

EVIDENCE FOR FUNCTION OF Ia MOLECULES ON GUT EPITHELIAL CELLS IN MAN

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The induction of an immune response at the mucosal level requires a series of complex interactions between lymphocyte subpopulations in Peyer's patches and mesenteric lymph nodes (1) once antigen has been introduced. It has been proposed that the major pathway of antigen entry and presentation to these local lymphoid populations is through specialized epithelium overlying Peyer's patches, the so-called M cells (2). The actual numbers of these M cells are limited, however, and although it has been suggested that certain specific antigens can be "attracted" to these areas (3), the vast numbers of luminal antigens make this a potentially inefficient process. Recently, several groups have described the presence of Ia molecules on the surface of gut epithelial cells in rat (4, 5), and more recently in man (6). These Ia⁺ epithelial cells were initially described only in inflamed bowel (7), but these observations have been extended to include normal bowel as well, using more sensitive immunohistochemical techniques (Mayer, L., P. Solomen, and R. Plous, manuscript in preparation). The presence of these immunoregulatory molecules raises the issue as to whether they can function and contribute to immune responses in the GI tract. Although the presence of Ia molecules alone does not ensure that cells can function adequately as accessory or antigen-presenting cells (8), there has been recent evidence that IFN- γ -induced Ia⁺ endothelial cells can replace macrophages or dendritic cells as stimulators in MLRs (8).

To determine whether Ia⁺ gut epithelial cells can function as accessory cells, and therefore potentially participate in mucosal immune responses, isolated epithelial cells were used in a series of experiments assessing antigen presentation and processing. We demonstrate that not only can these cells process and present antigen to primed T cells but that they selectively stimulate peripheral blood T cells of the suppressor/cytotoxic phenotype. These T8⁺ T cells appear to function as antigen nonspecific suppressor cells in various assay systems. These data may help explain the generally "suppressed" immunologic tone of the gut and may help in the understanding of the concept of oral tolerance.

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Materials and Methods

Cell Separation and Culture Conditions. Heparinized venous blood was obtained from normal control volunteers or preoperatively from patients undergoing bowel resection. Peripheral blood mononuclear cells and T/B separation were performed as previously described (9). Adherent monocytes were obtained by incubating 4×10^5 , 2×10^5 , and 2×10^4 non-T cells in 0.2 ml RPMI 1640 (Hazelton-Dutchland, Denver, PA), 5% human agammaglobulinemic serum, 1% penicillin/streptomycin (GIBCO Laboratories, Grand Island, NY), 2 mM glutamine (GIBCO Laboratories), henceforth termed culture medium (CM)¹, in triplicate microwell cultures (Linbro Chemical Co., Hamden, CT) for 45 min at 37°C in a humidified 5% CO₂ incubator. After 45 min, nonadherent cells were aspirated and the microwells were washed vigorously three times with PBS. In most experiments, plates with adherent cells were irradiated 3,000 rad. Peripheral blood T cells, devoid of contaminating monocytes, were isolated by counterflow elutriation as previously described (10) and pure populations of monocytes could be obtained in the later fractions for use in suspension cultures.

Isolation of Gut Epithelial Cells. Mucosal epithelial cells were obtained by a modification of the procedure used to isolate lamina propria and intraepithelial lymphocytes by Bull and Bookman (11). Briefly, surgically resected specimens were obtained sterilely from the operating room, washed extensively with HBSS (Gibco Laboratories) containing 1% penicillin/streptomycin, gentamicin (50 µg/ml Sigma Chemical Co., St. Louis, MO) and 1% fungizone (Gibco Laboratories). The mucosa was carefully dissected from the underlying submucosa, minced into small 2–3-mm pieces, and washed sequentially with calcium/magnesium-free HBSS (CMF-HBSS) containing antibiotics, 1 mM DTT in CMF-HBSS for 5 min, and again three times with CMF-HBSS. Mucosal pieces were then incubated in a sterile siliconized flask for 1 h in CMF-HBSS containing 100 mM EDTA and antibiotics on a platform shaker at 37°C. Epithelial cells and intraepithelial lymphocytes were liberated during this process. Remnant mucosal pieces were removed by passage through a large pore strainer. Epithelial cells were pelleted, resuspended in HBSS, and subjected sequentially to Ficoll Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and Percoll (Sigma Chemical Co.) density gradient centrifugation to improve viability and purity of the preparation. The resultant epithelial cells were 80–95% viable as determined by trypan blue exclusion (and in some cases confirmed by ethidium bromide staining analyzed by the cytofluorograph), <0.1% esterase-positive, <0.1% OKM1⁺ (Ortho Pharmaceutical, Raritan, NJ), <0.1 OKT6⁺, <1% sIg⁺, and 1–2% T3⁺ (Ortho Pharmaceutical). Epithelial cells were irradiated 3,000 rad as described above. In some experiments, epithelial cells were pulsed with tetanus toxoid (40 µg/ml, Connaught Laboratories, Ltd., Willowdale, Canada) in CM for 60–120 min at 37°C. After this incubation period, cells were washed and fixed with paraformaldehyde (0.1%) for 15 min and washed again before use (see below). There was no appreciable decrease in cell viability during this short incubation period.

