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A role for IL-4 in immunologically mediated enteropathy

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SUMMARY

A number of clinical enteropathies are associated with a local cell-mediated immune (CMI) response, and experimental evidence indicates that cytokines are responsible for the intestinal pathology. We show here that depletion of IL-4 using MoAb or a soluble form of the IL-4 receptor (IL-4R) prevents the jejunal manifestations of a proliferative form of murine graft-*versus*-host reaction (GVHR) characterized by crypt hyperplasia and recruitment of intraepithelial lymphocytes (IEL). Depletion of IL-4 did not prevent the appearance of villus atrophy in a destructive model of GVHR, and had no effect on any indices of systemic immunity. These results indicate that IL-4 may play a selective role in mediating proliferative aspects of intestinal immunopathology, and suggest that this cytokine may provide a useful target for immunotherapy.

Keywords enteropathy graft-*versus*-host reaction IL-4

INTRODUCTION

Treatment of immunopathology by depleting individual cytokines has the theoretical advantage of targetting only those immune mechanisms which play a pathogenic role in disease. However, if it is to be successful, the full range of such mediators and their modes of action must be ascertained in appropriate models.

A number of clinical enteropathies are associated with a local cell-mediated immune response, including coeliac disease, cow's milk protein intolerance, Crohn's disease and intestinal graft-*versus*-host disease after allogeneic bone marrow transplantation [1]. Previous studies using intestinal graft-*versus*-host reaction in mice as a model of enteropathy have suggested that the pathology is caused by soluble mediators released by $CD4^+$ T cells [2–4]. More recently, we and others have obtained evidence that a number of individual cytokines are important in immunologically mediated enteropathy, including interferon-gamma (IFN-γ) [5,6], tumour necrosis factor-alpha (TNF-α) [7–9], IL-1 [10,11] and IFN-α/β [12]. Although these findings support the idea that such mediators could provide targets for selective immunotherapy, the pathogenesis of enteropathy is complex, and none of the mediators identified so far can account for all the manifestations of the mucosal damage.

There is now considerable evidence that IL-4 is important for the effector functions of T cells, particularly cytotoxic T cells (CTL) and $CD4^+$ T cells of the Th2 subclass [13,14]. More specifically, it has been shown recently that IL-4 participates in the rejection of allografts in mice and that graft survival can be prolonged by depleting IL-4 *in vivo* using either antibodies or small amounts of a purified soluble form of the murine IL-4 receptor [15]. Here, we have used a similar approach to examine whether IL-4 is also important for enteropathy due to activated T cells.

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MATERIALS AND METHODS

Animals

Specified pathogen-free CBA and (CBA × BALB/c) F_1 mice were obtained from Harlan Olac Ltd (Bicester, UK) and were maintained under conventional conditions until first used at 6–8 weeks of age.

*Induction and assessment of graft-*versus*-host response*

In studies using unirradiated hosts, F_1 mice were injected intraperitoneally with 6×10^7 CBA spleen cells in RPMI 1640 (GIBCO BRL, Paisley, UK), or with RPMI alone as a control, and the progress of the systemic graft-*versus*-host response (GVHR) was monitored by the presence of splenomegaly. As described previously [16], this was expressed as the spleen index (SI), calculated for each GVHR mouse by the following formula:

$$SI = \frac{\text{relative spleen weight in GVHR mouse}}{\text{mean relative spleen weight in controls}} \text{(mg/10 g body wt)}$$

To induce a GVHR in irradiated hosts, (CBA × BALB/c)F_1 mice received 8·5 Gy γ-irradiation from a ^{57}cobalt source and, 24 h later, were injected intravenously with 10^7 CBA spleen cells. The progress of this GVHR was followed by measuring

weight loss and haematocrits. In this model, control mice received 10^7 syngeneic CBA spleen cells.

Inhibition of IL-4 in vivo

Mice were injected intraperitoneally with 1 mg of an ammonium sulphate fraction of monoclonal rat anti-mouse IL-4 ascites (11B11) 2 days before and 4 days after induction of GVHR, or with recombinant, soluble murine IL-4 receptor [15] diluted in PBS containing 1% human serum albumin (HSA) from the day before induction of GVHR and daily thereafter. Control mice received equivalent amounts of normal rat immunoglobulin (NRIg) or human IL-4R (which does not neutralize murine IL-4) in PBS/HSA.

