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Interferon Gamma and Interleukin 1, But Not Interferon Alfa, Inhibit Rotavirus Entry Into Human Intestinal Cell Lines

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Background & Aims: **Rotavirus, an important agent of gastroenteritis in children, causes diarrhea by infecting differentiated villus enterocytes in the small intestine. The aim of this study was to determine whether cytokines that can be expressed by mucosal cells have an effect on the rotavirus susceptibility of cultured human enterocytes.** ***Methods:*** **Caco-2 and HT-29 cells were pretreated with various cytokines before challenge with rotavirus.** ***Results:*** **Interleukin (IL)-1, interferon (IFN)-α, and IFN-γ pretreatment led to a dose-dependent resistance to rotavirus infection. Maximum effects occurred after 72 hours of pretreatment, whereas no detectable inhibition occurred with <12 hours of pretreatment. Liposomal transfection of single-shelled and double-shelled rotavirus particles bypassed the block to rotavirus replication in IFN-γ– and IL-1–treated but not IFN-α–treated cells. Binding studies with purified, metabolically labeled rotavirus showed no significant difference among IFN-γ– and IFN-α–treated and control Caco-2 cells. Viral entry into Caco-2 cells was significantly inhibited by IFN-γ and IL-1 but not IFN-α.** ***Conclusions:*** **IFN-α and IFN-γ induce rotavirus resistance by different mechanisms, suggesting that cytokines play a role in host defense against viral agents by changing the phenotype of intestinal epithelial cells.**

The intestinal epithelium serves as a barrier, protecting the host from noxious organisms and molecules and simultaneously facilitating absorption of essential nutrients. Recently a number of investigators have shown that enterocytes are active participants in intercellular cross-talk via cytokines with immune effector cells such as mononuclear cells and neutrophils. This interaction allows localized and specific modulation of epithelial and immune effector responses under varying conditions. For example, enterocytes release various cytokines, including interleukin (IL)-8, in response to invasive bacteria,[1,2] presumably to recruit neutrophils to sites of bacterial invasion. On the other hand, many cytokines originating in mononuclear cells, such as interferon (IFN)-γ and IL-4, are able to modulate intestinal epithelial barrier function and electrophysiology and to increase enterocyte surface expression of molecules such as major histocompatibility complex antigens.[3,4]

Rotavirus is one of the most common mammalian intestinal infections. Virtually all humans have been infected by 4 years of age, and primary infections are an important cause of childhood morbidity and mortality.[5] Under normal conditions, rotavirus exclusively infects enterocytes, primarily differentiated villus enterocytes in the small intestine. Recent studies have shown that although both T and B cells can participate in rotavirus clearance in mice, a functional B-cell response is required for immunity to subsequent infection.[6,7] Less information is available regarding nonspecific host defense mechanisms against rotavirus that may be important in preventing severe disease in naive hosts. A variety of cytokines, particularly the IFNs, have been shown to mediate antiviral states in various cells. The role of cytokines in host defense against mucosal viral pathogens has not been well characterized.

In this study, the effects of various cytokines on the rotavirus susceptibility phenotype of human intestinal cell lines, Caco-2 and HT-29, were examined. These cell lines, derived from human colonic carcinomas, have many features of small intestinal epithelium including the ability to form polarized monolayers with well-developed microvilli, expression of digestive enzymes such as sucrase-isomaltase and lactase, and the ability to transport water and ions toward the basolateral aspect.[8] They are also highly permissive for rotavirus.[9] Whether cytokines that are known to be secreted by mucosal mononuclear or epithelial cells modulate the susceptibility of enterocytes for rotavirus infection was examined.

Materials and Methods

Cytokines

All cytokines were purchased as lyophilized powders shipped, aliquoted, and stored according to the suppliers' rec-

Abbreviations used in this paper: IFN, interferon; IL, interleukin; MIC_{50}, mean inhibitory concentration; pfu, peroxidase focus units; RRV, rhesus rotavirus; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor.

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ommendations. Recombinant human IFN-γ (catalogue no. I 6507, 1.7×10^6 international reference units [U]/mg protein) and human lymphoblastoid IFN-α (catalogue no. I 9887, 1.6×10^8 U/mg protein) were obtained from Sigma Chemical Co. (St. Louis, MO). All other recombinant human cytokines were obtained from R&D Systems (Minneapolis, MN). Murine monoclonal antibody (immunoglobulin [Ig] G2a) directed against human IFN-γ, sheep antiserum against human IFN-α, cyclohexidine, and rabbit antiserum against IL-1 were also obtained from Sigma. Control antibodies for neutralization included a murine IgG2a monoclonal antibody (8G4) against astrovirus, antiastrovirus rabbit serum, and normal sheep serum.