Mixed Lymphocyte Responses. Autologous and allogeneic MLRs were performed as previously described (12) using either 2×10^5 , 10^5 , or 10^4 irradiated non-T cells, adherent cells, or epithelial cells as stimulators and either 10^5 autologous or allogeneic isolated T cells in CM. Control cultures of T cells alone or irradiated stimulators alone were included in each experiment. All cultures were performed in triplicate in 96-well round-bottomed microwell plates for 120 h at 37°C in a 5% CO₂ humidified incubator. During the last 18 hours of culture, 2 µCi [³H]thymidine (Schwarz-Mann, Orangeberg, NY) were added and cells were harvested onto glass fiber filter mats for counting as previously described (10). Counts were obtained and averaged by a scintillation counter (model 3801; Beckman Instruments, Fullerton, CA).

Processing of Soluble Antigen. Isolated T cells, monocytes, and epithelial cells were obtained from single patients undergoing bowel resection. These patients had recently undergone booster immunization with tetanus toxoid (TT) and demonstrated significant T cell proliferative responses to TT in vitro. Monocytes and epithelial cells were cultured

¹ Abbreviations used in this paper: CM, culture medium; CMF-HBSS, calcium/magnesium-free HBSS; GI, gastrointestinal; TT, tetanus toxoid.

with TT (40 $\mu\text{g}/\text{ml}$) in CM for 60 min at 37°C, washed three times in CM alone, fixed with paraformaldehyde, and washed again in CM before use. In some cases, monocyte and epithelial cells were pretreated (15 min before addition of TT), with chloroquine 0.1 mM (Sigma Chemical Co.) and washed extensively before exposure to TT. In some preliminary experiments, TT was directly added for 120 h to cultures of T cells and either monocytes or epithelial cells. Whichever protocol was used, the cultures were maintained for 120 h at 37°C in a 5% humidified incubator. As above, 2 μCi [^3H] thymidine were added during the last 18 h of culture. Cultures were harvested and counted as stated above. T cells in these studies were rigorously depleted of monocytes/dendritic cells, as described below and demonstrated no [^3H]thymidine incorporation upon stimulation with antigen (TT) or mitogen.

Immunofluorescence and Cell Staining. mAbs OKT3, T4, T8, M1 were obtained from Ortho Pharmaceutical. Rhodamine conjugated T4 and fluorescein conjugated T8 were obtained from Coulter Immunology (Hialeah, FL). mAbs VG2 (a framework anti-Ia) (13) and 9.3 (helper/cytotoxic T cell marker produced by Dr. John Hansen) were kindly provided by Dr. Shu Man Fu at the Oklahoma Medical Research Foundation, Oklahoma City, OK. 962, a rabbit heteroantiserum against human Ia (14), was developed at The Rockefeller University, New York. Fluorescein-conjugated goat anti-mouse Ig was obtained from Cappel Laboratories (Cochranville, PA).

Staining of MLR blasts after 48 h was performed as follows: 5×10^6 T cells were cocultured with 5×10^6 irradiated monocytes (or non-T cells) or epithelial cells in 5 ml CM in 25-cm² flasks (No. 3013; Falcon Labware, Oxnard, CA). After 48 h, cell mixtures were layered over a Percoll density gradient. Cells from the 30/50% interface (T cell blasts) were washed three times in PBS-BSA with 0.1% sodium azide and stained as previously described (15). Staining was determined by analysis on an Epics C (Coulter Electronics, Hialeah, FL) cytofluorograph gating on live cells and blasts. At least 5,000 cells were counted per specimen. In some experiments, stained blasts were counted on a Leitz fluorescent microscope (Leitz, Wetzlar, Federal Republic of Germany). At least 200 cells per sample were counted by this technique.

