Stimulation of Lamina Propria Lymphocytes by Intestinal Epithelial Cells: Evidence for Recognition of Nonclassical Restriction Elements

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Summary

We have addressed the restriction elements involved in the interaction of lamina propria lymphocytes (LPL) and intestinal epithelial cells using the model of primary mixed cell culture reaction. Whereas peripheral blood T cells proliferate in response to both allogeneic non T cells and class II antigen-bearing intestinal epithelial cells (non T cells >> epithelial cells), LP T cells appear to proliferate preferentially in response to intestinal epithelial cells. The interaction between these cells does not appear to be restricted by conventional products of the major histocompatibility complex as neither monoclonal antibodies to class I nor to class II antigens inhibit the mixed cell cultures, whereas they are inhibitory in conventional mixed lymphocyte reactions. Furthermore, treatment of epithelial cells with interferon γ fails to augment the cells' ability to induce proliferation of LPL while successfully enhancing proliferation of peripheral blood T cells in parallel cultures. Taken together, these data suggest that alternate restriction elements or mucosa-specific accessory molecules may exist on intestinal epithelial cells that are preferentially recognized by LPLs. Such a distinct regulatory network may be critical to the maintenance of immunologic homeostasis in the gut.

Materials and Methods

Specimens. Surgical specimens from patients undergoing bowel resection for cancer (10 cm away from tumor), Crohn's disease, ulcerative colitis, or other inflammatory diseases at the Mount Sinai Hospital, were used as a source of epithelial cells, intraepithelial lymphocytes (IELs), and LPLs.

Isolation of PB T and Non T Cells. Heparinized venous blood was obtained from normal donors, diluted 1:2 with PBS, and separated by Ficoll-Hyphaque (Pharmacia, Piscataway, NJ) density centrifugation to obtain isolated PBMC. Further separation of mononuclear cells into T and non T cell populations was performed by rosetting with sheep erythrocytes as previously described (19). Monocytes and dendritic cells were isolated by plastic adherence.

Isolation of LPLs. The method used to isolate LPLs was a modification of the original technique of Bull and Bookman (20). Briefly, surgical specimens were rinsed in PBS vigorously to remove loosely adherent material, and then gently rubbed with sterile gauze. The mucosa was then stripped off by careful dissection from the underlying sub-mucosa, minced, washed several times in calcium magnesium-free HBSS (CMF-HBSS) containing 1% penicillin-streptomycin, 1% fungizone, 50 μg/ml gentamicin (all from GIBCO BRL, Invitrogen, Carlsbad, CA) and cultured in complete medium (CM).
Gaithersburg, MD), and 10 mM Hepes (Sigma Chemical Co., St. Louis, MO). The tissue was then treated with 1 mM dithiothreitol (DTT, Sigma Chemical Co.) in RPMI 1640 containing 5% FCS (both from GIBCO BRL) for 15 min at 37°C. After washing, tissue pieces were placed in a siliconized flask in CMF-HBSS containing 0.75 mM EDTA (Sigma Chemical Co.) and maintained on an orbital shaker (100 rotations per min) at 37°C for a total of three 45-min incubations. At each time interval, the tissue was washed with CMF-HBSS to remove the loosely bound epithelial cells and liberated IELs. The remaining tissue was kept in RPMI for 15 min and further subjected to enzymatic digestion overnight at 37°C in 50 ml of antibiotic-antimycotic-supplemented RPMI containing 0.1 μg/ml DNase, 0.1 μg/ml soybean trypsin inhibitor (both from Sigma Chemical Co.) and 0.08 μg/ml collagenase (Boehringer Mannheim, Indianapolis, IN).

After 18 h, digested tissues were placed on a wire mesh and rubbed with a rubber policeman resulting in the liberation of lymphocytes from the lamina propria. This crude cell suspension was pelleted and resuspended in a 15-ml conical tube in 2.5 ml of 100% Percoll (Pharmacia) which had been adjusted to pH 7.4 with an osmolality of 290 mOsm. 2.5-ml layers of 60, 40, and 30% Percoll (prepared by diluting 100% Percoll with RPMI 1640) were successively layered on top to establish the gradient. The Percoll gradient was centrifuged at 1,500 rpm for 35 min at 22°C, and cells at the interface of the 60 and 40% Percoll layer were carefully collected to obtain the purified LPLs. The LPLs were typically >95% HLe-1+ with 2–5% contaminating epithelial cells.

