Reconstitution of Cultured Intestinal Epithelial Monolayers with a Mucosal-derived T Lymphocyte Cell Line

Modulation of Epithelial Phenotype Dependent on Lymphocyte–Basolateral Membrane Apposition

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Abstract

In vivo, epithelial cells which line the intestine are intimately associated with lymphocytes, termed intraepithelial lymphocytes. Previous studies have demonstrated that intraepithelial lymphocytes are present in the uninflamed mucosa, and become especially prominent in various human enteropathies including coeliac disease, tropical sprue, dermatitis herpetiformis, and giardiasis. Using the intestinal crypt cell line T84, and a previously well-defined human mucosa-derived lymphocyte (MDL) line with phenotypic features similar to (but not specific for) intraepithelial lymphocytes, we describe a co-culture model to study the functional sequellae of MDL-T84 cell interactions in vitro. A co-culture method was defined which permitted reconstitution of the paracellular spaces of physiologically confluent epithelial monolayers with MDL. Such co-cultures thus mimicked the correct geometry of intraepithelial lymphocytes–epithelial cell interactions. The presence of physiologically positioned MDL brought about specific and dramatic effects on intestinal epithelial monolayer function. In a dose-dependent fashion, the presence of MDL significantly attenuated barrier function (expressed as a decrease in monolayer resistance), decreased epithelial electrogenic Cl^- secretion, and modulated epithelial–neutrophil interactions. Such effects were not reproduced in monolayers similarly reconstituted with inert polystyrene beads equivalent in size to MDL. These MDL-elicited effects on epithelial function specifically required direct MDL apposition to the epithelial basolateral membrane. Furthermore, this specific form of MDL–epithelial basolateral contact released soluble factors which were able to confer the MDL-reconstituted phenotype on virgin epithelial monolayers in the absence of MDL. We have previously shown that many aspects of the MDL converted epithelial phenotype described here can be induced by IFN-γ. While IFN-γ, a cytokine produced by many lymphocytes including intraepithelial lymphocytes, was detectable in conditioned supernatants from co-cultures, it existed at concentrations insufficient to fully explain the physiologic effects observed here. (*J. Clin. Invest.* 1994. 788–796.) Key words: intestinal disease • inflammation • cytokine • neutrophil • electrolyte transport

Introduction

On surfaces exposed to the external environment, such as the intestine, the genito-urinary tract, and the lung, lymphocytes become compartmentalized to form the mucosal immune system. In the intestine, two major lymphocyte populations exist; those lymphocytes which localize to the subepithelial lamina propria, termed lamina propria lymphocytes (LPL),[1] and a second population of lymphocytes which remain associated with the basolateral membrane of intestinal epithelial cells, termed intraepithelial lymphocytes (IEL) (1). The role of IEL is not clearly understood. Acute inflammation and ulceration of the intestinal mucosa is rarely associated with increased accumulation of IEL. However, notable increases in the number of IEL is a prominent feature of chronic inflammatory conditions (2) and a number of enteropathies with a wide spectrum of etiological factors present with distinct increase in the number of IEL (3, 4). Moreover, several studies indicate that IEL of patients with Crohn's disease and ulcerative colitis may be activated in vivo and may contribute to the symptomology observed in these patients (5, 6).

Phenotypically, both lymphocyte populations present in the intestine, IEL and LPL, resemble memory T cells ($CD45RO^+$), and express predominantly the $\alpha\beta$–T cell receptor (1). In addition nearly all IEL and ~ 40% of LPL express the $\alpha^E\beta_7$-integrin, HML-1, an adherence molecule found on peripheral blood lymphocytes only after mitogenic stimulation, suggesting a role for this molecule on mucosal T cells (7, 8). It is likely that IEL in part function to recognize foreign or transformed self antigens presented by intestinal epithelial cells (9). Little is known about other potential functions of IEL at the level of the intestinal microenvironment.

Previous work has demonstrated that the intestinal mucosa

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1. *Abbreviations used in this paper:* IEL, intraepithelial lymphocytes; LPL, lamina propria lymphocytes; MDL, mucosal-derived lymphocyte; VIP, vasoactive intestinal peptide; Isc, short circuit current.

is an enriched source of the cytokines such as IFN-γ (10). IFN-γ is produced by IEL (11) and after epithelial preexposure to IFN-γ in vitro, epithelial function at several different levels is modulated, including diminished barrier function (12, 13, 14), increased flux of inert molecules (13), decreased Cl^- secretory responses to agonist stimulation (14, 15), enhanced expression of MHC class II molecules (15, 16, 17) and modulated interaction with neutrophils (12, 15). Although such data suggest that cytokines have the potential to modulate epithelial phenotype, it is not clear whether cytokine concentrations required for such effects are actually seen by the epithelial surface. Furthermore, cytokines and other cellular paracrine signals can have interacting effects ranging from negating to potentiating. Thus, to ascertain whether IEL actually influence epithelial phenotype, it appears crucial to obtain information from models in which intact cell types, both producing their plethora of bioactive compounds, inter-relate and do so in anatomically correct fashion.

In this study, we define a model which permits mechanistic studies of the sequellae of anatomically correct intestinal epithelial interactions with a human mucosal-derived lymphocyte line (MDL). This MDL line shares many phenotypic features of IEL, however, such features do not inequivocally identify them as IEL in origin, thus we conservatively refer to them as MDL. The results suggest that physiologically positioned MDLs extensively modulate the phenotype of intestinal epithelia. Moreover, such effects require direct interface of the MDL with the epithelial basolateral membrane and result in the release of bioactive factor(s) which confer the MDL-elicited phenotype on epithelial cells distant from the specific MDL-epithelial contact site.

Methods

Approximately 400 epithelial monolayers were used for the studies reported here.

