Clin Exp Immunol 1996; **105**:274–277

IL-4 enhances IEC-6 intestinal epithelial cell proliferation yet has no effect on IL-6 secretion

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(Accepted for publication 15 April 1996)

SUMMARY

Intestinal epithelial cells (IEC) form an important line of defence at the intestinal mucosa by providing a barrier to lumenal contents and also by their ability to secrete various inflammatory cytokines. Recently, several T cell-derived cytokines have been shown to regulate specific IEC functions. In this study, the effect of IL-4 on IEC proliferation and secretion of the inflammatory cytokine IL-6 was investigated using the non-transformed rat IEC-6 intestinal epithelial cell line. Recombinant rat (rr)IL-4 was found to enhance IEC-6 cell proliferation over 4 days of culture, and this enhancement was dose-dependent. Further studies using specific antibodies confirmed that IL-4 induced the effect and that the effect was not mediated by autocrine-produced transforming growth factor-alpha. However, IL-4 did not induce IL-6 secretion by the IEC-6 cells, nor did it alter IL-1β-induced IL-6 secretion. These results indicate that T cells may be capable of regulating IEC proliferation via the secretion of IL-4 without altering the capacity of the IEC to function in the inflammatory response by secreting IL-6.

Keywords IL-4 intestinal epithelial cell IEC-6 proliferation

INTRODUCTION

Intestinal epithelial cells (IEC) form the first line of defence against the onslaught of microbes and foreign antigens contained in the intestinal lumen. These cells not only form a continuous barrier to prevent the entry of microbes, they also mediate the transport of protective IgA via the polymeric immunoglobulin receptor and maintain a protective glycocalyx and mucus layer [1]. In addition, the constant turnover of IEC allows for the replenishment of damaged or infected cells [1], so that the control of IEC proliferation may be an important facet of the mucosal response to disease and infection. Isolated IEC and IEC cell lines have also been shown to be capable of secreting several proinflammatory cytokines such as the chemotactic factor IL-8 [2] and IL-6 [3–7], an inflammatory cytokine which is important in enhancing mucosal IgA responses [8]. These two cytokines can also alert the intestinal immune system to possible infections.

As such, the maintenance of the IEC layer is very important to the health of the intestine. However, several gastrointestinal diseases show pathologic changes in the epithelial cell layer such as villous atrophy and crypt cell hyperplasia. Studies using intestinal explants have shown that activation of T cells by specific antibody or mitogens resulted in crypt hyperplasia [9,10] and villous atrophy [9], suggesting a role for T cells or their secreted cytokines in these effects. Further, it has been suggested that the manifestations of villous atrophy are probably due to the T cell-derived cytokines interferon-gamma (IFN-γ) and tumour necrosis factor (TNF) [11], but the mechanisms involved in the induction of crypt cell hyperplasia are unknown.

Recently, Mowat & Widmer [12] have provided evidence that IL-4 may play a role in T cell-mediated enteropathy. Using the mouse intestinal graft-*versus*-host response (GVHR) as a model for intestinal enteropathy, they found that the intestinal crypt cell proliferative component of the GVHR was completely abolished by injections of an anti-IL-4 MoAb or soluble IL-4 receptor. These results clearly suggest that IL-4 may be responsible for the crypt cell hyperplasia in GVHR, and indicate that IL-4 may also play a role in this effect in other intestinal enteropathies.

Although the above study strongly suggests a role for IL-4 in inducing epithelial cell proliferation, direct evidence for this effect has not been shown. Also, the effect of IL-4 on other IEC functions, such as their capacity to act in response to inflammatory stimuli by producing IL-6, is unknown. Yet, the isolation of pure IEC populations and long-term culture *in vitro* are very difficult, making an assessment of the effect of IL-4 on isolated IEC impractical. In this study, we have used the non-transformed rat small intestinal crypt-like epithelial cell line IEC-6 [13] to show that IL-4 was capable of enhancing the proliferation of intestinal epithelial cells. In addition to this function of IL-4, we also provide evidence that IL-4 had no effect on IL-6 secretion by the IEC-6 cells, unlike monocytes in which IL-4 has been shown to inhibit secretion of this inflammatory cytokine [14,15]. These results suggest that T cells may enhance IEC proliferation while not

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affecting their capacity to function in the inflammatory response by secreting IL-6.

