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Experimental studies of immunologically mediated enteropathy

II. ROLE OF NATURAL KILLER CELLS IN THE INTESTINAL PHASE OF MURINE GRAFT-VERSUS-HOST REACTION

A. McI. MOWAT & M. V. FELSTEIN *Department of Bacteriology and Immunology, Western Infirmary, Glasgow*

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SUMMARY

This study has investigated whether natural killer (NK) cells play a protective or an effector role in unirradiated mice with graft-versus-host reaction (GvHR). Treatment of (CBA × BALB/c)F_1 mice with anti-asialo G_{M1} (AsG_{M1}) antibody produced a profound depletion of resting NK-cell activity and also inhibited the normal enhancement of NK activity found after induction of a GvHR with CBA spleen cells. Compared with normal hosts, mice treated with anti-AsG_{M1} developed less splenomegaly in GvHR and did not show the crypt hyperplasia normally found in this model of GvHR. Anti-AsG_{M1} also produced a small but significant reduction of intraepithelial lymphocyte (IEL) numbers in the jejunum of control mice. We conclude that intestinal NK cells are an essential component of the local delayed-type hypersensitivity (DTH) reaction which is responsible for the intestinal phase of GvHR in unirradiated mice.

INTRODUCTION

Graft-versus-host reactions in experimental animals provide useful models of immunologically mediated tissue damage (Gleichmann *et al.*, 1984; Bril & Benner, 1985), and we have used the intestinal phase of murine GvHR to investigate the pathogenesis of enteropathies with an immune basis that occur in man and domestic animals. These studies show that a GvHR in unirradiated (CBA × BALB/c)F_1 mice causes a proliferative enteropathy which reproduces the crypt hyperplasia and increased numbers of intraepithelial lymphocytes found in clinical disease (Mowat & Ferguson, 1981, 1982). However, this model does not produce the villus atrophy that is also typical of naturally occurring enteropathies. In addition, although we have shown that the intestinal phase of GvHR is induced by helper/inducer T cells (Mowat, Borland & Parrott, 1986), the pathogenic effector cells themselves have not been identified.

A characteristic feature of GvHR in unirradiated F_1 mice is generalized augmentation of NK-cell activity (Borland, Mowat & Parrott, 1983; Roy *et al.*, 1983; Kubota, Ishikawa & Saito, 1983) and we considered that investigating the role of NK cells in GvHR might help in understanding the intestinal pathology in two possible ways. Firstly, the enhanced NK activity by intestinal lymphocytes parallels the other features of enteropathy (Borland *et al.*, 1983) and is preceded by an anti-host delayed-type hypersensitivity (DTH) reaction (Mowat, Borland & Parrott, 1985). Thus, NK cells may conceivably act as pathogenic effectors in GvHR. Alternatively, recruitment of host NK cells in GvHR may reflect a similar mechanism to the NK-cell mediated resistance that F_1 animals show to parental bone marrow (Kiessling *et al.*, 1977; Dennert, Anderson & Warner, 1985). Therefore, NK-cell activation could be one reason why unirradiated F_1 mice with GvHR do not develop destructive tissue pathology that includes villus atrophy. In this study, we have examined whether NK cells play a protective or effector role in GvHR by using hosts that have been depleted of NK cells by *in vivo* treatment with anti-asialo G_{M1} antibody.

Correspondence: Dr A. McI. Mowat, Dept. of Bacteriology and Immunology, Western Infirmary, Glasgow G11 6NT, U.K.

MATERIALS AND METHODS

Mice

CBA (H-2^k), BALB/c (H-2^d) and (CBA × BALB/c)F_1 mice were bred and maintained in the department.

Treatment of mice with anti-asialo G_{M1} antiserum

Mice were injected intravenously with 0·2 ml rabbit anti-asialo G_{M1} antiserum (a kind gift from Dr N. Hanna, SK & F Research, Philadelphia, PA) diluted 1:50 in RPMI-1640 (Gibco Biocult, Paisley, Renfrewshire) 3 days before the induction of GvHR and at 3–4-day intervals thereafter (Charley *et al.*, 1983). Control mice were injected with 1:50 normal rabbit serum (NRS).

