

Neutrophil Migration across Cultured Intestinal Epithelial Monolayers Is Modulated by Epithelial Exposure to IFN- γ in a Highly Polarized Fashion

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Abstract. Neutrophil, or polymorphonuclear leukocyte (PMN), migration across intestinal epithelial barriers, such as occurs in many disease states, appears to result in modifications of epithelial barrier and ion transport functions (Nash, S., J. Stafford, and J. L. Madara. 1987. *J. Clin. Invest.* 80:1104–1113; Madara, J. L., C. A. Parkos, S. P. Colgan, R. J. MacLeod, S. Nash, J. B. Matthews, C. Delp, and W. I. Lencer. 1992. *J. Clin. Invest.* 89:1938–1944). Here we investigate the effects of epithelial exposure to IFN- γ on PMN migration across cultured monolayers of the human intestinal epithelial cell line T₈₄. Transepithelial migration of PMN was initially assessed in the apical-to-basolateral direction, since previous studies indicate general qualitative similarities between PMN migration in the apical-to-basolateral and in the basolateral-to-apical directions. In the apical-to-basolateral direction, epithelial exposure to IFN- γ markedly upregulated transepithelial migration of PMN in a dose- and time-dependent fashion as measured by both electrical and myeloperoxidase assays. This IFN- γ -elicited effect on transmigration was specifically due to a IFN- γ effect on epithelial cells and was not secondary to IFN- γ effects on epithelial tight junction permeability. Moreover, this IFN- γ effect was dependent on epithelial protein synthesis, and involved a pathway in which CD11b/18, but not ICAM-1 or CD11a/18, appeared to play a crucial role in PMN-epithelial adhesion. IFN- γ also substantially modified PMN transepithelial migration in the natural, basolateral-to-apical direction. The IFN- γ effect on naturally directed transmigration was also specifically due to an IFN- γ effect on epithelial cells,

showed comparable time and dose dependency to that of oppositely directed migration, was CD11b/18 dependent, and required epithelial protein synthesis. Additionally, however, important qualitative differences existed in how IFN- γ affected transmigration in the two directions. In contrast to apical-to-basolateral directed migration, IFN- γ markedly downregulated transepithelial migration of PMN in the natural direction. This downregulation of PMN migration in the natural direction, however, was not due to failure of PMN to move across filters and into monolayers. Indeed, IFN- γ exposure to epithelia increased the number of PMN which had moved into the basolateral space of the epithelium in naturally directed transmigration. These results represent the first detailed report of influences on PMN transepithelial migration by a cytokine, define conditions under which a qualitative difference in PMN transepithelial migration exists, and suggest that migration of PMN across epithelia in the natural direction may involve multiple steps which can be differentially regulated by cytokines. Specifically, it appears that in naturally directed migration, IFN- γ may enhance the retention time of the recruited PMN in the paracellular space below tight junctions, and does so, at least in part, by downregulating a PMN-epithelial interactive event required for subsequent transjunctional migration. We speculate that such retention of PMN at this specific anatomic location, the site wherein the transition from the external environment to the internal milieu occurs, may serve an important role in mucosal defense.

An early and crucial step in the acute inflammatory response is attachment of neutrophils (PMN)¹, or polymorphonuclear leukocytes to endothelial surfaces and subsequent emigration from the vasculature

(Springer, 1990; Osborn, 1990; Pober and Cotran, 1990). In inflammatory diseases of organs lined by columnar epithelia, however, the migration of PMN is not complete until PMN have also migrated across the epithelial surface (Yardley and Donowitz, 1977; Yardley, 1986). While substantial progress has been made in the understanding of PMN-endothelial interactions (Arnaout et al., 1988; Springer, 1990; Larson and Springer, 1990; Osborn, 1990;

1. *Abbreviations used in this paper:* ANOVA, analysis of variance; CE, cell equivalents; FMLP, n-formyl-methionyl-leucyl-phenylalanine; PMN, polymorphonuclear leukocytes.

Pober and Cotran, 1990; Ruoslahti, 1991), relatively limited information is available concerning PMN-epithelial interactions (Cramer et al., 1986; Nash et al., 1987, 1988; Migliorisi et al., 1988; Parkos et al., 1991, 1992). However, it is likely that such PMN-epithelial interactions may be of substantial importance. For example, we have recently described a neutrophil-derived product which elicits electrogenic Cl^- secretion (the basis of mucosal hydration and, when upregulated in the intestine, the basis of secretory diarrhea) (Donowitz and Welsh, 1987) from human crypt intestinal epithelial cell lines and from primary isolates of mammalian crypt cells (Nash et al., 1991; Madara et al., 1992). This neutrophil-derived secretagogue primarily expresses bioactivity when presented to the apical rather than the basolateral plasma membrane of intestinal epithelial cells. From such observations one can deduce that as a consequence of transepithelial migration of PMN in such organs, many important biological signals may be elicited.

PMN migration across intestinal epithelia is the hallmark of active intestinal inflammation and occurs in such disease states as ulcerative colitis, Crohn's disease, and infectious enterocolitis (Yardley and Donowitz, 1977; Yardley, 1986). Transepithelial migration of PMN is particularly evident in the intestinal crypt and eventuates in crypt abscess formation—a characteristic of severe inflammatory disease (Yardley and Donowitz, 1977; Yardley, 1986). In patients with inflammatory bowel disease, the degree of PMN transepithelial migration, assessed quantitatively, has been shown to correlate with patients' symptoms as well as with the degree of intestinal epithelial barrier dysfunction, as assessed quantitatively (Teahon et al., 1991). Studies of human mucosa in such diseases suggest that PMN transepithelial migration predates focal breakdown of the epithelial surface (Yardley and Donowitz, 1977; Yardley, 1986), and that defective epithelial barrier function also predates structural discontinuities in the mucosa (Hawker et al., 1980). As outlined above, such translocation of PMN across still-intact monolayers can, by exposure of apical membranes to a neutrophil-derived secretagogue (Nash et al., 1991; Madara et al., 1992), elicits fluid secretion from crypt epithelia.

We have previously modeled the event of PMN transmigration using human peripheral blood neutrophils and cultured, human-derived, intestinal epithelial monolayers derived from the cell line T_{84} (Nash et al., 1987; Parkos et al., 1991, 1992; Madara et al., 1993). Such monolayers are composed of columnar epithelial cells with features similar to those of natural crypt epithelia (Madara and Dharmasathaphorn, 1985; Madara et al., 1987). These cells join neighbors through apical circumferential tight junctions which maintain structure-function characteristics found in tight junctions of natural epithelia (Madara and Dharmasathaphorn, 1985). Furthermore, these cells have a polarized surface distribution of the pumps, channels, and cotransporters which allow manifestation of electrogenic Cl^- secretion (Dharmasathaphorn and Madara, 1990). Lastly, the regulation of ion transport events seen in T_{84} cells, both via direct manipulation of intracellular second messengers or indirectly through receptors for hormones or toxins (Dharmasathaphorn and Madara, 1990), closely recapitulates regulatory events seen in natural crypt epithelia. For these reasons, T_{84} cells are viewed as a model for human crypt epithelia—the site at which the bulk of PMN transepithelial migration occurs in a variety of active inflammatory intestinal diseases (Yardley and Donowitz, 1977; Yardley, 1986).

Our prior studies using this epithelium as a model for examination of PMN-intestinal epithelial interactions have indicated that PMN transmigration elicits a reversible decrease in transepithelial resistance due to impalement of intercellular tight junctions (Nash et al., 1987), and requires PMN β_2 -integrins, specifically CD11b/CD18 (Parkos et al., 1991, 1992). While we have shown that PMN transmigration is quantitatively more efficient in the physiological (basolateral-to-apical) direction (Parkos et al., 1991), qualitative differences in transepithelial migration, relating to the direction of transmigration, have generally not been found (Nash et al., 1987, 1988; Parkos et al., 1991).

As is the case with endothelial-PMN interactions, under natural inflammatory conditions, PMN-epithelial interactions often take place in the presence of lymphocyte-derived cytokines (Yardley and Donowitz, 1977; Yardley, 1986). While it is known that cytokines dramatically modulate the interactions of neutrophils with endothelial cells (Osborn, 1990; Pober and Cotran, 1990), essentially nothing is known concerning the potential for cytokine regulation of epithelial-neutrophil interactions. Others have shown that IFN- γ , the 40-kD homodimeric glycoprotein secreted by activated T cells (Trinchieri and Perussia, 1985), is a dominant cytokine in human intestinal mucosa and is particularly enriched in the intestine after antigenic challenge (Quiding et al., 1991). Thus, IFN- γ is likely an important cytokine in the intestinal epithelial microenvironment. Epithelia, including our model intestinal epithelial cell line (Madara and Stafford, 1989), appear to express receptors for IFN- γ . Lastly, IFN- γ appears capable of modulating the intestinal epithelial cell surface with which a transmigrating neutrophil must interface. For example, IFN- γ exposure elicits MHC class II antigen expression on intestinal epithelia (Mayer et al., 1991).

Here we examine the effects of IFN- γ on transepithelial migration of human neutrophils across monolayers of a cryptlike human intestinal epithelial cell line. IFN- γ is found to have substantial effects on PMN transepithelial migration, and thus, these data provide the first detailed description of cytokine modulation of neutrophil-epithelial interactions. We also find that the effects of IFN- γ on PMN transepithelial migration distinctly differ depending on the direction of migration of PMN across the epithelial monolayer. These latter data represent the first report of a qualitative difference in neutrophil-epithelial monolayer interactions relating to the polarity of the interaction.