Isolation of Epithelial Cells. Epithelial cells were obtained from two disperse (3 mg/ml; Boehringer Mannheim) treatments for 30 min each at 37°C as described previously (11). The cell suspensions resulting from dispase treatments were centrifuged on Percoll density gradients similar to the isolation of LPLs. The top 0/30% layer interface contained >95% pure epithelial cells. Puriﬁcation was confirmed by flow cytometric analysis (EPICS C; Coulter, Hialeah, FL) using anti-CD14, -CD3, -CD20, and L12 (antiepithelial cell) mAbs. Contaminating cells were predominantly CD3+ T cells (2–4%). Cells were washed three times in PBS, and viability was determined by trypsin blue exclusion. For both LPLs and epithelial cells, if viability was not >95%, the cells were not used in the studies.

Mixed Cell Culture Reaction. Allogeneic mixed cell culture reactions were performed as previously described (11) using freshly isolated LPLs (10⁵ cells/well) with varying numbers of irradiated epithelial cells or non T cells (1 × 10⁵, 5 × 10⁴, and 2.5 × 10⁴ cells/well) in RPMI, 5% human-γ-macroglobulin, 1% penicillin-streptomycin, and 2 mM glutamine, henceforth termed culture medium (CM). In each study, parallel assays were performed using PB T cells. Control cultures of LPL or PB T cells alone (negative control), irradiated stimulators alone (negative control), as well as T cells cultured in the presence of the nonspeciﬁc mitogen PHA (1 μg/ml; GIBCO BRL) (positive control) were included in each experiment. All cultures were performed in triplicate in 96-well round bottom microwell culture plates (Falcon, Oxnard, CA) for 120 h at 37°C in a 5% CO₂ humidified incubator. During the last 18 h of culture, 1 μCi [³H]thymidine (ICN Radiochemicals, Irvine, CA) was added, and cells were harvested onto glass fiber filter mats for counting as previously described (11) using a scintillation counter (model 3801; Beckman Instruments, Inc., Somerset, NJ). Whereas the initial studies used non T cells and epithelial cells from different donors (not reported here), all studies depicted in this article represent experiments where non T cells and IECs were derived from the same donor, eliminating the question of MHC matching of stimulator cells. Similarly, LPLs and PB T cells were derived from the same individual, allogeneic to the IEC/non T cells. The results under both conditions (i.e., whether IECs and non T cells were derived from different individuals or the same individual) were comparable.

Antibody Inhibition Studies. All antibodies used in these studies were purified from ascites by protein G column chromatography (Pharmacia). The anti-HLA-DR mAb VG2 (IgG1k) recognizing nonpolymorphic determinants, was obtained from Dr. Shu Man Fu (University of Virginia, Charlottesville, VA). L243 (anti-DR nonpolymorphic IgG1k), genox 3.53 (anti-DQ-IgG1k), and W6/32 (anti-class I-IgG1k) mAbs were obtained from the American Type Culture Collection (Rockville, MD). The anti-DP mAb, B7/21, was the kind gift of Drs. Barbara Bieter and Steven Burakoff (Dana Farber Cancer Institute, Boston, MA). These antibodies have previously been shown to inhibit conventional MLR cultures (anti-class II reagents) (21) and cytolytic T cell activation (W6/32) (22).

One million isolated epithelial cells or non T cells were preincubated with mAbs (10 μg/10⁵ cells) for 30 min at room temperature. This concentration was used since we had previously demonstrated that this was the optimal concentration for mixed cell culture reaction inhibition and staining. The cells were then washed three times in PBS, resuspended in CM, counted, and irradiated (3,000

Figure 1. (A) LPLs (from individual A, 10⁵ cells/well, triplicate cultures) were cocultured with varying concentrations (10⁵–2.5 × 10⁵) of either normal, isolated, irradiated (3,000 rad) IECs or non T cells from individual A for 120 h. [³H]Thymidine was added during the last 18 h and cells were processed for scintillation counting. (B) Similar to A except that PB T cells (from the same individual A as the LPL) are used as the responder population. This figure is representative of at least five experiments.

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The mAb treated or untreated irradiated cells were added to the responders cells (LPLs or PB T cells) in 96-well culture plates as described above for mixed cell culture reactions. The blocking activity of these antibodies was ascertained by comparing the proliferative responses induced by the antibody treated versus the untreated stimulator cells. Antibodies were not added directly to the wells as both mAbs to class II and I Ags may be inhibitory for T cell activation.

