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# Antibodies to IFN-γ prevent immunologically mediated intestinal damage in murine graft-versus-host reaction

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## SUMMARY

We have tested the hypothesis that interferon-gamma (IFN-γ) plays a role in the enteropathy of graft-versus-host reaction (GVHR) by treating host mice with a monoclonal antibody directed at this mediator. Two models of GVHR were examined. In the mild proliferative GVHR, which occurs in adult unirradiated (CBA × BALB/c)$F_1$ mice given parental spleen cells, anti-IFN-γ slightly inhibited the development of splenomegaly and the activation of natural killer (NK) cells in GVHR. Anti-IFN-γ had no effect on splenomegaly or generation of anti-host cytotoxic T lymphocytes (CTL) during the more severe GVHR in adult BDF hosts, but inhibited the weight loss and mortality normally found in this GVHR. Despite these variable effects on systemic GVHR, anti-IFN-γ treatment abolished the crypt hyperplasia and increased counts of intraepithelial lymphocytes (IEL) normally found in the jejunum of (CBA × BALB/c)$F_1$ mice with GVHR. In parallel, anti-IFN-γ-treated BDF1 mice with GVHR did not develop the villus atrophy and intense crypt hyperplasia found in untreated GVHR hosts. These results support the view that IFN-γ is essential for the development of enteropathy in GVHR and we propose that this mediator may also be involved in the pathogenesis of clinical enteropathies in man.


## INTRODUCTION

Several important human enteropathies, such as coeliac disease, cow's milk protein intolerance and certain parasite infections show a similar pattern of mucosal pathology and are associated with a local cell-mediated immune response (Ferguson & Mowat, 1980; Mowat, 1984). However, the immunological basis of the intestinal pathology has not been elucidated. The intestinal phase of graft-versus-host (GVHR) reaction in mice provides an experimental model which reproduces many of the characteristic features of the clinical enteropathies, including villus atrophy, crypt hyperplasia and increased numbers of intraepithelial lymphocytes (Hedberg, Reiser & Reilly, 1968; MacDonald & Ferguson, 1977; Mowat & Ferguson, 1981, 1982.

Intestinal GVHR occurs in two forms: a mild, proliferative disorder associated only with crypt hyperplasia and increased IEL counts, and a more severe destructive disease in which intense crypt hyperplasia progresses to villus atrophy (Mowat & Felstein 1989). Studies of both models have provided considerable indirect evidence that soluble mediators released during a local delayed-type hypersensitivity reaction are responsible for the intestinal damage. The mucosal alterations are almost entirely class II major histocompatibility complex (MHC)-restricted (Piguet, 1985; Mowat, Borland & Parrott, 1986) and are not closely correlated with the activity of cytotoxic T lymphocytes (Borland, Mowat & Parrott, 1983; Guy-Grand & Vassalli, 1986; Mowat *et al.*, 1988). Furthermore, the intestinal damage occurs as a bystander effect in grafts of gut that are syngeneic to the donor lymphocytes used to induce the GVHR (Elson, Reilly & Rosenberg, 1977; Mowat & Ferguson, 1981; Guy-Grand & Vassalli, 1986; Mowat *et al.*, 1988) and in the gut of parental strain mice made chimeric for $F_1$ bone marrow (Mowat, 1986). A role for soluble mediators has received more direct support from the finding that intestinal GVHR in mice can be prevented by administration of antibodies against tumour necrosis factor α (TNFα) (Piguet *et al.*, 1987). The possibility that IFN-γ might be a further cytokine involved in GVHR is suggested by the enhanced expression of class II MHC antigens on many tissues in GVHR (Guy-Grand & Vassalli, 1986), and in this study we have examined the role of IFN-γ in the enteropathy of GVHR, by depleting mice *in vivo* using a monoclonal antibody that neutralizes the biological activities of IFN-γ, including macrophage activation and induction of class II MHC expression (Spitalny & Havell, 1984; Vogel, Havell & Spitalny, 1986). This treatment prevented intestinal damage in both the proliferative and destructive forms of GVHR, indicating that IFN-γ is essential for GVHR-associated enteropathy.

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## MATERIALS AND METHODS

*Mice*

CBA ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), C57B1/6J (B6) ($H\text{-}2^b$) and (CBA × BALB/c)$F_1$ ($H\text{-}2^{k\times d}$) mice were bred and maintained in this department, while (C57B1/6 × DBA/2)$F_1$ (BDF1) ($H\text{-}2^{b\times d}$) mice were obtained from Harlan Olac, Bicester, Oxon. Mice were first used at 6–8 weeks of age.