Cell culture

Mucosal-derived lymphocytes. MDLs were isolated from human intestinal tissue as described previously and maintained by intermittent stimulation with phytohemogglutinin and irradiated feeder cells (8, 19). The phenotypic characteristics of these cells have been defined in great detail elsewhere (8, 19). In brief, the MDL were isolated as previously detailed and two color FACS analysis (8, 19), revealed that the $CD3^+$ cells express an $\alpha\beta$ TCR, were $CD8^+$, HML-1^+, and had a cell surface phenotype that included IL-$2R^+$ (p55), α^{1+} and $CD45RO^+$, consistent with an activated state. Expression of CD8 and not CD4 on the CD3+ cells confirmed that the T-lymphocytes isolated from stripped intestinal epithelia represented a highly purified MDL preparation (see references 8, 19 for details). The appearance of IL-$2R^+$ likely represents an in vitro acquisition of an activated phenotype.

T84 cells. T84 cells were grown and passaged, as previously described (18) in a 1:1 mixture of Dulbecco's modified Eagle's medium (DME) and Ham's F-12 medium supplemented with 14 mM $NaHCO_3$, 40 mg/l penicillin, 9 mg/l streptomycin, 8 mg/l ampicillin, 5% newborn calf serum and 15 mM Na^+-Hepes buffer, pH 7.5. T84 intestinal epithelial cells (passages 60–90) were split near confluency by incubating in 0.1% trypsin and 0.9 mM EDTA in Ca^{2+} and Mg^{2+} free phosphate buffered saline for 15–20 min. After trypsinization, epithelial cells were mixed with intraepithelial lymphocytes in three different ratios; namely, 0.3 lymphocytes per epithelial cell (0.3:1), 1 lymphocyte per epithelial cell (1:1) and 3 lymphocytes per epithelial cell (3:1). Epithelial/lymphocyte mixtures were plated on 0.33-cm^2 ring supported polycarbonate filters (Costar Corp., Cambridge MA). Co-cultures were grown in the above described media (1:1 mixture of DME and Ham's F-12 medium) to which human recombinant IL-2 was added at a final concentration of 2 nM. These epithelial–lymphocyte co-cultures were used 6–10 d after plating.

Morphologic studies

Confluent monolayers were used in morphologic studies using conventional microscopy as well as confocal microscopy. MDLs were localized and viewed by confocal after staining monolayers with rhodamine-phalloidin and/or OKT8 mAb (recognizes CD8+ lymphocytes) followed by FITC-labeled goat anti–mouse secondary mAb as described previously (19).

Counting of MDL within epithelial monolayers was performed on double-labeled monolayers (antibody OKT8 recognizing MDL CD8 and visualized with FITC-conjugated secondary antibody; rhodamine–phalloidin to recognize the apical perijunctional actin-myosin ring of T84 cells and therefore count T84 cell numbers). MDLs were counted on twenty different optical fields. T84 cells were counted on the same monolayers by first taking pictures of different optical fields followed by counting the number of T84 cells per field.

Physiologic assays

To measure currents, transepithelial potentials and resistance, a commercially available voltage clamp (Iowa Dual Voltage Clamps, Bioengineering, University of Iowa) interfaced with an equilibrated pair of calomel electrodes and a pair of Ag-AgCl electrodes was utilized as described in detail elsewhere (20). Stimulated short circuit current (Isc) was determined using the agonists forskolin (10 μM), carbachol (100 μM), or vasoactive intestinal peptide (VIP, 10 ng/ml). Using these values and Ohm's law (V = IR), tissue resistance and transepithelial current were calculated. System resistance was $< 5\%$ of total transepithelial resistance. Between different readings of one monolayer, $< 10\%$ variability in resistance and spontaneous transepithelial electrical potential exists (20).

To determine whether spatial rearrangement within epithelial monolayers induced by interspersed lymphocyte volume could account for the effects observed during co-culture, latex beads (11.9 ± 1.9 μm diameter, neutral charge; Seradyn Inc., Indianapolis, IN) were mixed with epithelial cells at the time of plating (three beads/epithelial cell). Subsequent imaging by phase microscopy verified the basolateral position of these beads. Monolayers co-incubated with latex beads were grown to confluency and electrophysiologically assessed as described above.

Furthermore, to determine whether cell–cell contact was essential for the observed effects, T84 cells were plated on 0.33-cm^2 ring supported polycarbonate filters, and MDL were placed in the reservoir below epithelial monolayers (i.e., no direct MDL-epithelial contact) at a concentration of 3 MDL per T84 cell. Monolayers were grown to confluency and electrophysiologically assessed as described above. In a subset of experiments, lymphocytes were settled for 2 or 4 d on the apical surface of previously confluent, polarized epithelial monolayers to permit contact between lymphocytes and the physiologically inappropriate membrane (apical membrane).

In other experiments, we determined whether a transferrable, soluble factor could account for the physiological effects observed in co-cultures. To test this, co-cultures were grown to confluency as described above. Media (termed conditioned media) from co-cultures of 3MDL:1T84 cell were harvested, filtered through a 0.2 μm filter and applied to virgin, confluent T84 monolayers. After 3 d, transepithelial resistance and stimulated short circuit current were determined and compared with control monolayers that were not exposed to conditioned media.

PMN transmigration assay

The PMN transepithelial migration assay has been previously described in detail (21, 22). Briefly, human PMNs were isolated from normal human volunteers and suspended in modified HBSS (without Ca^{2+} and Mg^{2+}) at a concentration of 5×10^7/ml. Before addition of PMN to the apical surface of monolayer co-cultures, monolayers were extensively

rinsed in HBSS and equilibrated at 37°C. Transmigration assays were performed in the presence of a 1 μM transepithelial gradient of the PMN chemoattractant fMLP (n-formyl-met-leu-phe; Sigma Chemical Co., St. Louis, MO). PMN transmigration was initiated by the addition of 2×10^6 PMN to the upper chamber (apical surface) of co-cultures and transmigration was allowed to proceed in the basolateral direction for 120 min. Transmigration was terminated by placing monolayers at 4°C for 30 min. Nonadherent PMNs were extensively washed from the apical surface of co-cultures. After removal of loosely adherent PMNs, transepithelial migration was quantitated by assaying for the PMN azurophilic granule marker myeloperoxidase. Monolayers were solubilized using a 0.5% solution of Triton-X 100 and transferred to a 96-well microtiter plate followed by the addition of 100 μl ABTS solution to each well. Color development was assayed at 405 nm on a microtiter plate reader (Molecular Devices, Menlo Park, CA) as described previously. The number of PMN were quantified in the reservoir bath (the number of PMN which had completely traversed the monolayer into the reservoir) as well as the number of PMN which remained associated with the epithelial monolayer (monolayer associated). The total number of transmigrating PMNs was expressed as the sum of the two measurements. PMN cell equivalents were estimated from a daily standard curve.