Assessment of intestinal GVHR

As described in detail elsewhere [4,16], mice were killed at intervals of 20–90 min after i.p. injection of 7·5 mg/kg colchicine to cause metaphase arrest. Villus and crypt lengths were measured by eyepiece micrometry in microdissected samples of jejunum which had been fixed in 75% ethanol/25% acetic acid and stained by the modified Feulgen reaction. Crypt cell production rates (CCPR) were determined in the same specimens by linear regression analysis of the accumulation of crypt metaphases against time after colchicine injection. Ten crypts and villi were assessed in each sample. Intraepithelial lymphocytes were counted in adjacent sections of jejunum which were fixed in formalin and stained with H&E and the numbers expressed as intraepithelial lymphocytes (IEL)/100 epithelial cells. A total of 600 epithelial cells was counted in each specimen.

Measurement of natural and specific cell-mediated cytotoxicity

As described previously [17], splenic natural killer (NK) cell activity was assayed against YAC-1 cells, while specific anti-host CTL activity was assayed against P815 (H-2^d) cells. Briefly, 100 μl spleen cells in RPMI 1640 supplemented with 5% newborn calf serum (NCS) (both GIBCO BRL, Paisley, UK) were added to the wells of V-bottomed microtitre plates (Costar, Northumbria Biologicals, Cramlington, UK), together with 100-μl aliquots of 2×10^4 target cells labelled for 1 h with Na^{51}chromate to give final effector : target (E : T) ratios of 50 : 1, 25 : 1 and 12·5 : 1. After 4 h incubation at 37°C in 5% CO_2 in air, 100 μl supernatant were removed from each well and the ^{51}Cr-specific radioactivity measured in a γ-counter. The per cent natural or specific cytotoxicity was calculated as follows:

$$\text{Per cent cytotoxicity} = \left(\frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}}\right) \times 100\%$$

In all experiments, 10% Triton X-100 (Sigma, Poole, UK) was used to obtain total release of ^{51}Cr, while spontaneous release was calculated using appropriate ratios of normal thymocytes or spleen cells in NK and CTL assays, respectively. Assays were performed in quadruplicate and variability between wells was normally $\leqslant 15\%$.

Statistical analysis

Groups of means ± s.d. were compared using Student's *t*-test, while CCPR were compared by covariance analysis.

RESULTS

Effects of IL-4 depletion on systemic progress of proliferative GVHR

In the first experiments, we examined the role of IL-4 in the proliferative form of GVHR which occurs in unirradiated (CBA × BALB/c) F_1 mice given CBA donor cells. This is a mild disorder whose intestinal phase reproduces the earliest stages of clinical enteropathy [2].

As we have shown previously [2,16], mice with this type of GVHR remained clinically well and exhibited no weight loss or other systemic signs of disease (data not shown). However, these animals developed very marked splenomegaly compared with controls, which peaked on day 8 (Table 1). Administration of anti-IL-4 MoAb or treatment with 1 μg or 10 μg/day soluble IL-4R had no significant effect on the development of splenomegaly. GVHR mice given 10 μg human IL-4R daily had identical splenomegaly to mice treated with normal immunoglobulin (data not shown).

There was also a large increase in NK activity in the spleen of GVHR mice compared with normal animals (Fig. 1). This was not affected by administering soluble IL-4R (Fig. 1). No specific anti-host CTL activity was found in GVHR mice, confirming previous findings in this model (data not shown).

Effects of depleting IL-4 on proliferative intestinal GVHR

Unirradiated mice with GVHR developed an enteropathy which was characterized by marked and significant increases in CCPR, crypt length and IEL count compared with controls (Figs 2a–c). There was no evidence of destructive pathology such as villus atrophy (Fig. 2d). These findings confirm previous results and indicate the entirely proliferative nature of this form of enteropathy [2,16].

Treatment of mice with anti-IL-4 antibody abolished the increase in IEL count normally found in GVHR and significantly reduced the crypt hyperplasia and hypertrophy (Fig. 2). Administration of soluble IL-4R had a dose-dependent ability to inhibit intestinal GVHR, with 10 μg/day IL-4R virtually abolishing all aspects of enteropathy and 1 μg/day significantly reducing the crypt hypertrophy, but having no significant effect on the increases in CCPR or IEL count.