Cells and Viruses

Rhesus rotavirus (RRV) (G3,P5[3]) and the Wa (G1, P1A[8]) strain of human rotavirus were grown in MA 104 cells and purified by hydrofluorocarbon extraction and isopycnic centrifugation as described previously.[10] Purified [^{35}S]-methionine–labeled rotavirus was prepared in the same fashion except that 4 hours after infection, the medium was replaced with methionine-free medium containing 100 μCi/mL [^{35}S]-methionine. HT-29 cells were a gift from John Barnard, Vanderbilt University. Caco-2 cells were obtained from American Type Tissue Collection (Rockville, MD). Cells were grown in RPMI medium supplemented with 10% fetal calf serum, L-glutamine, penicillin, and streptomycin in a 5% CO_2 incubator. Cells were grown in 96- or 24-well culture dishes. Unless otherwise stated, the cells were treated for the indicated times with cytokines at the stated concentrations in fresh media after reaching confluence 4 days after plating. Control cells were given fresh media without cytokine at the same time. Caco-2 cells, which were used 20 days after confluence for studies of differentiation effects, were fed fresh media every other day.

Cytotoxicity Assays

All cytokine treatments were tested for cytotoxicity on Caco 2-cells on 96-well plates by microscopic inspection for morphology, trypan blue exclusion, and MTT dye reduction. Cells treated with maximal doses (125 U/mL) of IFN-γ and IFN-α for 72 hours were pulse-labeled with [^{35}S]methionine, and total counts incorporated were compared with those of medium-treated cells. Specific protein incorporation was also examined by lysing the cells with Laemmli's sample buffer and performing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with fluorography.

Infection of Caco-2 Cells With Rotavirus

Monolayers in 96-well plates were washed with serum-free media and inoculated with dilutions (1:5000) of trypsin-activated virus at a concentration calculated to equal approximately 100 infectious units per well. Rotavirus stock was activated by treating with trypsin (type VIII; Sigma) at 5 μg/mL for 30 minutes at 37°C. Infection was allowed to proceed overnight at 37°C. Infected cells were identified by immunoperoxidase staining as previously described.[11] Briefly, monolayers were fixed with cold methanol and incubated in primary antibody (guinea pig antiserum to purified RRV rotavirus) at a 1:1000 dilution for 2 hours at 37°. After washing with phosphate-buffered saline, the cells were incubated for 1 hour with anti–guinea pig peroxidase conjugate (Kirkegarde and Perry, Gaithersburg, MD) and washed again before addition of substrate (3-amino-9-ethyl carbazole–H_2O_2 in *N*-methyl formamide). Infected cells stain a bright red-brown, and uninfected cells show no color using these reagents. The actual number of infected cells per well (2×10^4 total cells) was determined by counting all stained foci under an inverted microscope. All experimental conditions and controls were performed in triplicate in each experiment. Control infections varied <15% between and within each experiment. Each experiment was performed at least three times with each virus with similar results. Wa and RRV rotaviruses were used in all experiments with similar results.

Liposomal Transfection of Caco-2 Cells With Single- and Double-Shelled Rotavirus Particles

Single-shelled virus preparations were treated with 10 mmol/L ethylenediaminetetraacetic acid (EDTA) before use to ensure removal of outer capsid proteins. Such EDTA-treated preparations had no residual infectivity when inoculated on MA 104 cells without liposomes. Monolayers were washed with serum-free media and incubated at 37°C for 4 hours with a mixture containing 10 μg/mL Lipofectin (GIBCO/BRL, Gaithersburg, MD) and dilutions of CsCl-purified rotavirus double- or single-shelled particles in serum-free media as described previously.[12] The cells were then fed an equal volume of complete (10% fetal bovine serum) media and incubated overnight at 37°C. Infection was detected by immunoperoxidase staining.

Binding and Internalization of Radiolabeled RRV Rotavirus

Confluent Caco-2 monolayers in 24-well dishes were washed twice and chilled to 4°C. Metabolically [^{35}S]-methionine-labeled RRV (100,000 cpm/well, approximately 10^6 peroxidase focus units [pfu]/well, 10 pfu/cell) was added, and the monolayers were incubated at 4°C with gentle rocking for 1 hour. For measurement of binding, the monolayers were washed three times with cold serum-free media, lysed with 2% SDS, and counted as previously described.[12] Under these conditions, approximately 10%–20% of the radiolabeled virus bound to the monolayer. Internalization was determined by binding the ^{35}S-labeled virus as described, followed by warming to 37°C for 120 minutes. The cells were then treated with trypsin-EDTA (10× solution; Sigma) in saline for 30 minutes at 4°C, washed twice with ice-cold media containing 2 mmol/L phenylmethylsulfonyl fluoride and 10% fetal calf serum, lysed, and counted. Control experiments showed that 95% of virus bound at 4°C was removed under these conditions if the monolayers were not warmed.

