IL-4 and IFN-γ Increase Steady State Levels of Polymeric Ig Receptor mRNA in Human Airway and Intestinal Epithelial Cells[1]

Laynez W. Ackermann, Laura A. Wollenweber, and Gerene M. Denning[2]

Delivery of IgA to the mucosal surface occurs via transcytosis of polymeric IgA (pIgA) across the epithelium, a process mediated by the pIgR. Several factors increase pIgR expression in human epithelial cells, including IL-4 and IFN-γ. Using an RNase protection assay, we found that IL-4 and IFN-γ increase steady state levels of pIgR mRNA in both human intestinal (HT29) and airway (Calu-3) epithelial cells. Time course studies in HT29 clone 19A cells showed that with each cytokine alone and with both together: 1) there was a significant lag before mRNA levels increased; 2) maximal levels were not reached until 48–72 h after the addition of cytokines; 3) mRNA levels remained elevated in the continued presence of cytokines; and 4) addition of actinomycin D or removal of cytokines led to decreases in mRNA levels with a half-life of ~20–28 h. Cytokine-dependent increases in steady state levels of pIgR mRNA were inhibited by cycloheximide and by protein tyrosine kinase inhibitors but not by inhibitors of protein kinase C or cAMP-dependent protein kinase A. Both IFN-γ and IL-4 increased expression of the inducible transcription factor IFN regulatory factor-1 (IRF-1), but levels of IRF-1 only weakly correlated with levels of pIgR mRNA, suggesting that additional transcription factors are required. These studies provide additional insights into the mechanisms by which cytokines regulate expression of the pIgR, a central player in mucosal immunity. *The Journal of Immunology,* **1999, 162: 5112–5118.**

Immunoglobulin A, the primary Ig in mucosal fluids, is secreted by plasma cells present in the lamina propria of the intestine, airway, and genitourinary tract (1). Following secretion, the polymeric form of IgA (pIgA)[3] is transported by its receptor (pIgR) across the epithelium to the mucosal surface. Transcytosis of the receptor is regulated both by phosphorylation (2, 3) and by pIgA binding (4, 5). Subsequent to transcytosis, proteolytic cleavage of the receptor at the mucosal surface releases pIgA that is covalently linked by disulfide bonds to a portion of the receptor called secretory component (SC). The covalent complex of pIgA and SC is commonly referred to as secretory IgA. In addition to its role in transport, SC increases pIgA's half-life by protecting it from proteolysis (6, 7).

Studies indicate that the pIgR can transport both dimeric and tetrameric IgA, as well as immune complexes that contain pIgA (8). Thus, the receptor may play a dual role: providing mucosal pIgA as a defense against lumenal pathogens, as well as clearing pIgA-containing immune complexes from the submucosal space.

Several immunomodulatory factors increase release of SC (i.e., expression of the pIgR) by the human intestinal epithelial cell line HT29. These factors include TGF-β (9), TNF-α (10, 11), IFN-γ (12–14), and IL-4 (13, 14). Which of these factors is present at a given site likely depends upon both the population of cells at that site and the physiological state (healthy vs diseased) of the mucosal tissue (15).

We and others demonstrate that IL-4 and IFN-γ individually increase cell-associated SC and intact receptor. Furthermore, IFN-γ increases receptor expression by increasing steady state levels of pIgR mRNA (16). This IFN-dependent increase in expression is accompanied by activation of the transcription factor IFN regulatory factor-1 (IRF-1) (17). Moreover, in high-affinity pIgA binding studies, IL-4 and IFN-γ are synergistic (14). This synergy is due in large part to a synergistic increase in cell-associated pIgR (13).

Because the pIgR plays a central role in mucosal immunity, our laboratory is interested in how IL-4 and IFN-γ regulate pIgR expression. To address this question, we designed studies to characterize the effect of these cytokines on pIgR mRNA expression in human epithelial cells.

Materials and Methods

Materials

Human recombinant IL-4 and IFN-γ were obtained from R&D Systems (Minneapolis, MN). Actinomycin D-mannitol was purchased from Sigma (St. Louis, MO). The kinase inhibitors genistein, KT5720, staurosporine, and bisindolylmaleimide were obtained from Calbiochem (La Jolla, CA), and the inhibitor tyrphostin 23 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Abs against IRF-1 and c-Jun were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively.

Cell culture

HT29 clone 19A (18, 19), HT29 HTB38 (ATCC HTB38), and Calu-3 (ATCC 55-HTB) cells are human epithelial cell lines isolated from adenocarcinomas of the colon and lung, respectively. All cell lines were cultured in DMEM:Ham's F12 (1:1) supplemented with 10% FBS, 2 mM glutamine, and 500 U/ml each of penicillin and streptomycin. Cells were subcultured at a 1:10 (HT29) or a 1:4 (Calu-3) dilution. Fresh medium was added every 2–3 days and experiments were done when cultures were 70–90% confluent. Cytokine-dependent expression of the pIgR was reduced >50% if cytokines were added in fresh serum-containing medium or

Infectious Diseases Research Laboratory, Veterans Affairs Medical Center, and Department of Internal Medicine, University of Iowa, Iowa City, IA 52242

Received for publication June 24, 1998. Accepted for publication February 8, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

[2] Address correspondence and reprint requests to Dr. Gerene M. Denning, Infectious Diseases Research Laboratory, Building 3, Room 139, Veterans Affairs Medical Center, Highway 6 West, Iowa City, IA 52246. E-mail address: gdenning@blue.weeg.uiowa.edu

[3] Abbreviations used in this paper: pIgA, polymeric IgA; IRF-1, IFN regulatory factor-1; SC, secretory component; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; PTK, protein tyrosine kinase; RPA, RNase protection assay.

0022-1767/99/$02.00

if supplemental serum was added. The basis for this inhibition is currently unknown. Thus, cytokines were routinely added directly to the serum-containing growth medium 1–2 days after it was placed on the cultures.

RNase protection assay (RPA)

An insert for generating probe was made using an RT-PCR procedure available in the SuperScript II kit (Life Technologies, Gaithersburg, MD). The procedure was performed according to the manufacturer's instructions, using the following primers: 5′-CCC AGG ACC CTG GCT GAC CTC CAG G-3′ and 5′-GGC TAC GTC TCC AGC AAA TAT GCA G-3′. The insert was gel purified and subcloned into the pCRII plasmid (Invitrogen, Carlsbad, CA).