These results, which have been confirmed in an identical repeat experiment, indicate that IL-4 is essential for the development of enteropathy in proliferative GVHR.

Table 1. Role of IL-4 in the development of splenomegaly in (CBA × BALB/c)F_1 mice with proliferative graft-*versus*-host response (GVHR)

Group	Spleen index
NRIg GVHR	2·79 ± 0·32
Anti-IL-4 GVHR	2·70 ± 0·28
1 μg IL-4R GVHR	2·44 ± 0·32
10 μg IL-4R GVHR	2·56 ± 0·39

Results shown are mean spleen indices ± 1 s.d. for five mice/group on day 8 of GVHR in mice receiving normal rat IgG (NRIg) or treated with monoclonal anti-IL-4 antibody or soluble IL-4R.

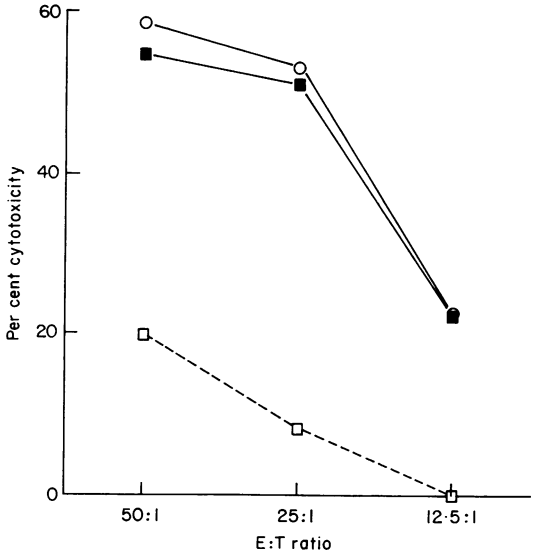

Fig. 1. Effects of depleting IL-4 on systemic graft-*versus*-host response (GVHR) in unirradiated (CBA x BALB/c) F_1 mice. Splenic natural killer (NK) cell activity on day 8 of GVHR, in controls and in mice with GVHR treated with 10 μg IL-4R daily or PBS/human serum albumin (HSA) as a diluent control. Results shown are per cent natural cytotoxicity for spleen cells pooled from three mice/group. □, Control; ○, GVHR; ■, 10 μg IL-4R GVHR.

Effects of depleting IL-4 on systemic aspects of destructive GVHR

The proliferative GVHR used in the first studies does not reproduce the villus atrophy which is characteristic of established clinical disease. As previous work has suggested that destructive mucosal pathology may reflect different mechanisms to that which causes the proliferative features [2,8,18], we thought it important to examine directly whether IL-4 was also required for destructive enteropathy in GVHR. To do this, we used the GVHR in irradiated hosts. In these experiments, we examined the role of IL-4 by treating host mice with 10 μg/day soluble IL-4R, as this was found to be the most effective regime for preventing proliferative GVHR.

As we have found previously [4], irradiated (CBA x BALB/c) F_1 mice given CBA spleen cells developed marked weight loss beginning 3 days after cell transfer (Fig. 3a). The weight loss by day 5 was significant (21·6 ± 0·6 g *versus* 23·4 ± 1·5 g; $P < 0·03$), and all GVHR mice died by days 6–7. The weight loss and mortality were not altered by treating host mice with soluble IL-4R (day 5, body wt 21·0 ± 0·9 g; $P < 0·01$ *versus* syngeneic controls).

A further characteristic of GVHR in irradiated mice was the appearance of specific anti-host CTL in the spleen, and this was not affected by treatment with soluble IL-4R (Fig. 3b).

Effects of depleting IL-4 on enteropathy in destructive GVHR

The GVHR in irradiated hosts produced crypt hyperplasia and hypertrophy which was more intense than that seen in unirradiated mice (Fig. 4). In addition, this was now associated with significant villus atrophy, indicating the presence of a destructive enteropathy (Fig. 4). This pathology was not dependent on the presence of IL-4, as identical changes were seen in GVHR mice treated with soluble IL-4R. Administering IL-4R had no harmful systemic or intestinal effects on irradiated mice which received syngeneic cells as controls. Similar findings were obtained in a replicate experiment.