Infectivity of Bound Rotavirus

Confluent Caco-2 monolayers that were mock-treated or pretreated with IFN-γ were washed with serum-free Dulbecco's modified Eagle medium and infected with RRV (5 pfu/cell) or Wa (0.5 pfu/cell) at 4°C for 1 hour. The monolayers were washed three more times, and the cell-associated infectious virus was recovered by freeze-thawing once and passing the lysate repeatedly through a 27-gauge needle. The resulting lysate was titrated on MA 104 cells by peroxidase focus counting.

Analysis of Data

All experiments were performed at least three times with results of typical experiments reported. Results were analyzed by comparison of means using Student's *t* test or by analysis of variance and are expressed as mean ±SD.

Results

Effects of Cytokine on Rotavirus Replication

Caco-2 cells were pretreated for 72 hours with various cytokines that are known to induce phenotypic responses in cultured intestinal epithelial cells[3,4,13] before challenge with rotavirus. IL-8 was also tested because it is released by intestinal epithelia after infection with rotaviruses[14] and invasive bacteria.[2] The relatively long pretreatment period, 72 hours, was chosen to provide maximum sensitivity in detecting cytokine effects on Caco-2 susceptibility to rotavirus. The results, summarized in Table 1, indicate that IL-1α and IL-1β induced a moderately rotavirus-resistant state in the Caco-2 cells in a dose-dependent manner (maximum inhibition, 70% of control), whereas IL-6 led to a reproducible decrease in infection of dubious physiological significance (maximum inhibition, 30%). Although synergy of effects of IL-1 and IL-6 in the expression of acute-phase proteins by Caco-2 cells has been observed,[13] no additive effects were noted when the cells were treated with both cytokines in varying concentrations in a "checkerboard" simultaneous titration (data not shown). IFN-γ and IFN-α pretreatment led to significant dose-dependent resistance to rotavirus infection (maximum inhibition, 99% and 90%, respectively). Similar results were obtained when cytokine-pretreated HT-29 cells were challenged with rotavirus (Table 1). IFN- and IL-1–induced rotavirus resistance was completely abrogated by the addition of appropriate neutralizing antibodies but not affected by addition of a control antibody (data not shown). IFN- and IL-1–induced rotavirus resistance was also abrogated by the presence of cycloheximide (5 μg/mL) during the first 18 of 24 hours of IFN or IL-1 treatment. None of the cytokines tested showed any evidence of toxicity to the cells at the concentrations used by gross morphology, trypan blue exclusion, or MTT reduction. Neither IL-1 nor IFNs had an effect on total counts per minute (cpm) of [^{35}S]methionine incorporated into the Caco-2 cells. In addition, treatment with IFN did not cause any gross change in the specific SDS-PAGE pattern of metabolically labeled Caco-2 proteins (data not shown).

Table 1. Effect of Various Cytokines on Wa Rotavirus Infection of Caco-2 and HT-29 Cells

Cytokine	Maximum inhibition (%)	MIC_{50}[a]	Maximum concentration tested
IL-1α	70 ± 4.5[b]	2 ng/mL	10 ng/mL
IL-1β	70 ± 4.2[b]	2 ng/mL	10 ng/mL
IL-2	None		20 ng/mL
IL-4	None		50 ng/mL
IL-6	30 ± 5.9[b]	[c]	50 ng/mL
TNF-α	None		20 ng/mL
TNF-β	None		20 ng/mL
IFN-α	90 ± 5.0[b]	15 U/mL	500 U/mL
IFN-γ	99.9 ± 0.2[b]	3 U/mL	125 U/mL
IL-1α (HT 29)	85 ± 52[b]	1 ng/mL	10 ng/mL
IFN-α (HT 29)	95 ± 3.0[b]	10 U/mL	500 U/mL
IFN-γ (HT 29)	89 ± 6.2[b]	10 U/mL	125 U/mL
IL-8	None		100 ng/mL
IL-1β + IL-6	70 ± 6.3[b]	2 ng/mL Il-1b	10 ng/mL + 10 ng/mL

NOTE. Caco-2 or HT-29 cells were treated with serial twofold dilutions of cytokines for 72 hours before challenge with 100 pfu/well of Wa rotavirus. Results are means of at least three replicate wells.
[a]Mean inhibitory concentration, concentration of cytokine required to reduce Wa rotavirus infection to 50% of control value.
[b]Significantly different from control wells (Student's *t* test, $P < 0.01$).
[c]Did not inhibit Wa to 50% of control.

