Intraepithelial lymphocytes coinduce nitric oxide synthase in intestinal epithelial cells

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Hoffman, Rosemary A. Intraepithelial lymphocytes coin-duce nitric oxide synthase in intestinal epithelial cells. Am J Physiol Gastron- test Liver Physiol 278: G886–G894, 2000.—The study of mucosal immunity has revealed that complex reciprocal interactions occur between intestinal intraepithe- lial lymphocytes (IEL) and intestinal epithelial cells (IEC). The present study focuses on the induction of inducible nitric oxide (NO) synthase in cocultures of freshly isolated rat IEL and the rat epithelial cell line IEC-18 after the addition of interleukin-1β (IL-1β), tumor necrosis factor-α, or lipopolysaccharide. When IEL and IEC were separated using Transwell chambers, NO synthesis was not induced, indicating that cell-cell contact was required. Culture of IEC-18 with IEL, even in the absence of inflammatory stimuli such as IL-1β, resulted in upregulation of class I and II antigens on IEC after 18 h, due to the interferon-γ (IFN-γ) that is constitutively produced by IEL. Addition of anti-IFN-γ antibody to the NO-producing cocultures resulted in inhibition of NO synthesis as well as the upregulation of class I and II antigen expression. These data indicate that IFN-γ production by IEL conditions IEC for the expression of other components of the inflammatory process.

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INTRAEPITHELIAL LYMPHOCYTES (IEL) and intestinal epithelial cells (IEC) display multiple reciprocal interactions because of their intimate association at various mucosal sites. One facet of this interaction is production of soluble mediators, and another aspect is cognate IEL-IEC interaction. IEL produce a wide variety of cytokines (see Ref. 1 for review), and investigators have shown that these cytokines modulate IEC function, including promotion of decreased barrier function and Cl− secretion mediated by interleukin (IL)-4 and interferon-γ (IFN-γ) (7) and modulation of epithelial cell restitution and IL-6 secretion by transforming growth factor-β (TGF-β) and IL-1β (12). It is also well documented that epithelial cells produce many cytokines (6), as well as a variety of chemokines (34), which can act as comitogens and chemotactic factors for lymphocytes. It is likely that cytokines produced by IEC influence IEL function in many ways that are yet to be defined. The proof of the importance of a fine balance in cytokine levels necessary for the maintenance of intestinal physiology is illustrated by the various intestinal pathologies described in cytokine-deficient mice such as IL-2 (24) and IL-10 (20) knockout mice.

Another aspect of IEL-IEC communication is via cell-cell contact, and several different cell surface molecules have been shown to mediate IEL-IEC interaction. Cepek et al. (5) have described a mechanism for adhesion of IEL to IEC via an integrin molecule on the T cell CD103 and the cadherin molecule on the epithelial cell. Another example of cognate interaction is the constitutive lytic activity displayed by CD8+ IEL (see Ref. 1 for review). Groh et al. (18) have identified the nonclassical major histocompatibility complex (MHC) class I chain-related gene A (MICA) and MICB as target molecules on IEC for the cytotoxic action of T cell receptor (TCR) γδ+ CD8+ IEL. Recently, the receptor for MICA, NKG2D, has been detected on natural killer cells as well as γδ TCR+ cells, indicating a role for these antigens in tumor surveillance (2, 32). Yio and Mayer (35) have described yet another cognate interaction mediated by the CD8 molecule on the IEL and the integrin-like molecule gp180 on the IEC. Recently, Yamamoto et al. (33) described the inhibitory effect of IEC membranes on anti-CD3-induced proliferation of IEL but not of peripheral T lymphocytes.

Because IEC constitutively express class I and II antigens, IEC also function as antigen-presenting cells (APC) in the classical interaction of the TCR on the T cell with either class I or II molecules on the APC. Recently, Nakazawa et al. (21) have shown that human IEC can express low levels of the costimulatory molecule CD86 on appropriately stimulated IEC, indicating that IEC could also invoke a primary immune response. Intestinal epithelial cells have also been shown to express nonclassical MHC molecules. Because these molecules display much less polymorphism than classical histocompatibility molecules and have a somewhat restricted tissue distribution, the T cells that are resident in epithelial sites may interact with these nonpolymorphic molecules (see Ref. 3 for review).