To make biotinylated RNA probe, the plasmid was linearized by treating with *Bam*HI. Transcription was then performed according to the manufacturer's instructions using the Ambion (Austin, TX) BIOTINscript kit. Briefly, the linearized plasmid was combined with buffer, ribonuclease inhibitor, T7 polymerase, and a nucleotide mixture containing biotin-14-CTP. Samples were then incubated at 37°C for 2 h, and subsequently treated with DNase I. The probe was gel purified by running the final mixture on a 5% polyacrylamide/8 M urea gel (1000 V, 1 h). Biotinylated m.w. markers and probe for 28S rRNA were similarly made using templates purchased from Ambion.

Total cellular RNA was prepared by scraping the cells into TRI REAGENT (Molecular Research Center, Cincinnati, OH) and incubating for 5 min at room temperature. The samples were extracted with chloroform, and the upper aqueous phase was transferred to a fresh tube. The RNA was precipitated by adding isopropanol and centrifuging at 20,000 × *g* for 30 min at 4°C. The pellet was washed once with 75% ethanol and dissolved in diethylpyrocarbonate-treated water.

The RPA was performed according to the manufacturer's directions, using the Ambion HybSpeed RPA kit. Briefly, 20–80 μg of cellular RNA was combined with ~2 ng each of pIgR and 28S rRNA biotinylated probe; the 28S rRNA probe was diluted ~1:30 with nonbiotinylated probe. For controls, 50 μg of yeast RNA was combined with probe and processed with and without addition of RNase. Ammonium acetate and ethanol were added to precipitate the RNA. Samples were centrifuged in a microfuge at 15,000 × *g* for 15 min and resuspended in HybSpeed Hybridization buffer (Ambion). Hybridization with the probe was accomplished by heating the samples at 95°C for 3 min then at 68°C for 10 min. Following hybridization, unprotected RNA was digested by adding HybSpeed Digestion buffer and RNase A/T1 mixture provided by the kit, and incubating at 37°C for 30 min. For RPAs using >40 μg of total RNA, twice the recommended concentration of RNase A/T1 was used. The protected RNA was then precipitated and separated on a 5% polyacrylamide/8 M urea gel (1200 V, 1.5 h), along with biotinylated m.w. standards. Samples and controls were electroblotted onto BrightStar-Plus (Ambion) nylon membrane (200 mA, 1 h) and UV-cross-linked to the membrane. Biotinylated RNA species were detected according to the manufacturer's directions, using streptavidin-alkaline phosphatase and the Ambion CDP-Star assay kit. The apparent m.w. of the species were as follows: uncut pIgR, 309; cut pIgR, 194; uncut 28S rRNA, 176; cut 28S rRNA, 111.

Band intensities for the pIgR mRNA were determined from the autoradiographs using the AlphaImager 2000 Digital Imaging System (Alpha Innotech, San Leandro, CA). A calibration film was used to identify band intensities that were within the linear range of the x-ray film. Control experiments indicated that the band intensities for pIgR mRNA were roughly linear within the range of total RNA (20–80 μg) used in our studies (Fig. 1). The need to use high amounts of total RNA to detect pIgR mRNA resulted in internal controls (28S rRNA) that were beyond the linear range. For this reason, we were unable to determine absolute values expressed as the ratio of the band intensities for pIgR and 28S rRNA. The 28S rRNA band intensities were used as a visual confirmation of RNA recovery and gel loading. To combine data, relative ratios of band intensities for the pIgR mRNA bands within individual experiments were determined. For direct comparisons, samples were run in parallel under identical conditions.

mRNA turnover studies

To determine mRNA stability, cells were first treated with and without the indicated cytokine(s) for 72 h. For studies on the effect of cytokine removal, the cells were then washed, repleted with medium without cytokine(s), and incubated for increasing times before harvesting the RNA. Conversely, to study turnover of pIgR mRNA in the continued presence of cytokines, actinomycin D (1 μg/ml) was added to inhibit further transcription, and the cells were incubated for increasing times up to 24 h. Actinomycin D was cytotoxic at times >24 h and, hence, longer incubation times were not tested. At the end of the chase period, total RNA was isolated from the cells and pIgR mRNA levels were measured using RPA.

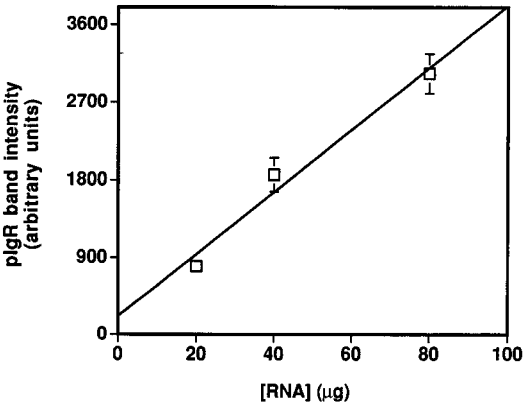

FIGURE 1. Band intensities for pIgR mRNA as a function of total RNA. HT29 cells (clone 19A and HTB38) were treated for 48 h with both IL-4 (10 ng/ml) and IFN-γ (200 U/ml) to stimulate maximal expression of pIgR mRNA. RNA was isolated, and total amounts of RNA (20–80 μg) were used in a nonradioactive RPA, as described in *Materials and Methods*. Band intensities in arbitrary units were determined using the AlphaImager 2000 Digital Imaging System. Data were combined from three independent experiments for each cell type and values represent the mean ± SEM.

Studies with protein biosynthesis and protein kinase inhibitors

For studies with cycloheximide, the inhibitor (2 mg/ml) was added 1 h before the addition of cytokines, and was removed after 5 h of cytokine treatment to avoid cytotoxic effects (16). For studies with protein kinase inhibitors, the indicated concentration of inhibitor was added for 1 h (24 h for tyrphostin 23) before addition of cytokines, and inhibitor was present throughout the subsequent incubations.

