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Experimental studies of immunologically mediated enteropathy: IV. Correlation between immune effector mechanisms and type of enteropathy during a GvHR in neonatal mice of different ages

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SUMMARY

We have used the intestinal phase of the graft-versus-host-reaction (GvHR) in unirradiated F_1 mice as a model for enteropathy due to cell-mediated immunity (CMI). Injection of neonatal (CBA × BALB/c)F_1 mice less than 48 h old with CBA spleen cells produced an acute GvHR, which was associated with runting and severe intestinal damage, characterized by villus atrophy. These animals developed specific cytotoxic T lymphocyte (CTL) activity and invariably died. In contrast, 7-day-old F_1 mice with GvHR developed a proliferative GvHR, characterized by intense splenomegaly, NK cell activation and intestinal crypt hyperplasia. These mice did not lose weight, had no villus atrophy or CTL activity and all recovered. A similar proliferative phase was also found to precede the established GvHR in 1–2-day-old hosts. Induction of a GvHR in 5-day-old hosts produced a disease with some characteristics of both proliferative and destructive GvHR, with some mice developing weight loss and villus atrophy, while others showed only crypt hyperplasia and NK cell activation. However, there was very little specific CTL activity in any of these animals. These results indicate that markedly different forms of GvHR can be induced in mice during the first week of life and that these are associated with different pathological effects. Although the immunological mechanisms which are activated may also differ between the types of GvHR, our findings support the hypothesis that intestinal damage which includes villus atrophy is merely a progressive form of the delayed type hypersensitivity responsible for a proliferative enteropathy.

Keywords enteropathy graft-versus-host reaction neonatal mice

INTRODUCTION

Graft-versus-host reactions (GvHR) in experimental animals provide a useful model of immunologically mediated tissue damage. Of particular note the intestinal lesions in murine GvHR have a pattern similar to that found in clinical conditions such as food sensitive enteropathies (FSE) and parasite infestations (Ferguson & Jarrett, 1975; Mowat, 1984), which include crypt hyperplasia and lymphocytic infiltration of the epithelium (Mowat & Ferguson, 1981; Mowat, 1984). However, our previous studies using the GvHR in unirradiated F_1 hybrid mice have not reproduced the villus atrophy which typifies the clinical disorders. Therefore we have sought models of GvHR which reproduce this form of mucosal pathology.

An acute GvHR in irradiated mice results in villus atrophy (Mowat *et al.*, 1987b), but investigation of the immunological mechanisms involved may be complicated by the additional effects which irradiation has on the gut. Severe intestinal damage also occurs during a GvHR in immature animals (MacDonald & Ferguson, 1977; Guy-Grand & Vassalli, 1986; Reilly & Kirsner, 1965)), but the immunopathogenesis of this model of enteropathy has not been studied in detail. Furthermore, interpretation of earlier studies is complicated by the fact that the degree of intestinal damage in neonatal mice with GvHR appears to vary with the age of the host animal which is used (MacDonald & Ferguson, 1977; Mowat & Ferguson, 1982). Nevertheless this difficulty also suggests that a GvHR in neonatal mice of different ages might provide a means of examining different forms of enteropathy in the same donor-host combination. In this way, it may be possible to examine whether different immune effector mechanisms are associated with different forms of intestinal pathology.

Therefore, in this report, we have examined the nature of the intestinal pathology which occurred after induction of a GvHR in (CBA × BALB/c)F_1 mice at different times during the first

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week of life, in parallel with assessments of clinical GvHD and the development of specific and non-specific cell mediated cytotoxicity *in vitro*.

MATERIALS AND METHODS

Animals

CBA (H-2^k) and BALB/c (H-2^d) mice were obtained from departmental stocks and (CBA × BALB/c)F_1 mice were bred from CBA female and BALB/c male mice.

Induction of graft-versus-host-reaction

A GvHR was induced in newborn (CBA × BALB/c)F_1 mice either within the first 48 h of life or at 5 days or 7 days of age by injection of 10^7 CBA spleen cells in 0·05 ml RPMI 1640 (Gibco Biocult) intraperitoneally (i.p.). Controls were uninjected littermates.