Induction of graft-versus-host reaction

Spleen cell suspensions were obtained from CBA mice by gently mincing spleens through a fine wire-mesh filter and washed three times in RPMI-1640 before counting and assessing viability by

phase-contrast microscopy. Viable spleen cells (6×10^7) were then injected intraperitoneally into recipient mice, while control mice received 0·2 ml RPMI-1640 i.p. only. The intensity of the GvHR was assessed by calculating the spleen index as follows:

$$\text{Spleen index} = \frac{\text{relative spleen weight in GvHR (mg/10 g body weight)}}{\text{mean control relative spleen weight}}.$$

Measurement of specific and natural cytotoxicity

Specific anti-host CTL activity was measured using P815 (H-2^d) mastocytoma cells, and NK-cell activity was measured using YAC-1 cells as described previously (Borland *et al.*, 1983). Spleen cells from GvHR and control mice were assayed against target cells labelled with ^{51}chromium at 50:1, 25:1 and 12·5:1 effector: target cell ratios in microcytoxicity assays. The percentage cytoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100\%.$$

Triton-X (10%; Sigma, Poole, Dorset) was used to obtain maximum release, while spontaneous release was measured using appropriate numbers of thymocytes from control F_1 mice.

Measurements of mucosal architecture

Villus and crypt lengths and crypt cell production rates were measured by microdissection of Feulgen-stained (Schiff Reagent; Difco, West Molesey, Surrey) jejunum as described previously (Mowat & Ferguson, 1981). Mice were killed at intervals of 20–100 min after the injection of 7·5 mg/kg colchicine (Sigma) i.p. to cause metaphase arrest, and pieces of jejunum were taken 10 cm from the pylorus. The CCPR was calculated from the slope of the regression line of metaphase accumulation against time. In each specimen 10 villi and crypts were measured. Intraepithelial lymphocytes were counted on adjacent sections of jejunum, stained with haematoxylin and eosin, and are expressed as IEL/100 epithelial cells.

Induction of allospecific cytotoxic T cells and delayed-type hypersensitivity

In order to induce H-2^d-specific CTL, CBA mice were immunized intraperitoneally with 10^7 P815 tumour cells 11 days before assaying spleen cells against P815 targets *in vitro*. H-2^d-specific DTH was induced by immunizing CBA mice with 10^7 (CBA × BALB/c)F_1 spleen cells intradermally into one footpad. Seven days later, DTH responses were assessed by measuring the increment in footpad thickness 24 hr after intradermal challenge with 10^7 F_1 spleen cells. Specific reactivity was calculated by subtracting the footpad response of unimmunized CBA mice to challenge with F_1 cells.

Statistics

Groups of means and standard deviations were compared by Student's *t*-test, while crypt cell production rates were compared by covariance analysis.

RESULTS

Progress of systemic GvHR in anti-asialo-treated mice

The effects of anti-asialo G_{M1} on GvHR were studied in four separate experiments, and no GvHR mice showed any clinical evidence of weight loss, skin disease or diarrhoea. The progress of the systemic GvHR was assessed by following the development of splenomegaly and the results of one representative experiment are shown in Fig. 1.

Peak splenomegaly was found in normal hosts with GvHR on Day 11 and, although anti-asialo-treated mice showed a similar time-course of GvHR, these animals had significantly less splenomegaly at all times (Fig. 1).

Specific and non-specific cytotoxicity in GvHR

In these studies, normal hosts with GvHR had enhanced spleen NK-cell activity compared with appropriate controls, and this paralleled the development of splenomegaly. As this confirms our previous findings in (CBA × BALB/c)F_1 mice with GvHR (Borland *et al.*, 1983), for simplicity, only the results pertaining to the mice used to study intestinal GvHR on Day 11 are shown here.