Additional studies were performed to characterize the time course and nature of the antiviral effect. Figure 1 shows the effect of different durations of IFN-α and IFN-γ pretreatment on rotavirus resistance in a typical experiment. Six-hour IFN treatment of Caco-2 cells had no significant effect on rotavirus infection. By 24 hours, rotavirus resistance was evident but required higher concentrations of IFN-γ (mean inhibitory concentration [MIC_{50}], 40 U/mL) compared with an MIC_{50} of approximately 3 U/mL for 72 hours of treatment. The resistance to rotavirus largely persisted even if cytokine was removed for 48 hours from cells that had been previously treated for 72 hours. Similar time course results were obtained for IL-1 treatment of Caco-2 cells (data not shown). Because rotavirus in vivo typically infects the most differentiated villus tip cells of the small intestine and because IFNs can affect cell differentiation, we studied the effect of the state of differentiation of the Caco-2 cells on the antiviral

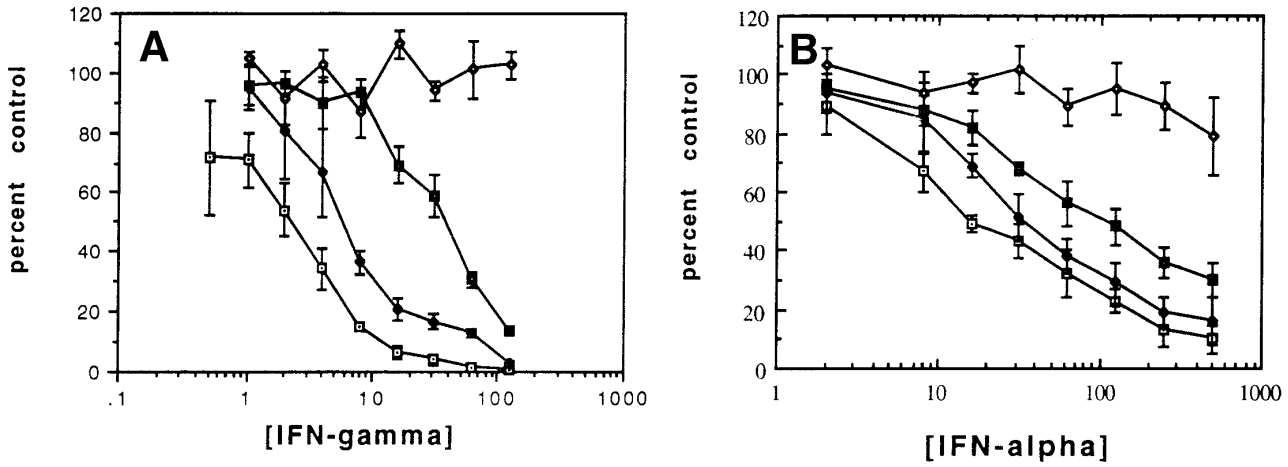

Figure 1. Effect of duration of IFN treatment of Caco-2 cells on inhibition of rotavirus replication. Caco-2 cells were pretreated with the indicated concentrations of (*A*) IFN-γ or (*B*) IFN-α for 72 (▣), 48 (◆), 24 (■), or 6 (◆) hours before challenge with Wa rotavirus. Infection was detected by immunoperoxidase staining. Results are expressed as the mean ±SD of three replicate wells.

state induced by IFN-γ and IFN-α. The differentiation of Caco-2 cells toward a phenotype characteristically increases similar to differentiated villus tip enterocytes with time after plating.[15] In our study, differentiated (20-day) Caco-2 cells expressed approximately 10-fold more alkaline phosphatase than the undifferentiated (4-day) Caco-2 cells. We treated monolayers of Caco-2 cells either 4 days (early confluence) or 20 days (differentiated) after plating with various concentrations of IFN and IL-1 for 48 hours before challenge with Wa rotavirus. Both IFNs and IL-1 induced rotavirus resistance in Caco-2 cells regardless of their differentiation status. The more differentiated cells were consistently significantly more sensitive to IFN-γ at lower concentrations of IFN tested (2–4 U/mL). No differences were noted in the effects of IFN-α or IL-1 on the two populations of Caco-2 cells. Interestingly, there were no significant differences in the rotavirus susceptibility of the media-treated control 4-day and 20-day Caco-2 cells.

Lipofection of Single- and Double-Shelled Rotavirus Particles Bypasses Rotavirus Resistance of IFN-γ– and IL-1–Treated Caco-2 Cells

Because most studies on the IFN-induced antiviral state have described inhibition of viral transcription or translation,[16] whether IFN treatment of Caco-2 cells also limited rotavirus replication at either of these steps of the viral life cycle was determined. Cationic liposomes were used to transfect viral particles into cells, a method previously used to induce productive rotavirus infection in naturally nonpermissive murine L-cell fibroblasts.[12] When single-shelled, noninfectious rotavirus particles were thus transfected into the IFN-γ–treated Caco-2 cells, there was virtually no inhibition of viral replication within the range of IFN-γ concentrations tested (Figure 2*A*). Because production of new viral antigen was being measured with the immunoperoxidase staining assay, the lack of inhibition observed indicates that under these conditions, IFN-γ treatment had no significant effect on rotavirus transcription or translation in Caco-2 cells.