Results

Normal LPLs Preferentially Respond to Epithelial Cells in MLRs. Conventional unidirectional allogeneic MLRs are characterized by the recognition of foreign class II Ag by alloreactive T cells. We have previously demonstrated that PB T cells proliferate in response to class II Ag-bearing intestinal epithelium (11). To determine whether the effects seen have potential physiologic relevance, we cocultured isolated LPLs (which interact with epithelial cells in vivo via fenestrations in the basement membrane [23]) with either allogeneic epithelial cells or allogeneic non T cells (from the same donor as the IECs). As seen in a representative experiment (representative of five experiments), normal LPL T cells (Fig. 1A), like PB T cells (Fig. 1B), proliferate in a dose-dependent fashion in response to allogeneic epithelial cell stimulators. However, in contrast to PB T cells (Fig. 1B), the proliferative response by LPLs (Fig. 1A) to non T cells was less than that seen with epithelial cells. Although the ability of LPL to proliferate in response to non T cells was variable in different experiments, non T cell stimulation of LPL proliferation was always lower when compared with epithelial cells. Furthermore, the source of epithelial cells did not appear to influence the results. Whereas epithelial cells from patients with inflammatory bowel diseases (IBD) were generally more stimulatory (as previously reported for PB T cultures; 24) than normal epithelial cells, responses to normal epithelial cells were consistently greater than those induced by non T cells. Since non T cells express class II Ag on their surface at greater density, and were matched for both class I and II MHC with the

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

Figure 2. (A) LP T cells (10^5/well triplicate cultures) isolated from actively inflamed tissue from a patient with Crohn's disease (CD) cocultured in MLR cultures with allogeneic epithelial cells (10^5) or non T cells (10^5). (B) LP T cells (10^5/well triplicate cultures) isolated from uninvolved tissue from a patient with Crohn's disease (CD) cocultured in MLR cultures with allogeneic epithelial cells (10^5) or non T cells (10^5). (C) LP T cells (10^5/well triplicate cultures) isolated from actively inflamed tissue from a patient with ulcerative colitis (UC) cocultured in MLR cultures with allogeneic epithelial cells (10^5) or non T cells (10^5). (D) T cells (10^5/well triplicate cultures) isolated from uninvolved tissue from a patient with ulcerative colitis (UC) cocultured in MLR cultures with allogeneic epithelial cells (10^5) or non T cells (10^5). Proliferation was assessed as described in Fig. 1. This figure is representative of at least 10 experiments.
epithelial cells, these data suggest that LPLs are recognizing a non class II Ag determinant on epithelial cells.

The preferential proliferation in response to epithelial cells was seen regardless of whether the LP T cells were derived from normal or IBD bowel. As seen in Fig. 2, LP T cells derived from Crohn's Disease (CD) (inflamed, A, uninfamed, B) or ulcerative colitis (UC) (inflamed, C, uninflamed, D), all preferentially proliferate in response to isolated epithelial cells regardless of whether the epithelial cells were derived from IBD or normal tissues.

One recognized difference between PB and LP T cells is that LPLs are enriched for memory and activated T cells (15). It is plausible, therefore, that the findings described above may relate to the recognition of luminal Ags that are bound, in vivo, to classical restriction elements on isolated epithelial cells. Given the heterogeneity of class I or II molecules, LPLs may be recognizing luminal Ags in the context of matched MHC molecules carried on IECs. Since we did not determine the MHC haplotypes of the cells studied, it is conceivable that appropriate Ag presentation through appropriate MHC is occurring in these cultures. To address this more directly, we performed an autologous mixed cell culture reaction using LP and PB T cells cocultured with either epithelial cells or non T cells from the same individual. If memory T cell activation was occurring in the LPL-epithelial cell interactions via presentation of luminal Ags, we would see an enhanced autologous mixed cell culture reaction in these cultures, but not in the non T cell-driven cultures. As seen in Fig. 3, proliferation of either LPLs or PB T cells occurred in the allogeneic cultures only, and no enhancement of the autologous mixed cell culture reaction was seen. Since this experiment maximizes the chances of appropriate memory T cell activation, it is unlikely that the results in the allogeneic system reflect such a scenario. Furthermore, these findings speak against the solitary role of an adhesion molecule to explain the observations described above.

One possible explanation for our findings could be that the isolation procedure used to purify LPLs had altered their ability to respond to non T cells. Although this should not account for the difference between epithelial cells and non T cells, it may reflect an alteration of recognition elements on LP T cells. To address this issue, we subjected PB T cells to the same isolation protocol used for LPLs. The enzyme-treated T cells were still capable of responding (slight reduction of <20%) to non T cells (Fig. 4) in an allogeneic MLR.