DLD1 cells (colonic carcinoma line obtained from the American Type Culture Collection, Rockville, MD) were stained after overnight adherence onto plastic cover slips by indirect immunofluorescence and analyzed using the fluorescent microscope. In most experiments, rIFN (50–500 U/ml) (kindly provided by Interferon Sciences, New Brunswick, NJ) was added 24–48 h before staining.

DLD1 Cell Line. DLD1 is an adherent malignant colonic adenocarcinoma cell line maintained in continuous culture in RPMI 1640 with 10% FCS and antibiotics.

For specific assays, flasks were trypsinized (Trypsin-EDTA; Sigma Chemical Co.) and allowed to reattach in flat-bottomed 96-well plates at varying cell numbers in medium for 18 h. When reattachment occurred, cells were either treated with IFN- γ for 48 h at 50–100 U/ml or left alone in medium. After washing to remove residual IFN- γ , plates were irradiated 3,000 rad and allogeneic, monocyte/dendritic cell-depleted T cells were added at 10^5 cells/well in 100 μl of CM for 120 h as described above for MLR.

Monocyte/Dendritic Cell Depletion and Lectin Stimulation. T cells were depleted of adherent cells by a combination of plastic adherence (16) for 45 min and carbonyl iron depletion (17). Alternatively, T cells were isolated from high density fractions of peripheral blood mononuclear cells isolated by counterflow elutriation (10). By either method the resultant T cell preparations were <0.1% esterase-positive, OKMI⁺, or T6⁺ and did not respond to PHA (1/100 dilution, Gibco Laboratories) or Con A (10 $\mu\text{g}/\text{ml}$; Sigma Chemical Co.).

Isolation of T Cell Subsets. Cultures of T cells alone, T cells with irradiated allogeneic adherent cells, or T cells with irradiated epithelial cells were maintained for 48 h in CM. After this initial period, the T cells were isolated by Ficoll-Hypaque density gradient centrifugation and separated into T4 and T8⁺ cell subpopulations by an indirect rosetting technique as previously described (9). Negatively selected cells were used for staining, and further cultured for proliferation assays and assays of helper/suppressor functions. Negatively selected populations were >96% pure for T4⁺ population (T8⁻ selection) and

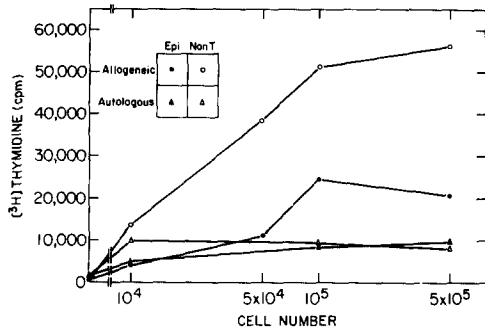


FIGURE 1. Graded numbers of irradiated epithelial (closed symbols) or non-T cells (open symbols) are added to 10^5 autologous (triangles) or allogeneic (circles) T cells for 5 d. After 120 h, $2 \mu\text{Ci}$ [^3H]thymidine are added and cells are harvested and counted after an additional 18-h incubation. Results are expressed as mean counts per minute (cpm).

>94% for T8⁺ populations (T4⁻ selection). Contaminating residual epithelial cells represented 2–4% of the negatively selected cells. Unseparated but mAb-treated T cells were used as controls in all experiments.

For proliferation assays, negatively selected cells were recultured in CM at 10^5 cells/well in triplicate cultures. After an additional 72-h culture period $1 \mu\text{Ci}$ of [^3H]thymidine was added and cells were harvested and counted as noted above.

Assays for helper and suppressor T cell function were performed as follows: Irradiated (3,000 rad) or nonirradiated isolated T cell subpopulations or untreated T cells were added back to primary MLR or secondary MLR cultures of combinations to which the T cells had been primed or to novel allogeneic combinations. Cultures were maintained and harvested as described above. Inhibition of T cell mitogenic responses by primed T cells was assessed in a similar fashion with control T cells or T cell subpopulations added to culture, in graded numbers, to allogeneic T cells stimulated with PHA ($1 \mu\text{g}/\text{ml}$) or Con A ($1 \mu\text{g}/\text{ml}$) for 72 h. [^3H]thymidine incorporation was measured in an additional 18-h period. Finally inhibition of T cell-dependent B cell differentiation was evaluated by addition of the various T cell populations to cultures of PBL and PWM ($1 \mu\text{g}/\text{ml}$). Ig secretion was measured by Elisa of culture supernatants after 10 d of culture as previously described (9).