Materials and Methods

Antibodies

Mouse ascitic fluids containing previously characterized functionally inhibitory mAb to CD18 (TS1/18, subclass IgG₁), CD11a (TS1/22, subclass IgG₁), CD11b (44a, subclass IgG_{2a}), or CD11c (L29, subclass IgG₁), were used as described previously (Parkos et al., 1991, 1992). A control mAb directed against CD10 (J5, subclass IgG_{2a}) and previously shown to bind PMN (Ritz et al., 1980; Arnaout et al., 1983), but not inhibit transmigration (Parkos et al., 1991), was used as a PMN binding control. All mAb were used at a 1:200 dilution in HBSS (containing Ca^{2+} and Mg^{2+} , with 10 mM HEPES, pH 7.4, Sigma Chem. Co., St. Louis, MO) during trans-

migration assays. Anti-ICAM-1 (CD54) mAb R6.5 (Fab fragment) and RRI/1 (Diamond et al., 1991) were generously provided by Dr. Robert Rothlein (Boehringer Ingelheim Pharmaceuticals).

Cell Culture

Approximately 500 epithelial monolayers were used for these studies. T₈₄ intestinal epithelial cells (passages 70–95) were grown and maintained as confluent monolayers on collagen-coated permeable supports as previously described in detail (Dharmasathaporn and Madara, 1990). Monolayers were grown on 0.33-cm² ring-supported polycarbonate filters (Costar Corp., Cambridge, MA) and utilized 6–14 d after plating as described previously (Madara et al., 1993). Inverted monolayers, used to study transmigration of PMN in the basolateral-to-apical direction were constructed as described before (Parkos et al., 1991, 1992; Madara et al., 1993). T₈₄ epithelial monolayers were exposed to recombinant human IFN- γ (10⁶ U/ml, Genentech, Inc., San Francisco, CA) for periods of time ranging from 2 to 72 h and at doses ranging from 1 to 1,000 U/ml. All incubation periods with IFN- γ included controls consisting of media containing no IFN- γ .

Transmigration Assay

The PMN transepithelial migration assay has been previously detailed (Nash et al., 1987, 1988; Parkos et al., 1991, 1992). Briefly, human PMN were isolated from normal human volunteers and suspended in modified HBSS (without Ca²⁺ and Mg²⁺, with 10 mM HEPES, pH 7.4, Sigma Chem. Co.) at a concentration of 5×10^7 ml. Before addition of PMN, T₈₄ monolayers were extensively rinsed in HBSS to remove residual serum components and IFN- γ . Transmigration assays were performed by the addition of PMN to the upper chambers after chemoattractant (1 μ M n-formyl-methionyl-leucyl-phenylalanine [FMLP]) was added to the opposing (lower) chambers. For apical-to-basolateral transmigration experiments, 2×10^6 PMN were added at time 0. However, we have previously shown that transmigration in the basolateral-to-apical direction, while qualitatively similar, is substantially more efficient than in the apical-to-basolateral direction (Parkos et al., 1991). Therefore, fivefold fewer PMN (4×10^5), unless otherwise noted, were added when transmigration proceeded in the basolateral-to-apical direction in order that baseline transmigration signals be equivalent in both directions (Parkos et al., 1991). To assess transepithelial resistance, the upper and lower reservoirs were interfaced with pairs of calomel and Ag-AgCl electrodes via 5% agar bridges. Resistance measurements were obtained before and at 5, 10, 30, 50, and 110 min after the addition of PMN using a dual voltage clamp (University of Iowa) as described before. Validation of and techniques to apply such standard electrical techniques on such monolayer "mini-preps" have been detailed elsewhere (Madara et al., 1993).

Divalent cation depletion experiments were performed as follows: epithelial monolayers were rinsed extensively in HBSS to remove residual serum and IFN- γ . Monolayers were next rinsed free of HBSS and exposed apically and basolaterally to 1 mM EDTA (Sigma Chem. Co.) diluted in modified HBSS for 20 min at 37°C. After 20 min, monolayers were repleted with Ca²⁺ and Mg²⁺ by rinsing and submerging in Ca²⁺ and Mg²⁺ containing HBSS. PMN were added within 15 min after the repletion of Ca²⁺ and Mg²⁺.

Cycloheximide (Sigma Chem. Co.), when used, was prepared fresh as a 2-mg/ml stock in 95% ethanol and diluted to 2 μ g/ml in media at a concentration which we have previously found to inhibit >85% of radiolabeled leucine incorporation into precipitable protein. All experiments were performed in a 37°C room to ensure that epithelial monolayers, solutions, plasticware, etc., were maintained at uniform 37°C temperature.

Transmigration was quantitated by assaying for the PMN azurophilic granule marker myeloperoxidase as described previously (Parkos et al., 1991, 1992). After each transmigration assay, nonadherent PMN were extensively washed from the surface of the monolayer and PMN cell equivalents, estimated from a daily standard curve, were assessed as the number of PMN associated with the monolayer, the number which had completely traversed the monolayer (i.e., into the reservoir bath), as well as the total number of transmigrating PMN (the sum of monolayer and reservoir-associated PMN). As we have previously described (Nash et al., 1987; Parkos et al., 1991), the location of monolayer-associated PMN (apical vs. within paracellular space vs. attached to filter supports) was judged morphologically.

Morphologic Studies

Monolayers were fixed in 2% glutaraldehyde in HBSS at the end of representative experiments ($t = 120$ min) and processed for 1 μ M Epoxy sections or stained for the localization of peroxidase as described previously (Nash et al., 1987).

ICAM-1 ELISA

Expression of ICAM-1 was assayed exactly as described by others (Rothlein et al., 1988) using RRI/1 (mouse ascites diluted 1:100 in 1% BSA) as a primary antibody on 1% paraformaldehyde-fixed T₈₄ epithelial monolayers in 96-well flat-bottomed microtiter plates (Costar Corp.). Negative controls included omitting the primary and/or secondary antibody. HL-60 cells were plated in triplicate on each plate, exposed to 10 ng/ml PMA for 3 d, and served as a positive control for ICAM-1 expression (Dustin et al., 1986).

PMN-Endothelial Adhesion Assay

Adherence of purified human PMN to confluent monolayers of human umbilical vein endothelial cells (a kind gift from Dr. Michael Gimbrone) was done as described previously (Charo et al., 1985) with minor modifications. After the final wash, 100 μ l ABTS solution (containing 1 mM 2,2'-azino-di-[3-ethyl] dithiazoline sulfonic acid with 10 mM H₂O₂ in 100 mM citrate buffer, pH 4.2) was added to each well and color development was assayed at 405 nm on a microtiter plate reader (Bio-Rad Laboratories, Richmond, CA). When used, mAb R6.5 Fab (50 μ g/ml) was preincubated with endothelial cells for 30 min at 25°C before addition of PMN.

Data Presentation

Since variations exist in both transepithelial resistance between groups of monolayers (baseline resistance range 650–1,500 ohm-cm²) and in PMN obtained from different donors, individual experiments were performed using large numbers of uniform groups of monolayers and PMN from single blood donors on individual days. PMN isolation was restricted to 12 different donors (repetitive donations) over the course of these studies. To account for monolayer-to-monolayer resistance differences within experiments, resistance data were normalized and expressed as percent baseline resistance, unless otherwise noted. Resistance time courses were compared by two-factor analysis of variance (ANOVA) and myeloperoxidase assay data were compared by Student's t test (Steele and Torrie, 1980). PMN transmigration results are represented as PMN cell equivalents (CE) derived from a daily standard PMN dilution curve. Monolayer-associated PMN are represented as the number of PMN CE per monolayer and reservoir-associated PMN (i.e., PMN which had completely traversed the monolayer into the lower chamber) are represented as the number of PMN CE/ml (total volume of 1 ml). Values are expressed as the mean and SEM of n experiments.

Results

IFN- γ Modulates PMN Migration in the Apical-to-Basolateral Direction

IFN- γ Upregulates Apical-to-Basolateral PMN Transmigration. As shown in Fig. 1, exposure of T₈₄ epithelial monolayers to 1,000 U/ml IFN- γ for 72 h results in significantly increased PMN transepithelial migration in the presence of a 1- μ M FMLP gradient. Pretreatment of epithelial monolayers with IFN- γ increased both monolayer-associated PMN CE ($3.7 \pm 0.87 \times 10^4$ for controls vs. $12.2 \pm 1.31 \times 10^4$ PMN CE/monolayer for IFN- γ -treated monolayers, $p < 0.001$, transmigration time = 110 min) as well as the number of PMN traversing the monolayer into the reservoir (8.2 ± 1.53 vs. $19.7 \pm 2.07 \times 10^4$ PMN CE/ml for control and IFN- γ -treated monolayers, respectively, $p < 0.001$). To further characterize this transmigration event, T₈₄ epithelial monolayers were exposed to IFN- γ for various time periods in the range of 2 to 72 h. As shown in Fig. 2 A, treatment of T₈₄ monolayers with 1,000 U/ml IFN- γ

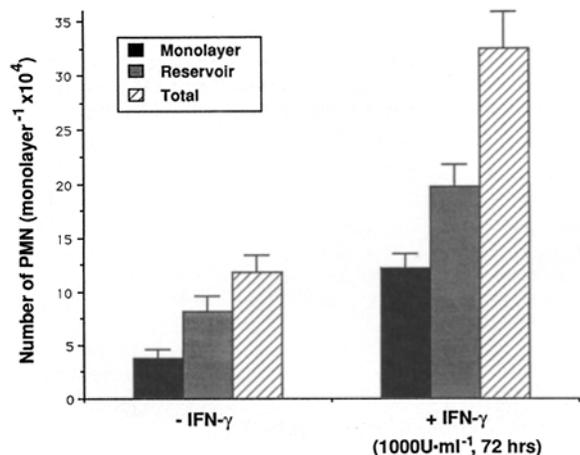


Figure 1. Effect of epithelial exposure to IFN- γ (1,000 U/ml, 72 h) on the number of PMN transigrating in the apical-to-basolateral direction. PMN at a density of 2×10^6 /monolayer were added to the apical surface of T₈₄ intestinal epithelial monolayers and driven to migrate basolaterally under the influence of a 1- μ M gradient of FLMP. Shown here are the results obtained from harvesting the PMN-specific enzyme myeloperoxidase from washed monolayers, lower reservoirs and total myeloperoxidase activity after 110 min, relative to a known standard number of PMN. Since tight junctions are the rate-limiting barrier to passive paracellular permeation, transmigration is defined as movement of PMN across the tight junction. Since monolayer-associated PMN were largely below the tight junction (see Results), total transmigration in the apical-to-basolateral direction equals the sum of PMN in the opposite reservoir plus monolayer PMN. Data are pooled from nine individual monolayers in each condition and results are expressed as the mean and SEM.