Antibodies to Class II Ags Fail to Inhibit LPL Response to Epithelial Cells. We have previously shown that antibodies to class II molecules inhibit epithelial cell–stimulated PB T cell MLR responses (11) when added directly to the culture well. Since non T cells fail to stimulate LPLs effectively, we wanted to determine whether LPLs were recognizing a non class II Ag on epithelial cells. Epithelial cells from normal individuals or patients with CD or UC were incubated with varying concentrations of the anti-DR mAb VG2 (10–0.1 µg/ml) for 30 min. After washing, the epithelial cells were cocultured with either LP or PB T cells in CM. In a representative set of experiments, the anti-DR pretreatment, but not an isotype-matched control mAb (data not shown), effectively inhibited the PB T cell response to CD epithelial cells and non T cells (Fig. 5 A). This same anti-DR mAb treatment, however, failed to block the LPL response (Fig. 5 B) to the same epithelial cells. In the experiment shown, since non T cell stimulation of LPLs was weak to start with, the anti-DR mAb treatment had little effect in these cultures. However, in other experiments where non T cell stimulation was evident, VG2 could inhibit LPL proliferation, although not completely. We further analyzed the role of class II Ags in these cultures by pretreating the IECs with antibodies to DQ (genox 3.53), DP (B7/21), and/or an additional anti-DR mAb (L243). These antibodies have previously been shown to inhibit conventional MLR cultures (21). As seen in Fig. 6, none of these antibodies, alone or in combination, inhibited LPL stimulation by IECs. Stimulation could be inhibited by the antiepithelial cell mAb LI2 previously defined for this property (25). These data support the concept that LPLs recognize a non class II determinant on epithelial cells, whereas PB T cells recognize class II Ags at least on IBD epithelium.

![Figure 3](image-url) **Figure 3.** LPL (10⁶) or PB T cells (10⁵) isolated from the same individual were cocultured with either irradiated autologous or allogeneic epithelial cells (10⁵). Proliferation was assayed as described in Fig. 1. This figure is representative of three experiments.
Figure 5. Epithelial cells (10^5) from patients with CD were incubated with the anti-DR mAb VG2, an IgG1 isotype control (data not shown), or medium alone for 30 min at 4°C and washed three times in PBS to remove unbound mAb. Treated and untreated epithelial cells were then cocultured with LPL (10^5) (A) or PB T cells (10^5) (B) in MLR cultures. Proliferation was assessed as described in Fig. 1. No inhibition by the IgG1 mAb was seen in any culture. This figure is representative of at least 10 experiments.

Figure 6. Normal epithelial cells (10^5) were incubated with the anti-DR mAb VG2 (anti-DR (A)), or L243 (anti-DR (B)), anti-DQ mAb genox 3.53, anti-DP mAb B7/21, alone or in combination, the anti-epithelial cell mAb L12, or medium alone for 30 min at 4°C and washed three times in PBS to remove unbound mAb. Treated and untreated epithelial cells were then cocultured with LPL (10^5) in mixed cell cultures. Proliferation was assessed as described in Fig. 1. No inhibition by an IgG1 control mAb was seen in any culture. This figure is representative of three experiments.

Figure 7. Isolated epithelial cells (10^5) were cocultured with IFN-γ (250 U/ml) for 18 h, washed, irradiated 3,000 rad, and cocultured with either LPL (10^5) or PB T cells (10^5) for 120 h. Proliferation was assessed as described in Fig. 1. This figure is representative of two experiments.

IFN-γ Fails to Upregulate or Alter the LPL Response to Epithelial Cells. IFN-γ is known to upregulate class II Ag expression on lymphoid cells (26) as well as on epithelium (27, 28). We cultured epithelial cells in the presence of IFN-γ (250 U/ml) for 18–24 h before use as stimulators in mixed cell cultures. We have previously shown that this concentration is effective at enhancing expression class II Ags on these cells (28). As seen in Fig. 7, despite IFN-γ treatment, no change in the proliferative response by LPLs was demonstrable. In parallel cultures, however, the response to these cells by PB T cells was augmented by IFN-γ. These data provide further support for a non class II Ag stimulator molecule present on normal and IBD epithelial cells.