PMN adhesion assay

The PMN–epithelial adhesion assay has been described in detail elsewhere (15). Briefly, co-culture monolayers (basolaterally positioned MDL only, see Results) or monolayers containing no MDL were extensively washed in HBSS followed by the addition of 2×10^6 PMN to the apical surface of monolayers. Plates were centrifuged at 800 r.p.m. for 4 min to uniformly settle PMN to the epithelial surface. After 10 min at 37°C, fMLP (10^{-8} M final concentration) was added and PMN were allowed to adhere for an additional 5 min. Under such conditions, PMN adhere only to the apical surface of the monolayer (Parkos Colgan, Bacarra, Nasrat, Delp-Archer, Carlson, Su, and Madara, manuscript submitted for publication) and thus do not have access to basolaterally positioned elements, here being MDL. Monolayers were immediately washed with HBSS and assayed for myeloperoxidase content as described above.

Cytokine quantitation

Interferon-γ (IFN-γ) was quantified from media conditioned with MDL:epithelial co-cultures using a commercially available Human Interferon-gamma ELISA kit (Endogen Inc., Boston, MA) following the manufacturer's instructions. Interleukin-8 (IL-8) was quantitated from conditioned media taken from MDL:epithelial co-cultures or from control monolayers (i.e., no MDL contact) using a capture ELISA as described previously (23). Rabbit anti–human IL-8 polyclonal antibody (10 μg/ml, Endogen Inc., Boston, MA) was attached to 96-well plates by overnight incubation at 4°C. Plates were blocked with 5% BSA and culture supernatants or IL-8 standards (Genentech Corp., South San Francisco, CA) were added for 2 h at 22°C. As a positive control, virgin T84 monolayers were exposed to PMA (500 ng/ml, 1 h, 37°C). After washing with HBSS, a goat anti–human IL-8 polyclonal antibody (10 μg/ml; R&D Systems, Minneapolis, MN) was added for 1 h at 22°C. Peroxidase-conjugated rabbit-anti-goat IgG was used as a detection antibody and color development was monitored at 405 nm after addition of ABTS substrate.

Data presentation

Results were analysed by comparison of means using Students' t test or by analysis of variance (ANOVA) and are expressed as mean±SEM.

Results

Morphology of mucosal-derived lymphocyte positioning within T84 monolayers. T84 intestinal epithelial cells were plated on 0.33-cm^2 ring supported polycarbonate filters as co-cultures with varying densities of MDL and the morphologic appearance of these monolayers was assessed using confocal microscopy. These images, shown in Fig. 1, revealed that co-cultures of T84 cells and lymphocytes existed as confluent epithelial monolayers with lymphocytes located adjacent to the basolateral surface of T84 cells, defined as being located subjunctionally. This finding is in agreement with that reported elsewhere on co-cultures of MDL with T84 cells grown on glass coverslips (19).

We next quantitated the number of MDLs associated with T84 monolayers plated at varying densities of MDL:T84 cells. As shown on Table I, with increasing plating densities of MDL, an increase in the number of basolaterally-associated MDL was observed (ANOVA $P < 0.05$). These results demonstrate that when epithelial cells are co-cultured with native MDL, resulting monolayers assume a physiologically relevant orientation.

Effect of monolayer reconstitution with MDL transepithelial resistance. T84 intestinal epithelial cells exhibit the unique feature of a high transepithelial resistance to passive ion flow (20). We therefore examined the effects of the presence of MDL in co-cultures with T84 cells on transepithelial resistance to passive ion flow. As shown in Fig. 2, the presence of MDLs brought about a dose-dependent decrease in resistance with increasing monolayer-associated MDL (baseline resistances were 970±56.3, 385±46.9, 180±21.4 ohm·cm^2, at plating densities of 0.3:1, 1:1, and 3:1, respectively, vs no MDL control T84 cells with a baseline resistance of 1284±63.4 ohm·cm^2, one way-ANOVA $P < 0.01$).

In a subset of experiments, we determined whether this fall in monolayer resistance with increasing MDL density could be explained by the spatial rearrangement of epithelial tight junctions. To answer this question, T84 epithelial cells were plated at high density (i.e., 3 beads/epithelial cell) with inert, neutral charged latex beads with a diameter similar to MDL (11.9 μm). The presence of latex beads (morphologically confirmed to be basolaterally positioned throughout the monolayer in a fashion analogous to MDL, data not shown) had only minimal physiologic effects on monolayer resistance (Fig. 2, resistance 919±56.0 ohm·cm^2).

This attenuation in baseline resistance by MDL was not explained by the exogenous addition of IL-2 (see Methods). To determine this, T84 epithelial monolayers were exposed to IL-2 at similar concentrations as added to co-cultures (2 nM) and were subsequently assessed for effects on transepithelial resistance. Compared with control monolayers, IL-2 did not affect baseline transepithelial resistance (baseline resistance 1,050±82.0 ohm·cm^2 for monolayers exposed to IL-2, 2 nM, 72h; vs control monolayers supporting a resistance of 1,230±125 ohm·cm^2, $n = 6$, NS), suggesting that exogenous addition of IL-2 used to establish co-cultures did not explain the physiologic effects observed during co-culture.