for various periods of time enhanced the usual transmigration-associated fall in transepithelial resistance ($p < 0.01$ vs. control at 12, 24, 48, and 72 h, respectively), indicative of PMN impalement of the T₈₄ tight junction (Nash et al.,

1987). However, we have previously shown that prolonged exposure of monolayers to IFN- γ also decreases baseline monolayer resistance (Madara and Stafford, 1989) and the same effect was noted here (344 ± 70 , 461 ± 20 , and 721 ± 27 ohm-cm² for exposure times of 72, 48, and 0 h, respectively, to 1,000 U/ml IFN- γ). However, the effects of IFN- γ on PMN transepithelial migration were not explained by a secondary effect of IFN- γ permeability since the effects on transmigration predated IFN- γ resistance alterations. As shown in Fig. 2 B, and as we have previously described (Madara and Stafford, 1989), baseline resistance is unaffected by monolayer exposure to 1,000 U/ml IFN- γ for 24 h (721 ± 27 vs. 715 ± 42 ohm-cm² for control and 24-h IFN- γ exposure, respectively, $p > 0.05$). In contrast (Fig. 2 B), the transepithelial resistance fall due to PMN transmigration (a biological assay which correlates with numbers of transmigration PMN [Nash et al., 1987; Parkos et al., 1991]) was significantly enhanced across monolayers which were exposed to 1,000 U/ml IFN- γ for 24 h ($60 \pm 5\%$ fall in resistance after 2 h in control vs. $76 \pm 2\%$ fall in resistance for IFN- γ -treated monolayers, $p < 0.01$). Corresponding to these data, as shown in Fig. 2 C, the number of transmigrated neutrophils, directly assessed quantitatively by myeloperoxidase, was greater even after a 24-h preexposure of monolayers to IFN- γ ($p < 0.001$, ANOVA). Fig. 2 C also confirms that the effects observed were not due to carryover of IFN- γ from preexposed and subsequently washed monolayers. As can be seen, transmigration is identical between monolayers unexposed to IFN- γ and monolayers incubated with IFN- γ for 2 h, washed, and then exposed to PMN. Such observations are important given that IFN- γ may be able to bind to some basement membrane components with high affinity (Lortat-Jacob et al., 1991). These, and supplementary data (vida infra), indicate the effects observed are due exclusively to IFN- γ -mediated modulation of monolayers, not neutrophils. The IFN- γ exposure/time-dependent enhancement of subsequent neutrophil transmigration was

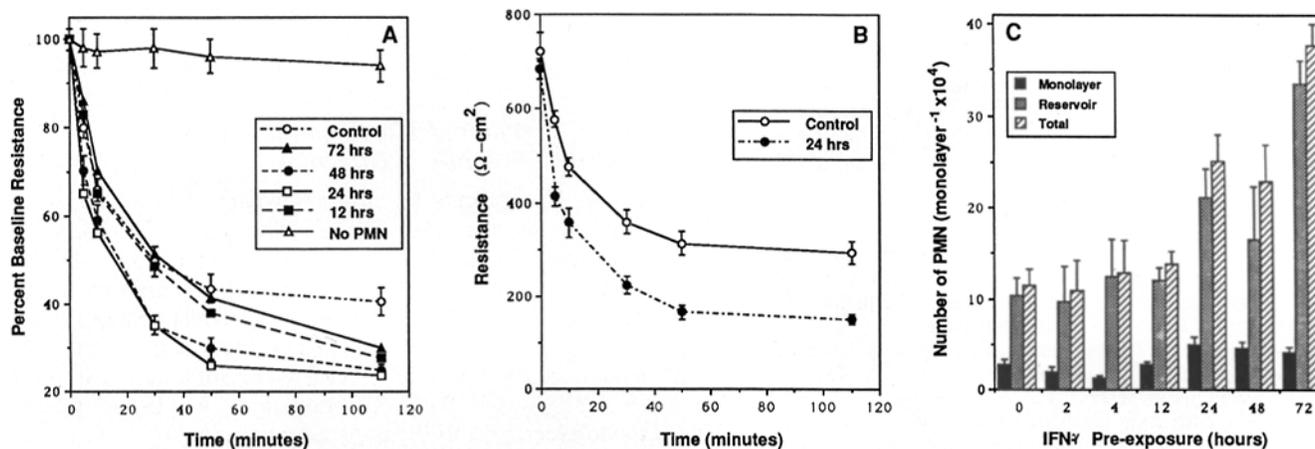


Figure 2. Effect of epithelial exposure to IFN- γ and PMN transmigration in the apical-to-basolateral direction. PMN were added to the apical surface of T₈₄ intestinal epithelial monolayers and driven to transigrate basolaterally under the influence of a 1- μ M gradient of FMLP. Shown here are (A) the normalized (expressed as % of baseline) results of the fall in transepithelial resistance during transmigration across epithelial monolayers preexposed to IFN- γ (1,000 U/ml) for various periods of time in the range of 12–72 h. Also shown is the negative control (no PMN added at time 0), (B) comparison of the true resistance time course of resistance fall after addition of PMN to the basolateral side of control monolayers and monolayers preexposed to IFN- γ (1,000 U/ml, 24 h), (C) quantitation of the number of transmigrating PMN across monolayers which were preexposed to IFN- γ (1,000 U/ml) for a variety of time periods in the range of 2–72 h. Results are presented as the mean and SEM of between 6 and 10 monolayers each.

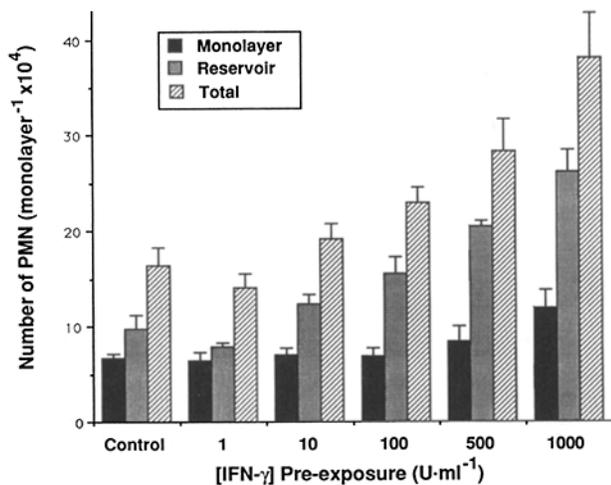


Figure 3. Effect of epithelial exposure to different concentrations of IFN- γ on the number of PMN transmigrating in the apical-to-basolateral direction. T₈₄ intestinal epithelial monolayers were exposed to five different concentrations of IFN- γ in the range of 1–1,000 U/ml for 72 h. PMN were added to the apical surface of T₈₄ intestinal epithelial monolayers and driven to migrate basolaterally under the influence of a 1- μ M gradient of FMLP. Shown here are the results obtained from harvesting the PMN-specific enzyme myeloperoxidase from washed monolayers, lower reservoirs and total myeloperoxidase activity after 110 min, relative to a known standard number of PMN. Data are pooled from 6–9 individual monolayers for each condition and results are expressed as the mean and SEM.

largely due to transmigrated PMN (11.5 ± 1.80 vs. 37.7 ± 2.36 PMN CE/ml for control and IFN-exposed monolayers at 72 h, respectively, $p < 0.001$), although, not surprisingly, a significant enhancement of the number of monolayer-associated PMN was also found ($2.76 \pm 0.57 \times 10^4$ PMN for controls vs. $4.11 \pm 0.56 \times 10^4$ PMN CE/monolayer for monolayers exposed to 1,000 U/ml IFN- γ for 72 h, $p < 0.01$). No significant increase in the number of transmigrating PMN were found at 2, 4, or 12 h of exposure (Fig. 2 C).

The dose response to 72 h of monolayer preexposure to IFN- γ is shown in Fig. 3. Exposure of epithelial monolayers to 1–1,000 U IFN- γ /ml elicited a progressive increase in the number of transmigrating PMN. IFN- γ -induced increases in PMN transmigration were again largely reflective of increased numbers of neutrophils moving to the opposite reservoir although a small increase in transiting PMN (i.e., monolayer associated) was again seen. As reported previously (Parkos et al., 1991), examination of 1- μ m T₈₄ epithelial monolayer sections revealed that PMN were only rarely associated with the apical epithelial surface (data not shown). Rather, the majority of monolayer-associated PMN were found associated with basal or lateral membranes of T₈₄ cells. Therefore, monolayer-associated PMN in this apical-to-basolateral assay had transmigrated across the tight junction barrier.

We next examined the effect of IFN- γ upregulation of PMN transepithelial migration as a function of applied PMN number. PMN were applied over an eightfold range in density to monolayers preexposed to IFN- γ (1,000 U/ml, 72 h). As shown in Fig. 4, an increase in the total number of transmigrating PMN after epithelial exposure to IFN- γ was maintained at all initial PMN concentrations (2×10^6 , $p <$

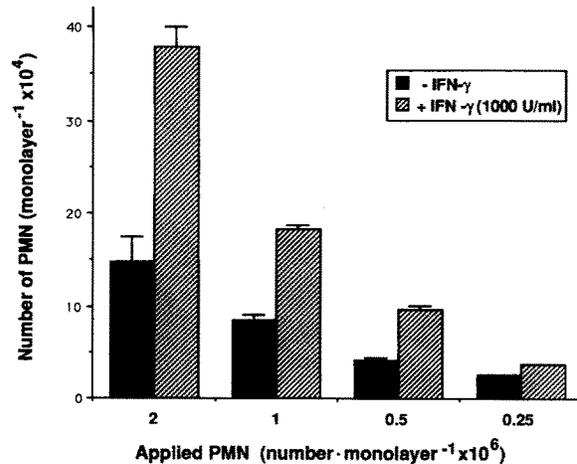


Figure 4. Effect of PMN density on the number of transmigrating PMN in the apical-to-basolateral direction. PMN at the indicated concentrations were layered on control T₈₄ epithelial monolayers and on monolayers which were preexposed to IFN- γ (1,000 U/ml, 72 h). Shown here are the total PMN number (the sum of monolayer and reservoir-associated PMN) migrating in response to a 1- μ M FMLP gradient after 110 min. Data were pooled from six monolayers each and are presented as the mean and SEM.