*Induction of GVHR*

A GVHR was induced in (CBA × BALB/c)$F_1$ mice by intraperitoneal injection of $6 \times 10^7$ viable CBA spleen cells, while the GVHR in BDF1 hosts was induced by intravenous injection of $10^8$ B6 spleen cells. Control mice received 0·2 ml medium only. The intensity of the systemic GVHR was assessed in (CBA × BALB/c)$F_1$ mice by measurements of spleen weights and the results expressed as the spleen index (Simonsen, 1962). Systemic GVHR in BDF1 mice was followed by assessing splenomegaly and by regular measurements of body weights.

*Cell-mediated cytotoxicity assays*

Spleen cells from mice with GVHR were assessed for specific CTL activity and natural killer cell activity as described previously (Borland *et al.*, 1983). Briefly, spleen cells from three mice/group were pooled in RPMI-1640 (Gibco BRL, Paisley, Renfrewshire) supplemented with 5% newborn calf serum (NCS) (Gibco BRL), and 100-$\mu$l aliquots added to the wells of V-bottomed microtitre plates (Titertek, Flow Labs, Irvine, Ayrshire) to give final effector:target (E:T) cell ratios of 50:1, 25:1 and 12·5:1. YAC-1, P815 [H-$2^d$) and EL-4 (H-$2^b$) tumour target cells were labelled for 45 min at 37° with 100 $\mu$Ci Na $^{51}$Cr/$5 \times 10^6$ cells/ml and $2 \times 10^4$ targets added to the assay plates in 100 $\mu$l aliquots. After culture for 4 hr at 37°, 100 $\mu$l supernatant were removed and $^{51}$Cr-specific radioactivity measured in a gamma-counter. The percentage cytotoxicity was calculated as follows:

% cytoxicity =

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100\%.$$

In CTL assays, spleen cells from control mice were used to obtain spontaneous release, while NK-inactive control thymocytes were used in NK cell assays. In all assays, 10% Triton-X (Sigma Ltd, Poole, Dorset) was used to obtain maximum release.

*Measurement of intestinal GVHR*

The enteropathy of GVHR was assessed in pieces of jejunum taken 10 cm from the pylorus, as described previously (MacDonald & Ferguson, 1977; Mowat & Ferguson, 1981, 1982). Villus lengths, crypt lengths and crypt cell production rates (CCPR) were measured by microdissection and morphometric analysis of jejunum fixed in 75% ethanol/25% acetic acid and stained in bulk with Schiff reagent (Difco Labs, West Molesey, Surrey). Mice were killed 20–100 min after injection of 7·5 mg/kg colchocine (Sigma) i.p. to cause metaphase arrest in dividing crypt cells, and the CCPR obtained by linear regression analysis of metaphase accumulation against time. Ten villi and crypts were assessed in each specimen. Intraepithelial lymphocytes were counted on adjacent, formalin-fixed sections of jejunum stained with H & E, and the results expressed as IEL/100 epithelial cells. Six hundred epithelial cells were counted on each section.

*Treatment of mice with monoclonal anti-IFN-γ antibodies*

Rat IgG anti-mouse IFN-γ monoclonal antibody-containing ascites was prepared by injecting BALB/c nude mice with $1{\cdot}5 \times 10^7$ R4-6A2 hybridoma cells (Spitalny & Havell, 1984; Vogel *et al.*, 1986) (obtained from Dr A. Morris, Dept. of Biological Sciences, University of Warwick) i.p, 7 days after priming with 0·5 ml Pristane (Sigma) i.p. The resulting ascites was tapped and the IgG fraction obtained by precipitation with 40% ammonium sulphate. Mice were injected with 0·2 ml of a 1:4 dilution of this material at 2–3 day intervals, beginning 1 day before induction of GVHR. Control mice received a similar preparation of IgG from normal rat serum diluted in saline.

*Statistics*

Results presented as means ± 1 SD were compared using Student's *t*-test, except for body weights which were compared using Wilcoxon's Rank Sum test. Crypt cell production rates were compared by covariance analysis.