A major role of the intestinal mucosa is to provide a barrier against invasion of the host by pathogens present in the intestinal lumen. The production of nitric oxide (NO) by epithelial cells might function as a host defense mechanism because NO displays microbiocidal properties (11). Thus the in vivo finding that inducible NO synthase (iNOS) is upregulated in the intestine after administration of an inflammatory agent such as lipopolysaccharide (LPS) (9, 25) is in accord with a host defense mechanism initiated by the many cytokines that are produced by an inflammatory stimu-
lus such as invasion by a pathogen. Furthermore, Witthoft et al. (31) have shown that enteroinvasive bacteria directly induce NO production in the human colon epithelial cell lines Caco-2 and HT-29. This laboratory (19) has recently shown that iNOS is constitutively present in the epithelial cells of the ileal mucosa of untreated rats and mice, and that SCID mice, devoid of mature T cells, express decreased levels of constitutive ileal iNOS. This finding led to the investigation of the role of IEL in iNOS expression in intestinal epithelial cells. The current study finds that in the presence of an inflammatory stimulus, IEL influence iNOS expression in epithelial cells through a mechanism that utilizes both soluble and cell-cell interaction.

MATERIALS AND METHODS

Animals. Sprague-Dawley, Lewis, and ACI male rats, weighing 150–174 g, were purchased from Harlan Sprague Dawley (Indianapolis, IN) and used for experimentation between 4 and 6 mo of age. Animals were housed in a specific pathogen-free animal facility and provided rodent chow and acidified water ad libitum.

Antibodies. For fluorescence-activated cell sorting (FACS) analysis, αβTCR-phycocerythrin (PE)(R73), γδTCR-FITC(V65), CD3-FITC(G4.18), CD4-FITC and PE(OX-35), CD8-FITC and PE(OX-8), CD45-FITC(OX-1), RT1A-FITC(OX-18), RT1B-FITC(OX-6), and CD54-FITC(EA29) were purchased from Pharmingen (San Diego, CA). For blocking studies, unlabeled FITC(OX-6), and CD54-FITC(1A29) were purchased from Becton Dickinson (Mountain View, CA). CD4, CD8, RT1A, RT1B, CD54, and CD11a antibodies were utilized (Biosource International, Camarillo, CA). Polyclonal anti-human IL-15 (Biosource International) was used as a control antibody for the polyclonal IFN-γ antibody.

Preparation of intraintestinal lymphocytes. Small IEL were harvested according to established procedures (8). The small intestine was flushed to remove luminal contents, the Peyer’s patches were removed, and the intestine was cut longitudinally and into 1-cm pieces that were stirred for 30 min at 37°C in 1 ml of thioerythritol-10% FCS in Ca2+- and Mg2+-free PBS. The cell population was filtered over a nylon wool column, and the lymphocytes were recovered from the interface of a 44%/67% Percoll gradient. The recovered cell population contained 75–85% cells that displayed the forward by side scatter properties characteristic of lymphocytes, and 95% of this lymphoid population was CD45+, indicating cells of hematopoietic origin (see Table 1 for further phenotypic analysis). For positive and negative sorting, the cells were stained with PE-labeled antibodies and sorted using a Becton Dickinson FACStar Plus. Medium used for cocultures was DMEM supplemented with 5% FCS, 1 mM L-glutamine, 0.1 U/ml insulin. DMEM supplemented with 5% FCS, 1 mM L-glutamine, and 0.1 U/ml insulin. New culture by weekly passage in DMEM (4.5 g/l glucose) containing 5% FCS, 1 mM L-glutamine, and 0.1 U/ml insulin. New culture by weekly passage in DMEM (4.5 g/l glucose) containing 5% FCS, 1 mM L-glutamine, and 0.1 U/ml insulin. New culture by weekly passage in DMEM (4.5 g/l glucose) containing 5% FCS, 1 mM L-glutamine, and 0.1 U/ml insulin.