Assessment of systemic GvHR

Clinical runting was assessed by weighing all the mice daily, while the intensity of the proliferative GvHR was assessed by the development of splenomegaly (Simonsen, 1962).

Measurement of specific and non-specific cytotoxicity

Natural and specific cytotoxic activity of F_1 spleen cells from GvHR and control mice was assayed in a microcytotoxicity assay against ^{51}Cr-labelled YAC-1 and P815 (H-2^d) target cells, respectively, as described elsewhere (Davies & Parrott, 1980; Borland, Mowat & Parrott, 1983). All assays were performed in quadruplicate and variation between wells was normally $< 15\%$.

Assessment of mucosal architecture

Villus lengths, crypt lengths and the crypt cell production rate (CCPR) were measured on samples of jejunum using a strathmokinetic technique employing colchicine and microdissection as described previously (MacDonald & Ferguson, 1977; Mowat & Ferguson, 1981).

Statistics

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

RESULTS

The relationship between intestinal pathology and immune effector mechanisms was examined by inducing a GvHR in (CBA × BALB/c)F_1 mice aged 48 h or less, or on the 5th or 7th day of life. Up to four individual experiments were conducted on these three groups of neonatal mice with essentially identical findings within each group. Therefore, for simplicity, only the results of one typical experiment from each series are presented.

Progress of systemic GvHR in neonatal (CBA × BALB/c)F_1 mice

(CBA × BALB/c)F_1 mice given 10^7 CBA spleen cells before 48 h of age developed a classical, acute GvHR, which was characterized by runting, diarrhoea and ruffled fur. GvHR mice began to lose weight rapidly after day 8 and, in most experiments, all died within 21 days of introducing the GvHR (Fig. 1a). These mice with GvHR had significant splenomegaly from day 8 until day

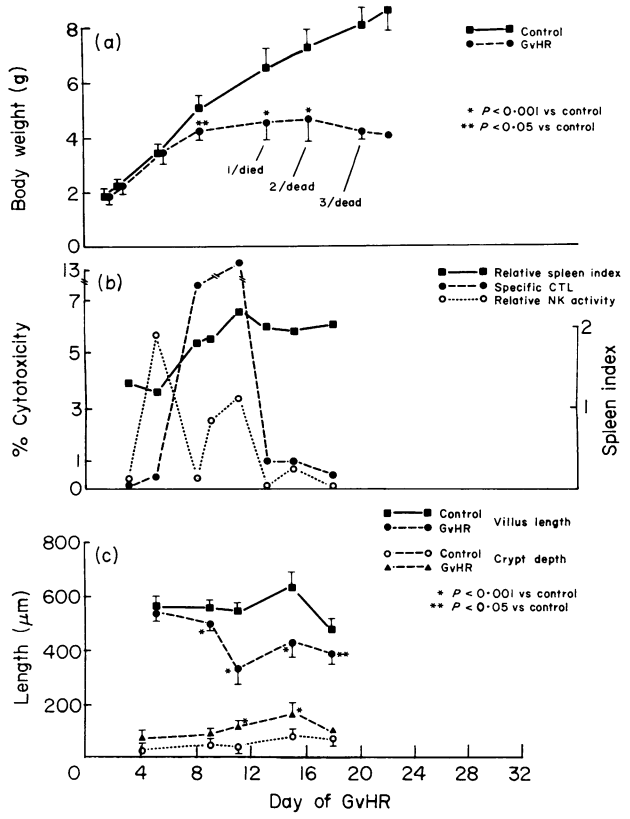

Fig. 1. Development of graft-versus-host reaction in 1–2-day-old neonatal (CBA × BALB/c)F_2 mice. (a) rate of growth in neonates with GvHR compared with littermate controls (b) spleen index, and natural killer cell and specific cytotoxic T lymphocyte activity against YAC-1 and P815 target cells, respectively, at intervals after induction of GvHR. (c) Mucosal architecture in mice with GvHR and in controls, assessed by villus and crypt lengths at intervals after the induction of GvHR. Results shown are the means (± 1 s.d.) for three mice/group, while cytotoxicity results are shown as the % lysis at 50:1 E:T ratio for spleen cells pooled from three mice/group.