Double-shelled, infectious rotavirus virions become transcriptionally activated when they lose their outer capsid.[17] Uncoating is therefore an essential early step in the rotavirus replicative cycle. If an uncoating defect was present in the IFN-treated Caco-2 cells, it would be expected that double-shelled particles are unable to infect the cells. To determine whether viral uncoating was inhibited by IFN-γ, similar liposomal transfections were performed with double-shelled rotavirus particles (Figure 2*B*). Like single-shelled particles, double-shelled particles were fully infectious in IFN-γ–treated cells if they were introduced into the cells via liposomes.

Parallel experiments showed that liposomal transfection of single- or double-shelled rotavirus particles bypassed the inhibition induced by IL-1 treatment of Caco-2 cells (Figure 3). Successful rotavirus lipofection of single- and double-shelled rotavirus particles was also performed in IFN-γ– and IL-1–treated HT-29 cells (data not shown).

Lipofection of Single-Shelled Rotavirus Particles Fails to Bypass Rotavirus Resistance of IFN-α–Treated Caco-2 Cells

Because IFN-α was also a potent inhibitor of rotavirus replication (Table 1), single-shelled rotavirus particles were transfected into IFN-α–treated cells in

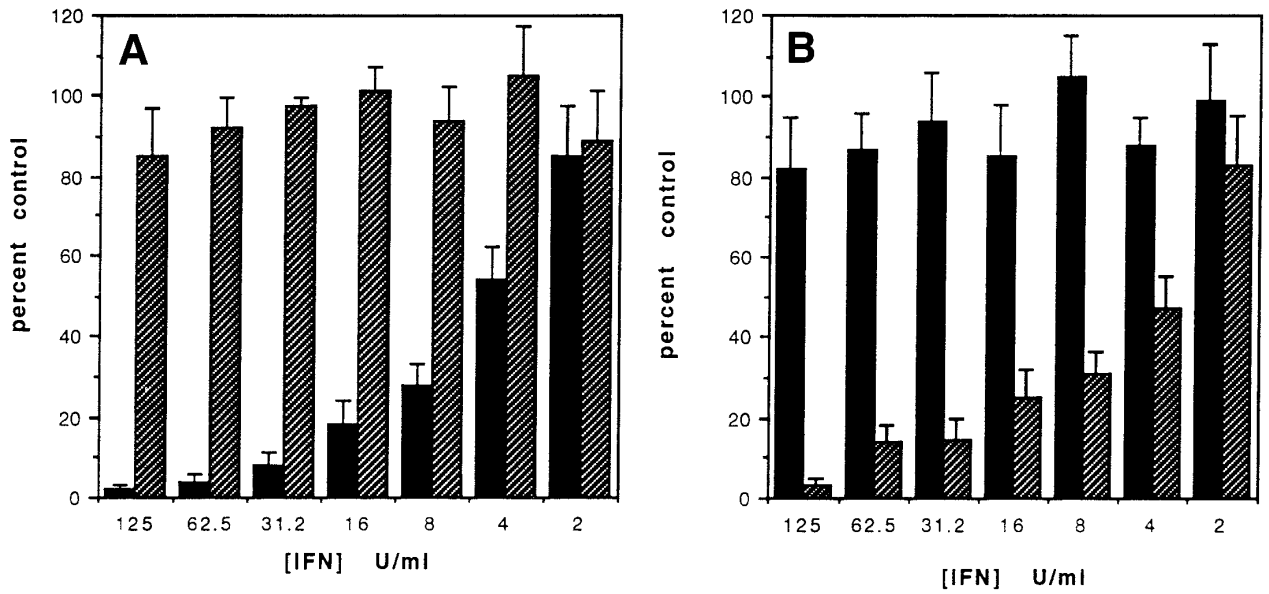

Figure 2. (*A*) Lipofection of single-shelled rotavirus particles restores rotavirus permissivity to IFN-γ–treated Caco-2 cells. Caco-2 cells were pretreated for 72 hours with the indicated concentrations of IFN-γ, washed, and infected with either purified double-shelled Wa rotavirus particles (■) or purified single-shelled Wa particles with Lipofectin (▨) as described. Results are expressed as percentage of media-treated control wells that were infected in the same manner (double-shelled particles or Lipofectin/single-shelled particles). (*B*) Lipofection of double-shelled rotavirus particles restores rotavirus permissivity to IFN-γ–treated Caco-2 cells. Caco-2 cells were pretreated for 72 hours with the indicated concentrations of IFN-γ, washed, and infected with either purified double-shelled Wa rotavirus particles (▨) or purified double-shelled Wa particles with Lipofectin (■) as described. Results are expressed as percentage of media-treated control wells that were infected in the same manner (double-shelled particles or Lipofectin/double-shelled particles).

an identical fashion. Unlike IFN-γ–pretreated cells, IFN-α pretreated Caco 2 cells remained resistant to rotavirus infection even with liposomal transfection (Figure 4). Double-shelled rotavirus particles also failed to bypass IFN-α rotavirus inhibition in Caco-2 or HT-29 cells (data not shown).