Western blot analysis of transcription factors

At the indicated time after the addition of cytokines, cells were washed with PBS, scraped into PBS, transferred to a microfuge tube, and pelleted by centrifuging at 2500 × *g* for 3 min. The cells were resuspended in PBS, an aliquot was taken to measure total cellular protein using the micro bicinchoninic acid assay (micro BCA; Pierce, Rockford, IL), and 5× SDS gel sample buffer was added to the remainder. Equal amounts of protein (250 μg) were separated on 7.5% SDS polyacrylamide gels, transferred to nitrocellulose, and subjected to Western blot analysis using the indicated Ab. Briefly, nonspecific binding was blocked by incubating the blots for 1 h at room temperature with nonfat dry milk (NFDM, 5% nonfat dry milk in PBS with 0.05% Tween 20). After each step the blots were washed four to five times with wash buffer (10 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20). The blots were sequentially incubated overnight at 4°C with polyclonal antitranscription factor Ab (1 μg/ml in NFDM) and for 1 h at room temperature with HRP-conjugated donkey anti-rabbit IgG (1:10,000 in NFDM; Amersham, Arlington Heights, IL). Ab binding was visualized using the Pierce SuperSignal reagent and autoradiography.

Results

Effect of IL-4 and IFN-γ on steady state levels of pIgR mRNA in HT29 cells

Previous studies by our laboratory using HT29 cells demonstrate that IL-4 and IFN-γ increase cell-associated pIgR and synergize at the level of pIgR protein (13). While earlier studies show that IFN-γ increases pIgR mRNA levels (16), no similar studies have been reported for IL-4. To determine whether the observed increases in pIgR protein are due to increased levels of mRNA, we measured steady state pIgR mRNA levels in control and cytokine-treated cells using a nonradioactive RPA. Levels of 28S rRNA were used as a measure of variability in sample recovery and gel loading.

Fig. 2*a* shows representative results using HT29 clone 19A cells treated for 48 h with and without individual cytokines or with both

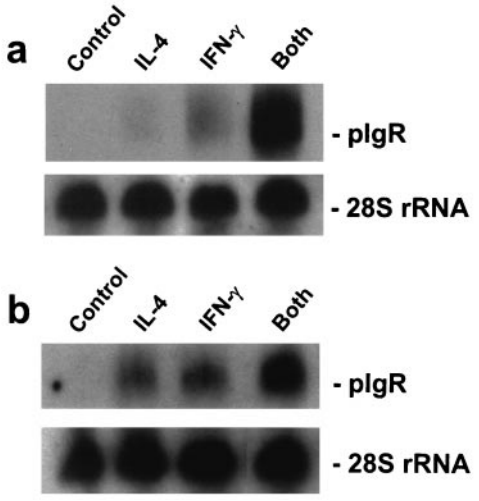

FIGURE 2. Cytokine-dependent increases in steady state levels of pIgR mRNA in HT29 cells. HT29 clone 19A (*a*) and HT29 HTB38 cells (*b*) were treated for 48 h without cytokines (Control), with 10 ng/ml IL-4, 200 U/ml IFN-γ, or both cytokines together (Both). Total RNA was isolated and pIgR mRNA was measured using RPA; results are representative for RPAs using 80 μg and 40 μg of total RNA for clone 19A and HTB38 cells, respectively. An internal control (28S rRNA) was used to verify sample recovery. Similar results were seen in two or more additional independent experiments.

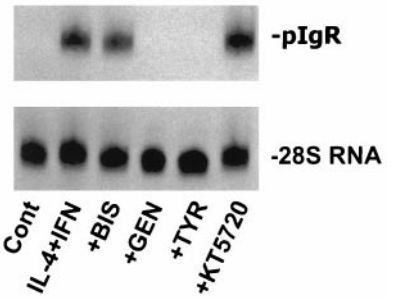

FIGURE 3. Effect of kinase inhibitors on cytokine-dependent increases in pIgR mRNA. HT29 clone 19A cells were pretreated for 1 h (24 h for tyrphostin) with and without the PKC inhibitor bisindolylmaleimide (300 nM, +BIS), the PKA inhibitor KT5720 (300 nM, +KT5720), or the PTK inhibitors genistein (300 μM, +GEN) and tyrphostin 23 (300 μM, +TYR). Cultures were then treated without (Control) and with IL-4 (10 ng/ml) and IFN-γ (200 U/ml) together for 24 h in the continued presence of inhibitor. At the end of the treatment period, total RNA was isolated, and pIgR mRNA and 28S rRNA (internal control) were determined using RPA. Similar results were seen in two other independent experiments.

cytokines together. Basal mRNA levels (Control) for these cells were consistently below the level of detection by this assay. Both IL-4 and IFN-γ individually increased mRNA levels in these cells and appeared to synergize when present together. Because of limitations in our ability to determine absolute values for pIgR mRNA, we compared the responses by arbitrarily defining the pIgR band intensity from IL-4-treated cells as 1.0. Combined data from ten independent experiments yielded values of 1.6 $\pm$ 0.3 and 12 $\pm$ 2.7 for cells treated with IFN-γ and with both cytokines together, respectively. These data suggest that, in a side-by-side comparison, IFN-γ and IL-4 potency are similar and that the two synergize. These results are consistent with results measuring pIgR protein (13) and pIgA binding (14). The observed synergy using maximal concentrations of cytokines suggests that IL-4 and IFN-γ increase pIgR expression through different signaling pathways.

When we tested an HT29 cell line recently obtained from American Type Culture Collection (ATCC HTB38; Manassas, VA) (Fig. 2*b*), we found that this cell line appeared to express higher levels of pIgR mRNA. Basal levels of pIgR mRNA were readily detectable in this cell line. Additionally, when we compared the pIgR band intensities for samples from HT29 HTB38 cells treated with IFN-γ alone and with both cytokines together with those for cells treated with IL-4 alone (band intensity defined as 1.0), we found ratios of 1.8 $\pm$ 0.37 and 2.1 $\pm$ 0.33, respectively (ten independent experiments). These data suggest that IL-4 and IFN-γ are somewhat less than additive in this cell type.