Table 1. Phenotypic analysis of Sprague-Dawley rat small intestinal IEL

<table>
<thead>
<tr>
<th>Antigen</th>
<th>% Single Positive</th>
<th>% Double Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>84 ± 3</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>35 ± 5</td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>77 ± 1</td>
<td></td>
</tr>
<tr>
<td>CD4, γδTCR</td>
<td>79 ± 2</td>
<td></td>
</tr>
<tr>
<td>γδTCR</td>
<td>7 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

IEL-18, and 100-mm petri dishes contain 10–15 × 10^6 IEC-18. Various concentrations of recombinant human IL-1β (Du Pont De Nemours, distributed by NCI), recombinant murine tumor necrosis factor-α (TNF-α) (Genzyme, Cambridge, MA), recombinant rat IFN-γ (GIBCO BRL, Gaithersburg, MD), or LPS (Escherichia coli 011B4, Sigma Chemical, St. Louis, MO) were added, and supernatant NO2 levels were determined on various days after initiation of coculture. Where indicated, the monolayers of IEC-18 were trypsinized and surface markers were assayed by staining with FITC-labeled antibodies and examined by FACS. For selected experiments, IEL were separated from IEC-18 using Transwell culture wells (Corning Costar, Cambridge, MA) with a 0.4-µM pore size membrane.

Preparation of peritoneal macrophage cultures. The peritoneal cavity of the rat was lavaged with 5% FCS-MEM. The cells were washed once, counted, and plated at 0.25, 1, and 2.0 × 10^6 peritoneal cells/well. After a 4-h incubation at 37°C, the monolayers were washed vigorously to remove nonadherent cells and used for experimentation.

Supernatant NO2 assay. A microplate assay was utilized, combining 0.05 ml of culture supernatant and 0.05 ml of NaNO2 (Sigma Chemical) was used as the standard, and samples were analyzed with a maximum velocity microplate reader. NO2 was a stable product in culture supernatants, and values are an indication of total amount of NO synthesized during the culture period. Supernatant NO2 levels were assayed at various times after initiation of culture, depending on whether the initiation of NO synthesis or total amount of NO synthesized was being determined in a particular experiment.

Western blot. Cell lysates were prepared by three rapid freeze-thaw cycles and centrifuged. The supernatant protein was quantitated using the Pierce bicinchoninic acid kit. Forty micrograms of protein were loaded per lane of a 8% polyacrylamide gel. The gel was transferred to a nitrocellulose membrane, incubated with rabbit polyclonal anti-MAC iNOS antibody (Transduction Laboratories, Lexington, KY), and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG.

RESULTS

Induction of NO synthesis by cytokines. To ascertain which stimuli or combinations of stimuli, added exog-
enously, induced NO synthesis in IEC-18, 25 ng/ml IFN-γ, 100 pg/ml TNF-α, 5 ng/ml IL-1β, and 10 µg/ml LPS were added singly or in combination to microtiter wells containing confluent monolayers of IEC-18. Supernatant NO2 levels from 48-h cultures are depicted in Fig. 1. Under the influence of any single stimulus, NO synthesis was not induced. The combination of IL-1β and IFN-γ resulted in induction of modest amounts of NO, whereas other double combinations of the stimuli were not effective. Under the influence of several combinations of triple stimuli, NO synthesis was variably induced, with the combination of IL-1β, TNF-α,

![Fig. 1. Induction of nitric oxide (NO) synthesis in intestinal epithelial cells (IEC-18) by various inflammatory stimuli.](http://apgp.physiology.org/)

and IFN-γ being the most effective and the combination of TNF-α, LPS, and IL-1β the least effective.

Induction of NO synthesis in IEL-IEC coculture. Various numbers of freshly isolated IEL were added to confluent monolayers of IEC-18 in microtiter wells in the presence of 5 ng/ml IL-1β. A constant number of IEL, splenocytes (spleen), or mesenteric lymph node lymphocytes (MLN) (2 × 10⁶) were added to confluent monolayers of IEC-18 in microtiter wells in the presence of 0.05, 0.5, and 5 ng/ml IL-1β. B, inset: NO2 levels in cocultures of hepatocytes and IEL plus various concentrations of IL-1β. Supernatant NO2 levels were assayed on day 3 of culture and are depicted as means ± SD of triplicate wells. Data are representative of 5 independent experiments.

![Fig. 2. Induction of NO synthesis in intraepithelial lymphocyte (IEL)-IEC-18 coculture.](http://apgp.physiology.org/)

A: various numbers of freshly isolated rat IEL (0.125, 0.25, 0.5, and 1.0 × 10⁶) were added to confluent monolayers of IEC-18 in microtiter wells in the presence of 5 ng/ml IL-1β. B: a constant number of IEL, splenocytes (spleen), or mesenteric lymph node lymphocytes (MLN) (2 × 10⁶) were added to confluent monolayers of IEC-18 in microtiter wells in the presence of 0.05, 0.5, and 5 ng/ml IL-1β. B, inset: NO2 levels in cocultures of hepatocytes and IEL plus various concentrations of IL-1β. Supernatant NO2 levels were assayed on day 3 of culture and are depicted as means ± SD of triplicate wells. Data are representative of 5 independent experiments.