Effect of monolayer reconstitution with MDL on epithelial electrogenic Cl^- secretion. T84 cells secrete Cl^- from apical membrane channels by a process termed electrogenic Cl^- secretion, reflected as the short circuit current, Isc, generated by the monolayer. To determine the effects of co-culturing T84 cells with MDL on Cl^- secretion, differing densities of MDL were plated with T84 cells as described above and subsequently assessed for their ability to generate a stimulated Isc. No significant effects on baseline, unstimulated currents were observed in co-cultured monolayers (5.3±0.72, 5.2±1.08, 3.4±1.06 μA/cm^2, at plating densities of 0.3:1, 1:1, and 3:1, respectively,

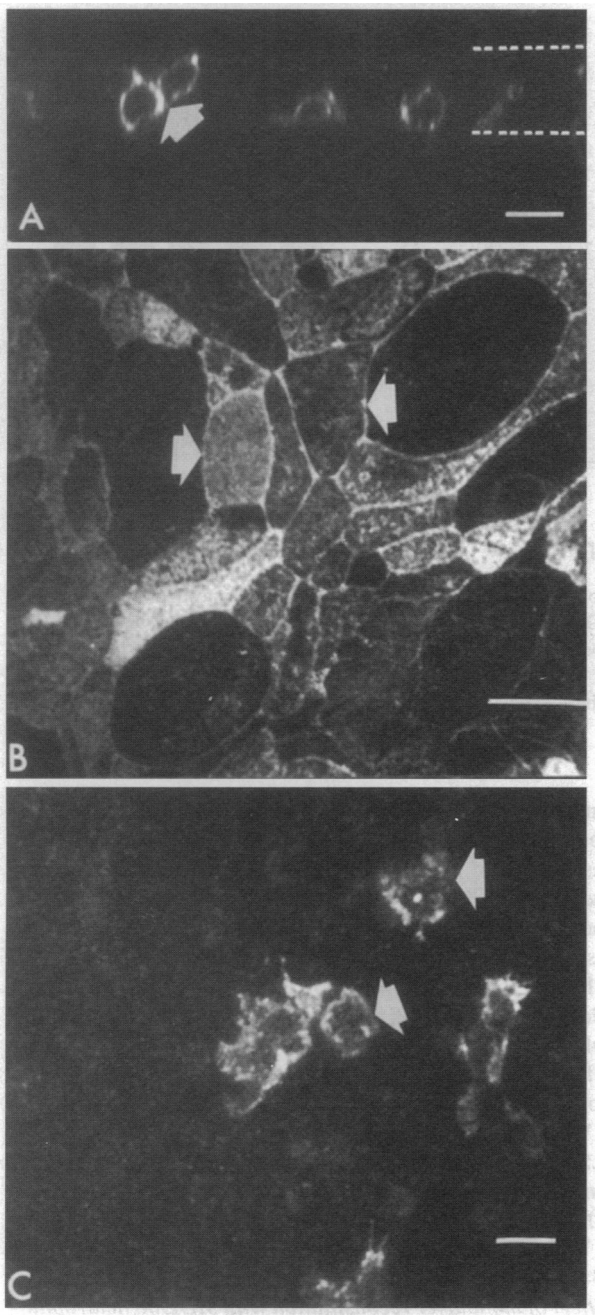

Figure 1. Localization of MDL in co-culture monolayers using confocal microscopy. T84 intestinal epithelial monolayers were co-cultured with human MDL at a ratio of 3MDL:T84 cell for 5 d as described in Methods. Monolayers were stained with rhodamine–phalloidin to label f-actin and lymphocytes were visualized using OKT8 (anti-CD8) followed by FITC-conjugated goat anti–mouse secondary Ab. (*A*) A confocal X-Z axis of co-culture monolayers revealing the presence of stained MDL within the plane of focus (*arrows*). The dashed lines to the right define the positions of the apical (*top*) and basal (*bottom*) surfaces of the T84 monolayer. The position of these membranes is constant across the panel (including the unmarked portion of the panel). (*B*) An en face view of the apical surface of double stained co-culture monolayers. The ring-like structures (*arrows*) represent perijunctional actin–myosin rings and define individual T84 cell apical membranes. The speckled apical labeling represents microvillus f-actin. At this plane, no MDLs were present. (*C*) An en face image taken at 6 μM into the monolayer revealing localization of MDL to the basolateral surface of T84 monolayers (*arrows*). Bars, ~ 10 μm.

Table I. Effect of Varying Plating Densities on Monolayer Morphology

Plating density	Mucosal-derived lymphocytes
*MDL:T84**	*mean±SEM number MDL/T84 cell*‡
0.3:1	1.12±0.197
1:1	1.73±0.218§
3:1	1.88±0.296§

* Plating density at the time of initial co-culturing. ‡ Results were obtained by counting the number of MDL found in 20 separate fields on two occasions. Results are expressed as the mean±SEM number MDL/field. Counting of MDLs was done on double labeled monolayers. MDLs were counted using the OKT8 antibody. T84 cells were counted on the same monolayers, using rhodamine phalloidin, by first taking pictures of three different optical fields and then counting the number of T84 cells per field. T84 numbers were consistent on both occasions. § significantly different compared with plating density of 0.3:1 by Student's *t* test, $P < 0.05$.

vs control T84 cells plated without MDL carrying a baseline Isc of 5.2±0.51 μ A/cm^2, NS).

When MDL:epithelial co-cultures were stimulated to secrete Cl^-, an attenuated Isc was evident with increasing MDL concentrations using three different agonists (Fig. 3). In response to forskolin stimulation (10 μM final concentration), a cAMP-mediated agonist, peak Isc tended to decrease with increasing MDL density. As shown in Fig. 3 *A*, a statistically significant decrease in Isc was apparent at an initial plating density of 3 MDL:1 T84 (65.6±2.15 vs 32.8±7.70 μA/cm^2 for control vs co-culture, respectively, $P < .01$). In contrast, the presence of

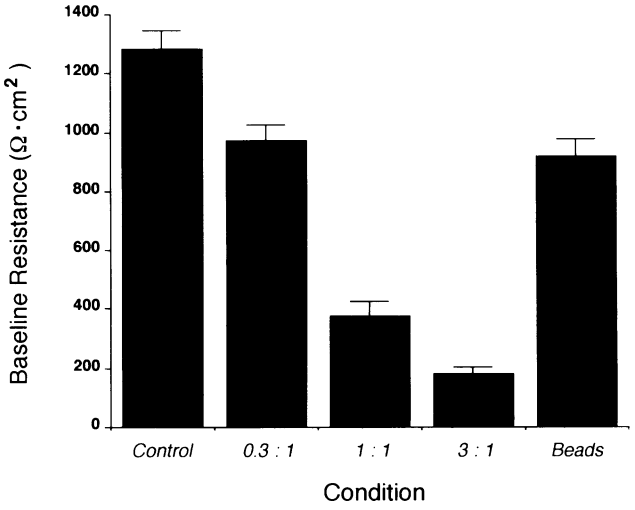

Figure 2. Epithelial:MDL co-culture decreases baseline transepithelial resistance. T84 intestinal epithelial cells were co-cultured with human intestinal MDL at indicated ratios (expressed as MDL per epithelial cell), or were co-cultured with latex beads of approximately equivalent volume at the highest concentration (i.e., three beads/epithelial cell). Monolayers were washed with HBSS and media was replaced with HBSS. Transepithelial resistance was measured by standard methods (see Methods). Data are pooled from 40 monolayers in each condition and results are expressed as the mean±SEM.