0.001; 1×10^6 , $p < 0.001$; 0.5×10^6 , $p < 0.001$; and 0.25×10^6 PMN/monolayer, $p < 0.01$).

To determine whether the basal secretion of a chemotactic substance could account for increased transmigration in the apical-to-basolateral direction, PMN were placed on the apical surface of control (monolayers not exposed to IFN- γ) and IFN- γ preexposed (1,000 U/ml, 72 h) T₈₄ epithelial monolayers in the absence of an FMLP gradient and allowed to incubate for 2 h at 37°C. Under these conditions, no observed increase in total PMN transmigration was evident (2.1 ± 0.09 vs. $2.3 \pm 0.18 \times 10^4$ for control and IFN- γ -exposed monolayers, respectively; $n = 6$, $p > 0.05$), indicating that IFN- γ pretreatment does not induce the secretion of a chemotactic factor potent enough to induce transepithelial migration of PMN.

As detailed above, IFN- γ -elicited upregulation of PMN migration across epithelial monolayers was apparent at 24 h – a time at which baseline resistance was unaffected by IFN- γ . We further verified that enhanced transmigration was not wholly dependent on subtle baseline or evolving effects on junctional permeability induced by this cytokine. To do this, PMN transmigration was examined using IFN- γ -exposed (1,000 U/ml, 72 h) and -unexposed monolayers in which the junctional barrier had been disrupted by exposure of epithelial monolayers to 1 mM EDTA in Ca²⁺/Mg²⁺-free buffer for 20 min. As in other cultured epithelia (Pitelka et al., 1983), treatment of T₈₄ epithelial monolayers with 1 mM EDTA induces a disruption of tight junctions and a fall in resistance to near background levels. Using the conditions outlined, we found we could return monolayers to physiologic buffers with a subsequent resistance recovery to pretreatment values over 2 h (data not shown). Thus, we were able to isolate IFN- γ effects on transmigration from general effects on junctional permeability. Divalent cation depletion brought about a significant increase in the number of transmigrating PMN across control monolayers (17.1 ± 1.31 vs.

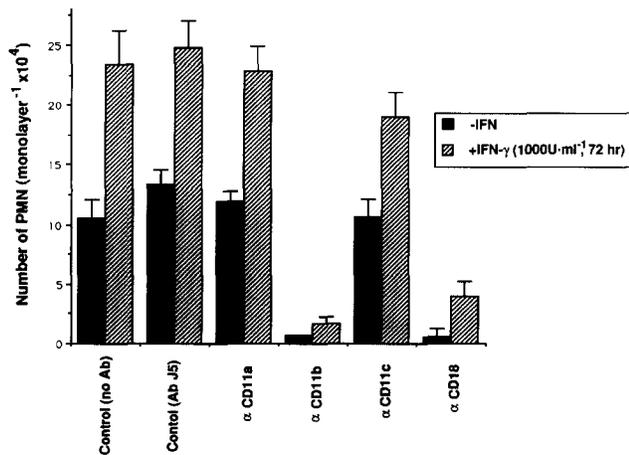


Figure 5. Effect of PMN antiintegrin antibodies on the number of PMN transmigrating in the apical-to-basolateral direction. PMN were incubated with the indicated antiintegrin antibodies at saturating concentrations, previously determined to be functionally inhibitory (see Materials and Methods), and layered on control epithelial monolayers or on monolayers which were exposed to IFN- γ (1,000 U/ml, 72 h). Also shown is the PMN binding control (mAb J5, see Materials and Methods) and the control in the absence of antibody. Data were pooled from between 6 and 9 monolayers for each condition and presented as the mean and SEM.

9.6 ± 0.11 PMN CE/ml with and without EDTA pretreatment, respectively; $p < 0.001$) and across monolayers preexposed to IFN- γ (26.4 ± 2.95 vs. 21.7 ± 1.83 PMN CE/ml with and without EDTA pretreatment, respectively; $p < 0.025$). Under conditions of EDTA pretreatment, the significant enhancement of PMN transmigration induced by preexposure of monolayers to IFN- γ was still clearly apparent (17.1 ± 1.31 vs. 26.4 ± 2.95 PMN CE/ml for controls and IFN- γ -exposed monolayers, respectively; $p < 0.025$). These results were obtained from a condition in which the junctional permeability barrier was near maximally abolished. This evidence, in conjunction with the IFN- γ enhancement of transmigration seen at 24 h and other evidence summarized in the second paragraph of the discussion, strongly suggests that the observed upregulation of transmigration induced by IFN- γ in the apical-to-basolateral direction is independent of junctional permeability changes brought about by this cytokine.

IFN- γ -induced Upregulation of Apical-To-Basolateral Transmigration is CD11b/CD18 Dependent. As shown previously (Parkos et al., 1991), PMN migration across cytokine untreated monolayers is dependent on the PMN β_2 integrin CD11b/CD18. To investigate whether IFN- γ -upregulated PMN transmigration also involves a pathway dependent on the β_2 integrins, epithelial monolayers were exposed to IFN- γ (1,000 U/ml, 72 h) and subsequently assessed for PMN transmigration in the presence of functionally inhibitory concentrations of β_2 -integrin monoclonal antibodies. As shown in Fig. 5, the presence of functionally inhibitory concentrations of mAbs to CD11b and CD18 inhibited the number of transmigrating PMN by $95 \pm 2\%$ and $96 \pm 7\%$, respectively, in control monolayers. Moreover, in epithelial monolayers preexposed to IFN- γ (1,000 U/ml, 72 h), anti-CD18 and anti-CD11b mAbs inhibited this PMN transmigration event by $84 \pm 7\%$ and $93 \pm 6\%$,

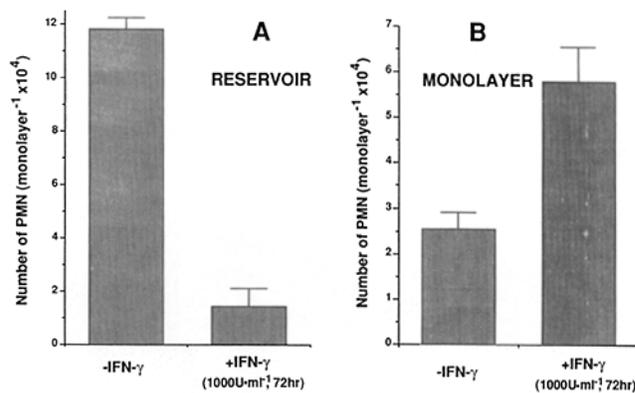


Figure 6. Effect of epithelial exposure to IFN- γ (1,000 U/ml, 72 h) on the number of PMN transmigrating in the basolateral-to-apical direction. PMN at a density of 4×10^5 /monolayer were added to the basolateral surface of T₈₄ intestinal epithelial monolayers (i.e. inverted monolayers) and driven to migrate apically under the influence of a 1- μ M gradient of FMLP. Shown here are the results obtained from harvesting the PMN-specific enzyme myeloperoxidase from lower reservoirs (A) and washed monolayers (B) after 110 min, relative to a known standard number of PMN. Since tight junctions are the rate-limiting barrier to passive paracellular permeation, transmigration is defined as movement of PMN across the tight junction. Since monolayer-associated PMN were largely below the tight junction (see Results), total transmigration in the basolateral-to-apical direction equates with PMN in the opposite reservoir only. Data are pooled from 6–9 individual monolayers in each condition and results are expressed as the mean and SEM.

respectively. Transmigration in the presence of mAbs to CD10 (J5, PMN binding control), CD11a, or CD11c were not different from control in monolayers untreated or preexposed to IFN- γ ($p > 0.05$). These data indicate that increased PMN transmigration in the apical-to-basolateral direction elicited by epithelial exposure to IFN- γ also largely involves a pathway which is dependent on the PMN β_2 -integrin CD11b/CD18.

IFN- γ Effects on PMN Transmigration in the Basolateral-to-Apical Direction

IFN- γ Downregulates PMN Transmigration in the Basolateral-to-Apical Direction. We have previously detected quantitative, but not qualitative, differences in PMN trans-epithelial migration depending on the direction of migration. We next prepared inverted monolayers, which permit basolateral-to-apical directed transmigration, to assess whether IFN- γ pretreatment of epithelial monolayers exhibited polarized effects on subsequent PMN transmigration. As shown in Fig. 6, exposure of monolayers to IFN- γ (1,000 U/ml, 72 h) also markedly altered PMN transmigration in the basolateral-to-apical direction, but surprisingly did so in a fashion which differed qualitatively from the result in the apical-to-basolateral direction. Transmigration of PMN in this direction was significantly decreased compared to untreated control epithelial monolayers (11.8 ± 0.46 vs. 1.46 ± 0.68 PMN/ml for control and IFN- γ -treated monolayers, respectively; $p < 0.001$, Fig. 6 A). Furthermore, pretreatment of epithelial monolayers with IFN- γ induced more than a 100% increase in monolayer-associated PMN (2.54 ± 0.15 vs. 5.79 ± 0.76 PMN/monolayer for control and IFN- γ -treated monolayers, respectively; $p < 0.05$, Fig. 6 B).