Finally, to verify these quantitative differences, we compared results from each cell line treated in parallel under identical conditions. Consistent with our results using individual cell lines, pIgR mRNA levels in clone 19A cells treated with individual cytokines were lower than those in HT29 HTB38 cells using 80 μg of total RNA and were undetectable at RNA concentrations ($<$40 μg) where signals using HTB38 cells were still observed (data not shown). In contrast, the pIgR mRNA band intensities for cells treated with both cytokines together were similar for the two cell lines in this RNA concentration range (20–80 μg): the ratio of clone 19A to HTB38 was 0.99 $\pm$ 0.12 (five independent experiments). This suggests that both cell lines can express similar maximal levels of pIgR mRNA but that individual cytokines are less potent in clone 19A cells. The reason for this difference in potency remains to be determined.

Effect of kinase inhibitors on cytokine-dependent increases in pIgR mRNA

Previous studies by our laboratory demonstrate that cytokine-dependent increases in cell-associated pIgR protein are prevented by inhibitors of protein tyrosine kinases (PTKs) but not by inhibitors of cAMP-dependent protein kinase A (PKA) or protein kinase C (PKC) (13). Because PTKs are also components of pathways that regulate gene expression posttranscriptionally (20) and because studies by our laboratory demonstrate that PTK inhibitors can differentially affect mRNA and protein levels (21), it was necessary to verify that the effect of these inhibitors on pIgR protein levels reflected effects on pIgR mRNA levels. To test this hypothesis, HT29 clone 19A cells were pretreated with inhibitor as described in *Materials and Methods* and then with cytokines in the presence and absence of inhibitor for an additional 24 h. At the end of the treatment period, mRNA levels were determined (Fig. 3). The tyrosine kinase inhibitors genistein and tyrphostin prevented the cytokine-dependent increase in pIgR mRNA. In contrast, the PKC inhibitor bisindolylmaleimide and the PKA inhibitor KT5720 had no effect. These data support the hypothesis that activation of PTKs regulates cytokine-dependent pIgR expression at the steady state level of pIgR mRNA.

Time course of cytokine-dependent increases in pIgR mRNA

We next examined the time dependence of pIgR mRNA induction by treating cells for increasing times with IL-4, IFN-γ, or both together. Because of variability from experiment to experiment, values were normalized by arbitrarily defining the maximum pIgR band intensity within each experiment as 100%. Combined data from three independent experiments (mean $\pm$ SEM) for each condition are shown in Fig. 4. Overall, a similar time dependence was observed, although maximal levels were observed at earlier times with IFN-γ alone (48 h) than with IL-4 alone or with both cytokines together (72 h). As previously reported for IFN-γ (16), there was a lag following addition of IL-4 or both cytokines together before mRNA levels began to rise. In some experiments, bands

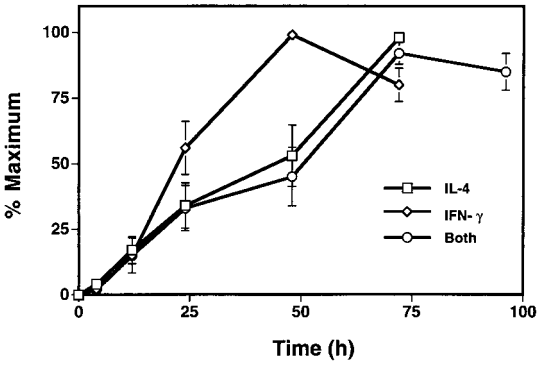

FIGURE 4. Time course studies of cytokine-dependent increases in pIgR mRNA. HT29 clone 19A cells were treated for the indicated time with IL-4 (10 ng/ml), IFN-γ (200 U/ml), or both cytokines together. At the end of each time period, total RNA was isolated and pIgR mRNA and 28S rRNA (internal control) were determined using RPA. Data are expressed as percent maximum, where the highest pIgR band intensity within a given experiment was defined as 100%. Values represent the mean ± SEM for combined data from three independent experiments for each condition.

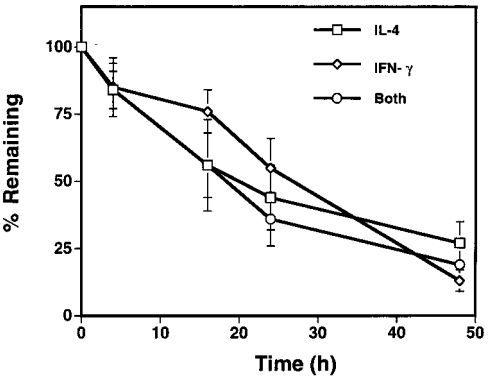

FIGURE 5. pIgR mRNA stability. HT29 clone 19A cells were treated with IL-4 (10 ng/ml), IFN-γ (200 U/ml), or both cytokines together for 72 h. Cytokines were then removed, the cells were washed, and cultures were incubated in complete medium for the indicated time. At the end of each incubation period, total RNA was isolated, and pIgR mRNA and 28S rRNA (internal control) were determined using RPA. Data are expressed as percent remaining, where the pIgR band intensity at 0 h of chase was defined as 100%. Values represent the mean ± SEM for combined data from three independent experiments for each condition.

were barely detectable at the 12 h time point. Additionally, mRNA levels remained elevated in the continued presence of cytokines; times greater than 96 h were not tested. This time course mirrors the one that we observed for pIgR protein levels in these cells (13). A similar lag before pIgR mRNA levels increased was observed with HT29 HTB38 cells (data not shown).

Turnover of pIgR mRNA

Because basal levels of pIgR mRNA were at or below our level of detection, we were unable to determine whether cytokines alter mRNA stability relative to unstimulated cells. We could, however, determine whether there were differences in mRNA stability between IL-4- and IFN-γ-treated cells and whether increases in mRNA stability might account for the synergy observed in clone 19A cells. To determine the effect of cytokines on pIgR mRNA stability, clone 19A cells were treated with cytokines for 72 h to achieve maximal steady state levels. Following cytokine treatment, two approaches were used. To determine the effect of cytokine removal, cells to be chased in the absence of cytokines were washed once with Hanks' balanced saline solution and repleted with complete medium. Conversely, to examine mRNA stability in the continued presence of cytokines, cells were chased in the presence of 1 μg/ml actinomycin D. To combine data from multiple experiments, we defined the band intensity for the pIgR mRNA at time zero in cytokine-treated cells as 100% and expressed the data at increasing times as % remaining. Results for combined data from three independent experiments (mean ± SEM) for each condition are shown in Fig. 5.