MATERIALS AND METHODS

Reagents

A recombinant rat (rr)IL-4 containing culture supernatant from a Chinese hamster ovary (CHO) cell line transfected with a construct for rat IL-4 [16] was obtained from Serotec (Washington, DC). A recombinant human IL-1β was purchased from R&D Systems (Minneapolis, MN). An IgG1 mouse anti-rat IL-4 MoAb (MRC OX-81) was also obtained from Serotec and a rabbit anti-rat transforming growth factor-alpha (TGF-α) serum was obtained from Research & Diagnostic Antibodies (Berkley, CA). A control purified mouse IgG1 was obtained from Sigma Chemical Co. (St Louis, MO).

Cell culture

The rat intestinal epithelial cell line IEC-6 was obtained from the American Type Culture Collection (CRL 1592; Rockville, MD). Cultures of the IEC-6 cells were maintained in high glucose (4·5%) Dulbecco's modified Eagle's medium containing 5% fetal calf serum (FCS; Hyclone Labs, Inc., Logan, UT), 0·1 U/ml bovine insulin (Sigma), 2 mM L-glutamine, 25 U/ml penicillin, and 25 μg/ml streptomycin (complete DMEM). All experiments were performed on cells at or before the 20th passage.

The IEC-6 cell cultures were initiated at 2×10^5 cells/well in 12-well culture plates with 5% FCS complete DMEM and incubated at 37°C in a 90% air–10% CO_2 humid atmosphere. After 2 days, the culture medium was removed and adherent cells were washed gently in serum-free medium before addition of 1·5 ml of 1% FCS complete DMEM. Test cultures contained various percentages (v:v) of rrIL-4 containing supernatant and/or IL-1β at 1 ng/ml as indicated. All cultures were done in triplicate. After 1–4 days of incubation, the culture supernatants were removed and adherent IEC-6 cells were treated with trypsin and EDTA (Sigma). The cells were then diluted in serum-containing medium and counted using a haemacytometer.

In some experiments, the rrIL-4 was neutralized by adding 10 μg/ml of an anti-rat IL-4 MoAb to aliquots of fresh 1% FCS complete DMEM containing the rrIL-4 and incubating at 37°C for 30 min before adding to the IEC-6 cells. Aliquots of medium alone, rrIL-4-containing medium, or medium with rrIL-4 and a purified mouse IgG at 10 μg/ml were also included as controls.

In some instances, culture supernatants were collected on day 2 for determination of IL-6 content by a proliferative bioassay using the IL-6-dependent mouse 7TD1 hybridoma [3,17]. Proliferation of the 7TD1 cells was measured by the MTT colorimetric assay [18] and the results were compared with a standard generated using recombinant mouse IL-6. IL-6 levels are expressed in units, where 1 U is defined as the reciprocal of the dilution giving half-maximal proliferation of the 7TD1 cells.

Statistical analysis

Student's *t*-test or ANOVA and Fisher's least-significant difference test were used to determine significant differences between mean values, as appropriate.

RESULTS

Effect of IL-4 on IEC-6 cell proliferation

When the IEC-6 cells were cultured with the rrIL-4-containing supernatant at 0·1% of the total volume, IEC-6 cell proliferation was enhanced, especially after 3 and 4 days of culture (Fig. 1). A similar pattern of enhancement was seen in six separate experiments, and the average calculated population doubling time was determined to be $1{\cdot}4 \pm 0{\cdot}1$ days (mean $\pm$ s.e.m.) for the IL-4-treated cultures compared with $2{\cdot}3 \pm 0{\cdot}4$ days for control cultures. The results in Table 1 show that the effect of IL-4 on IEC-6 cell proliferation was also dose-dependent, with a maximum at $\approx$ 0·1% of the IL-4-containing supernatant.

However, since the stimulus used was an IL-4-containing culture supernatant, other factors in the supernatant could have accounted for the enhanced proliferation seen. Therefore, 4-day cultures were prepared as before, except that the medium containing the rrIL-4 was pre-incubated with an anti-rat IL-4 MoAb before addition to the IEC-6 cells (Table 2). As controls, medium alone, medium containing rrIL-4, and medium containing rrIL-4 and purified normal mouse IgG1 were treated in a similar manner. As shown in Table 2, pretreatment of the medium with the anti-IL-4 antibody neutralized the added rrIL-4 and prevented the enhancement in IEC-6 cell proliferation, confirming that the effect was due to IL-4.

In addition, experiments were also performed where IEC-6 cell-derived TGF-α was neutralized by the addition of an anti-rat TGF-α antiserum (Research & Diagnostic Antibodies, Berkley, CA). Addition of the anti-TGF-α antiserum to 4-day cultures of IEC-6 cells grown with rrIL-4 did not affect the proliferation of the IEC-6 cells compared with cultures containing IL-4 only (data not shown).