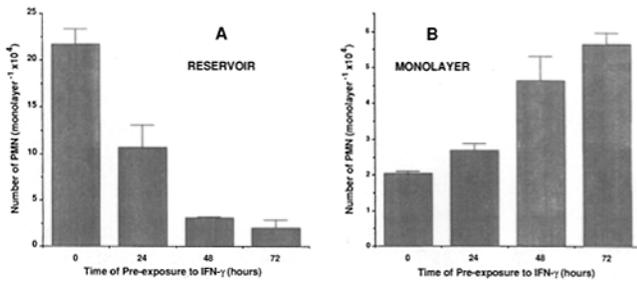


Figure 7. Effect of epithelial exposure to IFN- γ on PMN transmigration in the basolateral-to-apical direction. PMN were added to the basolateral surface of T₈₄ intestinal epithelial monolayers and driven to transmigrate apically under the influence of the 1- μ M gradient of FMLP. Shown here is quantitation of the number of transmigrating PMN across monolayers which were preexposed to IFN- γ (1,000 U/ml) for a variety of time periods in the range of 24–72 h. PMN migration was determined by assaying for reservoir (A) and monolayer-associated myeloperoxidase (B) against a standard number of PMN. Results are presented as the mean and SEM of between 6 and 9 monolayers per condition.

Examination of 1- μ m sections of such monolayers again revealed that monolayer-associated PMN were uniformly associated with the basolateral space of the monolayer (not the apical surface of the monolayer or the filter support; data not shown).

A time course of IFN- γ exposure (1,000 U/ml) was next performed for basolateral-to-apical directed migration (Fig. 7). A progressive decrease in the number of transmigrating PMN resulted with increasing periods of exposure to IFN- γ (Fig. 7 A, $p < 0.001$, ANOVA). In contrast, as shown in Fig. 7 B, IFN- γ treatment elicited a significant increase in monolayer-associated PMN with increasing time of epithelial exposure to IFN- γ (2.05 ± 0.06 , 2.69 ± 0.18 , 4.19 ± 0.67 , and 5.64 ± 1.16 PMN/monolayer, for exposure times of 0, 24, 48, and 72 h, respectively; $p < 0.025$ for 48 and 72 h). Comparable results were obtained from dose-response experiments (all 72 h preexposure, Fig. 8). Increasing concentrations of IFN- γ resulted in a progressive decrease in transmigrated PMN (Fig. 8 A). Again, this diminished trans-

migratory response in the basolateral-to-apical direction was accompanied by an increase in monolayer-associated PMN (Fig. 8 B, $p < 0.001$ and $p < 0.025$, respectively, for transmigrated and monolayer-associated PMN numbers, both ANOVA).

To determine whether the kinetics of PMN migration in the basolateral-to-apical direction differed after epithelial exposure to IFN- γ , monolayers were harvested at various time points during transmigration and assayed for PMN by myeloperoxidase content. As shown in Fig. 9 A, the number of transmigrating PMN increased with increasing experimental time across both control monolayers and monolayers which were preexposed to IFN- γ (ANOVA, $p < 0.01$ for controls and $p < 0.05$ for IFN- γ preexposed monolayers). As depicted in Fig. 9 A, decreased PMN migration across monolayers preexposed to IFN- γ was apparent by 60 min ($p < 0.01$) and progressively decreased at 85 ($p < 0.001$) and 120 min ($p < 0.001$). As shown in Fig. 9 B, monolayer-associated PMN did not change over the 2-h experimental period in either control or IFN- γ preexposed monolayers (ANOVA, $p > 0.05$ for both). Compared with control monolayers, increased PMN association to monolayers preexposed to IFN- γ was apparent at all time periods of monolayer harvest ($p < 0.01$ for all time periods).

As depicted in Fig. 9 C, after epithelial exposure to 1,000 U/ml IFN- γ , a decrease in the number of transmigrating PMN in the basolateral-to-apical direction was apparent over a 20-fold range in initial PMN concentrations (ANOVA, $p < 0.01$). Moreover, a greater number of monolayer-associated PMN were apparent in monolayers preexposed to IFN- γ (Fig. 9 D, ANOVA, $p < 0.05$). These data indicate that decreased PMN transmigration in the basolateral-to-apical direction after epithelial IFN- γ exposure is independent of initial PMN concentrations and is apparent at various time periods during the course of the experiment.

Morphologic Correlates of PMN Transmigration in the Basolateral-to-Apical Direction. We were able to morphologically demonstrate increased accumulation of monolayer-associated PMN after epithelial exposure to IFN- γ (Fig. 10). PMN were highlighted by localizing PMN endogenous per-

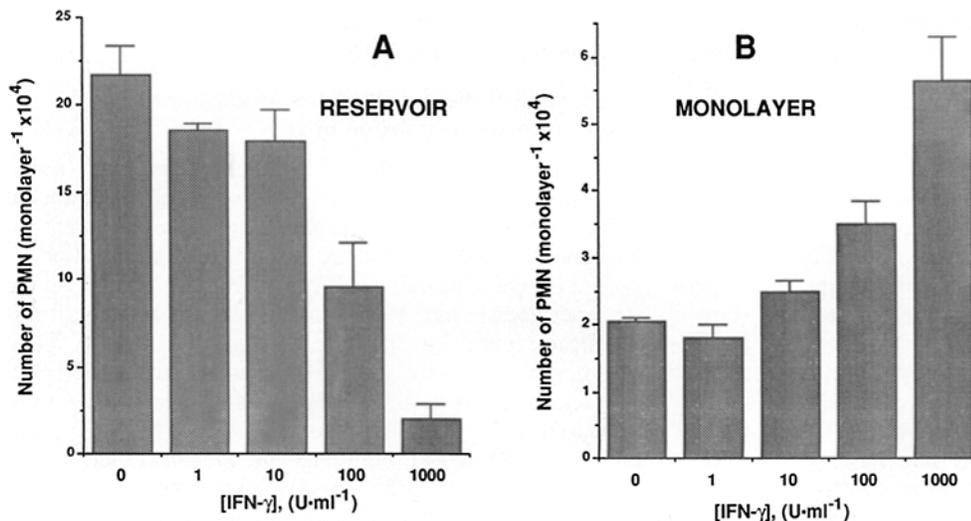


Figure 8. Effect of epithelial exposure to different concentrations of IFN- γ on the number of PMN transmigrating in the basolateral-to-apical direction. T₈₄ intestinal epithelial monolayers were exposed to four different concentrations of IFN- γ in the range of 1–1,000 U/ml for 72 h. PMN were added to the basolateral surface of T₈₄ intestinal epithelial monolayers and driven to migrate apically under the influence of a 1- μ M gradient of FMLP. Shown here are the results obtained from harvesting the PMN-specific enzyme myeloperoxidase from lower reservoirs (A) and washed mono-

layers (B) after 110 min against a known standard number of PMN. Data are pooled from 6–9 individual monolayers for each condition and results are expressed as the mean and SEM.

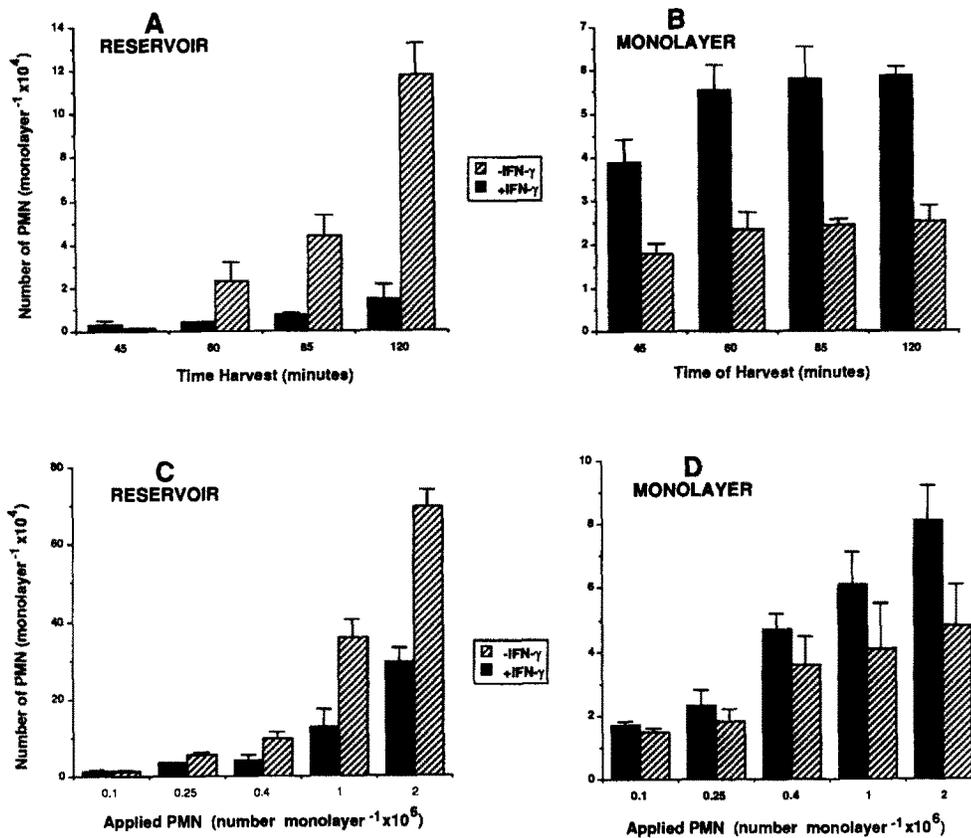


Figure 9. Effect of time of monolayer harvest and applied PMN density on the number of transmigrating PMN in the basolateral-to-apical direction. (A and B) PMN at the indicated concentrations were layered on control T₈₄ epithelial monolayers and on monolayers which were preexposed to IFN- γ (1,000 U/ml, 72 h). Shown here are the number of PMN migrating into the reservoir (A) and the number of monolayer-associated PMN (B) in response to a 1- μ M transepithelial FMLP gradient after 110 min. (C and D) Control monolayers and monolayers preexposed to IFN- γ (1,000 U/ml) were harvested at the indicated time periods during the transmigration assay. Shown here are the number of PMN migrating into the reservoir at each indicated time period (C) and the number of monolayer-associated PMN at each time period (D) in response to a 1- μ M transepithelial FMLP gradient after 110 min. Data were pooled from six monolayers each and are presented as the mean and SEM.

oxidases in en face-stained monolayers (Nash et al., 1987) 1 h after the addition of PMN under chemotactic conditions (4×10^5 PMN added to the basolateral aspect of monolayers and driven to transmigrate by a 10^{-6} M gradient of FMLP). The density of PMN was dramatically increased in monolayers which were preexposed to IFN- γ (Fig. 10 C) compared with control monolayers (Fig. 10 A). These results correlate with the enzymatic accumulation of myeloperoxidase after epithelial exposure to IFN- γ (vide supra).