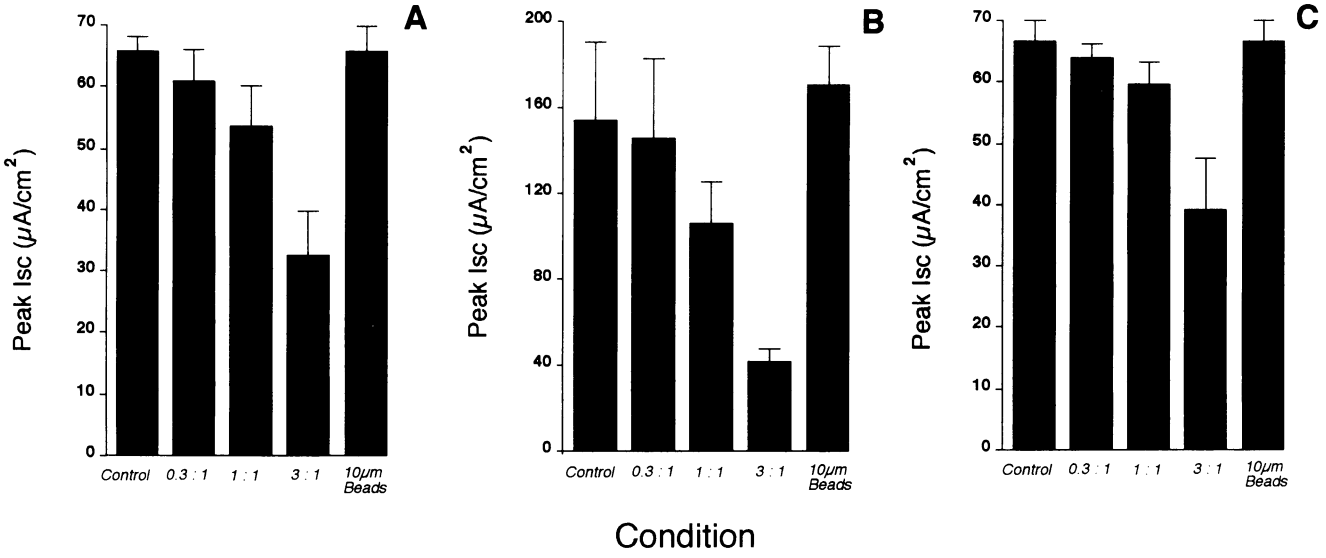

Figure 3. Epithelial:MDL co-culture attenuates epithelial electrogenic Cl^- secretion to a variety of specific agonists. T84 intestinal epithelial cells were co-cultured with human intestinal MDL at indicated ratios (expressed as MDL per epithelial cell). Monolayers were washed with HBSS and media was replaced with HBSS prior to the addition of forskolin (10 μM final concentration, *A*), carbachol (100 μM final concentration, *B*) or vasoactive intestinal peptide (0.3 nM final concentration, *C*). Short circuit current (Isc) was measured by standard methods (see Methods). Data are pooled from nine monolayers in each condition and results are expressed as the mean±SEM peak Isc.

equivolume basolaterally located latex beads at 3 beads/T84 cell during co-culture did not attenuate forskolin-stimulated current (65.6±2.15 vs 67.4±3.45 μA/cm^2 for no MDL control and 3 beads:1T84, respectively, NS). A comparison of the Isc dose response to forskolin (10 μM to 10 mM) is shown in Fig. 4

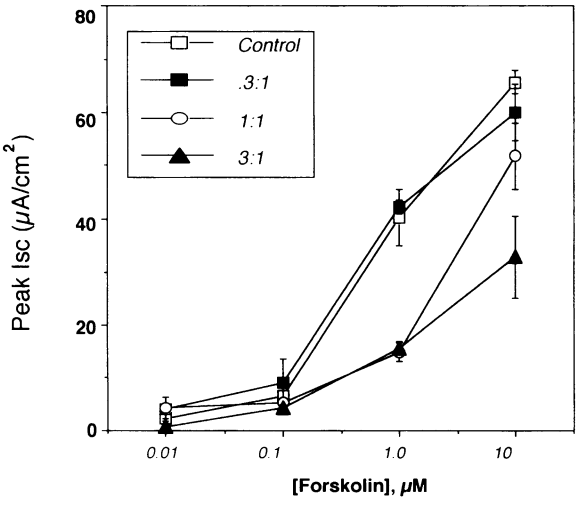

Figure 4. Effect of increasing concentration of forskolin on electrogenic Cl^- secretion from MDL–epithelial co-culture monolayers. T84 intestinal epithelial cells were co-cultured with human intestinal MDL at indicated ratios (expressed as MDL per epithelial cell). Monolayers were washed with HBSS and media was replaced with HBSS prior to the addition of forskolin (range 0.01–10 μM final concentration). Short circuit current (Isc) was measured by standard methods (see Materials and Methods). Data are pooled from 9 monolayers in each condition and results are expressed as the mean±SEM peak Isc.

and indicates a shift in the dose response with increasing MDL concentration during co-culture (2-factor ANOVA, $P < .01$).