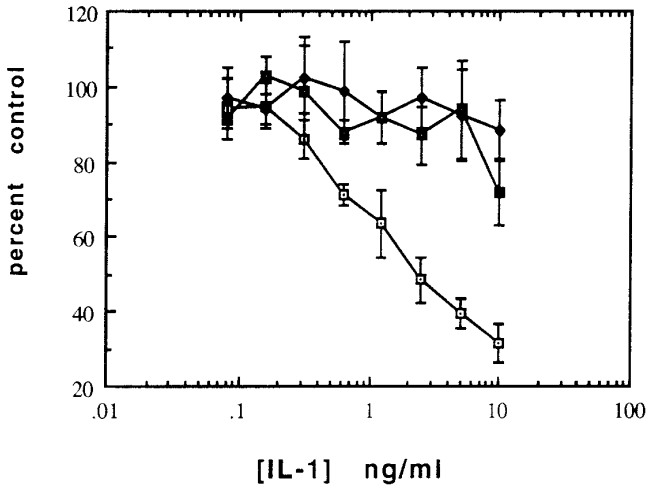

Figure 3. Lipofection of single- or double-shelled rotavirus particles restores rotavirus permissivity to IL-1–treated Caco-2 cells. Caco-2 cells were pretreated for 72 hours with the indicated concentrations of IL-1, washed, and infected with either purified double-shelled Wa rotavirus particles (⊡) or purified single-shelled (◆) or double-shelled (◘) Wa rotavirus particles with Lipofectin. Results are expressed as percentage of media-treated control wells that were infected in the same manner (double-shelled particles or Lipofectin/single-shelled particles).

Neither IFN-γ, IL-1, nor IFN-α Has a Significant Effect on Rotavirus Binding to Caco-2 Cells

Because uncoating, transcription, and translation were not blocked in the IFN-γ– and IL-1–treated cells, it was deduced that inhibition must be caused by a very early step in viral replication. To identify this early step, binding studies were performed using purified, metabolically labeled RRV rotavirus. Results of a typical experiment are shown in Table 2. No significant difference in radiolabeled virus binding between control and IL-1–, IFN-α–, and IFN-γ–treated cells could be observed. Because many of the purified rotavirus particles may not be capable of initiating infection, it was possible that IFN reduced the binding of the infectious fraction of rotavirus particles. Therefore, the binding of infectious particles of both RRV and Wa rotavirus was also measured using previously described methods.[12] As can be seen from Table 2, this approach failed to show any differences between cytokine and media-treated cells.

IFN-γ and IL-1, But Not IFN-α, Reduces Rotavirus Internalization by Caco-2 Cells

To examine the entry of virus particles into IFN-γ–treated Caco-2 cells, metabolically labeled RRV rota-

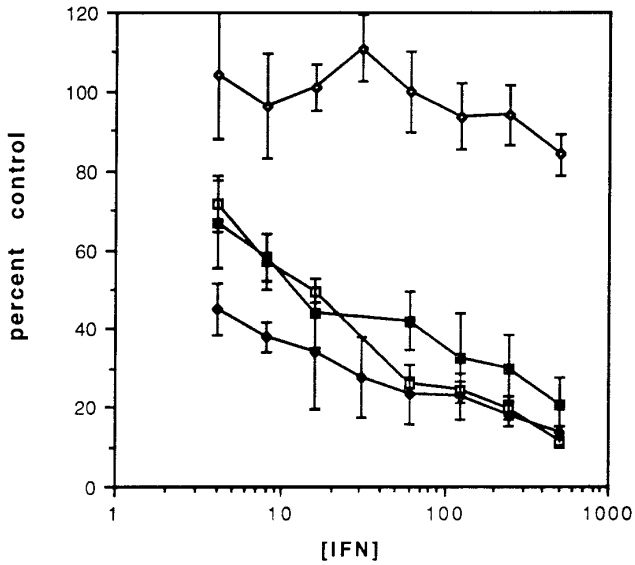

Figure 4. Lipofectin of single-shelled rotavirus particles fails to restore rotavirus permissivity to IFN-α–treated Caco-2 cells. Caco-2 cells were pretreated for 72 hours with the indicated concentrations of IFN-γ or IFN-α, washed, and infected with either purified double-shelled Wa rotavirus particles (control: IFN-α [□]; IFN-γ [◆]) or purified single-shelled Wa particles with Lipofectin (IFN-α [■] and IFN-γ [◆]). Results are expressed as percentage of media-treated control wells that were infected in the same manner (double-shelled particles or Lipofectin/single-shelled particles).