1- μ m sections of monolayers from transmigration experiments in the serosal-to-mucosal direction showed increased accumulation of PMN in monolayers which were preexposed to IFN- γ (Fig. 10 D) compared with control T₈₄ epithelial monolayers (Fig. 10 C). PMN associated with T₈₄ monolayers/filters were largely monolayer associated.

Effect of Antibodies to PMN β_2 -Integrins on IFN- γ Modulation in the Basolateral-to-Apical Direction. To determine whether decreased PMN transmigration in the basolateral-to-apical direction after IFN- γ exposure could be further influenced by mAbs to PMN β_2 -integrins, transmigration across inverted monolayers was assessed in the presence of functionally inhibitory concentrations of β_2 -integrin mAbs, as described above. As shown in Fig. 11 A, the presence of anti-CD18 and anti-CD11b mAbs decreased PMN transmigration by $85 \pm 4\%$ and $90 \pm 5\%$, respectively, compared with the PMN binding control (J5) across control epithelial monolayers, and by $51 \pm 3\%$ and $53 \pm 6\%$, respectively, across epithelial monolayers which had been preexposed to IFN- γ (1,000 U/ml, 72 h). The number

of monolayer-associated PMN tended to decrease in control monolayers in the presence of anti-CD18 ($p < 0.01$) and anti-CD11b mAbs ($p < 0.05$) (Fig. 11 B). The presence of anti-CD18 and anti-CD11b mAbs had no effect on the number of PMN associated with monolayers preexposed to IFN- γ . The presence of mAbs to CD11a and CD11c during transmigration did not significantly decrease the number of transmigrating PMN or the number of monolayer-associated PMN. These results indicate that the PMN β_2 -integrin CD11b/CD18 is a necessary component for PMN transmigration across IFN- γ -treated epithelial monolayers.

Effect of Cycloheximide on the Modulation of PMN Transepithelial Migration by IFN- γ

To determine whether the effects of IFN- γ on PMN transmigration required the synthesis of new protein by epithelial cells, transmigration was assessed in both the apical-to-basolateral and the basolateral-to-apical directions after exposure of epithelial cells to IFN- γ (1,000 U/ml, 72 h) in the presence and absence of 2 μ g/ml cycloheximide. In performing these experiments, and as shown in Fig. 12 A, we found that monolayers were able to maintain baseline electrical characteristics, such as high transepithelial resistance, even after a 72-h exposure to cycloheximide ($1,148 \pm 63$ ohm-cm² vs. $1,184 \pm 81$ ohm-cm² for cycloheximide-treated control monolayers, $p > 0.05$). We also noted that, interestingly, the drop in transepithelial resistance after prolonged exposure to IFN- γ (1,000 U/ml, 72 h) which we have previously described (Madara and Stafford, 1989), is largely cy-

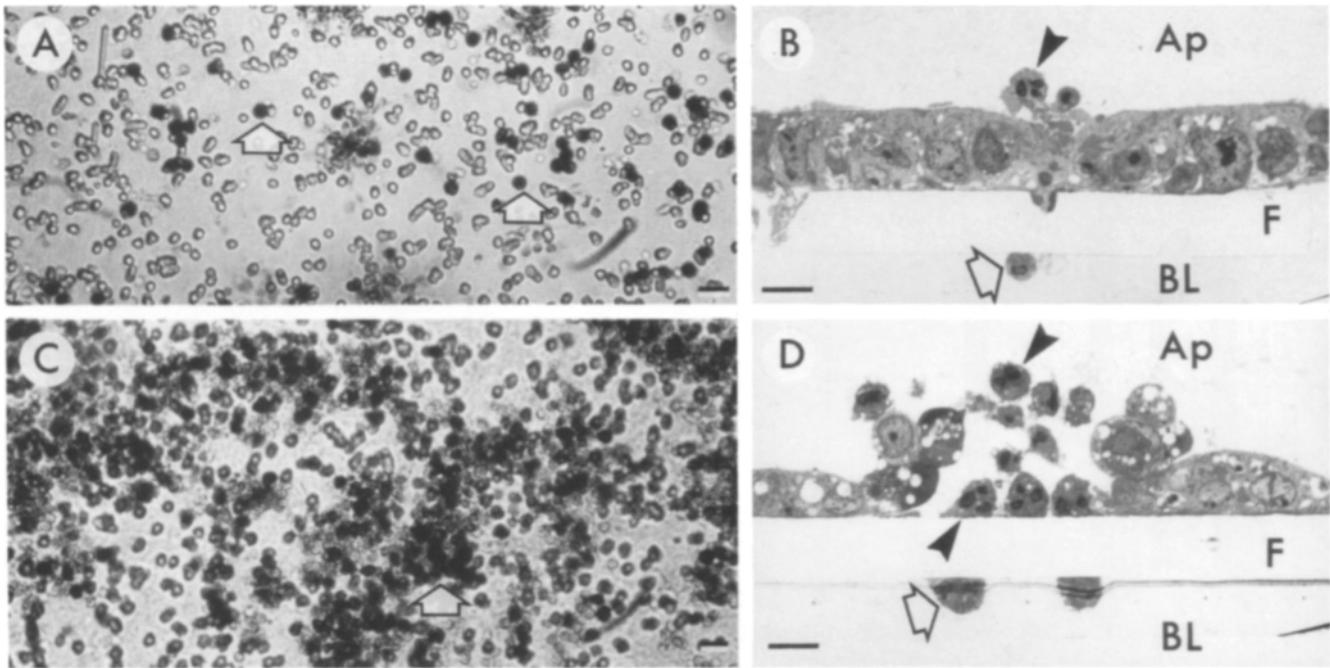


Figure 10. Epithelial preexposure to IFN- γ increases monolayer-associated PMN after basolateral-to-apical directed transmigration. T₈₄ epithelial monolayers were exposed to IFN- γ (1,000 U/ml, 72 h) followed by the addition of PMN to the basolateral side under chemotactic conditions (1×10^5 PMN/monolayer, 10^{-6} M FMLP gradient). *A* (control) and *C* (IFN- γ exposed) demonstrate en face views of monolayers obtained from a mid-epithelial plane of the monolayers (Nash et al., 1987). PMN (arrows) are highlighted by peroxidase histochemistry and appear as discrete black cells. The open circles focally displaying a "stacked coin" appearance represent the 5- μ m pores in the polycarbonate filter. *B* (control) and *D* (IFN- γ exposed) demonstrate 1- μ m sections of T₈₄ monolayers. The apical (Ap) and basolateral (BL) reservoirs are marked as are the polycarbonate filters (F). PMN display characteristic lobated nuclei and can be morphologically distinguished both within/above the monolayer (arrowhead) and below the filter (arrows). IFN- γ markedly enhances the number of monolayer associated PMN (*A* vs. *C*). The majority of monolayer/filter associated PMN after IFN- γ exposure are above, not below, the filter. At sites of transmigration of large numbers of PMN the monolayer is focally disrupted (*D*). Bars, $\sim 10 \mu\text{m}$.

cloheximide sensitive (Fig. 12 *A*). With basolateral-to-apical directed transmigration, cycloheximide inhibited both the IFN- γ -induced downregulation of PMN transepithelial migration and the upregulation of PMN migration into the monolayer (Fig. 12 *B*). Finally, with apical-to-basolateral directed PMN migration, upregulation of transepithelial migration by IFN- γ was again inhibited (Fig. 12 *C*). These data suggest that new protein synthesis is, in part, necessary for the IFN- γ modulations of PMN transepithelial migration in either direction.

Effects of IFN- γ on PMN Transepithelial Migration Can Not Be Explained by IFN- γ Modulation of ICAM-1

The expression of ICAM-1 is known to be modulated in some cell types by IFN- γ and other inflammatory cytokines (Dustin et al., 1986). Moreover, ICAM-1 is a putative receptor for CD11b/CD18 (Diamond et al., 1991), and this epitope is bound and functionally inhibited by the mAb R6.5 (Diamond et al., 1991). To determine whether ICAM-1 regulation by IFN- γ could explain the results found above, we performed apical-to-basolateral and basolateral-to-apical directed transmigration experiments in the presence of the anti-ICAM-1 mAb R6.5 (Fab fragments, 50 $\mu\text{g}/\text{ml}$) after epithelial exposure to IFN- γ (1,000 U/ml, 72 h). The presence of R6.5 during transmigration failed to significantly alter the pattern of PMN transmigration regulation by IFN- γ in the basolateral-to-apical (total of $4.7 \pm 0.50 \times 10^4$ PMN CE

transmigrated in the presence of R6.5 Fab while $5.4 \pm 0.61 \times 10^4$ PMN CE transmigrated in the absence of R6.5 Fab, $n = 4$ each, NS) or in the apical-to-basolateral direction (total of $20.4 \pm 2.36 \times 10^4$ PMN CE transmigrated in the presence of R6.5 Fab while $19.5 \pm 1.16 \times 10^4$ PMN CE transmigrated in the absence of R6.5 Fab, $n = 6$ each, NS). As a positive control for the anti-ICAM-1 mAb, PMN adhesion to endothelial monolayers was assessed in the presence and absence of R6.5 Fab (50 $\mu\text{g}/\text{ml}$). In these experiments, R6.5 Fab blocked the adhesion of PMN to endothelial monolayers by 72% ($23.7 \pm 2.83 \times 10^4$ PMN CE/monolayer bound for controls and $6.71 \pm 1.85 \times 10^4$ PMN CE in the presence of R6.5 Fab, $p < 0.001$).