## RESULTS

Our previous work had indicated that the full manifestations of intestinal GVHR developed progressively over the course of the disease (Mowat & Ferguson, 1982; Mowat *et al.*, 1988; Felstein & Mowat, 1988). Although we wished to determine the role of IFN-γ throughout this spectrum of enteropathy, formal time-course studies were precluded by the limited availability of anti-IFN-γ antibodies. Therefore, we examined the individual aspects of GVHR-induced enteropathy by using single-time-points in two separate models of GVHR, which were known to represent distinct forms of immunopathology.

### Effects of anti-IFN-γ on proliferative enteropathy in GVHR

The mild end of the spectrum of enteropathy was examined first, by inducing a GVHR in adult, unirradiated (CBA × BALB/c)$F_1$ mice by transfer of CBA spleen cells. As we have detailed previously, this produces a moderately intense GVHR which is characterized by lymphoid hyperplasia. Host mice do not show clinical signs of disease, do not lose weight and all recover within 2–3 weeks (Mowat & Ferguson, 1981; 1982). Representative systemic features of this GVHR found in the current study are illustrated in Table 1, where it can be seen that at the peak of the GVHR on Day 8, mice treated with normal rat IgG had significant splenomegaly and enhanced activity of splenic NK cells compared with controls. In contrast, specific anti-host

**Table 1.** Effects of anti-IFN-γ on systemic GVHR in (CBA × BALB/c)$F_1$ mice

| Group | Anti-IFN-γ | Spleen index | NK activity (%) |
|---|---|---|---|
| Control | – | — | 18·3 |
| GVHR | – | 2·44 ± 0·31 | 26·5 |
| Control | + | — | 13·4 |
| GVHR | + | 1·88 ± 0·02* | 19·8 |

Splenomegaly and NK cell activation at the peak of GVHR on Day 8. Results shown are mean spleen indices ± 1 SD for four mice/group, while the NK cell activity is the percentage cytotoxicity against YAC-1 target cells at 50:1 E:T cell ratios, using spleen cells pooled from four mice/group.

* $P < 0{\cdot}02$ vs. untreated GVHR.

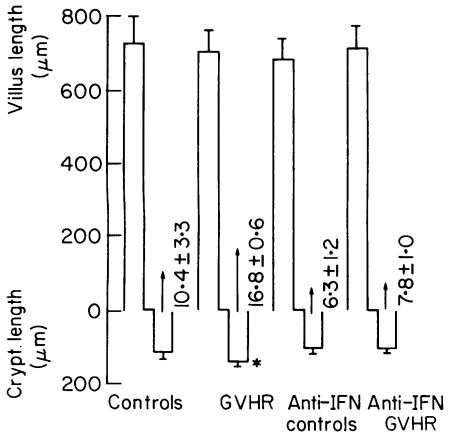


**Figure 1.** Effects of anti-IFN-γ on intestinal GVHR in (CBA × BALB/c)$F_1$ mice. Crypt lengths, villus lengths and crypt cell production rates (CCPR) measured in the jejunum on Day 8 of GVHR. Bars represent villus and crypt lengths, while arrows represent the CCPR and the results shown are means ± 1 SD for five to six mice/group. * $P < 0{\cdot}01$.

cytotoxic T cells were not detectable in mice with this GVHR (data not shown). In parallel with these systemic changes, the jejunum of (CBA × BALB/c)$F_1$ mice with GVHR showed significant increases in crypt length (Fig. 1) and IEL count (Fig. 2), as well as a 60% increase in CCPR (Fig. 1) compared with controls. No villus atrophy was found in mice with GVHR (Fig. 1). All these findings confirm our previous work on this model of GVHR and indicate that it represents an entirely proliferative form of immunopathology.

Treatment of host mice with anti-IFN-γ depressed the systemic effects of this proliferative GVHR, as assessed by the studies performed at the peak of the GVHR on Day 8. Splenomegaly was significantly less in these mice than in control GVHR mice and their NK activity was similar to that found in normal control animals (Table 1). Nevertheless, anti-IFN-γ treated mice had significant splenomegaly as well as enhanced NK cell activity compared with their controls. Although similar, mildly inhibitory effects of anti-IFN-γ were found at other times of this GVHR (data not shown), anti-IFN-γ treatment abolished all evidence of intestinal GVHR in (CBA × BALB/c)$F_1$ hosts. These animals had values for crypt length, CCPR (Fig. 1) and IEL count (Fig. 2), which were identical to those found in