![Graph](http://apgp.physiology.org/)

With IL1β

Without IL1β

![Graph](http://apgp.physiology.org/)

IL18 (ng/ml)

IEL

SPLEEN

MLN

Supernatant NO2 levels were assayed on day 3 of culture and are depicted as means ± SD of triplicate wells. Data are representative of 5 independent experiments.
for IEL in the cocultures, NO was not induced, indicating that a specific interaction between IEL and IEC was necessary for induction of NO synthesis. Similarly, when the IEL population was cocultured with hepatocytes freshly harvested from Sprague-Dawley rats, NO synthesis was not induced (Fig. 2B, inset), even though stimulation with a cytokine mixture of IFN-γ, LPS, IL-1β, and TNF-α did result in NO synthesis by the hepatocytes (59.0 µM NO₂, data not shown). These data indicate that IEL, but not mesenteric lymph node lymphocytes or splenocytes, coinduce NO synthesis in IEC and that another epithelial cell such as the hepatocyte does not serve as a target NO-producing cell when cocultured with IEL.

IEL coinduction of NO synthesis in peritoneal macrophages. Because the IEC-18 cell line was derived from an outbred rat (23) and the Sprague-Dawley rats used as a source of IEL in these experiments are also outbred, it was important to confirm that IEL could induce NO synthesis in a completely syngeneic system. Therefore, various concentrations of peritoneal macrophages from a Lewis rat were allowed to adhere to culture dishes for 3–4 h and washed free of nonadherent cells, and freshly harvested Lewis IEL were added to these cultures. As can be seen in Fig. 3, addition of IEL resulted in enhanced supernatant NO₂ levels. Because IEL have been shown to constitutively produce IFN-γ and peritoneal macrophages have been shown to produce NO in response to IFN-γ, NO synthesis by peritoneal macrophages in the presence of anti-IFN-γ antibody was examined. A modest decrease (from 33 to 22 µM NO₂) in NO synthesis was observed in cultures where 50 µg/ml of antibody were added, indicating that some of the NO synthesis in the cocultures could be attributed to IFN-γ (Fig. 3, inset). Addition of 125 ng/ml of IFN-γ to peritoneal macrophage culture resulted in 13.0 µM NO₂ in the supernatant (data not shown), illustrating that peritoneal macrophages synthesized less NO in response to addition of exogenous IFN-γ than when cocultured with IEL.

Influence of other inflammatory stimuli on coinduction of NO synthesis in IEL-IEC cultures. To determine whether stimuli other than IL-1β resulted in coinduction of NO synthesis, IEL-IEC-18 cocultures were established in the presence of various concentrations of TNF-α, LPS, or IFN-γ. As can be seen in Fig. 4, at concentrations of 100 pg/ml TNF-α and 10 µg/ml LPS, NO synthesis was also induced. However, in the presence of IFN-γ, even at high concentrations such as 125 ng/ml, NO synthesis was not induced. This experiment revealed that IFN-γ alone was an insufficient stimulus for induction of NO synthesis in IEC-18.

Western blot for iNOS protein. To ascertain that iNOS protein was present in the induced cultures, IEC-18 monolayers were established in 100-mm petri dishes and stimulated with 5 ng/ml IL-1b and/or 25 ng/ml IFN-γ or by the addition of 2 x 10⁵ IEL in the presence of IL-1b and/or IFN-γ. On day 2 of culture, supernatant NO₂ levels were determined, lysates of the cultured cells were prepared, and a Western blot was performed (Fig. 5). It is clear that iNOS protein was not present in IEC-18 control cell cultures or in cultures that were treated with IL-1β, IFN-γ, IEL, or IEL plus IFN-γ. Supernatant NO₂ levels of 1.3 µM or less were measured in these cultures. When IEC-18 was stimulated with a combination of IL-1β and IFN-γ, an NO₂ level of 10.1 µM was measured and a faint band at 130 kDa was present. However, when IEC-18 was cultured with IEL and IL-1β, a band migrating at 130 kDa was detected along with enhanced supernatant NO₂ levels (43.0 µM). Therefore, the amount of iNOS protein present in cocultures of IEL and IEC-18 plus IL-1β correlated well with the supernatant NO₂ levels.