These results illustrate several points. First, pIgR mRNA is relatively long-lived under each of these conditions. In one experiment with both cytokines, we observed increased levels of pIgR mRNA relative to control 72 h after cytokine removal. In addition, the turnover rate was roughly similar whether cytokines were removed or actinomycin D was added (data not shown). This suggests that continued signaling by cytokines is required to maintain elevated levels of pIgR mRNA and that removal of cytokines may lead to a rapid loss of new transcription. These time course data also mirror our observations of pIgR protein levels. Finally, if effects on mRNA stability contribute to synergy, then the turnover rate of pIgR mRNA in cells treated with both cytokines together should be measureably less than in cells treated with each cytokine alone. Our observation that the turnover rate was approximately the same for individual cytokines (21–28 h) as for both together (19 h) suggests that the mechanism of synergy observed in HT29 clone 19A cells does not involve major differences in mRNA stability, although we can't rule out some contribution by this effect.

Effect of cycloheximide on increases in pIgR mRNA

Previous studies show that protein biosynthesis is required for IFN-dependent induction of pIgR mRNA (16). To determine whether this is the case for IL-4, we treated cells with cycloheximide as described in *Materials and Methods*, then with cytokines for 24 h. Fig. 6 shows that cycloheximide inhibits the cytokine-dependent increase in pIgR expression when either cytokine is present alone and when both are present together. These data indicate that protein biosynthesis is required for induction of pIgR mRNA by both IL-4 and IFN-γ. Similarly, cytokine-dependent increases in pIgR mRNA were inhibited by cycloheximide in HT29 HTB38 cells (data not shown). A requirement for protein biosynthesis is consistent with the lag that is observed in time course studies.

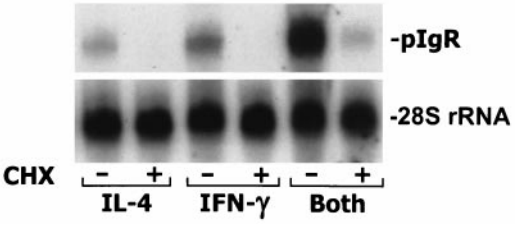

FIGURE 6. Effect of cycloheximide on cytokine-dependent increases in pIgR mRNA. HT29 clone 19A cells were pretreated with and without 2 mg/ml cycloheximide (CHX) for 1 h. The indicated cytokine(s) were then added: 10 ng/ml IL-4, 200 U/ml IFN-γ. Cycloheximide was removed after 6 h, and cultures were treated with and without cytokines for a total of 24 h. At the end of the treatment period, total RNA was isolated, and pIgR mRNA and 28S rRNA (internal control) were determined using RPA. Similar results were seen in two other independent experiments.

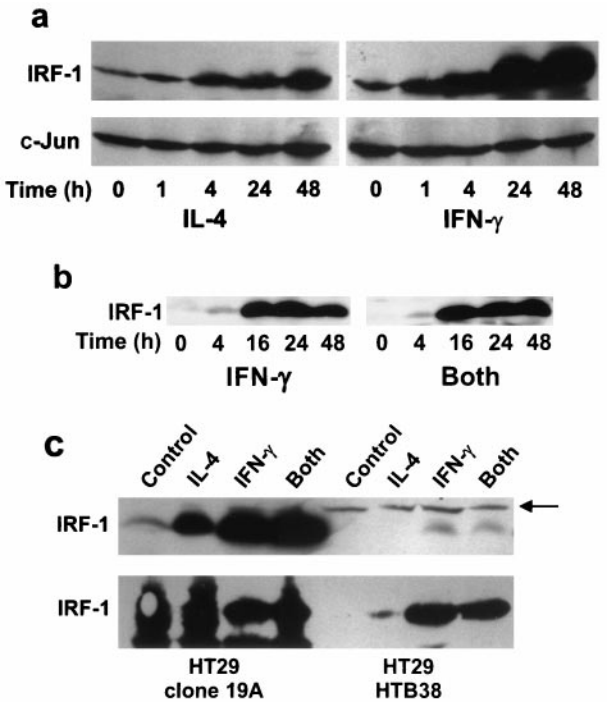

FIGURE 7. Effect of cytokines on expression of IRF-1 and c-Jun. *a*, HT29 clone 19A cells were treated with IL-4 (10 ng/ml) or IFN-γ (200 U/ml) for the indicated time. *b*, HT29 clone 19A cells were treated with IFN-γ or both cytokines together for the indicated time. *c*, HT29 clone 19A and HTB38 cells were treated in parallel without (Control) and with IL-4, IFN-γ, or both cytokines together for 24 h and processed in parallel under identical conditions. Arrow indicates a nonspecific band seen in some but not all blots. *a*, *b*, and *c*, (*upper panel*), Cells were harvested and whole cell extracts (250 μg total protein) were subjected to Western blot analysis using Abs against IRF-1, as described in *Materials and Methods*. *a*, Blots were reprobed with Ab against c-Jun. *c* (*lower panel*), Whole cell extracts (350 μg total protein) were subjected to Western blot analysis with anti-IRF-1 and exposed for longer times. Similar results in each case were seen in two other independent experiments.

Effect of cytokines on the inducible transcription factor IRF-1

Earlier studies report a lag before IFN-γ stimulates an increase in pIgR mRNA and that this increase is inhibited by cycloheximide (16). Our studies show similar characteristics in cells treated with IL-4 alone and in cells treated with IL-4 plus IFN-γ. One explanation for these observations is a requirement for biosynthesis (induction) of transcription factors. Consistent with this hypothesis are the studies by Piskurich et al. (17) who find that IFN-γ increases mRNA levels of the inducible transcription factor IRF-1 and increases its transcriptional activity. No studies to date have examined the effects of IL-4 on IRF-1 in any cell type.

To determine whether IL-4 induces IRF-1 in HT29 cells, whether induction is different in cells treated with both cytokines together compared with each cytokine alone, and to characterize the time course for any observed changes, we treated HT29 clone 19A and HTB38 cells with cytokine(s) for increasing times and subjected whole cell extracts to Western blot analysis using polyclonal anti-IRF-1. To facilitate direct comparisons, samples from each cell type were run in parallel under identical conditions. Representative results from these experiments are shown in Fig. 7.