As noted above, at this time, NRS-treated mice with GvHR had enhanced spleen NK activity compared with controls (Fig. 2), while the efficacy of the anti-asialo G_{M1} treatment protocol was substantiated by the markedly reduced NK activity found in control mice treated with the antibody. Induction of a GvHR in these mice also produced an increase in NK activity, but this only attained the levels found in normal (CBA × BALB/c)F_1 mice without GvHR. Similar results were found throughout the GvHR in anti-asialo-treated mice, and specific anti-host CTL activity was never found in any GvHR mouse (results not shown).

Intestinal phase of GvHR

The intestinal phase of GvHR was assessed by counts of IEL and by measurements of mucosal architecture around the peak of GvHR on Day 11.

As anticipated, induction of a GvHR in normal (CBA × BALB/c)F_1 produced a significant increase in IEL count, compared with controls (Fig. 3), while control mice given anti-asialo G_{M1} had a slight, but significant, reduction in IEL counts compared with normal animals (10·2 ± 2·1 vs 13·5 ± 1·7,

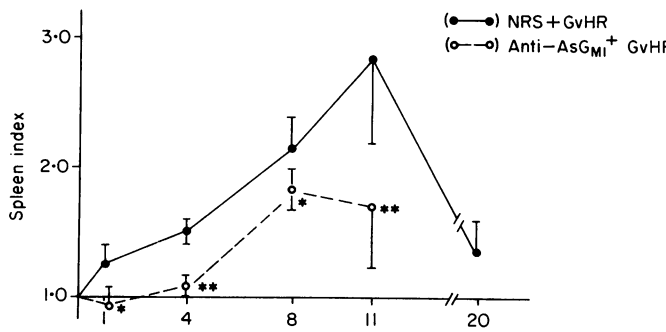

Figure 1. Effect of anti-asialo G_{M1} antibody on the evolution of systemic GvHR in unirradiated (CBA × BALB/c)F_1 mice. Development of splenomegaly at intervals after injection of 6×10^7 CBA spleen cells i.p. in hosts treated every 3–4 days with 0·2 ml NRS or 1:50 rabbit anti-AsG$_{M1}$ i.v. Results are mean spleen index ± 1 standard deviation for three to four mice/group (*$P < 0.05$, **$P < 0.01$ vs NRS GvHR).

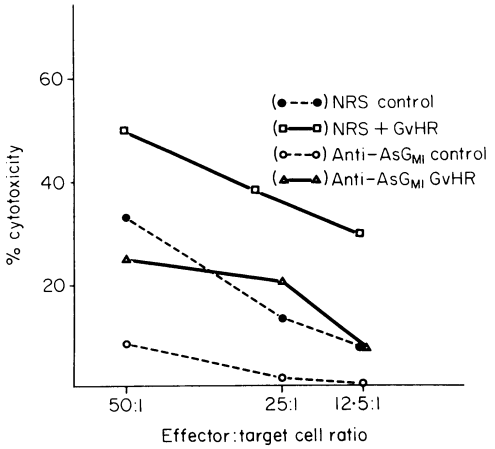

Figure 2. Effect of anti-asialo G_{M1} on NK-cell activity during a GvHR in unirradiated (CBA × BALB/c)F_1 mice. Spleen NK-cell activity against YAC-1 targets in NRS- or anti-AsG_{M1}-treated mice on Day 11 of the GvHR, and in appropriate controls. Results shown are the percentage lysis obtained at 50:1, 25:1 and 12·5:1 E:T ratios using cells pooled from three to four mice/group.

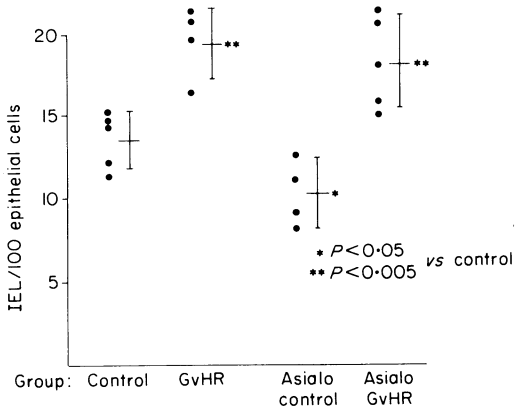

Figure 3. Effect of anti-asialo G_{M1} on the intestinal phase of GvHR in unirradiated (CBA × BALB/c)F_1 mice. Intraepithelial lymphocyte counts in the jejunum of NRS- or anti-AsG_{M1}-treated mice on Day 11 of the GvHR, and in appropriate controls. Results shown are the mean numbers of IEL/100 epithelial cells ± 1 standard deviation for four to five mice/group.