Effect of separation of IEL from IEC-18 on NO synthesis. To determine whether cell-cell interaction was required for coinduction of NO synthesis, IEL were added to the upper compartment of a Transwell culture system that was inserted into a 22-mm well containing a confluent IEC-18 monolayer. IL-1β (5 ng/ml) was added to the culture system, and supernatant NO₂ was determined on day 2 of culture. As can be seen in Table 2, NO was definitely not induced in cultures where IEL were separated from IEC-18, even though IL-1β came in contact with both cell types. This result clearly demonstrated that cell contact between the IEL and IEC was required for NO synthesis to ensue.

Because IEL have been shown to constitutively express IFN-γ, we measured the level of this cytokine in the culture supernatants and found picogram levels of IFN-γ, regardless of whether the IEL were in contact with or separated from the IEC-18. Similarly, class I expression was upregulated on IEC-18 obtained from cultures with exogenously added IFN-γ as well as IEC-18 cocultured with IEL, regardless of whether cell-cell contact between IEC-18 and IEL was established. Uptregulation of class II antigen was also observed, although higher levels of class II were seen in
those cultures where cell-cell contact between IEL and IEC-18 was established, especially in the presence of IL-1β. Treatment of IEC-18 with IL-1β alone does not result in increased expression of class I or II on IEC-18 (unpublished observation). This experiment separated the upregulation of class I and, to a lesser extent, class II on IEC-18, which did not require cell-cell contact, from the NO synthesis that was not observed unless IEC-18-IEL contact was established.

To rule out the possibility that a soluble factor was present in a culture system containing IEL, IEC-18, and IL-1β that was not produced when IEL and IEC-18 were separated from each other by a membrane, supernatants were transferred from cultures where NO synthesis had been induced to naive IEC-18 cultures. NO synthesis was monitored in the secondary culture and an increase in supernatant NO2 was not observed, indicating that a soluble mediator capable of inducing NO synthesis was not produced in the cultures where NO was coinduced by IEL (data not shown).

Determination of which IEL subpopulation is responsible for inducing NO synthesis. The IEL population is a complex mixture of CD3+ IEL that express either CD4 or CD8 (Table 1). To determine whether a subpopulation of IEL was responsible for iNOS induction, IEL

![Fig. 4. Effect of other inflammatory stimuli on induction of NO synthesis in IEL-IEC-18 cultures. Freshly harvested rat IEL (2 x 10^6) were added to confluent monolayers of IEC-18 in microtiter wells in the presence of 10-fold dilutions of TNF-α (A), LPS (B), or IFN-γ (C). Supernatant NO2 levels were assayed on day 2 of culture and are expressed as means ± SD of triplicate wells. Gray bars, NO2 levels in presence of IEL. Black bars, levels in absence of IEL. Data are representative of 3 individual experiments.]

![Fig. 5. Western blot for inducible NO synthase protein. Confluent monolayers of IEC-18 were established in 100-mm petri dishes. Cultures were stimulated with either 5 ng/ml IL-1β or 25 ng/ml IFN-γ or both in the presence and absence of 2 x 10^7 freshly harvested IEL in a final culture volume of 10 ml of complete medium. On day 2 of culture, supernatant NO2 levels were assayed, lysates of the cultured cells were prepared, and a Western blot was performed. Lysates prepared from RAW 264.7 cells stimulated with LPS served as the positive control, and lysates from the EL4 lymphoma cell line served as the negative control.

**Table 2. Characterization of IEL-IEC-18 cocultures using the Transwell culture system**

<table>
<thead>
<tr>
<th>Below Filter</th>
<th>Above Filter</th>
<th>IL-1β</th>
<th>NO2, µM</th>
<th>IFN-γ, pg/ml</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEC-18</td>
<td></td>
<td></td>
<td>0.6 ± 0.2</td>
<td>ND</td>
<td>3.2</td>
</tr>
<tr>
<td>IEC-18 + IFN-γ</td>
<td></td>
<td></td>
<td>1.2 ± 0.3</td>
<td>300 ± 15</td>
<td>20.4</td>
</tr>
<tr>
<td>IEC-18 + IEL</td>
<td></td>
<td></td>
<td>0.9 ± 0.2</td>
<td>300 ± 15</td>
<td>20.4</td>
</tr>
<tr>
<td>IEC-18</td>
<td>IEL</td>
<td></td>
<td>1.4 ± 0.2</td>
<td>250 ± 50</td>
<td>16.7</td>
</tr>
<tr>
<td>IEC-18 + IEL</td>
<td>Yes</td>
<td></td>
<td>30.4 ± 2.3</td>
<td>350 ± 15</td>
<td>17.2</td>
</tr>
<tr>
<td>IEC-18</td>
<td>IEL</td>
<td>Yes</td>
<td>0.9 ± 0.1</td>
<td>250 ± 17</td>
<td>19.3</td>
</tr>
</tbody>
</table>