18 (Fig. 1b) and this was particularly notable in view of the marked weight loss which occurred at the later stages of the GvHR.

In contrast to these findings, mice injected with parental spleen cells at 7 days of age did not develop a lethal GvHR and had no evidence of clinical GvHR. There was also no evidence of weight loss (Fig. 2a). However, these GvHR mice developed intense splenomegaly which first appeared on day 9 with a maximum spleen index of 3·4 by day 12 and falling to 1·3 by 30 days (Fig. 2b).

The GvHR in 5-day-old mice was a more variable disease, although most of these neonates with GvHR developed normally until day 14 with little or no evidence of weight loss compared with controls (Fig. 3a). Thereafter, some animals showed evidence of clinical runting, weight loss and diarrhoea and a small proportion of these mice died, usually between days 17 and 24 of GvHR. Nevertheless, other mice in the same group continued to grow normally. Five-day-old mice with GvHR developed moderate splenomegaly which peaked around day 13 (Fig. 3b). Interestingly, by day 18, mice which developed signs of runting had less splenomegaly than their non-runting littermates with GvHR. This was more apparent on day 24, when

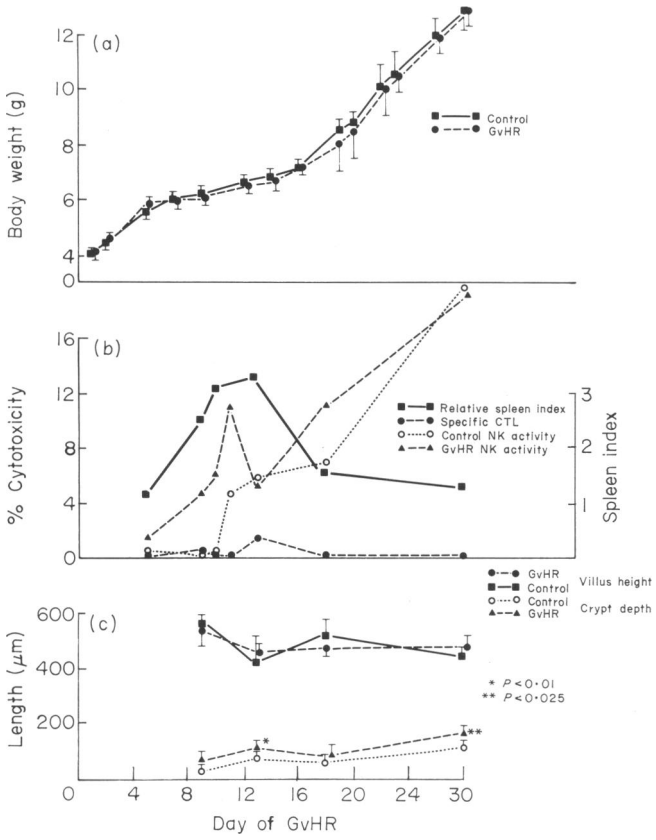

Fig. 2. Development of graft-versus-host reactions in 7-day-old (CBA-×BALB/c)F_1 mice. (a) Rate of growth in neonates with GvHR compared with littermate controls. (B) Spleen index, and natural killer cell and specific cytotoxic T lymphocyte activity against YAC-1 and P815 target cells, respectively, at intervals after induction of GvHR. (c) Mucosal architecture in mice with GvHR and in controls, assessed by villus and crypt lengths at intervals after the induction of GvHR. Results shown are the means (+1 s.d.) for three mice/group, while cytotoxicity results are shown as the % lysis at 50:1 E:T ratio for spleen cells pooled from three mice/group.