Results

Normal Epithelial Cells Constitutively Expressing Ia Act as Stimulators in Autologous and Allogeneic MLR. The recent descriptions of Ia molecules on normal GI tract epithelial cells in rat (4) and more recently in man (Mayer, L., P. Solomen, and R. Plous, manuscript in preparation) have raised questions as to their function in mucosal immune responses. To address these questions, a series of experiments were performed to assess the ability of these epithelial cells to present and process antigens. This was initially approached by performing MLRs where class II antigens on the cell surface can stimulate alloreactive T cells putatively without the requirement for further processing. Epithelial cells and isolated monocytes/dendritic cells or non-T cells were obtained from patients undergoing laparotomy and intestinal resection for noninflammatory disease (i.e., colon carcinoma, colonic inertia, volvulus, arteriovenous malformations, familial polyposis) of the bowel. These cells were compared for their ability to act as stimulators in either allogeneic or autologous MLR. As can be seen in Fig. 1, the addition of increasing concentrations of non-T cells to cultures of allogeneic T cells resulted in significant [^3H]thymidine uptake by these T cells after 120 h in culture. In contrast, marked, albeit somewhat lower, proliferative responses were seen using irradiated epithelial cells as stimulator cells. This difference in degree of stimulation appeared to correlate with Ia density, HLA-

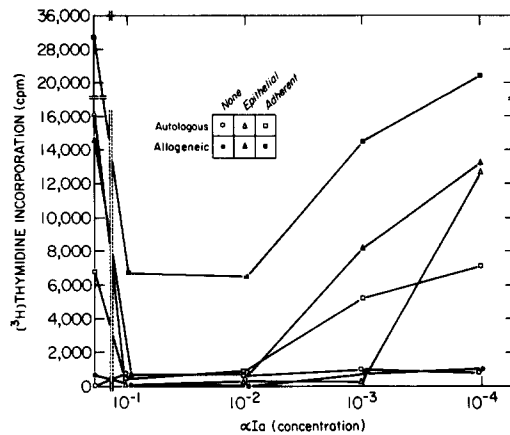


FIGURE 2. Graded doses of rabbit hetero-anti-Ia antibody 962 are added to cultures of 10^5 autologous (closed symbols) or allogeneic (open symbols) T cells with or without irradiated 10^5 epithelial cells (triangles) or 10^5 adherent cells (squares). Cultures are pulsed, harvested, and counted as in Fig. 1.

DR being expressed at significantly reduced but present levels on normal epithelial cells. Still, there was a consistent, statistically significant, increase in [^3H] thymidine incorporation in these experiments (10,000–50,000 cpm). It should be noted that this degree of stimulation was evident whether or not T cells had been vigorously depleted of adherent cells or Ia⁺ cells (in some experiments) by elutriation or complement lysis. In our preliminary studies the degree of depletion of accessory cells in the responder T cell population was confirmed by the inability of these cells to respond to Con A or PHA. Thus, contaminating accessory cells in the responder population did not appear to play a role in the responses described. Both monocyte/dendritic cells and epithelial cells were stimulatory in autologous MLR as well (Fig. 1). Although the degree of stimulation with either cell population was much lower than in the allogeneic system, they were consistent with values previously reported in man. Addition of rabbit heteroantibody 962 (anti-human class II) to the MLR cultures completely abrogated the stimulatory capabilities of both the adherent cells and epithelial cells (Fig. 2) in a dose-dependent manner. These effects were seen in both autologous and allogeneic MLRs, suggesting that the stimulatory molecules in both cases were Ia. Addition of preimmune rabbit serum to these cultures did not result in any inhibition (data not shown).

Epithelial Cells Can Process and Present Soluble TT to Immunocompetent T Cells. Epithelial cells, peripheral blood adherent cells, and T cells were obtained from two patients (recently reimmunized with TT) who were undergoing bowel resection for benign disease. PBMC from both patients were induced to proliferate in the presence of TT (40 $\mu\text{g}/\text{ml}$), however, as seen in Fig. 3 (representative of both patients), vigorous depletion of B cells, adherent cells, and phagocytic cells from the PBMC resulted in the loss of the TT-induced T cell proliferative response. In addition, in each case these accessory cell-depleted T cells did not respond to PHA or Con A. Addition of either adherent cells or isolated epithelial cells back to these monocyte/dendritic cell-depleted T cell cultures restored the ability of the T cell to respond to TT. The T/accessory cell ratio was equivalent in either system (10:1–20:1) suggesting that both accessory cell types were equally efficient in processing and presenting antigen. The ability to restore T cell responses to TT was inhibited by pretreatment of either accessory cell population