Effect of IL-4 on IL-6 secretion by IEC-6 cells

Previous studies have shown that IEC-6 cells are capable of producing high levels of IL-6 when stimulated by various cytokines [3–5]. Since IL-4 was found to have an effect on IEC-6 cell proliferation, we next determined the effect of IL-4 on IL-6 secretion by unstimulated and IL-1β-stimulated IEC-6 cells. As

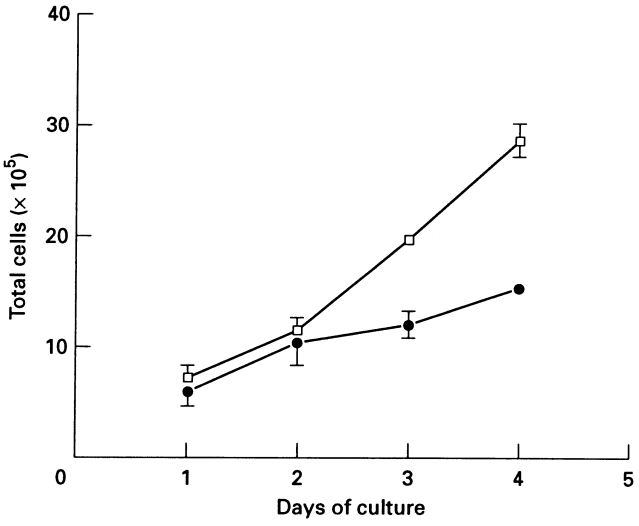

Fig. 1. IL-4 enhances IEC-6 cell proliferation. Triplicate wells of IEC-6 cells (2×10^5 cells/well) were cultured for 1–4 days with medium alone (●) or medium containing 0·1% of a rrIL-4-containing supernatant (□). On the day indicated, the culture supernatants were removed and adherent cells were removed by treatment with trypsin and EDTA for counting using a haemacytometer. This figure is representative of six separate experiments.

Table 1. The dosage effect of IL-4 on IEC-6 cell proliferation

Condition*	Total cell numbers ($\times 10^5$)†
Control	$14{\cdot}6 \pm 1{\cdot}4$
0·001% IL-4	$16{\cdot}2 \pm 0{\cdot}7$
0·01% IL-4	$22{\cdot}4 \pm 3{\cdot}1$‡
0·1% IL-4	$26{\cdot}6 \pm 3{\cdot}2$‡
1·0% IL-4	$27{\cdot}1 \pm 2{\cdot}3$‡

* The IEC-6 cells (2×10^5 cells/well) were cultured 2 days before adding fresh medium with or without an rrIL-4-containing supernatant to yield 1·0–0·001% (v : v). On day 4, culture supernatants were removed and the remaining adherent cells were treated with trypsin and EDTA before diluting and counting using a haemacytometer.

† Values are the mean ± s.d. for three separate cultures. These values are representative of four separate experiments.

‡ Significant difference from control cultures ($P < 0{\cdot}05$).

shown in Table 3, rrIL-4 alone had no effect on IL-6 secretion by IEC-6 cells. The rrIL-4 was also found to have no effect on IL-6 secretion by IL-1β-stimulated cells.

DISCUSSION

Intestinal epithelial cells have recently been imputed to have a potentially important role in the immune response at the intestinal mucosa. Isolated IEC and cell lines are known to produce IL-6 [3–7] and IL-8 [2] as well as a variety of other cytokines. In addition, several IEC functions can be regulated by both macrophage/monocyte-derived and T cell-derived cytokines. In this study we have focused on the effect of IL-4 on two well characterized IEC functions: proliferation and IL-6 secretion.

The Caco-2, HT-29, and T84 colonic carcinoma epithelial cell lines have been shown to express mRNA for the IL-4 receptor [19]. IL-4 has also been found to enhance the expression of the polymeric immunoglobulin receptor (secretory component) on HT-29 cells [20] and decrease the barrier function of T84 cells

Table 2. An anti-rat IL-4 antibody neutralizes the effect of IL-4 on IEC-6 cell proliferation

Condition*	Total cell numbers ($\times 10^5$)†
Control	$13{\cdot}4 \pm 1{\cdot}8$
+ IL-4	$21{\cdot}1 \pm 2{\cdot}6$‡
+ IL-4 and anti-IL-4	$14{\cdot}8 \pm 0{\cdot}5$
+ IL-4 and mouse IgGl	$18{\cdot}7 \pm 3{\cdot}0$‡

* The IEC-6 cells were cultured as in Table 1 for 4 days with or without 0·1% rrIL-4-containing supernatant. Before addition, the fresh medium was incubated for 30 min with or without 10 μg/ml of a mouse anti-rat IL-4 MoAb or normal mouse IgGl. On day 4, the cells were collected and counted as in Table 1.