For clone 19A cells (Fig. 7*a*, *upper panel*), we observed readily detectable basal levels of IRF-1 (0 h). Moreover, consistent with previous studies, IFN-γ caused a marked increase in IRF-1 levels. This increase was appreciable at 1 h, appeared to be maximal by 8 h, and remained elevated in the continued presence of cytokine. Times greater than 48 h were not tested. While less marked, IL-4 also increased IRF-1 levels in these cells with a time course similar to that of IFN-γ. In parallel cultures, results with both cytokines together mirrored those with IFN-γ alone (Fig. 7*b*). This observation suggests that the synergy observed between IFN-γ and IL-4 in these cells is not due to a marked change in the time course nor in the magnitude of IRF-1 expression. If cytokines were removed following induction of IRF-1, levels of the protein fell within 4 h of cytokine removal and were reduced to basal levels by 16 h (data not shown).

We speculated that higher levels of pIgR expression by HT29 HTB38 cells might reflect higher levels of IRF-1. Unexpectedly, we found that when run in parallel under identical conditions, clone 19A cells expressed considerably higher levels of IRF-1 (Fig. 7*c*) than did HTB38 cells. To detect IRF-1 levels in IL-4-treated HTB38 cells, both higher protein concentrations and longer exposure times were required (Fig. 7*c*, *lower panel*). This weak correlation between IRF-1 protein levels and pIgR mRNA levels suggests to us that while IRF-1 may contribute to pIgR expression, other factors may play an equal or greater role. These data also indicate that the lesser effect of IL-4 and IFN-γ on pIgR expression in HT29 clone 19A vs HTB38 cells is not a general characteristic of the cytokine response in these two cell lines.

Effect of cytokines on the inducible transcription factor c-Jun

While data support a role for IRF-1 in regulating pIgR expression (17), it is not clear whether IRF-1 induction can fully account for the lag observed before pIgR mRNA levels increase and/or for the effect of cycloheximide. For this reason, we continue to direct studies toward identifying the basis that underlies the requirement for protein biosynthesis and the lag that is observed.

Putative binding sites for the inducible transcription factor AP-1 are found in the 5′ flanking sequences of the pIgR gene (22). Whether these binding sites contribute to cytokine-dependent regulation of pIgR expression remains to be determined. Because one of the members of the AP-1 family, c-Jun, shows a delayed pattern of induction in some cell types (23), we hypothesized that c-Jun induction might contribute to the kinetics of the pIgR response and to the requirement for protein biosynthesis. To test this hypothesis, we reprobed our IRF-1 Western blots with anti-c-Jun Abs (Fig. 7*a*, *lower panel*). Detectable levels of c-Jun were observed in unstimulated cells (0 h). In contrast to IRF-1, however, neither cytokine alone nor both together (data not shown) increased levels of c-Jun protein over the time period of our study. These data suggest that IL-4 and IFN-γ do not influence expression of c-Jun in HT29 cells over these time periods. However, the data do not rule out a role for AP-1 in regulation of pIgR expression since phosphorylation of AP-1 family members that are already present could activate AP-1 transcriptional activity (23).

IL-4 and IFN-γ increase pIgR mRNA levels in human airway epithelial cells

Studies of cytokine-dependent regulation of pIgR expression have focused primarily on intestinal epithelial cells. Since the pIgR likely plays an important role in mucosal immunity in the airway, we wished to examine expression of the pIgR in airway epithelial cells. To do this, we determined the effect of cytokines on expression of pIgR mRNA using the human airway epithelial cell line Calu-3 (Fig. 8). Previous studies show that IFN-γ increases transcytosis of dimeric IgA by these cells (24). Results with Calu-3 cells were similar to those with HT29 HTB38 cells in that basal expression was easily detectable and band intensities were similar

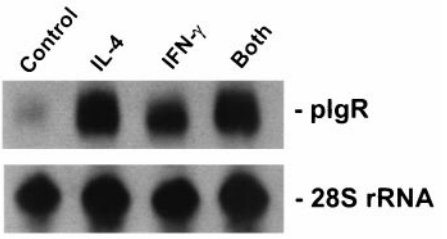

FIGURE 8. Effect of cytokines on steady state levels of pIgR mRNA in Calu-3 cells. Cells were treated for 48 h without (Control) and with IL-4 (10 ng/ml), IFN-γ (200 U/ml), or both cytokines together. Total RNA was isolated and pIgR mRNA and 28S rRNA (internal control) were determined using RPA (40 μg total RNA). Similar results were seen in two other independent experiments.

when samples from each cell type were run in parallel under identical conditions. Moreover, like in HT29 cells, cycloheximide blocked the cytokine-dependent increase in pIgR mRNA in Calu-3 cells (data not shown). Finally, IRF-1 protein levels in Calu-3 cells were increased by cytokines and, when run in parallel, were intermediate between HT29 clone 19A and HTB38 cell lines (data not shown). These data suggest that cytokine-dependent regulation is similar in airway and intestinal epithelial cells.

Discussion

Only a few studies have examined the molecular mechanisms that regulate basal (25) as well as cytokine-dependent pIgR mRNA expression in human epithelial cells (17, 26, 27). Our studies are the first to show that IL-4 increases pIgR mRNA levels in HT29 cells and that both IL-4 and IFN-γ increase pIgR mRNA expression in Calu-3 cells. Moreover, a close correlation between pIgR mRNA and protein levels under all of our experimental conditions suggests that the major mechanism for regulating pIgR expression is at the level of transcription and/or mRNA stability.

In addition, our results using HT29 cells indicate that regulation of pIgR expression by each cytokine alone and by both together share common features. First, similar time courses of induction were observed, including a lag before mRNA levels increase. This lag is consistent with the requirement for protein biosynthesis in each case. Next, a similar mRNA turnover rate was observed for each cytokine alone and for both together. These data suggest that neither cytokine has a unique effect on mRNA stability and that synergy occurs at the level of transcription rather than at the level of mRNA stability. Third, increases in pIgR mRNA in response to each cytokine can be blocked by PTK inhibitors, suggesting a role for PTKs in regulating pIgR transcription and/or mRNA stability. Finally, each cytokine can increase IRF-1 protein levels: IRF-1 is implicated in IFN-dependent regulation of pIgR expression (17). These common characteristics, however, should not obscure the fact that synergy/additivity suggests that there are differences between the mechanisms by which IL-4 and IFN-γ regulate pIgR expression in these cells. Other evidence for mechanistic differences are the observations that butyrate (26) and vitamin A (27) differentially affect the response to each cytokine.