Values are means ± SD of 3 separate experiments. Intestinal epithelial cells (IEC-18) were seeded in 22-mm tissue culture wells and allowed to grow to confluence. Costar Transwell culture inserts were put in place, and 2 x 10^6 freshly harvested IEL were added either directly to the IEC-18 monolayer or to the Transwell insert. Interleukin-1β (IL-1β) at 5 ng/ml or interferon-γ (IFN-γ) at 25 ng/ml was added to the indicated cultures. On day 2 of culture, supernatants were harvested for subsequent analysis of NO2 and IFN-γ levels. IEC-18 were trypsinized and stained for class I and II antigens. MFI, median fluorescence intensity; ND, not detected.
were treated with CD4-PE antibody and sorted by flow cytometry techniques. The sorted populations were then added to IEC-18 cultures in the presence of IL-1β and supernatant NO2 assessed on day 4. As depicted in Fig. 6, depletion of CD4+ IEL resulted in almost complete abrogation of NO synthesis. Addition of positively sorted CD4+ IEL resulted in induction of NO synthesis at cell concentrations that were ineffective with an unseparated population of IEL. Clearly, these results showed that the CD4+ IEL subpopulation was capable of coinducing NO synthesis.

Effect of IFN-γ antibody on IEL-mediated coinduction of NO synthesis and class I and II expression. Because IFN-γ mediated upregulation of class II molecules on IEC-18 and the CD4+ subpopulation of IEL was an effective coinducer of iNOS in IEC-18, blocking of class II upregulation may possibly prevent NO synthesis in this coculture system. Therefore, confluent monolayers of IEC-18 were established in 22-mm culture wells and 2 x 10^6 IEL were added, along with 5 ng/ml IL-1β, in the presence of various concentrations of polyclonal IFN-γ antibody. Cohort cultures received the control antibody, polyclonal anti-human IL-15. On day 2 of culture, supernatant NO2 levels were determined, cells were trypsinized, and expression of class I and II antigens on IEC-18 was determined by FACS analysis (expressed as median fluorescence intensity, MFI). As depicted in Fig. 7A, addition of IL-15 antibody had little effect on class I and II expression, whereas addition of IFN-γ antibody (Fig. 7B) resulted in ~50% reduction in class I MFI and 80% reduction in class II MFI. As seen in Fig. 7B, inset, anti-IL-15 did not inhibit NO synthesis, whereas anti-IFN-γ completely inhibited NO synthesis. These results suggest that IFN-γ plays a crucial role in the coinduction of NO synthesis by IEL.

Effect of class I and II blocking antibodies on coinduction of NO synthesis by IEL and mixed lymphocyte reaction. To determine whether blocking of class II antigen with antibody would prevent induction of NO synthesis in this culture system, various concentrations of either anti-IL-15 or -IFN-γ antibody. On day 2 of culture, supernatant NO2 levels were assayed, and cell monolayers were trypsinized and stained for class I (filled bars) and II (open bars) antigen expression. A and B: median fluorescence intensity (MFI) of antigen expression. B, inset: supernatant NO2 levels are depicted. MFI levels of antibody isotype control were always <2 on a log scale. Data are representative of 3 separate experiments. Class I and II MFI with 12.5 µg/ml anti-IL-15 vs. anti-IFN-γ were significantly different (P < 0.01) by unpaired Student’s t-test.

Fig. 6. Effect of CD4+ and CD4-depleted IEL on coinduction of NO synthesis. Freshly harvested rat IEL were treated with phycoerythrin-labeled CD4 antibody and sorted. CD4-depleted and CD4-positively sorted IEL were added to confluent monolayers of IEC-18 in the presence and absence of 5 ng/ml IL-1β. Control IEL consisted of unmanipulated IEL population. Supernatant NO2 levels were determined on day 4 of culture and are represented as means ± SD of triplicate determinations. Data are representative of 4 individual experiments.