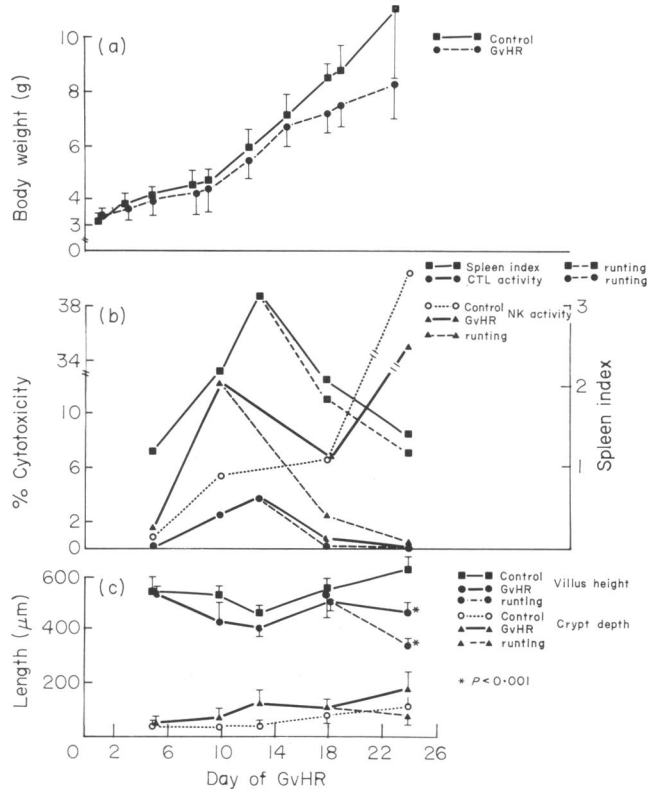

Fig. 3. Development of graft-versus-host reaction in 5-day-old (CBA-×BALB/c)F_1 mice. (a) Rate of growth in neonates with GvHR compared with littermate controls. (b) Spleen index, and natural killer cell and specific cytotoxic T lymphocyte activity against YAC-1 and P815 target cells, respectively, at intervals after induction of GvHR. (c) Mucosal architecture in mice with GvHR and in controls, assessed by villus and crypt lengths at intervals after the induction of GvHR. Results shown are the means (+1 s.d.) for three mice/group, while cytotoxicity results are shown as the % lysis at 50:1 E:T ratio for spleen cells pooled from three mice/group.

runting mice usually had spleen indices < 1·3 compared with 1·4 in non-runting littermates. After day 18 in these experiments, samples from mice which lost weight were processed separately from those of non-runting littermates and the results are presented separately.

We next investigated whether these differences in systemic GvHR in mice of different ages reflected different immune effector mechanisms.

Development of specific and non-specific cytotoxicity

Specific anti-host CTL activity and NK cell activity were chosen as immune parameters, as we had shown previously that these features reflected distinct forms of GvHR-associated immunity (Borland *et al.*, 1983; Mowat *et al.*, 1987a). Both activities were measured at intervals during at least four experiments with essentially identical results. Once again, only one representative series of results for each group is shown.

In the experiment using 1–2-day-old mice, control animals showed little or no splenic NK activity throughout the course of the study. In contrast, mice with GvHR had a small peak of NK activity on day 5, which declined over the next few days, although detectable levels were present until day 11 (Fig. 1b). Although these levels were very low, NK activity is normally not detectable in mice of this age (Murgita & Wigzell, 1981; Koo, Peppard & Hatzfield, 1982), and therefore any cytotoxicity above background must be considered significant. Furthermore, identical results were obtained on each occasion these experiments were performed (data not shown). Coinciding with this loss of NK activity, specific anti-host cytotoxicity appeared at 8–9 days and increased rapidly to a peak of 13·5% by day 11 (Fig. 1b). Thereafter, as the clinical condition of the GvHR mice deteriorated in the third week, only very low levels of specific CTL activity were found.

In the studies of GvHR induced in 7-day-old mice, spleen NK activity appeared in controls by day 10–11 (17–18 days of age) and continued to increase steadily thereafter (Fig. 2b). By day 5, mice with GvHR already had detectable NK activity and had consistently higher NK activity than controls until day 18. By day 30, adult levels of NK activity had developed in both the GvHR and control mice. Little or no specific CTL activity could be detected at any time (Fig. 2b).