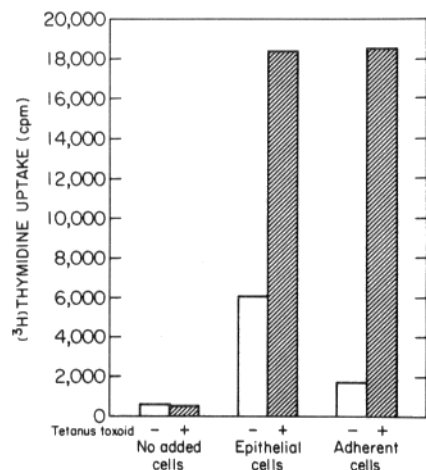


FIGURE 3. 10^5 severely monocyte/dendritic cell-depleted tetanus-primed T cells are cultured with or without TT ($40 \mu\text{g/ml}$) in the presence or absence of 10^5 autologous epithelial or adherent cells. Cultures are pulsed, harvested, and counted as described in Fig. 1.

with glutaraldehyde or paraformaldehyde (0.1%). In addition, pretreatment of epithelial cells or adherent cells with chloroquine (0.1 mM), followed by antigen pulsing with TT, blocked the ability of these cells to process antigen and restore the T cell response to TT (data not shown). Thus it appears that isolated gut epithelial cells can present soluble antigen efficiently, comparable to conventional accessory cells.

The effects demonstrated are not likely to be due to contaminating macrophages or dendritic cells since (a) morphologically, the isolated, density gradient, separated epithelial cells do not resemble macrophages or dendritic cells; (b) there are $<0.1\%$ M1⁺ and T6⁺ cells present by indirect immunofluorescence and the cells are DR⁺, DQ⁺, DP⁻ in contrast to adherent cells, which express all three class II molecules (although not 100%); and (c) one would have expected a more rapid decay in stimulatory capability by the epithelial cell preparations if the responsible cells were contaminating dendritic cells. However, since this possibility remained, we obtained the colonic epithelial cell line DLD1 to assess its ability to perform functions ascribed to the epithelial cells above.

DLD1 Cell Line Expresses Ia and Can Act as a Stimulator in MLR. DLD1 cell line cells growing as an adherent monolayer were trypsinized and recultured on plastic coverslips in preparation for cell staining. After 18 h the cells had readhered and were washed to remove dead cells. Fresh CM alone or CM containing IFN- γ (50–5000 U/ml) was layered on top for 48 h, at which time the coverslips were washed and stained for the presence of Ia by indirect immunofluorescence. As seen in Table I, cells cultured in medium alone were negative for HLA-DR. In contrast, cells cultured in the presence of IFN- γ for 48 h were strongly positive for DR in high percentages. This effect could be inhibited by the addition of an anti-IFN- γ antibody KAM-2. These IFN- γ -induced DLD1 cells were irradiated and used as stimulators in MLR cultures. These cells significantly stimulated allogeneic T cells, whereas uninduced DLD1 cells were nonstimulatory (Table II). Since this cell line is devoid of other contaminating stimulator cells, and the responder T cells were depleted of all accessory cells, the effects seen must be due to the epithelial cell itself.

TABLE I
Induction of HLA-DR on Colonic Adenocarcinoma Cell Line DLD-1

IFN- γ *	Anti-IFN \dagger	Fluorescence	Staining intensity
U/ml		%	
500	—	79.6	+++
50	—	56.2	++
5	—	1.4	+/-
0	—	0	—
500	+ (500 U)	0	—

10^5 DLD-1 cells were adhered onto plastic coverslips for 18 h at 37°C. After adherence, cells were cultured in the presence or absence of IFN- γ in varying concentrations for an additional 48-h period. After this incubation, the coverslips were washed and stained for the presence of HLA-DR by indirect immunofluorescence. Percent positive as well as staining intensity was assessed using a fluorescence microscope. This experiment is representative of multiple stainings.

* Recombinant IFN- γ at varying concentrations was added after the cells had adhered to the plastic coverslips.

\dagger Anti-IFN- γ mAb KAM-2 was added (+) along with recombinant IFN at the onset of the second culture period. Neutralizing units are indicated in the parenthesis.