† Values are the mean ± s.d. of three separate cultures.

‡ Significant difference from control cultures ($P < 0{\cdot}05$).

Table 3. Effect of IL-4 on the secretion of IL-6 by IEC-6 cells

Condition*	Units of IL-6/10^5 cells†
Control	$8{\cdot}9 \pm 0{\cdot}8$
IL-4	$5{\cdot}3 \pm 0{\cdot}4$
IL-1β	$110{\cdot}8 \pm 17{\cdot}3$
IL-1β + IL-4	$91{\cdot}3 \pm 10{\cdot}6$

* IEC-6 cells (2×10^5 cells/well) were cultured 2 days before adding fresh medium containing 1 ng/ml rhIL-1β and/or a rrIL-4-containing supernatant to yield 0·1% (v : v). The culture supernatants were collected 2 days later for determination of IL-6 content and the remaining cells were counted using a haemacytometer.

† Values are the mean ± s.e.m. for six separate experiments.

[21], suggesting that IEC can be functionally regulated by IL-4. In the present study, we have found that IL-4 could also stimulate IEC-6 cell proliferation, suggesting a role for this cytokine in the maintenance of the mucosal epithelial layer. Although the IEC-6 cell line is well suited for this study, as it is a non-transformed crypt-like IEC cell line, an attempt was also made to determine the effect of IL-4 on the proliferation of the HT-29 colonic carcinoma cell line. Unfortunately, the proliferation rate of the HT-29 cells was much higher than the IEC-6 cell line, so that only a slight but non-significant increase in proliferation was noted.

The proliferation of IEC cell lines can also be enhanced by TGF-α, which is known to be produced by IEC [22,23]. However, the addition of an anti-TGF-α antiserum did not alter IL-4-induced enhanced proliferation of IEC-6 cells, suggesting that the enhancement was not due to a secondary effect of IL-4 on TGF-α production. IEC are also capable of producing TGF-β, which can suppress proliferation [24], yet studies using reverse transcriptase-polymerase chain reaction suggested that TGF-β mRNA levels were not affected by IL-4 treatment (data not shown). These results suggest that the effect of IL-4 on IEC proliferation may be direct, and not via regulation of a secondary cytokine signal.

IL-4 is not the only T cell cytokine which has been found to regulate IEC proliferation. One recent study has shown that a subset of intraepithelial T cells bearing the $\gamma\delta$ T cell receptor were capable of secreting keratinocyte growth factor, a cytokine which could enhance epithelial cell proliferation [25]. However, IL-4 is unique at this point, in that it has been shown to be produced at the intestinal mucosa during intestinal diseases such as shigellosis [26] and experimental GVHR [12]. Indeed, the enhancing effect of IL-4 on IEC-6 cell proliferation directly supports the conclusion that IL-4 was responsible for the crypt cell hyperplasia induced by the experimental GVHR.

In addition to the functions of IL-4 discussed above, recent studies have shown that IL-4 can inhibit the production of several proinflammatory cytokines such as IL-1β, TNF-α and IL-6 by monocytes [14,15]. This suggests that IL-4 may be a potent anti-inflammatory agent and a mechanism by which T cells could control the inflammatory response. Several reports have shown that isolated IEC or cell lines could secrete high levels of IL-6 [3–7], especially when stimulated by inflammatory cytokines such as

IL-1β or TNF-α. Since T cell regulation of IL-6 secretion by IEC could be important in the mucosal inflammatory response, we have also determined the effect of IL-4 on IEC-6 cell IL-6 secretion. However, unlike monocytes, IEC-6 cell IL-6 secretion was found to be insensitive to regulation by IL-4, suggesting that the regulation of IEC-derived IL-6 secretion may be distinct from that of monocytes. This finding, along with a previous study which has shown that IL-8 production by IEC cell lines is also unaffected by IL-4 [2], suggests that IL-4 may not regulate the capacity of the IEC to function in the mucosal inflammatory response by producing these two cytokines, a potentially important function for these cells in many digestive tract diseases. However, the effect of IL-4 on the production of other inflammatory factors by IEC remains to be determined.

ACKNOWLEDGMENTS

The authors would like to thank Darren Esposito for his technical help in this study. This study was supported by US PHS grant DK 44617.

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