As shown in Fig. 3 *B*, using the Ca^{2+}-mediated agonist carbachol (100 μM final concentration), peak Isc decreased as a function of MDL density (one-way ANOVA, $P < 0.005$), with significant effects at an MDL:T84 concentration of 3:1 ($P < .01$), whereas the presence of latex beads at 3 beads/T84 cell during co-culture did not result in an attenuated carbachol-stimulated current (154.6±32.15 vs 163.4±21.45 μA/cm^2 for no MDL control and 3 beads:1T84, respectively, NS). Stimulation of electrogenic Cl^- secretion by a receptor-mediated agonist, VIP (10 ng/ml) revealed an attenuated Isc at the high MDL:T84 plating densities (Fig. 3 *C*, 66.3±3.25 vs 36.4±11.45 μA/cm^2 for no MDL control and 3 MDL:T84, respectively, $P < 0.01$) while latex beads did not influence VIP-mediated currents (66.3±3.25 vs 67.9±4.43 μA/cm^2 for no MDL control and 3 beads/T84, respectively, $P < 0.01$).

Effect of monolayer reconstitution with MDL on neutrophil–epithelial interaction. We have previously demonstrated that human PMNs can be induced to migrate across T84 intestinal epithelial monolayers in the presence of a transepithelial gradient of the PMN chemoattractant n-formyl-met-leu-phe (fMLP, 1 μM final concentration). As shown in Fig. 5 *A*, co-cultures of lymphocytes and intestinal epithelial cells supported increased fMLP-stimulated PMN transepithelial migration in the apical to basolateral direction. Increased PMN migration was reflected primarily as an increase in the number of PMNs completely traversing the monolayer into the reservoir (4.3±0.94, 5.4±0.82, 8.2±1.60 PMN CE/ml for co-cultures of 0.3, 1 and 3 MDLs per T84, respectively, vs 4.4±0.89 for no MDL control, $P < 0.025$ by ANOVA). The presence of MDL during co-culture did not affect the number of monolayer-associated PMN at any co-culture plating density (one-way ANOVA, $P > 0.05$).

We have recently reported a method in which fMLP-stimu-

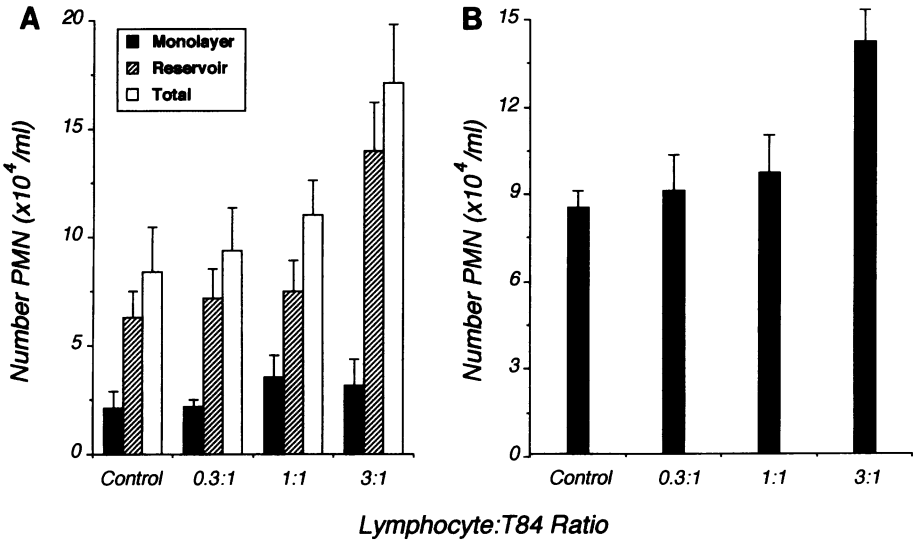

Figure 5. Presence of human MDL in co-culture with intestinal epithelial cells modulates subsequent neutrophil-epithelial interactions. T84 intestinal epithelial cells were co-cultured with human intestinal MDL at indicated ratios (expressed as MDL per epithelial cell). Monolayers were washed with HBSS and media was replaced with HBSS. PMN transmigration (*A*), or adhesion (*B*) was initiated by adding PMN (2×10^6/monolayer) to the surface of monolayers and allowed to proceed as described in Methods. The number of PMNs was quantitated by enzymatic quantitation of myeloperoxidase according to a daily PMN standard. Data are pooled from 6–9 monolayers in each condition and results are expressed as the mean±SEM number of PMN.

lated PMN adhesion to T84 intestinal epithelial cells can be assessed independent of PMN transepithelial migration (15). To assess whether co-cultures of MDL and T84 cells modulate PMN adhesion, co-culture monolayers at different densities were prepared as described above followed by the addition of PMN to reconstituted epithelial monolayers. As shown in Fig. 5 *B*, the presence of MDL in co-cultures increased PMN adhesion to the apical surface of co-culture monolayers. This effect was especially prominent at the highest MDL:T84 density (4.4±0.64 vs 7.9±0.86 PMN CE/ml for no MDL control and 3 MDL per T84, respectively, $P < 0.025$).

Recently, it was shown that T84 cells produce and secrete IL-8 (23), a potent PMN chemoattractant. To determine whether the presence of MDL in co-cultures could induce the production and secretion of IL-8, culture supernatants were assayed for IL-8 during MDL-epithelial co-culture. Our results reveal that the presence of MDL in co-cultures did not liberate T84 secretion of IL-8 above baseline values (1.6±0.44 ng/ml for controls vs. 1.3±0.29, 1.7±0.53, 1.2±0.28 ng/ml for co-cultures of 0.3:1, 1:1, and 3:1 lymphocytes/T84 cell, respectively, all $P > 0.05$ compared to controls). As a positive control, stimulation of control T84 monolayer IL-8 production with PMA (500 ng/ml) brought about a significant increase in detectable IL-8 (4.2±0.58 ng/ml, all $P < 0.01$). These results indicate that the production of IL-8 by T84 cells during co-culture with MDL is an unlikely explanation for the results observed here.