It seems likely that biosynthesis of transcription factors accounts, at least in part, for the observed lag that precedes the increase in pIgR mRNA levels and for inhibition by cycloheximide. However, our results suggest that cytokine-dependent changes in IRF-1 expression may not fully account for these observations. This includes the observation that cycloheximide inhibits cytokine-dependent increases in pIgR mRNA in HT29 HTB38 cells, despite the relatively low levels of IRF-1. Similarly, induction of the AP-1 family member c-Jun cannot account for the lag, for the requirement for protein biosynthesis, nor for synergy, since we saw no change in the expression of c-Jun under the conditions of our study. Whether induction of other members of the AP-1 family accounts for the observed lag remains to be determined. In addition to induction of transcription factors, alternative mechanisms to explain the lag and the effect of cycloheximide must also be considered. Potential mechanisms include biosynthesis of secreted factors that regulate pIgR expression, increased expression of the receptors for IL-4 and/or IFN-γ, and biosynthesis of proteins that regulate transcription, mRNA stability, and/or translation of the pIgR.

PTK inhibitors prevent cytokine-dependent increases in pIgR mRNA. There are at least two general signaling pathways that could explain this effect. First, both IL-4 and IFN-γ activate signaling pathways that include members of the Janus family of tyrosine kinases (JAKs) (28, 29). JAKs, in turn, phosphorylate and activate members of the STAT family of transcription factors (30). The JAK-STAT pathway regulates the expression of numerous immunomodulatory factors (31). While cytokines can activate overlapping groups of JAK proteins, each cytokine activates specific STATs. It seems likely that the JAK-STAT pathway contributes to cytokine-dependent IRF-1 expression (32) and, thus, indirectly to pIgR expression. Additionally, while there are no reported STAT binding sites in the pIgR promoter (22), STATs may bind to sites in the promoter that were not identified based on known consensus sequences, may bind to other transcription factors that regulate pIgR expression, and/or may regulate pIgR transcription indirectly by modulating expression of other regulatory proteins.

A second major group of cellular signaling pathways are the mitogen-activated protein kinase (MAPK) pathways (33). There are three distinct MAPK pathways, those for extracellular signal-regulated kinase (ERK), c-Jun terminal kinase (JNK), and for p38; PTKs are early intermediates in each of these pathways. These pathways, in turn, have been shown to activate transcription factors for which there are putative binding sites in the pIgR promoter, specifically the transcription factors NF-κB (20) and AP-1 (34). Since transcription factors, in addition to IRF-1, are likely required for maximal expression of the pIgR, it seems reasonable to explore a possible role for NF-κB and AP-1 and for signaling pathways that regulate their activity.

With respect to the physiological relevance of our studies, it should be noted that there are several reported subpopulations of T cells in the intestine and that each of these subpopulations exhibits a different pattern of cytokine expression; intraepithelial lymphocytes secrete more IFN-γ than IL-4, while the opposite is true for lamina propria lymphocytes (15). Additionally, different types of challenge (bacterial, antigenic, viral, parasitic) can stimulate a different pattern of cytokine secretion. Finally, there are differences between the intestine and the airway in T cell subpopulations and responses (35). Thus, regulation of pIgR expression in vivo likely involves the coordinate action of numerous immunomodulatory factors, among them IL-4 and IFN-γ. The relative contribution of specific immunomodulatory factors to pIgR expression is likely determined by the mucosal tissue of interest, by the population of resident immune cells at a particular site, and by the physiological state (healthy vs diseased) of the tissue.

Understanding the complex regulation of this critically important protein will require additional studies both in vivo and in vitro. By examining the molecular mechanisms by which IL-4 and IFN-γ regulate pIgR expression using human airway and intestinal epithelial cell lines, our studies contribute toward our general understanding of pIg-mediated mucosal immunity.