virus was allowed to bind to IL-1–, IFN-γ–, IFN-α–, and mock-treated monolayers. After 1 hour of binding at 4°C, the monolayers were washed and warmed to 37°C for 2 hours. Surface-bound virus was removed with trypsin-EDTA, and internalized counts per minute were determined by scintillation counting. The results showed 90% inhibition of rotavirus internalization in cells that had been pretreated with 75 U/mL IFN-γ (Figure 1). This is quite similar to the level of inhibition of viral replication found with similar doses of IFN-γ (Figure 2) and suggests that poor internalization of virus is the major block to rotavirus replication in IFN-γ–treated Caco-2 cells. Similarly, IL-1–treated Caco-2 cells showed decreased internalization of the labeled virus. On the other hand, IFN-α–treated Caco-2 cells (250 U/mL) internalized rotavirus particles efficiently (90% of control).

Discussion

In vivo, rotavirus infects intestinal epithelial cells that are intimately associated with mononuclear cells located within and below the epithelium. These mononuclear components of the intestinal mucosal immune system interact with the epithelial cells in a complex fashion. Intestinal epithelial cells have been shown to express various cytokines including tumor necrosis factor α (TNF-α), IL-1, IL-8, IL-10, monocyte chemotactic protein 1, and granulocyte-macrophage colony–stimulating factor, either constitutively[1] or in response to stimuli such as TNF-α, IL-1, viruses,[14] or invasive bacteria.[2] Furthermore, intestinal epithelial cells have been shown to exhibit various physiological or phenotypic responses to a variety of cytokines including TNF-α, IFN-γ, IL-1, IL-4, and IL-6.[3,4,13] Thus, cytokines can function as local autocrine or paracrine regulators of both mucosal and immune function. Because many cytokines have potentiating, redundant, or antagonizing effects on various cells, the in vivo interactions between epithelial and lymphoid cells are extremely complex.

To identify potential modifiers of enterocyte susceptibility to rotavirus in vivo, the effects of selected cytokines on rotavirus susceptibility of enterocytes were studied by pretreating monolayers of cultured intestinal epithelial cells. The initial findings indicated that certain cytokines, particularly IL-1, IFN-α, and IFN-γ, could downregulate the rotavirus permissivity of Caco-2 cells (Table 1). The experiments cannot exclude the possibility that other cytokine-like substances could be released by enterocytes in response to IFN or IL-1 and mediate the antiviral effects.

The time course of development of the antiviral state (onset at approximately 12 hours, peak at 72 hours; Figure 1) was similar to that observed with the development of IFN-γ– or IL-4–induced changes in enterocyte phenotype such as decreased stimulated Cl^- secretion and major histocompatibility complex expression.[3,4] This time course is also compatible with previously described IFN-induced antiviral states. Such a time course suggests the need for a change in gene expression for the observed phenotypic response. In fact, cyclohexidine-mediated inhibition of protein synthesis prevented development of the antiviral state. Although most of the experiments were performed after 48–72 hours of cytokine exposure, substantial inhibition of rotavirus replication was evident after 24 hours.

Table 2. Effects of IFN on Rotavirus Binding to Caco-2 Cells

Virus	Control	IFN-γ	IFN-α	IL-1
^{35}S RRV (cpm bound)	10,050 ± 420	10,330 ± 620	9760 ± 720	11,250 ± 810
RRV (pfu bound)	13,500 ± 450	14,200 ± 820	12,900 ± 560	12,980 ± 210
Wa (pfu bound)	530 ± 60	490 ± 110	480 ± 80	560 ± 100

NOTE. Binding studies were performed as described in Materials and Methods; results are expressed as mean ± SD for four replicate wells.

It is not surprising to observe that IFNs induce strong antiviral states in Caco-2 cells, given that IFN was originally discovered, described, and named for its antiviral effect.[18] Type I IFNs (such as IFN-α) are believed to inhibit viral translation by several pathways including inactivation of the eukaryotic initiation factor 2α by phosphorylation via an induced kinase. Thus, in early studies of type I IFN–induced antiviral states, transfection of viral RNA failed to cause cellular infection.[19] Previous experiments in which liposomal transfection of rotavirus failed in IFN-α–treated Caco-2 cells confirm those results (Figure 4).

The antiviral properties of IFN-γ (type II IFN) have been studied less than those of the type I IFNs such as IFN-α. IFN-γ binds to a distinct receptor and apparently induces a distinct antiviral state.[19–23] The fact that antiviral synergy for types I and II IFNs can be demonstrated suggests that different mechanisms exist for each class of IFN.[24] In the present experiments, the antiviral state of IFN-γ– and IL-1–treated cells could be overcome by liposomal transfer of single- or double-shelled rotavirus particles (Figures 2 and 3). Because this procedure resulted in control levels of transcription and translation of new viral antigen as detected by our immunoperoxidase assay, it can be concluded that inhibition of viral transcription and/or translation is not important in the IFN-γ– or IL-1–induced rotavirus resistance. The production of new viral antigen by transfected cells also shows that the antiviral effect was not caused by nonspecific cytotoxic effects of the cytokines. Studies of viral binding and entry into cytokine-treated cells showed that most of the IFN-γ– and IL-1–induced viral resistance can be accounted for by failure of bound rotavirus to enter the cell (Figure 5).