These results correlate with our conclusion that IFN- γ does not significantly modulate apical ICAM-1 expression at times in which apical-to-basolateral directed transmigration was affected, as determined by ELISA on adherent T₈₄ epithelial cells. T₈₄ epithelial cells preexposed to 1,000 U/ml IFN- γ did not show significantly increased ICAM-1 expression (mean OD 0.51 ± 0.088 , 0.53 ± 0.085 , 0.61 ± 0.071 at 72, 48, and 24 h IFN- γ exposure, respectively, $n = 6$) compared with control monolayers (mean OD 0.49 ± 0.063 , $n = 6$). In contrast, ICAM-1 expression by adherent HL-60 cells was readily measured in this assay (mean OD 1.05 ± 0.065 , $n = 6$). Moreover, preexposure of human endothelial monolayers to IFN- γ (500 U/ml, 48 h) resulted in a $260 \pm 39\%$ increase in OD ($n = 6$, $p < 0.001$) under the conditions used here.

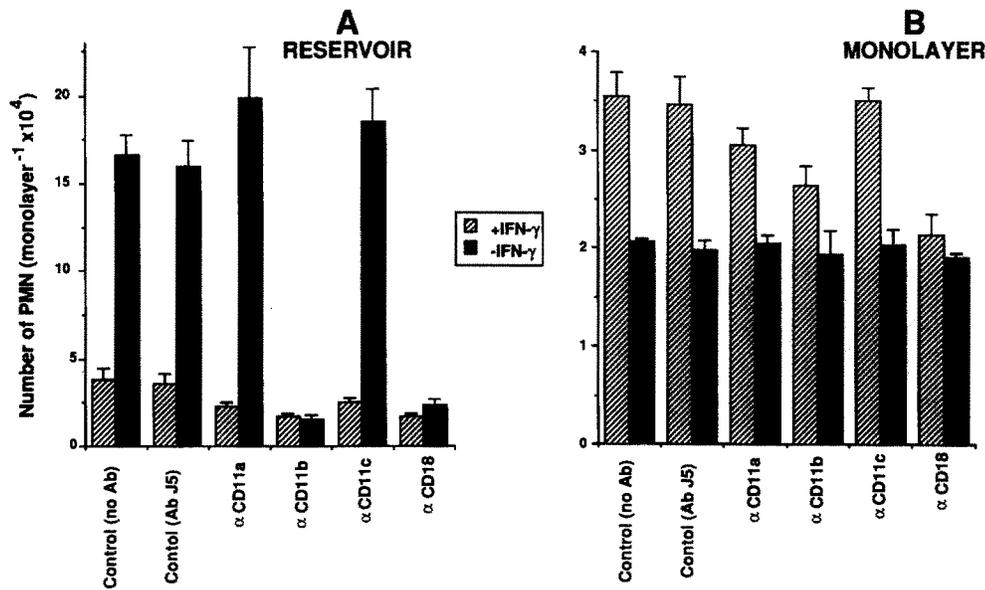


Figure 11. Effect of PMN anti-integrin antibodies on the number of PMN transmigrating in the basolateral-to-apical direction. PMN were preincubated with the indicated anti-integrin antibodies at saturating concentrations previously determined to be functionally inhibitory (see Materials and Methods), and layered on control epithelial monolayers or on monolayers which were exposed to IFN- γ (1,000 U/ml, 72 h). Also shown is the PMN binding control (J5, see Materials and Methods) and the no-antibody control. The results are presented as the number of PMN migrating into the reservoir (A) and the number of monolayer-associated PMN (B). Data were pooled from six monolayers for each condition and presented as the mean and SEM.

Discussion

We report two general and major new findings. First, to our knowledge this is the first detailed report showing that a cytokine can modulate PMN transmigration across confluent epithelial monolayers. Second, we report for the first time a condition (IFN- γ exposure) which elicits effects on PMN transmigration that qualitatively differ depending on the direction of migration.

IFN- γ Modulates PMN Transepithelial Migration in the Apical-to-Basolateral Direction

Studies of PMN transepithelial migration have largely focused on this nonphysiologically directed migration for tech-

nical reasons (Evans et al., 1983; Cramer et al., 1986; Milks et al., 1986; Nash et al., 1987; Migliorisi et al., 1988; Parkos et al., 1991). However, we have also recently shown that, while the efficiency of transmigration does vary depending on the direction in which PMN cross monolayers, in several qualitative ways transmigration is similar in both directions (i.e., ability of transmigration to proceed, specific chemotactic gradients required, and CD11b/18 dependence) (Parkos et al., 1991). Using this apical-to-basolateral directed transmigration assay as a screening procedure, we find that the cytokine IFN- γ , which is richly expressed in the intestinal mucosa of humans (Quiding et al., 1991), exerts major effects on transepithelial migration. Namely, transepithelial migration of PMN in this direction is markedly up-

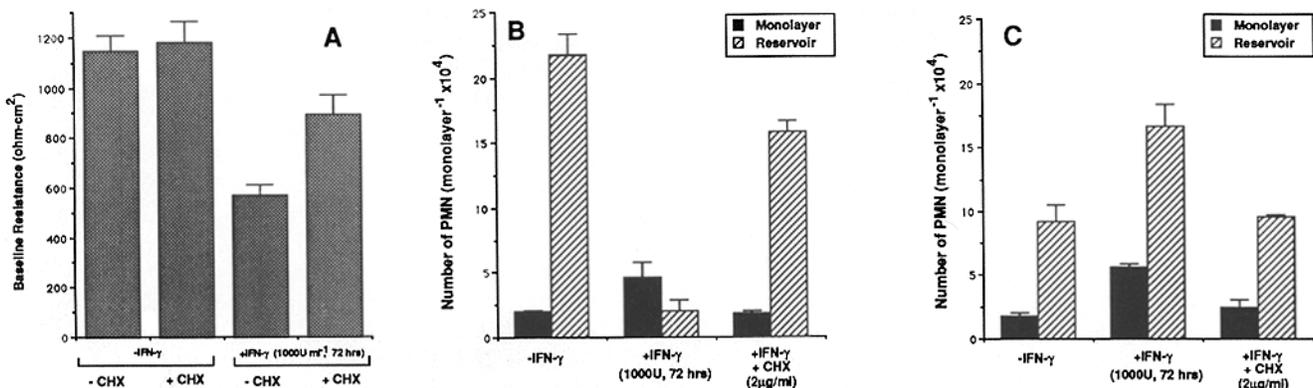


Figure 12. Effect of cycloheximide on IFN- γ modulation of PMN transmigration. T₈₄ intestinal epithelial monolayers were incubated in the presence or absence of 2 μ g/ml cycloheximide and in the presence or absence IFN- γ (1,000 U/ml, 72 h) to determine what effect inhibition of new protein synthesis has on IFN- γ modulation of PMN transmigration. Shown here are the true baseline resistances (A), results from the myeloperoxidase assay in the basolateral-to-apical direction (B), and in the apical-to-basolateral direction (C). No differences existed in the number of transmigrating PMN between controls treated and untreated with cycloheximide. Data were pooled from six monolayers in each condition and presented as the mean and SEM.

regulated as a consequence of epithelial exposure to IFN- γ . Furthermore, such upregulation is apparent both throughout the time course of transmigration and with varying densities of applied PMN, and the degree to which the response is upregulated varies as a function of dose and time of epithelial exposure to IFN- γ . Although we have previously shown that IFN- γ decreases tight junction resistance (Madara and Stafford, 1989), it is unlikely that this decrease in resistance accounts for the observed enhancement of transmigration for several reasons. First, IFN- γ -mediated enhancement of transmigration was still apparent after both control and IFN- γ -treated monolayers were exposed, before transmigration, to Ca²⁺-depleting conditions which open tight junctions. Second, the IFN- γ -elicited enhancement of transmigration was only seen with apical-to-basolateral directed PMN migration. Third, the enhancement of transmigration occurred in monolayers exposed to IFN- γ for periods of time insufficient to elicit alterations in baseline resistance. Lastly, even though gross perturbations of tight junctions, such as those elicited by Ca²⁺ depletion, modestly enhanced transmigration. Monolayers exposed to even 1,000 U/ml IFN- γ for 72 h maintain resistances generally exceeding 250–300 ohm-cm² (Madara and Stafford, 1989), and we have previously shown that modulation of epithelial resistance in the range of 200–2,000 ohm-cm² has little effect on the efficiency of PMN transmigration (Nash et al., 1987). This is likely due to the fact that the relationship between resistance and the flux of solutes, such as those in the size-range of FMLP is asymptotic (Madara and Dharmasathaphorn, 1985; Hecht et al., 1988); that is, at higher resistance values (>200–250 ohm-cm²), large changes in resistance are accompanied by only modest changes in solute flux. The reasons for this are clear when one considers the behavior of a circuit composed of parallel resistances as we have discussed in the past (Madara and Dharmasathaphorn, 1985). Such sieving characteristics have other implications for the movement of PMN across epithelia (vide infra).

In this study, we show that the IFN- γ -elicited effects on apical-to-basolateral PMN transmigration are dependent on new protein synthesis by the epithelial cells. Such findings imply that either a soluble or membrane surface protein induced by IFN- γ exposure may, by direct or indirect means, modulate PMN-epithelial interactions. Since IFN- γ effects are apparent after extensive washing of monolayers, and since the effects of IFN- γ on transmigration cannot be transferred to an IFN- γ unexposed monolayer (i.e., by separately collecting and applying apical and basolateral solutions to new monolayers; our unpublished observations), it is unlikely that the observed effects are due to release of a soluble factor. We therefore speculate that an IFN- γ -elicited alteration in epithelial membrane surface protein(s) may serve as the basis for the observed upregulation of apical-to-basolateral directed PMN migration brought about by IFN- γ . The interaction of PMN with epithelial cells during transmigration is likely to be a complex, multistep process consisting of sequential adhesion, deadhesion, and spreading events such as has been proposed for transendothelial migration (Larson and Springer, 1990; Osborn, 1990; Pober and Cotran, 1990; Springer, 1990). The antiintegrin mAb experiments suggest, however, that the additional component of transmigration elicited by IFN- γ exposure involves a pathway in which CD11b/18-mediated PMN-epithelial interac-

tion plays a critical role. These results are comparable to transmigration in the basal state, in which CD11b/18 is required. Evidence to date suggests that the epithelial counter-receptor for CD11b/18 required for transmigration in the basal state is not any of the known counterreceptors for this integrin (Parkos et al., 1991). Similarly, we now find that apical CD54 (ICAM-1), a potential counterreceptor for this integrin (Diamond et al., 1991) is neither upregulated by IFN- γ over the time course in which enhanced transmigration occurs, nor does R6.5 Fab, which blocks CD11b/18-CD54-associated binding (Diamond et al., 1991), interfere with upregulated transmigration in either direction. This observation, coupled with the lack of a requirement for serum factors in IFN- γ -upregulated transmigration, indicates that if new CD11b/18 counterreceptors are induced on the epithelial surface by IFN- γ , they are also likely to be previously unrecognized ligands for this integrin. These data, however, do not rule out the possibility that ICAM-1 could be upregulated on the basolateral surface of T₈₄ epithelial monolayers.