References

1. Kimball, J. 1986. Immunoglobulin A. In *Introduction to Immunology*. Macmillan Publishing Company, New York. p. 216.
2. Casanova, J. E., P. P. Breitfeld, S. A. Ross, and K. E. Mostov. 1990. Phosphorylation of the polymeric immunoglobulin receptor is required for its efficient transcytosis. *Science 248:742.*
3. Luton, F., M. Cardone, M. Zhang, and K. Mostov. 1998. Role of tyrosine phosphorylation in ligand-induced regulation of transcytosis of the polymeric Ig receptor. *Mol. Biol. Cell 9:1787.*
4. Song, W., M. Bomsel, J. Casanova, J. P. Vaerman, and K. Mostov. 1994. Stimulation of transcytosis of the polymeric immunoglobulin receptor by dimeric IgA. *Proc. Natl. Acad. Sci. USA 91:163.*
5. Singer, K., and K. Mostov. 1998. Dimerization of the polymeric immunoglobulin receptor controls its transcytotic trafficking. *Mol. Biol. Cell 9:901.*
6. Mostov, K. E., and G. Blobel. 1983. Biosynthesis, processing, and function of secretory component. *Methods Enzymol. 98:458.*
7. Crottet, P., and B. Corthesy. 1998. Secretory component delays the conversion of secretory IgA into antigen-binding competent $F(ab')_2$: A possible implication for mucosal defense. *J. Immunol. 161:5445.*
8. Kaetzel, C. S., J. K. Robinson, and M. E. Lamm. 1994. Epithelial transcytosis of monomeric IgA and IgG cross-linked through antigen to polymeric IgA: a role for monomeric antibodies in the mucosal immune system. *J. Immunol. 152:72.*
9. McGee, D., W. Aicher, and J. Eldridge. 1991. Transforming growth factor-β enhances secretory component and major histocompatibility complex class I. *Cytokine 3:543.*
10. Kavale, D., D. Lovhaug, L. Sollid, and P. Brandtzaeg. 1988. Tumor necrosis factor-α up-regulates expression of secretory component, the epithelial receptor for polymeric Ig. *J. Immunol. 140:3086.*
11. Moro, I., T. Takahashi, T. Iwase, M. Asano, N. Takenouchi, S. Nishimura, I. Kudo, P. Krajci, P. Brandtzaeg, Z. Moldoveanu, and J. Mestecky. 1995. Expression of SC, IL-6 and TGF-β1 in epithelial cell lines. In *Advances in Mucosal Immunology*. J. Mestecky, ed. Plenum Press, New York, p. 175.
12. Sollid, L. M., D. Kvale, P. Brandtzaeg, G. Markussen, and E. Thorsby. 1987. Interferon-γ enhances expression of secretory component, the epithelial receptor for polymeric immunoglobulins. *J. Immunol. 138:4303.*
13. Denning, G. 1996. IL-4 and IFN-γ synergistically increase total polymeric IgA receptor levels in human intestinal epithelial cells. *J. Immunol. 156:4807.*
14. Phillips, J. O., M. P. Everson, Z. Moldoveanu, C. Lue, and J. Mestecky. 1990. Synergistic effect of IL-4 and IFN-γ on the expression of polymeric Ig receptor (secretory component) and IgA binding by human epithelial cells. *J. Immunol. 145:1740.*
15. Abreu-Martin, M., and S. Targan. 1996. Regulation of immune responses of the intestinal mucosa. *Crit. Rev. Immunol. 16:277.*
16. Piskurich, J. F., J. A. France, C. M. Tamer, C. A. Willmer, C. S. Kaetzel, and D. M. Kaetzel. 1993. Interferon-γ induces polymeric immunoglobulin receptor mRNA in human intestinal epithelial cells by a protein synthesis dependent mechanism. *Mol. Immunol. 30:413.*
17. Piskurich, J., K. Youngman, K. Phillips, P. Hempen, M. Blanchard, J. France, and C. Kaetzel. 1997. Transcriptional regulation of the human polymeric immunoglobulin receptor gene by interferon-γ. *Mol. Immunol. 34:75.*
18. Augeron, C., and C. L. Laboisse. 1984. Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. *Cancer Res. 44:3961.*
19. Denning, G. M., R. A. Clark, and M. J. Welsh. 1994. cAMP and inositol 1,4,5-trisphosphate increase Ca^{2+} in HT-29 cells by activating different Ca^{2+} influx pathways. *Am. J. Physiol.* 267:C776.
20. Schulze-Osthoff, K., D. Ferrari, K. Riehemann, and S. Wesselborg. 1997. Regulation of NF-κB activation by MAP kinase cascades. *Immunobiology 198:35.*
21. Denning, G., L. Wollenweber, M. Railsback, C. Cox, L. Stoll, and B. Britigan. 1998. *Pseudomonas pyocyanin* increases interleukin-8 expression by human airway epithelial cells. *Infect. Immun. 66:5777.*
22. Verrijdt, G., J. Swinnen, B. Peeters, G. Verhoeven, W. Rombauts, and F. Claessens. 1997. Characterization of the human secretory component gene promoter. *Biochim. Biophys. Acta 1350:147.*
23. Foletta, V., D. Segal, and D. Cohen. 1998. Transcriptional regulation in the immune system: all roads lead to AP-1. *J. Leukocyte Biol. 63:139.*
24. Loman, S., J. Radl, H. Jansen, T. Out, and R. Lutter. 1997. Vectorial transport of dimeric IgA by the Calu-3 human lung epithelial cell line: upregulation by IFN-γ. *Am. J. Physiol. 272:L951.*
25. Johansen, F.-E., B. Bosloven, P. Krajci, and P. Brandtzaeg. 1998. A composite DNA element in the promoter of the polymeric immunoglobulin receptor regulates its constitutive expression. *Eur. J. Immunol. 28:1161.*
26. Kvale, D., and P. Brandtzaeg. 1995. Butyrate differentially affects constitutive and cytokine-induced expression of HLA molecules, secretory component (SC), and ICAM-1 in a colonic epithelial cell line (HT29, clone m3). In *Advances in Mucosal Immunology* J. Mestecky, ed. Plenum Press, New York, p. 183.
27. Sarkar, J., N. Gangopadhyay, Z. Moldoveanu, J. Mestecky, and C. Stephensen. 1998. Vitamin A is required for regulation of polymeric immunoglobulin receptor (pIgR) expression by interleukin-4 and interferon-γ in a human intestinal epithelial cell line. *J. Nutr. 128:1063.*
28. Ihle, J. 1994. The Janus kinase family and signaling through members of the cytokine receptor superfamily. *Proc. Soc. Exp. Biol. Med. 206:268.*
29. Murata, T., P. Noguchi, and R. Puri. 1996. Receptors for interleukin (IL)-4 do not associate with the common γ chain, and IL-4 induces the phosphorylation of Jak2 tyrosine kinase in human colon carcinoma cells. *J. Biol. Chem. 270:30829.*
30. Ihle, J. 1996. STATs: Signal transducers and activators of transcription. *Cell 84:331.*
31. Ihle, J., B. Witthuhn, F. Quelle, K. Yamamoto, W. Thierfelder, B. Kreider, and O. Silvennoinen. 1994. Signaling by the cytokine receptor superfamily: JAKs and STATs. *Trends Biochem. Sci. 19:222.*
32. Sims, S., Y. Cha, M. Romine, P.-Q. Gao, K. Gottlieb, and A. Deisseroth. 1993. A novel interferon-inducible domain: Structural and functional analysis of the human interferon regulatory factor 1 gene promoter. *Mol. Cell. Biol. 13:690.*
33. Ahn, N. 1993. The MAP kinase cascade: discovery of a new signal transduction pathway. *Mol. Cell. Biochem. 127:201.*
34. Whitmarsh, A., and R. Davis. 1996. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J. Mol. Med. 74: 589.*
35. Jahnsen, F., I. Farstad, J. Aansen, and P. Branddtzaeg. 1998. Phenotypic distribution of T cells in human nasal mucosa differs from that in the gut. *Am. J. Respir. Cell Mol. Biol. 18:392.*