IFN-induced resistance to entry of invasive bacteria has been noted previously.[25] Whitiker-Dowling et al. described type I IFN-induced reduction of vesicular stomatitis virus cell entry in some (but not all) murine fibroblast cell lines, but the effect was much less pronounced and required much higher doses of IFN.[26,27] Thus, a potent and novel mechanism for an IFN-γ–induced antiviral state may be applicable to other cells and viruses in the present study. In fact, IFN-γ–pretreated Caco 2 cells are equally resistant to astrovirus, a completely unrelated agent of viral diarrhea, and serotype 1 (Lang) reovirus (D. Bass, unpublished observations).

The barrier to cell entry of rotavirus in IFN-γ– and IL-1–treated Caco 2 cells is unclear. Rotaviruses (unlike astroviruses and reoviruses) infect cells by an endocytosis-independent direct membrane penetration.[11,12,28] IFN-γ has been reported to induce changes in cellular membrane lipid composition[29,30] and changes in cellular cytoskeletal elements,[31–33] either of which may be relevant to this mechanism of cellular resistance. We have previously described modification of cellular susceptibility to rotavirus by cytoskeletal inhibitors.[28] In these studies, cytochalasin B and D were capable of enhancing susceptibility of normally resistant murine L cells to rotavirus. Neither cytochalasins, Taxol, nor cholcine had any ability to inhibit rotavirus infection of permissive cell lines.

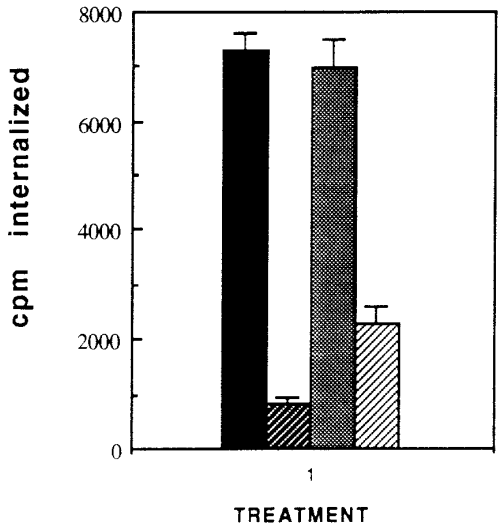

Figure 5. Internalization of [^{35}S]methionine-labeled rotavirus on cytokine and media-treated Caco-2 cells. Caco-2 cells were treated with 75 U/mL IFN-γ (▨), 200 U/mL IFN-α (▩), or 5 ng/mL IL-1 (▨) or were mock-treated (■) for 72 hours before incubation at 4°C for 1 hour with 100,000 cpm labeled rotavirus. The monolayers were then washed and warmed to 37°C for 2 hours. Extracellular virus was removed with trypsin-EDTA as described; the cells were washed and dissolved with Laemmli's sample buffer, and residual radioactivity was determined by scintillation counting.

Our studies do not directly address the in vivo role of IFN and other cytokines in host defense against enteric viral pathogens. In previous human and animal studies of rotavirus gastroenteritis, type I IFN was detected both locally and systemically at levels comparable to or higher than those used in these experiments.[34–36] Exogenous IFN-α has been reported to be therapeutic in rotavirus diarrhea in piglets and calves.[37,38] IL-1 has been found in both normal and inflamed intestinal tissue at levels ranging from 0.7 ng/mL for normal tissue to 50 ng/mL in tissue from active colitis.[39] Elevated intestinal levels of IL-1 messenger RNA have been detected during rotavirus infection of mice in a number of murine strains (R. Shaw, personal communication, December 1996). Basal levels of IFN-γ similar to those used in this study are readily detectable in noninflamed intestinal tissues.[40] Viral infection could induce increased intestinal levels of IFN-γ in vivo by natural killer cells or rotavirus-specific T cells that have been shown to secrete IFN-γ in the

presence of rotavirus antigen in vitro.[41,42] Rotavirus-specific CD8 T cells can mediate clearance of rotavirus from chronically infected immunodeficient mice; IFN-γ could be a component of that clearance.[6,7,43]

The true in vivo role of IFN and other cytokines in host defense against rotavirus remains to be clarified in ongoing experiments. The in vitro data of this study show that several cytokines are capable of strongly modifying the permissivity of enterocytes to viral infection. Cytokine modulation of enterocyte phenotype is probably important in maintaining intestinal integrity in the presence of a variety of gut luminal pathogens including viruses such as rotavirus.

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