Although the mechanisms by which gut cytotoxic re-
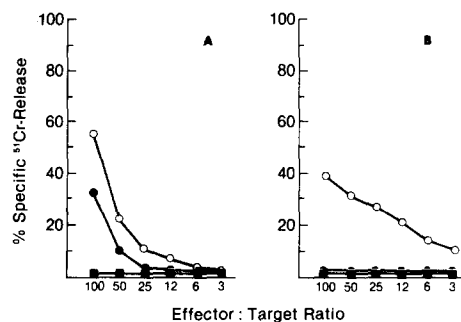
FIGURE 3.   Abrogation of NK cytotoxic activity in mice after in vivo treatment with anti-asialo GM-1 antibody. Gut IEL (*A*) and splenic lymphocytes (*B*) from EL-4-primed BALB/c antibody-treated mice were tested for cytotoxic activity against EL-4 (O), P815 (●), and YAC-1 (■) target cells.

TABLE I

Cold Target Competition of P815 Killing by Gut IEL

^{51}Cr-labeled target cell	E/T* ratio	Percent specific lysis of P815 in presence of cold target competitor‡				
		None	EL-4	YAC-1	P815	SP2/0
P815	100:1	71	74	66	20	28
	50:1	32	25	17	0	0

* Ratio of gut IEL effector cells to ^{51}Cr-labeled P815 target cells.
‡ Unlabeled target cells were added to ^{51}Cr-labeled P815 at a ratio of 3:1.

sponses are controlled have not been determined as yet, regulation may be under the influence of lymphokines elaborated during the course of an antigen-specific response. Our data further define the types of cytotoxic cells that make up the IEL, demonstrating the simultaneous appearance of three distinct effector populations: cytotoxic T lymphocytes, natural killer cells, and spontaneous cytotoxic cells. Finally, the findings reported here describing the in vivo activation of NK and SC responses correlate with recent studies (9, 10, 14, 15) demonstrating the generation of these activities in vitro using human and murine CTL.

Summary

We have examined cytotoxic responses of lymphocytes derived from the gut epithelium of mice primed systemically and enterically with alloantigens. Both gut intraepithelial (IEL) and splenic lymphocytes from alloantigen-primed mice were found to contain antigen-specific cytotoxic T cell activity. However, after priming, gut IEL also developed high levels of natural killer and spontaneous cytotoxic cell activities. We suggest that this nonspecific activation of additional cytotoxic effector populations during an antigen-specific response is an important host immune defense within the intestinal mucosa.

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