MDL-elicited alteration of epithelial phenotype requires correct anatomic positioning of MDL. To determine whether the MDL modulation of epithelial phenotype required correct anatomical positioning of MDL, two series of experiments were performed. First, MDL were plated in the reservoir below epithelial monolayers (i.e., no direct MDL:epithelial cell contact) at ratios of 0.3:1, 1:1, or 3:1 MDL per T84 cell. Resistances of these monolayers were 1287±62.9, 1042±101.2, and 1688±53.1 ohm·cm^2, respectively, while control monolayers without MDL established a transepithelial resistance of 1284±63.4 ohm·cm^2 ($P > 0.05$ by ANOVA). We next used gravitational settling of MDL onto the apical surface of postconfluent monolayers to determine whether the resulting improperly polarized MDL-epithelial apposition would also elicit the MDL modulated epithelial phenotype. Apical addition of MDL at the various densities (0.3:1, 1:1, and 3:1) for 4 d brought about no significant decrease in transepithelial resistance (1174±50.6, 1481±39.5, and 1338±59.1 ohm·cm^2, respectively, compared with control monolayers without MDL; 1326±32.7 ohm·cm^2, $P > 0.05$ by ANOVA). Similarly, and in contrast to MDL reconstituted monolayers, MDL positioned either in the basolateral reservoir (i.e., no direct MDL basolateral membrane contact) or directly on the apical surface of postconfluent epithelial monolayers had no effect on agonist-elicited Cl^- secretion. Indeed, monolayers exposed to 3MDL:T84 cell from the basolateral reservoir and subsequently stimulated with forskolin (10 μM) generated a Isc of 41.9±3.05 μA/cm^2 vs. no MDL control monolayers of 46.9±6.24 μA/cm^2 ($P > 0.05$). Apically positioned MDL (3 MDL:T84 cell) stimulated with forskolin (10 μM) resulted in a Isc of 59.2±6.05 μA/cm^2 compared to no MDL control monolayers of 66.3±7.24 μA/cm^2 ($P > 0.05$). These data suggest that the physiologic attenuation in transepithelial resistance by MDL requires direct MDL interaction with epithelial basolateral membranes. Importantly these latter findings also indicate that direct apposition of MDL with epithelial membranes does not by itself lead to detectable alterations in epithelial function.

Lastly, conditioned media from 3MDL:T84 co-cultures were pooled and transferred to virgin, confluent T84 monolayers and resistance was assessed 3 d later. These experiments demonstrated a significant drop in transepithelial resistance (376±23.7 ohm·cm^2 for monolayers fed MDL-T84 cell conditioned media vs 1284±63.4 ohm·cm^2 for controls, $n = 6$, $P < 0.001$), and an attenuation in forskolin-stimulated (10 μM) Isc (10.4±2.03 μA/cm^2 for monolayers fed MDL-T84 cell conditioned media vs 23.5±2.79 ohm·cm^2 for no MDL controls, $n = 6$, $P < 0.01$), thus indicating that the factor(s) responsible for decreased transepithelial resistance associated with MDL reconstitution of monolayers is/are soluble, are produced only after basolateral membrane contact with MDL, and can influence epithelial phenotype in distant non MDL-associated sites (i.e., can be transferred to MDL free monolayers).

Quantitation of IFN-γ elicited from MDL reconstituted monolayers. We have previously demonstrated (12, 13, 15),

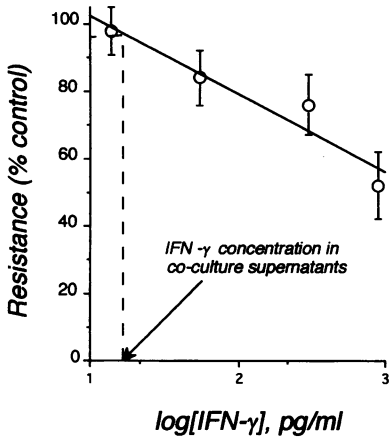

Figure 6. Correlation of IFN-γ effects on transepithelial resistance with quantitated cytokine levels found in co-culture supernatants. T84 intestinal epithelial cells were exposed to human recombinant IFN-γ at indicated concentrations for 48 h. Monolayers were washed with HBSS and media was replaced with HBSS. Transepithelial resistance was measured by standard methods. Also indicated is the concentration of IFN-γ quantitated from 3MDL:T84 co-cultures (*arrow*).

and others have confirmed (14, 24), that epithelial exposure to recombinant IFN-γ in vitro induces physiologic effects similar to those observed here, including modulation of epithelial barrier function (12, 13, 14, 15, 24), attenuation of Cl- secretion (14, 15) and modulation of PMN transmigration and adhesion (12, 15). Thus, we next assayed conditioned supernatants from MDL:epithelial cultures for human IFN-γ production using a quantitative ELISA. IFN-γ was easily detectable in supernatants from co-culture and increased with increasing MDL concentrations (0.7 ± 0.20, 2.6 ± 3.43, 17.2 ± 1.52 pg/ml for 0.3:1, 1:1, and 3:1 lymphocytes:T84 concentrations, respectively, $n = 8-12$ each). As shown in Fig. 6, addition of exogenous cytokine to virgin T84 monolayers, as we have shown before (12, 13), results in a concentration-dependent decrease in transepithelial resistance. As indicated in Fig. 6, the concentration of IFN-γ quantitated from 3:1 co-culture supernatants does not explain the decrease in barrier function observed in the above described results. Indeed, linear regression suggests that concentrations of cytokine in supernatants would attenuate barrier function by $< 5\%$ (see Fig. 6), indicating that other factors likely play a role in the above observed results. Similarly, IFN-γ concentrations at least an order of magnitude greater than that measured in the conditioned media here are required to produce the effects of IFN-γ on epithelial Cl^- secretion and neutrophil adherence (15). It is important, however, to consider that cytokine not detectable in the bulk flow could play a major role in the physiologic changes observed here. Attempts to negate such effects using anti-IFN-γ antibodies have been unsuccessful (data not shown).

Discussion

We demonstrate here that cultured monolayers established from human intestinal epithelial cell lines can be successfully reconstituted with MDL cell lines derived from human intestinal epithelia. MDLs in this reconstituted system are positioned above the epithelial basement membrane, below the apical intercellular tight junction, and in direct apposition to epithelial basement membranes as is appropriate physiologically.