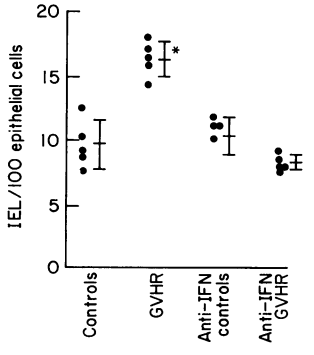


**Figure 2.** Effects of anti-IFN-γ on intestinal GVHR in (CBA × BALB/c)$F_1$ mice. Intraepithelial lymphocyte counts in the jejunum on Day 8 of GVHR. Results shown are means ± 1 SD for five to six mice/group. * $P < 0{\cdot}001$.

**Table 2.** Effects of anti-IFN-γ on systemic GVHR in BDF1 mice

| Group | Spleen index | Minimum weight (g) |
|---|---|---|
| Control | — | 27·5 ± 3·0 |
| GVHR | 3·1 ± 0·49 | 23·9 ± 4·1 |
| Anti-IFN-γ GVHR | 4·3 ± 0·74* | 28·1 ± 1·2** |

Splenomegaly measured on Day 7 of GVHR and body weights measured at the time of maximum weight loss by GVHR on Day 33. Results are mean ± 1 SD for four (splenomegaly) or seven (body weights) mice/group.

* $P < 0{\cdot}05$; ** $P < 0{\cdot}02$ vs. untreated GVHR mice.

control animals. Once again, no villus atrophy was found. Anti-IFN-γ itself had no significant effects on intestinal architecture or IEL counts. Identical results were obtained in two further experiments in this model and they indicate that IFN-γ is essential for the intestinal consequences of a proliferative GVHR.

### Effects of anti-IFN-γ on destructive enteropathy in GVHR

The results described above indicate a potentially important role for IFN-γ in T lymphocyte-mediated enteropathy. Nevertheless, the GVHR in (CBA × BALB/c)$F_1$ mice is a mild disorder which does not reproduce the villus atrophy normally found in association with crypt hyperplasia in naturally occurring enteropathies associated with local CMI. Therefore, we investigated the requirement for IFN-γ in a more intense form of GVHR. To this end, a GVHR was induced in adult, unirradiated BDF1 mice by transfer of $10^8$ B6 spleen cells. This produces an acute, wasting disease quite different from that described above, with the generation of distinct immune effector mechanisms (Gleichmann *et al.*, 1984).

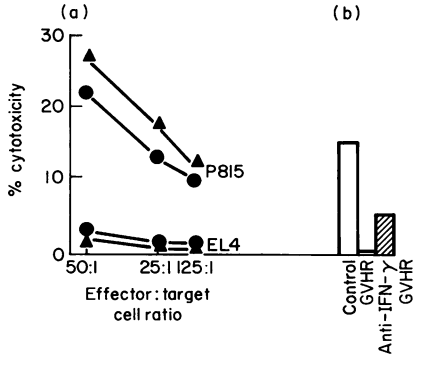


**Figure 3.** Effects of anti-IFN-γ on systemic immunity during GVHR in BDF1 mice. (a) Specific CTL activity measured against $^{51}Cr$-labelled P815 (H-$2^d$) tumour target cells at different E:T ratios using spleen cells pooled from three to four mice/group on Day 9 of GVHR. No killing of non-specific EL-4 (H-$2^d$) targets was observed (●) GVHR; (▲) anti-IFN-γ GVHR. (b) NK cell activity measured at 50:1 E:T ratios in the spleens of the same mice.

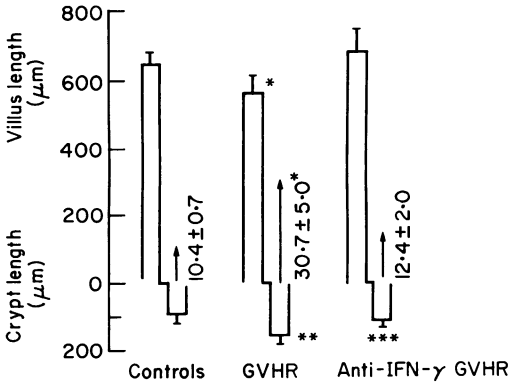


**Figure 4.** Effects of anti-IFN-γ on destructive intestinal GVHR in BDF1 mice. Villus lengths, crypt lengths and CCPR in the jejunum of mice on Day 28 of GVHR. Results shown are means ± 1 SD for five to six mice/group. * $P<0{\cdot}01$; ** $P<0{\cdot}001$; *** $P<0{\cdot}05$ vs. control.