DISCUSSION

The results presented here show that IL-4 is critical for the enteropathy of GVHR. This effect is selective, being restricted to the proliferative aspects of the disorder and having no influence on the systemic immune response. This is the first demonstration that IL-4 is involved in tissue-specific immuno-

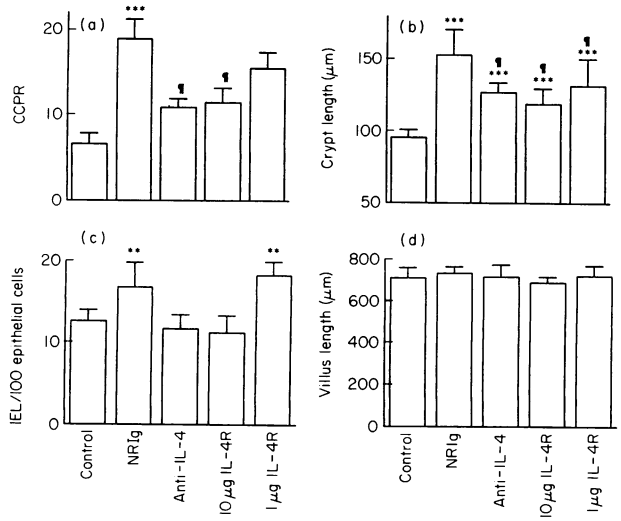

Fig. 2. Effects of depleting IL-4 on intestinal pathology in proliferative graft-*versus*-host response (GVHR). Results shown are the mean crypt cell production rate (CCPR) (a), crypt length (b), intraepithelial lymphocyte (IEL) count (c) and villus length (d) ± 1 s.d. in the jejunum on day 8 of GVHR for five to six mice/group. GVHR mice received either anti-IL-4 antibody, 1 or 10 μg/day soluble IL-4R, or normal rat immunoglobulin (NRIg) as a control. ** $P < 0·005$ *versus* all other groups; *** $P < 0·001$ *versus* controls; ¶ $P < 0·05$ *versus* NRIg GVHR.

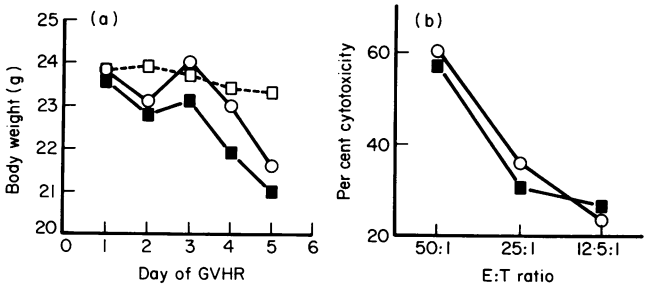

Fig. 3. Effects of depleting IL-4 on systemic graft-*versus*-host response (GVHR) in irradiated (CBA x BALB/c) F_1 mice. Development of weight loss (a) and anti-host-specific CTL activity (b) in GVHR mice treated with 10 μg/day IL-4R or with diluent control. Control mice were irradiated and received syngeneic spleen cells. Body weights are the mean of five mice/group, while CTL activity was measured in spleens pooled from two mice/group on day 5 of GVHR. (a) □, Control; ○, GVHR; ■, IL-4R GVHR. (b) ○, GVHR; ■, 10 μg IL-4R GVHR.

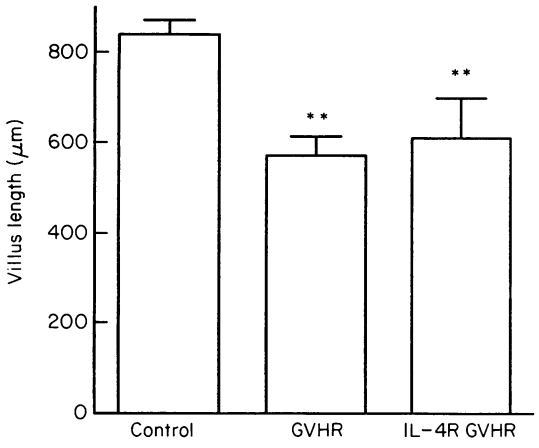

Fig. 4. Effects of depleting IL-4 on destructive intestinal graft-*versus*-host response (GVHR). Villus lengths in the jejunum on day 5 of GVHR in mice treated with IL-4R or diluent control and in irradiated control mice given syngeneic spleen cells. ** $P < 0{\cdot}01$ *versus* controls.

pathology, and suggests that this cytokine could prove a useful target for immunotherapy *in vivo*.