TABLE II
DR⁺ DLD-1 Cells Are Capable of Stimulating an Allogeneic MLR

T cells	IFN- γ \dagger	Added DLD-1 cells* (^3H)thymidine incorporation)			
		None	10^5	10^4	10^3
<i>n</i>		<i>cpm</i>			
10^5	—	179	287	214	123
10^5	500	179	34,234	13,238	3,423
10^5	50	179	12,888	3,445	1,209
0	—	—	122	138	189

* DLD-1 cells were adhered onto flat-bottomed 96-well plates at varying cell concentrations over 18 h. After this initial culture period, the cells were then left in medium alone or cultured for an additional 48 h with IFN- γ (500 or 50 U/ml). MLR cultures of responder allogeneic T cells and irradiated (6,000 rad) adherent DLD-1 cells were established in CM and maintained for 120 h at 37°C. 1 μCi [^3H]thymidine was added to these cultures during the last 18 h as described in the Materials and Methods section. This experiment is representative of three such cultures.

\dagger Recombinant IFN- γ was added to DLD-1 cultures for 48 h, removed by washing, and the cells were irradiated before establishing MLR cultures.

Epithelial Cells Selectively Stimulate T8⁺ Suppressor Cells. Since it has been reported that adherent cell-stimulated MLR activate all classes of functional T cells (18, 19), it was of interest to determine whether epithelial cells could stimulate similar T cell populations or whether it would be more restricted in its stimulatory capacity. As seen in Table III, when T cells were cultured in medium alone for 48 h, T4⁺ and T8⁺ T cell populations remained relatively stable (a 2:1 ratio). When T cells were stimulated with irradiated adherent cells this ratio was maintained. However, when irradiated normal epithelial cells were used as the stimulator in MLR there was a greater percentage of T8⁺ T cells. Similar results

TABLE III
*Epithelial Cells Stimulate T Cells of the
 Suppressor/Cytotoxic Phenotype*

MLR [‡]	Staining*		
	T3	T4	T8
		%	
T cells only	97.8	66.4	22.5
T × adherent cells	94.2	58.9	25.1
T × epithelial cells	92.3	14.5	67.8

* After 48 h in culture, T cells were isolated by Ficoll/Hypaque density gradient centrifugation and stained with mAbs recognizing all T cells (T3) or the helper (T4) or suppressor/cytotoxic (T8) subpopulations by direct immunofluorescence. Percent positive staining was assessed by cytofluorographic analysis gating for blasts and live cells.

[‡] T cells alone or equal numbers of T cells and irradiated allogeneic adherent (T × adherent cells) or epithelial cells (T × epithelial cells) were cultured for 48 h in CM. Although this table represents stimulation of T cells in an MLR, similar data were obtained in TT-stimulated T cell cultures.

were obtained in autologous MLRs and antigen-driven systems as well (data not shown). Furthermore, when T8⁺ cells from epithelial cell-stimulated cultures were simultaneously stained with the monoclonal 9.3, which recognizes the cytotoxic T cell in the T8⁺ population, no dual staining was noted. This was in contrast to those T8⁺ cells stimulated by adherent cells, in the MLR cultures, where most bore the 9.3 antigen and were indicative of cytotoxic T cells.

The data above suggested that the proliferating T cell in our system was of the T8⁺, 9.3⁻ subset. Since changes in staining patterns may not accurately reflect proliferation, T cells stimulated with allogeneic adherent cells or epithelial cells were separated into T4 and T8⁺ populations by indirect rosetting, after 48 h of culture. These isolated populations were maintained in culture for an additional 72 h at which time 1 μ Ci ³H was added for the final 18 h. As seen in Table IV, the T8⁺ T cells incorporated a significantly greater amount of isotope with a stimulation index of 13.7, whereas the T4⁺ T cell population had a much lower proliferative index (3.7). Thus it appears that the staining data reflect a selective proliferation of the T8⁺ T cell subpopulation.

Next we determined whether the staining and proliferation data correlated with the functional capabilities of these isolated T cells. In contrast to adherent cell-stimulated T8⁺ T cells, which were cytotoxic or suppressive in an antigen-restricted manner (Table V), T8⁺ T cells generated by epithelial cell stimulation mediated potent antigen nonspecific suppression of both primary, secondary, and unrelated MLRs (Table V), and demonstrated no evidence of cytotoxicity (data not shown). These potent antigen nonspecific suppressors could also inhibit mitogen-induced T cell proliferation as well as B cell differentiation in the presence of T cells and PWM (Table VI).