Modulation of epithelial function by MDL. Soluble lymphocyte-derived products, such as the dominant mucosal cytokine IFN-γ (10, 11) can modulate the function of intestinal epithelia in vitro (12–17, 24), and likely in vivo (10), when added in pure form. However, it is difficult to access how such experimental results correspond to events which might occur when intact epithelial cells and MDL interface. Intact cells likely release a plethora of biologically active compounds into the local microenvironment, engage potential activating adhesion ligands including CD11a and $\alpha^E\beta_7$ mediated adhesion, and release potentiating, synergystic, or negating signals. For these reasons, we have taken the approach of accessing the functional sequellae of intact MDL–epithelial interactions on assembled, vectorially transporting epithelial monolayers. Our results indicate that MDL contact with basolateral membranes results in the release of signal(s) which elicit a broad phenotype rearrangement of the epithelial surface. First, epithelial "barrier" function is diminished as judged by very sensitive electrical assays. Although such alterations are indicative of an altered epithelial phenotype, it is less clear how functionally significant these alterations may be. In high resistance monolayers such as T84 (18, 20), the relationship between transepithelial resistance and tight junction permeability to inert hydrophilic solutes is asymptotic, with very little change in solute flux above ~ 250 ohm·cm^2 (4). Thus, only at the lowest resistances observed in this study, was junctional solute permeability affected. On the other hand, the most dramatic effects were noted at actual MDL:T84 cell ratios which were low relative to that which can occur during disease states (25). Thus, it is likely that significant attenuations in junctional solute permeability (as opposed to resistance) occur only in states in which the MDL:T84 cell ratios are high. In contrast, Isc, the measure of Cl^- secretion used in this study, correlates well with actual net serosal-to-mucosal Cl^- flux (18). Thus, the large attenuation of Cl^- secretion seen with even low MDL:T84 cell densities suggests that basolateral epithelial–MDL contact has a damping effect on intestinal fluid and electrolyte secretion. Teliologically, such effects could serve to downregulate fluid secretion in chronic phases of intestinal inflammation, states in which MDL densities may be the greatest (26, 27). Lastly, reconstituted MDL–basolateral epithelial contacts elicit a profound alteration in subsequent PMN–epithelial interactions. Under these conditions, the rate at which PMN migrate into epithelial monolayers is enhanced—an effect which can not be attributed to alterations in epithelial barrier function given the resistances observed here (22). This latter point is reinforced by the observation that PMN adhesion assays suggest that reconstituted monolayers support increased expression of PMN ligands on the epithelial surface. In contrast to the above findings, addition of activated peripheral blood T cells to T84 cells resulted in the death of the latter (data not shown).

Based on our previously published data (12, 13, 15), as well as that published by others (14, 16, 17, 24), we have suggested that the epithelial surface phenotype may dynamically shift between classic epithelial states (barrier function, vectorial fluid, and electrolyte transport) and immune accessory states (PMN adhesive ligands, increased MHC Class I and II expression). Furthermore, relative shifts between these states can be induced by MDL-derived cytokines such as IFN-γ (15, 16, 17). Such a hypothesis has been previously proposed by us (15) and others (14) and suggests that MDL may influence the epithelial surface

phenotype within this transport-immune accessory function spectrum.

Nature of signal elicited by MDL–epithelial basolateral contact. Fortuitously, we find that the signal(s) responsible for the above outlined epithelial surface phenotype switch is transferrable in a cell-free conditioned supernatant derived from MDL reconstituted monolayers. Such data indicate that the signal(s) resulting from MDL–epithelial contact is active not only within the epithelial microenvironment, but also in its bulk phase. Since MDLs separated from the basolateral compartment by permeable filters (see Results) elicit no significant signal for epithelial phenotype switch, it is likely that the trigger event for signal liberation is direct interaction of MDL with epithelial basolateral membrane. Similarly, direct contact between MDL and apical membranes of T84 monolayers did not alter monolayer phenotype. The functionally significant MDL-basolateral membrane interactions could, in turn, be influenced by microenvironmental factors. For example, intestinal epithelia, including T84 cells, secrete TGF-β_1, a cytokine which induces surface expression of the IEL integrin $\alpha^E\beta_7$ for which an unidentified epithelial counter-receptor exists on the basolateral membrane (8, 19).

The conditioned media transfer experiments described here do not clearly identify the cell type (MDL vs epithelia) which releases the signal responsible for the alteration in epithelial surface phenotype. However, since the phenotype described is similar to that elicited by epithelial exposure to IFN-γ, we assessed whether release of this cytokine represented the bioactivity sought. Although MDL–epithelial contact was found to induce IFN-γ release, the quantity released resulted in a bulk phase concentration $\sim$ 2 logs lower than that necessary to demonstrate the effect by exogenous addition of cytokine. It is possible that IFN-γ at such low concentrations could be potentiated by an accessory cytokine such as has been described previously in other bioassays (28). However, TNF-α appears to be the major potentiating cytokine for IFN-γ, and we have previously shown that no such potentiating interactions exist for the T84 cell assays used (13). Furthermore, experiments performed using neutralizing antibodies for IFN-γ and conditioned media transfer experiments have not reliably negated the observed transferred bioactivity (unpublished observation). Similarly, although IFN-γ induces enhanced neutrophil adhesion to (15), and migration into (12) T84 monolayers, bulk phase concentrations of IFN-γ were determined to be nearly two orders of magnitude lower than would be expected to elicit this physiologic effect. Interleukin-8, a cytokine which can be induced from intestinal epithelial cells such as T84 (23), and exhibits neutrophil chemotactic activity (25), was also determined not to be responsible for the modulation of PMN-epithelial interactions by MDL-reconstituted monolayers. Having shown that obvious candidate cytokine signals are not responsible for the MDL-elicited alterations in surface phenotype, we are currently using fractionation approaches to identify the bioactivities responsible for our observations.

These data indicate that MDL, when physiologically positioned within intestinal epithelial monolayers, can dramatically alter the profile of epithelial surface functions. We speculate such MDL regulation of epithelial function may play a role in the baseline physiology of monolayer function in vivo and in its regulation in disease states in which MDL are present at increased density or in altered states of activation.

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