Fig. 7. Effect of IFN-γ antibody on NO synthesis and class I and II expression. Confluent monolayers of IEC-18 were established in 22-mm tissue culture wells, and 2 x 10^6 IEL were added in the presence of 5 ng/ml IL-1β and various concentrations of either anti-IL-15 or -IFN-γ antibody. On day 2 of culture, supernatant NO2 levels were assayed, and cell monolayers were trypsinized and stained for class I (filled bars) and II (open bars) antigen expression. A and B: median fluorescence intensity (MFI) of antigen expression. B, inset: supernatant NO2 levels are depicted. MFI levels of antibody isotype control were always <2 on a log scale. Data are representative of 3 separate experiments. Class I and II MFI with 12.5 µg/ml anti-IL-15 vs. anti-IFN-γ were significantly different (P < 0.01) by unpaired Student’s t-test.
Table 3. Comparison of blocking effect of class I and II antibodies on IEL coinduced NO synthesis and MLR

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IEC-18+IEL+IL-1β</th>
<th>Lewis×ACI</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>NO2, µM</td>
<td>([3H]Thd, cpm/10⁵)</td>
</tr>
<tr>
<td>None</td>
<td>21.0±2.0</td>
<td>17.9±3.2</td>
</tr>
<tr>
<td>Class I, µg/ml</td>
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<td></td>
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<tr>
<td>0.1</td>
<td>16.9±0.8</td>
<td>12.1±1.3</td>
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<tr>
<td>1.0</td>
<td>23.5±2.0</td>
<td>4.5±0.9</td>
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<tr>
<td>10.0</td>
<td>20.1±2.6</td>
<td>5.5±2.0</td>
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<tr>
<td>Class II, µg/ml</td>
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<tr>
<td>0.1</td>
<td>22.6±1.5</td>
<td>1.3±0.2</td>
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<tr>
<td>1.0</td>
<td>22.8±0.6</td>
<td>1.1±0.4</td>
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<tr>
<td>10.0</td>
<td>22.1±0.3</td>
<td>1.4±0.5</td>
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</table>

Values are means ± SD of 3 separate experiments. Various concentrations of class I and class II antibodies were added to flat-bottomed microtiter wells containing confluent monolayers of IEC-18 and 2x10⁶ IEL in the presence of 5 ng/ml IL-1β. On day 2 of culture, supernatant NO2 levels were assayed. The same concentrations of antibodies were added to round-bottom microtiter wells containing 2x10⁶ Lewis rat lymph node lymphocytes and irradiated ACI lymph node lymphocytes. ([3H]Thymidine ([3H]TdR) uptake was measured on day 4 of culture. cpm, Counts per minute.

DISCUSSION

Previous study has shown that iNOS is upregulated in various inflammatory bowel diseases including colitis and Crohn’s disease (26) as well as in animal models of colitis (13), although the exact mechanism of induction is unknown. It is assumed that NO synthesis is triggered by exposure of the epithelial cell to the multiple inflammatory cytokines characteristic of these disease processes, much like that observed in vitro on stimulation of various cell types with cytokines. The current study provides evidence that IEL can coinduce iNOS in epithelial cells in the presence of a single added inflammatory cytokine, indicating that induction of iNOS may occur under more subtle inflammatory conditions as well as under the influence of multiple inflammatory cytokines. Because we have also observed that IEL can coinduce NO synthesis in peritoneal macrophages, iNOS coinduction by CD4⁺-activated lymphocytes may be relevant in inflammatory conditions at various sites, in addition to the intestinal epithelial layer.