Cramer et al. (1986) have reported that serum components, likely iC3b, when added to epithelial monolayers at the time of transmigration can markedly affect adherence of PMN to monolayers and modestly affect transepithelial transmigration of PMN. Preabsorption studies revealed that these serum components were extracted by epithelial cells within 30 min at 37°C. Since serum (5% newborn calf) was last placed on the T₈₄ monolayers used here 72 h before experimentation, and since iC3b covalently linked to surfaces is unstable and further degraded to covalently attached products not known to be CD11b/18 ligands, it is unlikely that the effects we report are secondary to IFN- γ stimulation of a process which activates iC3b binding to the T₈₄ cell surface.

IFN- γ Modulates PMN Transmigration in the Physiological (Basolateral-to-Apical) Direction

IFN- γ treatment of epithelial monolayers affected PMN transmigration in a remarkably polarized fashion. Indeed, this serves as the first reported qualitative difference in PMN transepithelial migration related to the direction of migration. The observed IFN- γ -elicited downregulation of PMN transmigration in the physiological direction was surprising in light of the opposite effects observed in apical-to-basolateral directed transmigration. Such downregulation in the physiological direction occurred over a wide range of applied PMN densities, showed graded dose-response characteristics and time course effects, was dependent on protein synthesis, and was present at various times during the transmigration experimental period. While the net effect of epithelial exposure to IFN- γ on basolateral-to-apical directed PMN transmigration was opposite to that of apical-to-basolateral transmigration, some similarities existed. The movement of PMN into the monolayer and subsequent PMN association with the basolateral membrane of epithelial cells appeared to be enhanced by IFN- γ . However, the subsequent movement across the tight junction and into the apical reservoir was impeded, resulting in a net decrease in total PMN transmigration. Experiments using transfer of conditioned media, alluded to above, as well as monolayer washing experiments, again suggest that soluble factors were not responsible for these IFN- γ -elicited effects. Thus, it would

not be surprising if IFN- γ -elicited alterations in the surface characteristics of epithelial cells again accounted for the observed effects. Several possibilities, currently under investigation, exist to explain these results. First, IFN- γ could differentially regulate two steps in the sequence of PMN-epithelial interactions which occur with migration in the physiological direction. An initial associative step could be upregulated, while a secondary step before junctional impalement could be downregulated, resulting in enhanced movement into the monolayer but overall downregulation of PMN transmigration. Alternatively, downregulation of a required prejunctional step could simply result in observed accumulation of PMN in the epithelial basolateral compartment. Other possibilities include induction of new ligands for PMN or modulation of de-adhesive steps.

While the polarized differences in IFN- γ effects on PMN transmigration are of interest, it is clear that basolateral-to-apical directed transmigration is physiologically most relevant. In this regard, the observed results are of potential benefit. The overall effect of IFN- γ exposure for physiologically directed migration is to enhance the movement of PMN into the subjunctional paracellular space and to prolong the residence of the PMN at this site, even in the presence of a significant transjunctional chemotactic gradient. As a result, the residence time of the PMN is prolonged at a most crucial defensive position: the anatomic subsite at which separation of internal from external (luminal) compartments exist.

Consideration of the Effects of Epithelial Tight Junction Permeability on PMN Transmigration

Junctional permeability is a crucial consideration in such experiments since, unlike the case in Boyden chambers, or the highly permeable endothelial lining of the majority of the microcirculation, epithelial tight junctions have more restrictive permeability characteristics. For example, the transepithelial resistance of even "leaky" epithelia is on the order of 100 ohm-cm², while many epithelia have resistances of several hundred to several thousand ohm-cm² (Powell, 1981). In contrast, cable analysis studies of the mesenteric microvasculature suggests that even physiologically confluent endothelium (except for that of the brain which has resistance >1,000 ohm-cm²) has an average resistance of only a few ohm-cm² (on average, <2 ohm-cm²; Crone and Christensen, 1981). As a consequence of the capacity of epithelia to behave as very restrictive barriers to the passive transepithelial flux of hydrophilic solutes, one finds that even for solutes with hydrodynamic radii in the range of 10 Å, diffusional flux across epithelial monolayers, such as those used here, is <10 nM/h/cm² (Madara and Dharmasathaphorn, 1985). Indeed, even in the low resistance state induced by prolonged IFN- γ exposure, inulin fluxes, while increased sixfold from baseline, are <10 nM/h/cm² (Madara and Stafford, 1989). It is also clear that the intercellular tight junction, which in epithelia is continuous, is the specific anatomic subsite at which the rate-limiting barrier to passive paracellular diffusion resides. Such severe restriction of movement of hydrophilic chemotactic substances in the size range of *N*-formylated peptides implies that across the very narrow zone of the epithelial tight junction (<200 nM), extremely steep chemotactic gradients can be supported. As a consequence, one would expect that, to provide an equivalent driving force, larger chemotactic gradients would be

required across epithelia than across filters (i.e., Boyden chamber) or confluent endothelia. Similarly, any standard chemotactic gradient might more successfully drive migration across endothelia than across epithelia. Indeed, as shown in this study, when Ca²⁺ depletion experiments were used to produce discontinuous tight junctions and reduce resistances to near that of endothelia, enhancement of transmigration was found.

Comparative Evaluation of Transendothelial and Transepithelial Migration of PMN

As outlined in the introduction and as derived from model systems, PMN interactions with epithelia, including specific interactions with epithelial apical membranes after transmigration, modulate epithelial function. Hence, a better understanding of the rules governing PMN-epithelial interactions and transmigration are crucial to the understanding of active inflammation at mucosal surfaces. Additionally, if the rules vary between transepithelial and transendothelial migration of PMN, possibilities for new (and perhaps even more specific) therapeutic strategies would exist. For this reason, it is useful to consider what appears to be emerging differences in transmigration across these two crucial barriers. This comparison chiefly relies on studies derived from model human intestinal epithelium, and it is possible that organ- and site-specific differences in PMN-epithelial interactions exist. Also, since basal endothelial-neutrophil interactions are difficult to access because of small signal size, this comparison largely relies on data obtained from cytokine-activated endothelial cells.

We have found no differences in baseline electrical parameters across T₈₄ monolayers nor differences in subsequent PMN transmigration in monolayers kept in strict endotoxin-free conditions and subsequently exposed to endotoxin for various periods up to 6 h (Nash, S., J. L. Madara, and J. Stafford, unpublished observations). In contrast, endothelial cells are extremely sensitive to endotoxin and respond by expression of endothelial surface markers which define the activated state of enhanced PMN adherence (Pohlman et al., 1987; Wellicome et al., 1990). Since endotoxin constantly bathes intestinal epithelia under physiological conditions, endothelial exposure to endotoxin primarily occurs in association with adjacent bacterial disease. Such differences in endotoxin effects on these systems would, in a teleological sense, seem wholly appropriate. Differences in the participation of integrins may also exist between endothelia and epithelia. β_2 -integrin-mediated adhesion of PMN to endothelial cells appears to be a required step in transendothelial migration of PMN (Larson and Springer, 1990; Osborn, 1990; Pober and Cotran, 1990; Springer, 1990). However, with endothelia, depending on the conditions of activation of endothelial cells and PMN, CD11a/18 or CD11b/18 contribute in significant fashion to PMN interactions (Smith et al., 1989). In contrast, under basal or IFN- γ -induced states, we find no evidence for a significant role of CD11a/18 in PMN transmigration across our model intestinal epithelium. Substantial differences (as well as similarities) exist in model endothelial and model intestinal epithelial responses to IFN- γ . In both models, IFN- γ alters F-actin distribution (Stolpen et al., 1986; Madara and Stafford, 1989), and induces expression of MHC class II antigens on the plasma membrane (Collins et al., 1988; Mayer

et al., 1991). In the epithelial model, the observed alterations in F-actin distribution parallel alterations in tight junction permeability (Madara and Stafford, 1989), although it has not been assessed whether comparable functional changes occur with the comparable cytoskeletal alterations noted in endothelia. However, while IFN- γ induces CD54 (ICAM-1) expression on endothelia (Pober et al., 1986), we find no evidence for IFN- γ -mediated induction of apical ICAM-1 in our intestinal epithelial model. Of interest, in vivo studies in mice have shown that the epithelium for which T₈₄ cells serve as a model—colonic crypt cells—are not induced to express MHC class II equivalents by IFN- γ , even though some other epithelia (including villus enterocytes) are so induced (Momberg et al., 1986). Finally, IFN- γ substantially influences PMN-epithelial interactions, and does so in a highly polarized fashion, but does not appear to substantially effect PMN-endothelial interactions unless coexposed to other cytokines (Pober and Cotran, 1990). In aggregate, the above data suggest that substantial differences will exist between PMN interactions with endothelia and epithelia. Since PMN-epithelial interactions occurring in transepithelial migration likely appear to contribute to mucosal dysfunction in active inflammatory states (vida supra), a better understanding of the unique rules governing epithelial-neutrophil interactions may open the door to more tissue-specific therapies in the future.

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