P<0·05). Nevertheless, induction of a GvHR in anti-asialo-treated mice still led to an increase in IEL count, and these mice had IEL counts approaching those found in normal mice with GvHR (18·0 ± 2·8 and 19·4 ± 2·2, respectively).

NRS-treated (CBA × BALB/c)F_1 mice with GvHR also had significant increases in both CCPR (Fig. 4) (22·9 ± 4·5 vs 10·3 ± 1·1, P<0·025) and in crypt length (141·9 ± 5·8 μm vs 111·1 ± 5·7 μm, P<0·001) compared with controls. As we have shown previously (Mowat & Ferguson, 1981), villus atrophy was not a feature of this form of GvHR. In contrast to these findings, anti-asialo-treated mice with GvHR had CCPR and crypt lengths that were identical to those in appropriate controls. Again, no villus atrophy was found. Interestingly, both groups of anti-asialo-treated mice had significantly longer crypts (126·6 ± 3·8 μm in controls; 122·3 ± 8·5 μm in GvHR, p<0·025 vs NRS controls) than normal controls, as well as a slightly increased CCPR.

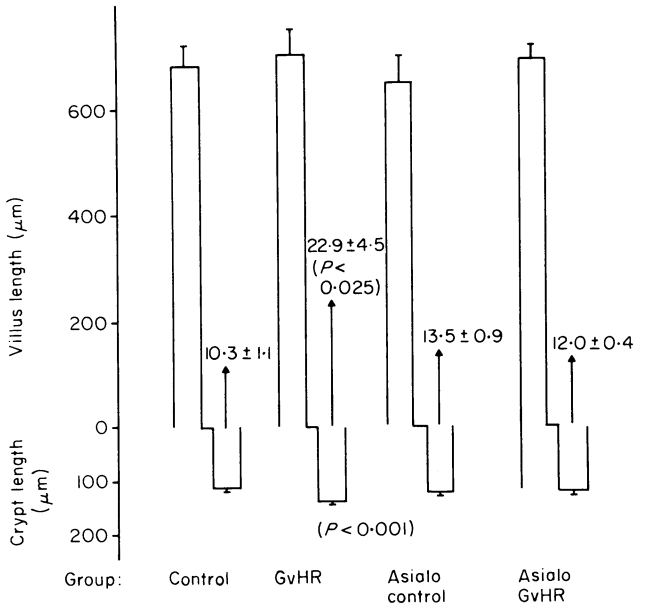

Figure 4. Effect of anti-asialo G_{M1} on the intestinal phase of GvHR in unirradiated (CBA × BALB/c)F_1 mice. Mucosal architecture in the jejunum of NRS- (control) or anti-AsG_{M1}-treated mice on Day 11 of the GvHR, and in appropriate controls. Bars represent mean villus and crypt lengths ± 1 standard deviation, and arrows show CCPR for four to five mice/group.

Thus, treatment of mice with anti-asialo G_{M1} does not prevent the increased infiltration of IEL that occurs during GvHR, but abolishes the crypt hyperplasia that usually characterizes the intestinal lesions.

Effects of anti-asialo G_{M1} on specific CTL and DTH activity

Although repeated injections of anti-asialo G_{M1} produced clear depression of NK activity *in vivo*, it was also necessary to assess its effects on other indices of cellular immunity. CBA mice treated with anti-asialo G_{M1} before and during immunization with P815 cells had a reduced ability to generate specific CTL compared with control mice (Fig. 5a). However, similar treatment with anti-asialo G_{M1} had no effect on the ability of intradermally immunized mice to generate specific DTH responses to (CBA × BALB/c)F_1 spleen cells (Fig. 5b).