In the present study, BDF1 mice treated with normal IgG and given $10^8$ B6 spleen cells began to lose weight during the second week of GVHR and this continued for up to 4–5 weeks thereafter (data not shown). In addition, two out of eight GVHR mice died and most showed clinical signs typical of an acute GVHR, including ruffled fur, abnormal gait and hunched posture. Although significant splenomegaly occurred during the initial phase of this GVHR (Table 2), this feature became more variable at later times (data not shown), as others have also reported (Gleichman *et al.*, 1984). One further feature of this model of GVHR which differed from the disease in (CBA × BALB/c)$F_1$ hosts was the generation of specific anti-host CTL (Fig. 3). This was accompanied by the complete disappearance of NK cell activity (Fig. 3), a phenomenon reported by others using this GVHR which has been ascribed to lysis of host NK cells by the CTL (Kubota, Ishikawa & Saito, 1983; Knobloch & Dennert, 1988).

Initial studies indicated that severe enteropathy occurred in BDF1 mice with GVHR and that it became most intense during the period of maximum weight loss (A. McI. Mowat and M. V. Felstein, manuscript submitted). Therefore, in the present experiment, we chose Day 28 of GVHR to examine the intestinal pathology. At this time, unmanipulated BDF1 mice with GVHRR had very marked enteropathy, with large increases in crypt length and CCPR compared with controls (Fig. 4). Furthermore, these mice had the additional feature of significant villus atrophy. An increased IEL count is not a feature of established GVHR in this model (A. McI. Mowat and M. V. Felstein, manuscript submitted; data not shown).

Although this acute GVHR in BDF1 hosts is clearly a much more severe disorder than that found in (CBA × BALB/c)$F_1$ hosts, it was partially suppressed by anti-IFN-γ therapy. None of the BDF1 mice with GVHR given anti-IFN-γ died, and despite a minor and transient reversal of weight gain in the second week, anti-IFN-treated hosts were always heavier than unmanipulated GVHR hosts (data not shown). At the time of maximal weight loss, this difference was highly significant (Table 2). Control mice treated with monoclonal anti-IFN-γ showed no adverse effects and gained weight at the same rate as normal controls. Splenomegaly was marked in anti-IFN-γ-treated mice during the early stages of GVHR and this was actually higher than that in unmanipulated GVHR mice (Table 2). At later times, splenomegaly again became variable and no consistent effects of anti-IFN-γ were discernible (data not shown). Anti-IFN-γ also did not prevent the generation of anti-host CTL in GVHR, and identical levels of CTL were found in both groups of GVHR mice (Fig. 3). However, in contrast to normal GVHR hosts, anti-IFN-γ-treated mice with GVHR retained some NK cell function, even though this was markedly less than that found in normal controls (Fig. 3).

Thus, anti-IFN-γ had only a limited ability to inhibit systemic GVHR in BDF1 mice. Nevertheless, this therapy virtually abolished the intestinal pathology in the same hosts. Mice treated with antiIFN-γ had no villus atrophy and had a CCPR that was identical to that in controls (Fig. 4). In addition, although some mice had crypt lengthening, overall this was minimal compared with that found in all control mice with GVHR (Fig. 4). As an identical pattern of effects of anti-IFN-γ was found when this experiment was repeated, we conclude that IFN-γ is essential for destructive enteropathy in this model of acute GVHR.

## DISCUSSION

The results presented here show that treatment of mice with anti-IFN-γ antibody abolishes virtually all the intestinal pathology which usually occurs in two distinct models of GVHR. This inhibition of intestinal GVHR occurred despite a lesser effect of anti-IFN-γ on systemic GVHR, and our findings indicate that IFN-γ has an important role in this form of enteropathy.