All the features of proliferative GVHR, including crypt hyperplasia, crypt hypertrophy and increased infiltration of the epithelium by lymphocytes required the presence of endogenous IL-4, as they were inhibited by administering anti-IL-4 MoAb or by small amounts of soluble receptor in a dose-dependent fashion. In contrast, IL-4 did not appear to be required for the systemic aspects of GVHR, including splenomegaly, activation of NK cells, weight loss or specific CTL activity. Together, these findings suggest that IL-4 may play a selective role in the proliferative pathology which typifies early or mild GVHR and that the intestine is particularly sensitive to the activity of this cytokine. Our study of intestinal immunopathology extends a previous report that depletion of IL-4 using similar doses of the same receptor preparation used here prolonged survival of neovascularized cardiac allografts in mice [15] and that IL-4 is essential for autoimmunity in chronic murine GVHR [19,20]. It would be of interest to examine the role of IL-4 in GVHR-induced pathology in tissues other than the gut, and to determine whether IL-4 plays a role in other forms of immunologically mediated enteropathy, such as that found in intestinal parasite infections associated with increased production of IL-4 [21].

In contrast to its effects on proliferative GVHR, depletion of IL-4 *in vivo* did not prevent the development of destructive intestinal GVHR, as typified by the presence of villus atrophy. We cannot formally exclude the possibility that this difference may simply reflect the fact that mice with severe GVHR produce relatively larger amounts of IL-4 and that this cannot be neutralized *in vivo* using the protocol we used. However, we consider this unlikely, as we have not been able to detect IL-4 production in any model of acute murine GVHR (Williamson & Garside, unpublished observations). In addition, we would have anticipated that daily administration of 10 μg IL-4R would have at least ameliorated intestinal GVHR in irradiated mice in view of its profound effects on the equivalent pathology in proliferative GVHR. Finally, the current findings are consistent with other evidence that the different aspects of intestinal GVHR are due to the activities of a number of distinct cytokines, including IFN-γ, TNF-α, IL-1 and IFN-α/β. Of these, IFN-γ is essential for the entire spectrum of enteropathy [5], while IL-1 and IFN-α/β play contributory, but non-essential roles in both proliferative and destructive enteropathy [10,12]. In contrast, TNF-α seems to be important only for the late, destructive phase of disease [8,18,22], thus playing an opposite role to that identified for IL-4 in the current work.

The ways in which IL-4 could contribute to epithelial pathology in GVHR are unknown. Previous work indicates that crypt stem cells are the primary targets of cytokines in GVHR [2], and hence IL-4 may interact directly with these cells to produce hyperplasia. Alternatively, IL-4 may promote pathology via its ability to stimulate other lymphoid cells [13,14,23]. Of particular interest, cytolytically active $CD8\alpha^+\beta^-$ T cells appear to be selective targets for the action of IL-4 [13,23]. This is consistent with our observation that IL-4 was required for the GVHR-mediated recruitment of IEL, a population dominated by cells with these unusual properties [24]. IL-4 can also stimulate macrophages [23], and macrophage products are critically important in intestinal GVHR [8,10,25]. Distinguishing between these possible effects requires further investigation. We have also not identified the source of the IL-4 required for intestinal GVHR, but assume that it is derived from donor-derived T cells. As we have been unable to detect IL-4 production by spleen or mesenteric lymph node cells from mice with GVHR (Steel & Garside, unpublished observations), we believe that the IL-4-producing cells are present in the mucosa itself. However, this needs direct proof at the local level using appropriate molecular or ELISPOT techniques.

Our studies are further encouraging evidence that depletion of individual mediators may be useful for modulating immunopathology *in vivo*. In particular, they underline the therapeutic potential of inhibiting cytokine function by administering small amounts of antagonists to receptors such as the IL-1R, IL-4R and TNFR [15,26–28]. Of particular interest is our finding that depleting IL-4 inhibited the intestinal consequences of GVHR without altering indices of systemic immunity. If these tissue-specific benefits in the absence of significant systemic immunosuppression can be extended to other models, cytokine-targetted immunotherapy may offer significant advantages over conventional treatment regimes.

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