Discussion

Using freshly isolated intestinal epithelial cells or an epithelial cell line devoid of contaminating mononuclear cells, we have been able to demonstrate that Ia⁺

TABLE IV
*Normal Epithelial Cells Induce the Proliferation of Suppressor
 T Cells in MLR*

Control cells*	[³ H]Thymi- dine incor- poration	MLR cells [‡]	[³ H]Thymi- dine incor- poration
	<i>cpm</i>		<i>cpm</i>
T only	1,792	T × epithelial cells	9,304
T only Leu-2 ⁻	1,344	T × epithelial Leu-2 ⁻	4,931
T only Leu-3 ⁻	801	T × epithelial Leu-3 ⁻	10,975

* T cells were cultured in CM alone for 48 h and isolated into Leu-2⁻(T4⁺) or Leu-3⁻(T8⁺) subpopulations by an indirect rosetting technique. 10⁵ cells were then recultured for an additional 72 h and pulsed with [³H]thymidine for the final 18 h of culture.

[‡] T cells were cocultured with equal numbers of irradiated epithelial cells for 48 h and subsequently isolated into T4⁺ and T8⁺ subpopulations as stated above. Stimulation index was determined using the negatively selected population as background control.

TABLE V
Epithelial Cell-stimulated T Cells Mediate Antigen-nonspecific Suppression

MLR*	[³ H]Thymidine incorporation with added T cells [‡]		
	None	T and epithelial cells [§]	T and adherent cells [¶]
		<i>cpm</i> [¶]	
FR "T" × Mc epithelial cells	23,164	463	6,388
FR "T" × Mc adherent cells	44,321	324	29,244
FR "T" × LA adherent cells	68,129	1,625	61,683
FR "T" × FR adherent cells	11,324	902	10,443

* MLR cultures using the same responder T cell population in each case with different stimulator cells.

[‡] Mixed lymphocyte cultures were performed using either allogeneic or autologous epithelial cells or adherent cells as stimulator cells with T cells for 48 h. Activated T cells were harvested from these cultures by isolation on Ficoll/Hypaque density gradients and added back to primary MLR cultures of the original pairing (in this experiment, FR "T" and Mc epithelial or adherent cells) or to an irrelevant MLR. In this representative experiment, activated T cells were added back at 10⁵/well. In other experiments, T cells could be used at 10⁴ and 10³/well with evidence of similar suppressor cell activity.

[§] Activated T cells derived from the MLR-FR "T" and Mc epithelial cells.

[¶] Activated T cells derived from the MLR-FR "T" and Mc adherent cells.

[¶] As per Table II.

epithelial cells can function as accessory cells in regulating immune responses. Thus, like Ia⁺ monocytes, dendritic cells, and B cells, all capable of processing and presenting soluble antigens, epithelial cells appear to be able to take up, process, and present antigens to immunocompetent T cells. These cells are also effective stimulators in both autologous and allogeneic MLR. This capacity appears to be linked to the expression of Ia molecules on the epithelial cell surface since a rabbit heteroantibody, 962, directed against human Ia molecules

TABLE VI
Epithelial Cell-activated T Cells Suppress PWM-driven B Cell Differentiation

Cells	PWM	Ig secretion after addition of T cells			
		None	10 ⁵	10 ⁴	10 ³
PBL 10 ⁵	-	1.2	1.9	1.2	1.5
PBL 10 ⁵	+	216.2	1.3	21.9	133.9

Activated T cells harvested from MLR cultures of T cells stimulated with irradiated epithelial cells were added in varying concentrations to cultures of PBL (10⁵/well) in the presence or absence of PWM (1 µg/ml). Cultures were maintained for 10 d. Supernatants were harvested from these cultures at day 10 and assayed for total Ig secretion by ELISA as described in Materials and Methods. Marked suppression was noted with T cells derived from epithelial cell-driven MLRs. No suppression was noted with T cells from adherent cell-stimulated cultures (10⁵, 456.3; 10⁴, 404.5; 10³, 337.8 µg/ml for PBL and PWM). Comparable results were obtained regardless of the PBL preparation used.

(14) suppresses this stimulatory capacity. More importantly, these same epithelial cells, pulsed with TT, can process and present this antigen to tetanus-primed T cells in a manner comparable to control monocytes/dendritic cells. Since freshly isolated epithelial cells from gut tissue could potentially be contaminated with dendritic cells and/or macrophages, and could account for the findings described above, a colonic epithelial cell line, DLD1, was tested. This cell line could be induced to express HLA-DR with IFN-γ and, as such, significantly stimulated alloreactive adherent cell-depleted T cells.