Other workers have detected production of IFN-γ by lymphoid cells from mice with GVHR (Cleveland, Annable & Klimpel, 1988) and anti-IFN-γ prevents the enhanced protection against *Listeria* normally found in GVHR (Leist, Heuchel & Zinkernagel, 1988). Nevertheless, our study is the first to demonstrate a role for this mediator in the pathological consequences of GVHR. The mechanisms by which IFN-γ acts in intestinal GVHR remain to be elucidated, but could reflect either a direct effect on the gut or may be secondary to its ability to activate immune effector cells, such as macrophages or NK cells (Spitalny & Havell, 1984; Trinchieri & Perussia, 1986; Leist *et al.*, 1988). Both these cell types could damage the intestine by lytic attack on enterocytes or, alternatively, by acting as sources of further inflammatory mediators. This possibility is supported by the enhanced production of IFN-α/β in GVHR (Cleveland *et al.*, 1988) and by the demonstration that treatment of mice with antibodies to TNFα also prevents intestinal pathology in GVHR (Piguet *et al*, 1987). We are currently examining the inter-relationships between these different mediators in GVHR.

CTL do not appear to be required for the effects of IFN-γ in intestinal GVHR, as treatment of BDF1 mice with anti-IFN-γ prevented intestinal GVHR but had no effect on the appearance of anti-host CTL. This result is entirely consistent with our previous findings that CTL are not necessary for enteropathy in GVHR (Mowat & Ferguson, 1981; Borland *et al.*, 1983; Mowat *et al.*, 1988; Felstein & Mowat, 1988) and we conclude that CTL are merely an epiphenomenon of this particular form of pathology. Nevertheless, the ability of anti-IFN-γ to prevent Moloney sarcoma virus-induced tumours does seem to reflect an inhibition of virus-specific CTL activity (Zanovello *et al.*, 1988). Therefore, we cannot exclude a possible role for interaction between IFN-γ and CTL in other forms of GVHR-induced immunopathology.

Our studies raise the possibility that IFN-γ may be directly harmful to the intestine. Although this can only be proved by studies using enterocytes *in vitro*, it is consistent with the wide range of direct, pathogenic effects which IFN-γ has on many other cell types, including thyroid cells, pancreatic islet cells, fibroblasts and endothelium (Trinchieri & Perussia, 1986; Weetman & Rees, 1988; Campbell, Iscaro & Harrison, 1988). Further support for a direct effect of IFN-γ on the gut comes from the increased expression of class II MHC antigens by enterocytes in GVHR (Barclay & Mason, 1982; Guy-Grand & Vassalli, 1986). Indeed, it is possible that the role of IFN-γ in immune-mediated enteropathy is to render epithelial cells more sensitive to other immune effector cells because of aberrant expression of class II MHC molecules. It would be of interest to determine whether anti-IFN-γ therapy prevents this feature of intestinal GVHR.

Our study supports earlier work showing that anti-IFN-γ can inhibit allograft responses in mice (Landolfo *et al.*, 1985) and so indicates that this therapy may be of general use in preventing immune-mediated tissue damage. Furthermore, if confirmed by more detailed study, our results suggest that anti-IFN-γ may be more effective at preventing the local intestinal rather than the systemic consequences of GVHR. This finding has two general implications. First, it indicates that intestinal epithelial cells may be unusually sensitive to the biological effects of IFN-γ, suggesting that this cytokine may be involved in remodelling the intestine under many physiological and pathological conditions. As the pathology of intestinal GVHR is similar to that found in several, naturally occurring enteropathies (Ferguson & Mowat, 1980; Mowat, 1984; Mowat & Felstein, 1989), IFN-γ-dependent mechanisms may also be responsible for the crypt hyperplasia and villus atrophy found in these conditions. Intestinal damage due to GVHR disease also remains an important complication of allogeneic bone marrow transplantation (Slavin & Woodruff, 1976) and it would be of interest to examine for IFN-γ production in the intestine of patients with all these disorders.

The second implication of our work is that therapy with anti-IFN-γ antibodies may ultimately prove to be a more selective treatment for immunologically mediated diseases which currently require agents with generalized immunosuppressive effects. Interestingly, others using anti-IFN-γ antibodies to study alloreactivity and autoimmunity in mice have suggested that positive effects of IFN-γ on local immunopathology may be associated with an ability to inhibit systemic inflammatory responses (Landolfo *et al.*, 1985; Billau *et al.*, 1988). This apparently paradoxical effect obviously requires further investigation. Nevertheless, if our findings that anti-IFN-γ prevented enteropathy but had few effects on systemic immune responsiveness in GVHR can be extended to other forms of immunopathology, this approach could provide a potent and specific immunotherapy for many important disorders.

## ACKNOWLEDGMENT

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