DISCUSSION

The results presented here show that depletion of NK cells by treating host mice with anti-asialo G_{M1} antibody is associated with a diminished level of systemic GvHR after injection of parental spleen cells. Furthermore, anti-asialo-treated mice do not show the crypt hyperplasia that normally characterizes the intestinal phase of GvHR in unirradiated mice, indicating that NK cells may act as effector cells in the intestinal phase of GvHR in unirradiated mice.

Previous studies had shown that intestinal and peripheral NK-cell activity was enhanced in unirradiated mice with GvHR (Borland *et al.*, 1983; Roy *et al.*, 1983; Kubota *et al.*, 1983). Therefore, the aim of the present investigation was to determine whether these NK cells were recruited as non-specific effectors in

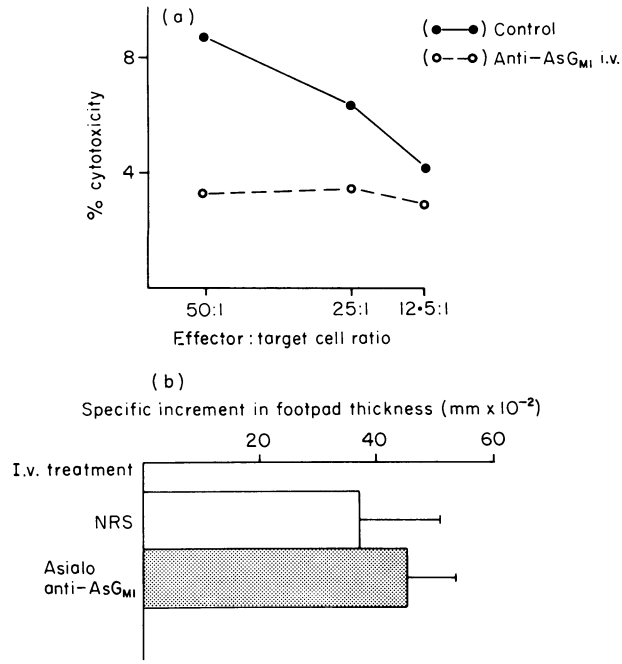

Figure 5. Effect of anti-asialo G_{M1} on allospecific CTL and DTH responses. (a) Specific anti-H-2^d CTL activity in spleens of NRS- or anti-AsG_{M1}-treated mice, 11 days after immunization with 10^7 P815 (H-2^d) tumour cells i.p. Results are the percentage lysis of P815 targets at 50:1, 25:1 and 12·5:1 E:T ratios, using spleen cells pooled from three mice/group. (b) Anti-H-2^d specific DTH responses in NRS- or anti-AsG_{M1}-treated CBA mice, measured 7 days after immunization with (CBA × BALB/c)F_1 spleen cells. Results shown are mean specific increments in footpad thickness ± 1 standard deviation, 24 hr after intradermal challenge with 10^7 F_1 spleen cells for six mice/group.

GvHR, or were mediating a form of F_1 hybrid resistance against the parental donor cells (Kiessling *et al.*, 1977; Dennert *et al.*, 1985).

In this report, it was confirmed that repeated injections of anti-AsG_{M1} antibody leads to profound depletion of resting NK-cell activity (Charley *et al.*, 1983; Habu *et al.*, 1981; Stitz *et al.*, 1986). In addition, we showed that although a GvHR produces some increase in NK activity in anti-asialo-treated mice, the levels do not approach those found in untreated mice. Furthermore, in comparison to normal F_1 mice, anti-asialo-treated mice with GvHR had consistently less splenomegaly and did not show the increases in crypt length and CCPR that characterize the intestinal lesions in this model of GvHR. These findings extend an earlier report that anti-AsG_{M1} treatment prevents the development of systemic, lethal GvHR in irradiated mice (Charley *et al.*, 1983), and we conclude that, under normal circumstances, AsG_{M1}^+ cells contribute to the lymphoid hyperplasia of GvHR in unirradiated mice. In addition, our findings indicate that AsG_{M1}^+ cells are an essential component of the local immune response which causes the intestinal crypt hyperplasia in GvHR.