There is accumulating evidence to suggest that several cells of nonmonocyte or lymphoid lineage can express surface Ia molecules. Initially such molecules were described on endothelial cells in tissues undergoing graft rejection (20), however subsequently, HLA-DR was detected on keratinocytes (21), thymic epithelium (22), thyroid epithelium (23), and intestinal epithelial cells in rat and man (4-7). The rationale for the presence of these molecules has been less clear. Recent studies (8) using IFN-γ-induced Ia⁺ endothelial cells have demonstrated that these cells can act as stimulators in MLR and can replace monocytes in monocyte-dependent T cell mitogen responses. However, it was evident in these studies that the mere presence of Ia molecules was not sufficient for these functional capabilities, as Ia⁺ fibroblasts were nonstimulatory in any assay system. Our finding that Ia⁺ epithelial cells can effectively function as accessory cells suggests that these cells, the first line of defense in the gastrointestinal (GI) tract, may play an important role in local mucosal immune responses. In fact, by sheer numbers, these cells might be a more efficient means of processing luminal antigens than M cells. Previous studies (2, 3) have suggested that specialized epithelium, the M cell, overlying Peyer's patches, are the major cells responsible for antigen sampling from the gut, transporting antigens to local lymphoid populations. This may be true for specific antigens for which receptors may be present on the M cells, i.e., reovirus (3), but this type of interaction might result in a different immune response than that seen with epithelial cells.

What appears to be the most interesting finding is that related to the population of T cells specifically stimulated by epithelial cells. In either epithelial cell-driven autologous, allogeneic MLR, or antigen-specific systems, T8⁺ T cells make up the major proliferating T cell. These T8⁺ cells function effectively as antigen-

nonspecific suppressor cells in contrast to antigen-restricted suppressors seen in monocyte/dendritic cell driven MLRs (18). These findings are of particular interest in that they may help explain certain phenomena of mucosal immunity such as oral tolerance (24, 25) and the controlled ongoing chronic inflammation of the GI tract. In addition, it is quite intriguing to note that virtually all of the intraepithelial lymphocytes, so-called theliolymphocytes, are T8⁺ (26). Although the function of these cells is still unclear, they may either be attracted to surface epithelium or, as has been recently suggested (27), may play a major role in induction of Ia molecules on local epithelial cells. Since these cells are transient, passing through the epithelial cell layer, a chemotactic factor for or selective stimulation of T8⁺ T cells produced by the epithelial cell appear to be plausible explanations. Thus far, we have been unable to reproduce the T8⁺ T cell proliferation using epithelial cell supernatants, although this certainly does not rule out a chemotactic factor. With regard to selective stimulation of these cells, one would have to propose a distinct T8⁺ T cell-specific stimulatory molecule on the surface of the epithelial cell. This would not be HLA-DR itself since T8⁺ cells are usually thought to be class I-restricted and the epithelial cell DR appears to be analogous to that found on monocyte/dendritic cells. We have found that epithelial cells weakly express DQ and DP (DQ>>>DP), in contrast to the monocyte (data not shown), but we have found epithelial cells from certain disease states (inflammatory bowel disease) that are equally poor expressors of DP and DQ and appear to stimulate T4⁺ cells selectively (Mayer, L., and D. Eisenhardt, manuscript in preparation). Thus, direct stimulation of T8⁺ T cells does not appear to be related to D region molecules. However, there may still be alternative stimulatory molecules present on normal epithelial cells. Indeed, our data in patients with inflammatory bowel disease suggest that this may be the case. Studies attempting to define these molecules are currently in progress.

Taken together, our data suggest that there may be a significant role for the Ia⁺ gut epithelial cell in mucosal immunity. The findings with the epithelial cell line, as well as the selective T8⁺ T cell stimulation by epithelial cells make it less likely that contaminating accessory cells account for our results. In fact, the induction of antigen-nonspecific suppressor cells can certainly help to explain the dichotomy between systemic and mucosal stimulation. Controlled immunologic responsiveness is much more appropriate for a GI tract overwhelmed by foreign antigens. The lack of such control could result in unchecked immune responses and inflammation, which may be the case in certain intestinal inflammatory diseases.

Summary

Using freshly isolated Ia⁺ gut epithelial cells we have been able to demonstrate that these cells can function as accessory cells in an immune response. The cells can act as stimulators in both autologous and allogeneic MLRs. More importantly, these cells are capable of taking up the soluble antigen, tetanus toxoid, processing it, and presenting it to tetanus-primed T cells. These functions appear to relate to the presence of surface Ia in that a hetero-anti-Ia antibody can block these effects. Noteworthy is the finding that the subpopulation of T cells stimulated when epithelial cells are used as accessory cells is the T8⁺,9.3⁻ T cell. These cells

function as potent antigen-nonspecific suppressor cells in both MLR, T cell antigen responses, and induction of B cell differentiation by PWM. These findings have significant implications in local gut immune responses and may help explain several poorly characterized phenomena of mucosal immunity.

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