When 5-day-old mice were used as hosts for GvHR, NK activity appeared in controls around days 10–11, while in mice

Table 1. Crypt cell production in the jejunum of (CBA × BALB/c)F_1 mice with GvHR, and in littermate controls (means ± 1 s.d. for four mice per group were used in each experiment)

		Crypt cell production rate	
Age of host (days)	Day of GvHR	Control	GvHR
1–2	5	4·2+0·5	7·5+1·2
	9	8·8+4·1	4·4+1·3
	15	12·6+2·5	6·2+4·0
5	5	0·7+0·4	5·3+0·6‡
	10	3·3+1·1	7·8+1·7
	24	9·5+1·7	35·0+0†
7	9	3·2+1·0	8·7+0·9‡
	18	7·0+1·5	13·2+1·6*

* $P<0·05$.
† $P<0·01$.
‡ $P<0·02$.

with GvHR, some NK activity was detectable by day 5 and was markedly increased compared with controls by day 10 (Fig. 3b 12·2% vs 5·4%). Thereafter, the pattern of NK activity in GvHR mice depended on whether mice developed overt runting. Thus, NK activity in GvHR mice which did not lose weight developed in parallel with that in controls (Fig. 3b), while in mice which runted, NK activity became less than controls (day 18: 2·5% vs 6·6%), and eventually disappeared completely. Although small, these differences were found in three similar experiments (data not shown). Interestingly, only a low level of specific CTL activity was present on day 10 of the GvHR and was never found thereafter.

Thus the development of acute lethal GvHR in 1–2-day-old mice is associated with a transient enhancement of NK activity, followed by the development of marked specific CTL activity which parallels the onset of clinical disease. In contrast, 7-day-old mice with proliferative GvHR have prolonged activation of NK cell activity, but no specific CTL are detectable. The intermediate form of GvHR in 5-day-old mice showed some features in common with both acute and proliferative GvHR.

Mucosal phase of GvHR

Intestinal GvHR in 1–2-day-old hosts. Villus atrophy was a consistent feature of the GvHR in 1–2-day-old neonates and was already present when the mice first began to runt on day 9 (493·4 ± 13 μm vs 563·3 ± 11 μm for controls, $P<0·005$) (Fig. 1c). This became even more marked by day 15, when all the mice with GvHR were extremely ill and had significant CTL activity. At both times, mice with GvHR had significantly longer crypts than controls (90·6 ± 6 μm vs 57·2 ± 6 μm, $P<0·001$ on day 9 and 162·5 ± 35 μm vs 80·5 ± 6 μm, $P<0·05$ on day 15). In parallel with these alterations, mice with GvHR had an increased crypt cell mitotic activity at the earliest time examined (Table 1) but thereafter GvHR mice had levels of CCPR which were roughly half those in control mice.

Intestinal GvHR in 7-day-old hosts. No evidence of villus atrophy was found at any time during this GvHR (Fig. 2c). However, these mice had significant crypt lengthening compared with controls from day 9 (Fig. 2c) (85·6 ± 11 μm vs 44·8 ± 3·8 μm for controls, $P<0·01$) until day 30 (152·5 ± 16 μm vs 113·9 ± 9 μm for controls, $P<0·02$). In addition, these animals had a significantly enhanced CCPR compared with controls during the same period (Table 1).

Intestinal GvHR in 5-day-old hosts. As before, this group of mice developed a form of intestinal GvHR which had features in common with both the proliferative and destructive enteropathies described above. On day 13, the fact that some GvHR animals would runt was already reflected in a slight overall decrease in villus length compared with controls (Fig. 3c) (400·6 ± 27 μm vs 466·6 ± 11·5 μm, $P<0·05$). Thereafter, mice which were runting developed progressive villus atrophy which became extremely marked by day 24 (346 ± 11 μm vs 625·4 ± 44 μm for controls, $P<0·001$). Mice which did not runt had no evidence of villus atrophy until day 24, when their villus lengths were significantly shorter ($P<0·001$) than controls, although these were still significantly greater than those of their runting littermates (453·0 ± 32 μm vs 346 ± 11 μm, $P<0·02$). All GvHR mice had crypt lengthening on day 13 (Fig. 3c) and this was maintained in non-runting mice until day 24 (174·1 ± 62 μm vs 133·2 ± 31 μm for controls). In contrast, on day 18, crypt lengths in runting mice were no greater than those of controls and by day 24, crypt lengths in runting mice were shorter than control values (93·6 ± 5 μm vs 133·2 + 31 μm for controls). In parallel, all mice with GvHR had an increased CCPR on days 5 and 10 (Table 1) and, in non-runting mice, this became even more marked by day 24. At this time crypt cell mitotic activity was absent in runting mice with GvHR.