Discussion

Despite the expression of class II Ags on normal IECs (7–12), IEC fail to function in a manner similar to that of
between monocytes/macrophages/dendritic cells and intestinal pressor cells (11). This suggests that unique differences exist between monocytes/macrophages/dendritic cells and intestinal epithelium. In this study, we document that LPLs are also capable of recognizing allogeneic epithelial cells (inducing proliferation), but in contrast to conventional MLRs, this effect does not appear to be regulated via classical class II Ag-TCR interactions. First, LPLs proliferate better to epithelial cells than to conventional MLR stimulators (macrophage/dendritic cells) from the same individual. Second, antibodies to class II Ags (DR, DP, DQ), singly or in combination, fail to inhibit LPL proliferation. Furthermore, upregulation of class II Ags by IFN-γ fails to alter the response of LPLs to IECs. Other investigators have documented that LPLs are distinct from circulating T cells. Whereas they proliferate in response to mitogens, they fail to proliferate in response to specific Ags (15), even when primed. In this setting, the Ag receptor appears to be intact since Ag-specific stimulation does induce the production of cytokines. It is presumed that the Ags used in these studies are presented to LPLs by tissue macrophages or dendritic cells. However, no specific assessment of LPL-epithelial cell interactions has been performed to date.

Our initial studies using normal IECs as APCs revealed a selective activation of CD8+ PB T cells by normal epithelial cells. This finding appears to represent the presence of an alternate stimulatory pathway for T cell activation. In a more physiologic system, LPLs and epithelial cells, this effect may be magnified. There is a reversal in the response pattern to allogeneic stimulators cells by PB T cells (non T cells >> epithelial cells) when compared with LP T cells (epithelial cells >> non T cells). As yet, we have been unable to document either the T cell subpopulations that are specifically proliferating in these cultures, or whether anti-CD8 mAbs are inhibitory. These studies are currently in progress.

Mixed lymphocyte reactivity is generally reflective of a response to a polymorphic product of the MHC. Typically, the response by alloreactive T cells is directed against a foreign class II molecule that may be perceived as self plus Ag (29). Alternative stimulatory molecules have not been clearly defined. From our studies it is clear that the response to epithelial cells is not class I (25) or II Ag related since non T cells bearing the same surface MHC (albeit in varying densities) are either less or nonstimulatory. In addition, one cannot explain our data by invoking classical Ag presentation since autologous class II Ag-bearing epithelial cells fail to activate LPLs to a level comparable with that of allogeneic epithelial cells. The stimulatory molecule in LPL-epithelial cell MLRs would, like class I or II Ags, have to be polymorphic since a common stimulatory molecule, such as one stimulating via CD2, would result in the stimulation of both autologous and allogeneic cells. In our studies, only stimulation of allogeneic cells is seen. Novel polymorphic determinants would therefore need to be proposed. Several possibilities exist. First there is growing evidence that heat shock proteins can bind peptide Ags and, if expressed on the cell surface, may serve as restriction elements for immune responses (30). In fact there have been numerous reports of IELs capable of responding to mycobacterial heat shock protein (31). Second, other molecules of potential interest include the class I-like molecule CD1d which has been recently identified on murine and human epithelium (32, 33). Whereas CD1d is not polymorphic, its similarity to class I Ag may render it capable of serving as a restriction element. Using mAbs to CD1d, we have been able to inhibit PB T cell-normal epithelial cell MLRs (34). However, this mAb failed to inhibit LPL-IEC mixed cell cultures (34).

In contrast to LPLs, we are unable to demonstrate induction of proliferation of IELs by allogeneic epithelial cells (data not shown). We are, however, using an allogeneic mixed cell culture system so that the data generated with LPLs or IELs may not truly reflect the interactions required for antigen-specific responses. Such studies are difficult to perform in humans since parenteral immunization would not result in T cell priming in the GI tract and oral priming may not result in activation of T cells at an accessible site (i.e., resection site). Directed immunization would be required to address this tissue.

Alternate pathways of T cell activation have been clearly defined. These include stimulation via CD2 and accessory stimulation via CD28, CD45, CD4, and CD8 (37–39). We have proposed that cross-linking CD8 by a novel ligand on epithelial cells may result in the activation and proliferation of these cells. However, many of the activation stimuli do not function in a vacuum, and it is conceivable that coactivation events (mucosa-specific adhesion molecules) are required. Our system provides an avenue to explore such activation events. Whereas several alternate activation pathways for T
cells have been described, it is not clear whether one of these pathways, or alternatively a novel pathway, is involved in LPL activation. Such differences could explain the unique environment of the intestinal tract and much of the phenomenology, such as oral tolerance, that is associated with it.

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