The lineage of the AsG_{M1}^+ cell implicated in GvHR has not been firmly established, and it is important to note that other lymphoid cells, including T lymphocytes, can express the AsG_{M1} marker (Stein, Schwarting, Marcus, 1978; Nakano *et al.*, 1980; Suttles, Schwarting & Stout, 1986). In addition, the pattern of expression of AsG_{M1} by intestinal lymphoid cells has not been studied in detail. Nevertheless, we consider that our results reflect depletion of NK cells rather than T cells for the following reasons. Firstly, in comparison to T cells, NK cells are more readily eliminated by anti-ASG_{M1} *in vivo* and *in vitro* (Kasai *et al.*, 1980; Suttles *et al.*, 1986; Stitz *et al.*, 1986). Secondly, although the present study confirmed other work that anti-AsG_{M1} inhibits the generation of CTL *in vivo* (Stitz *et al.*, 1986), anti-asialo-treated mice mounted entirely normal DTH responses. These findings are therefore important in view of our previous demonstration that a local DTH response is the principal effector mechanism in the intestinal phase of GvHR in unirradiated (CBA × BALB/c)F_1 mice, and that CTL are not involved (Mowat & Ferguson, 1981; Borland *et al.*, 1983; Mowat *et al.*, 1986). The concept that NK cells may act as non-specific effectors of an intestinal DTH response is supported by the fact that the enhanced NK activity by intestinal lymphocytes parallels the other features of intestinal pathology in GvHR (Borland *et al.*, 1983) and is immediately preceded by a systemic anti-host DTH response (Mowat *et al.*, 1985). Furthermore, purified NK cells can produce a variety of lymphokines *in vitro* (Handa *et al.*, 1983; Kasahara *et al.*, 1983), and we propose that non-specifically recruited NK cells may contribute to the mucosal lesions of GvHR by releasing some of the lymphokines thought to be critical for the pathogenesis of intestinal GvHR (Elson, Reilly & Rosenberg, 1977; Mowat & Ferguson, 1981). However, this hypothesis needs to be confirmed by examining a GvHR in animals depleted of NK cells by monospecific reagents such as anti-NK1 (Hackett *et al.*, 1986).

Although this study did not identify the AsG_{M1}^+ cell responsible for the mucosal alterations, it is tempting to speculate that IEL are important for this phenomenon. A small proportion of IEL carry the AsG_{M1} marker (Carman *et al.*, 1986), and mice treated with anti-AsG_{M1} *in vivo* had a significant depletion of IEL as well as a defective mucosal response to GvHR. During the GvHR, there is a parallel increase in the number and activity of IEL (Borland *et al.*, 1983), and IEL exhibit a higher mitotic index than normal (K. Ziegler and A. Ferguson, personal communication). IEL have been shown to transfer specific DTH *in vivo* (Shields & Parrott, 1985) and can produce lymphokines *in vitro* (Dillon & MacDonald, 1986; Wilson, Stokes & Bourne, 1986) and, together, these findings support the idea that activation of IEL in GvHR may contribute to the local DTH reaction in the mucosa.

In conclusion, the present study indicates that NK cells do not protect the host from the pathological consequences of a GvHR, but may be an important non-specific component of immunological tissue damage. This concept is supported by the presence of NK-cell infiltrates in rejecting allografts (Nemlander, Saksela & Häyry, 1986) and by the fact that depletion of NK cells inhibits the induction of autoimmune diabetes in rats (Like *et al.*, 1986). Nevertheless, others have suggested that removal of NK cells does not alter a lethal GvHR across class II MHC differences in irradiated mice (Korngold & Sprent, 1985), and further studies are required to elucidate the exact role of NK cells in immunologically mediated tissue pathology.

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