Although much effort has been focused on the functions of CD8⁺ IEL, including their constitutive lytic function, proliferation in response to gp180 on the epithelial cell (35) and production of keratinocyte growth factor by the TCR γδ subset (4), relatively little is known about the functions of the CD4⁺ IEL. However, attention has been focused on the CD4⁺ T cell by experiments that describe a role for CD4⁺ memory Th1 cells in the mediation of intestinal inflammatory disease. It has been demonstrated that the CD4⁺, CD45RB⁺, Th1-like subpopulation of lymphocytes obtained from lymph nodes causes wasting disease and hyperplasia of the colonic mucosa when injected into SCID mice. In contrast, the CD4⁺, CD45RB⁺, Th2-like lymph node population did not cause inflammatory bowel disease in SCID mice and could actually abrogate the effects of the CD45RB⁺ population via the production of TGF-β (22). The present study shows the ability of the CD4⁺ IEL subpopulation to coinduce NO synthesis in epithelial cells, revealing a proinflammatory role for the CD4⁺ IEL subset utilizing an in vitro system. More study is needed to determine whether subpopulations of IEL have different functions, for example the CD4⁺CD8⁺αβ⁻ vs. the CD4⁺CD8⁺αβ⁺ subsets. Recent experiments (unpublished observations) staining for intracellular cytokines reveal that both CD4⁺ and CD8⁺ IEL stain for IFN-γ, indicating that either cell population could be producing the IFN-γ required for NO synthesis in this coculture system. Another role for CD4⁺CD8⁺ as well as CD4⁺CD8⁺ IEL is that of provision of B cell help via production of IL-4 and IL-5 in an IEL-B cell culture system (15, 16). Recent study by Todd et al. (30) indicates that different subpopulations of IEL are recovered depending on the isolation procedure used, demonstrating that IEL populations utilized for study must be carefully characterized.

The cellsurface molecules on IEL and IEC that mediate the cell-cell interaction required for iNOS induction remain unidentified. The splenocyte and mesenteric lymph node lymphocyte populations, which contain ~30% CD4⁺ lymphocytes, did not serve to coinduce NO synthesis in IEC-18, indicating that a molecule present on activated lymphocytes such as IEL is necessary for iNOS-coinducing function. As far as the contribution of the target NO producing cell in this system of cell-cell interaction, there must also be a requisite molecule on this cell population because hepatocytes, cells that readily produce NO in response to multiple cytokine stimuli (10), did not produce NO when cocultured with IEL plus IL-1β (Fig. 2B, inset). The fact that peritoneal macrophages can serve as target NO-producing cells indicates that the molecule that the IEL reacts with on the NO-producing cell may not be restricted to the IEC or perhaps more than one molecule can serve as a target molecule for the IEL. The complete lack of effect of the blocking antibodies on NO synthesis in the IEL-IEC-18 cocultures, although the same antibodies (Table 3) very effectively block the rat mixed lymphocyte reaction, indicates that different cell surface molecules are mediating these two interactions.

This experimental coculture system also provides the important indication that iNOS may be coinduced in many cell types via the combination of a cytokine that by itself does not induce iNOS but in the presence of an activated lymphocyte will induce iNOS. In a similar system using mouse macrophages as target NO-producing cells, Tao and Stout (28) have shown that coculture of resting Th1 lymphocyte clones but not Th2 clones with IFN-γ-primed macrophages will induce NO in the macrophages in an antigen-specific manner (i.e., in the presence of the protein to which the clones have
been sensitized and an H-2 compatible macrophage), presumably due to the cytokines produced by the Th1 clone on antigen recognition. However, plasma membrane fractions obtained from either activated Th1 or Th2 clones, but not resting clones, could induce NO in IFN-γ-primed macrophages in an antigen-nonspecific manner. Tian et al. (29) also demonstrated that plasma membranes from activated T cells enhanced NO production by IFN-γ- and TNF-α-stimulated macrophages, and this enhancement was partially inhibited by antibodies to CD40L and LFA-1. These data provide evidence that activated T cells, in particular CD4+ T cells, have a membrane determinant that can promote NO synthesis in an appropriately primed macrophage. The CD4+ IEL are an activated population and may display a determinant similar to that which others have found on activated T cell clones.

These experiments have illustrated the complex interactions between IEL and IEC that presumably are necessary to preserve optimal intestinal function. This paradigm was promoted by Fujihashi et al. (14) to describe interactions between components of gut-associated lymphoid tissue and the parenchymal cells of the gut. In the presently described system, coculture of IEL with the IEC-18 epithelial cell line results in upregulation of class I and II antigen expression on IEC, a condition that reflects normal intestinal physiology (27). Because of the activated status of IEL, this cell population may be constitutively producing other mediators that may maintain levels of antigens on IEC that facilitate IEL-IEC interaction. In this setting, on exposure to an inflammatory stimulus such as IL-1β or TNF-α, NO is produced, perhaps by both the pure cytokine pathway and the CD4+ IEL coinduction pathway.

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