DISCUSSION

The results presented here show that the type of systemic and intestinal GvHR in neonatal mice depends on the age of the host when the GvHR is induced. Thus, the GvHR in neonatal (CBA × BALB/c)F_1 mice varied from an acute, lethal disease in 1–2-day-old mice, associated with weight loss, specific CTL activity and villus atrophy, to an entirely proliferative disease in older hosts, typified by increased NK activity and crypt hyperplasia. This approach has enabled us to examine all aspects of GvHR-induced intestinal pathology in the same strain of host mice and with the same inoculum of donor lymphoid cells.

Neonatal mice with GvHR induced at 7 days of age developed an entirely proliferative disease with no weight loss and none of the mice died. The proliferative nature of the GvHR was evidenced by increased splenic NK activity, as well as intestinal crypt hyperplasia and no specific anti-host CTL activity could be detected. In addition, none of these mice developed villus atrophy. This pattern of GvHR is identical to that found in adult unirradiated mice and there is evidence that the intestinal damage in this form of GvHR is due to a local DTH response (Elson, Reilly & Rosenberg, 1977; Mowat & Ferguson, 1981; Mowat, 1984).

In sharp contrast, 1-2-day-old mice developed an acute GvHR, with weight loss and runting and this was invariably fatal. The onset of runting was associated with the appearance of specific anti-host CTL and, in the intestine, a reduction in CCPR and crypt depth was accompanied by villus atrophy. However, this destructive GvHR was preceded by an early proliferative phase, characterized by a transient rise in NK activity and an increased CCPR, similar to that found in older mice with GvHR (Borland *et al.*, 1983; Kubota, Ishikawa &

Saito, 1983; Mowat, Borland & Parrott, 1985; and see above). This biphasic pattern is identical to the evolution of GvHR in irradiated mice with GvHR (Mowat *et al.*, 1987b), indicating that a destructive GvHR is not merely an artefact induced by irradiation, but reflects a rapidly progressive immune response in immunologically deficient hosts.

These features are consistent with the hypothesis that a local DTH response may be responsible for the proliferative features of crypt hyperplasia and NK cell activation, but specific CTL cause the more severe forms of tissue destruction. However, our results using 5-day-old neonatal mice, argue against this idea. Mice of this age developed an extremely varied form of GvHR which ranged from an acute, lethal disorder to an entirely proliferative disease. Thus, some of these animals lost weight and eventually died, but others developed normally. Furthermore, while all the mice had an early, proliferative GvHR, with crypt hyperplasia and NK cell activation, this was maintained only in those GvHR mice which did not run, in contrast with mice which runted who developed villus atrophy, decreased crypt cell turnover and crypt shortening. Nevertheless, in contrast to 1–2-day-old mice with similar destructive lesions, very little specific CTL activity could be detected in 5-day-old mice with GvHR, suggesting that the villus atrophy and systemic deterioration are not necessarily due to CTL. This conclusion is also supported by recent results in irradiated mice with GvHR which show that villus atrophy can develop as a bystander phenomenon in intestinal grafts which are syngeneic to the donor CTL (Guy-Grand *et al.*, 1986; Mowat *et al.*, 1987b). Together, these features suggest that a destructive enteropathy is merely a more severe form of the same immune response which is responsible for proliferative pathology and does not reflect a distinct immune mechanism. As noted above, we consider that this is a local DTH response and also suggest that a similar mechanism may also be responsible for the mucosal pathology of clinical FSE. One important aspect of these studies in that they underline how susceptible the young neonate is to a pathogenic intestinal immune response, probably because during the first week of life, immaturity of either the immune system or the intestine allows the local response to progress. The intestinal phase of murine GvHR not only provides an experimental means of dissecting the immunopathogenesis of clinical FSE, but may also help understand why such disorders are more commonly encountered in infancy.

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