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Depletion of Asialo-GM1$^+$ cells from the F_1 recipient mice prior to irradiation and transfusion of parental spleen cells prevents mortality to acute graft-versus-host disease and induction of anti-host specific cytotoxic T cells

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SUMMARY

Graft-versus-host disease (GVHD) was induced in irradiated (750 rad) (CBA × C57BL/6)F_1 hybrid mice by an intravenous injection of 30×10^6 CBA spleen cells and 5×10^6 syngeneic F1 bone marrow cells. The GVHD resulted in the death of 80% of recipients within 9 days. However, when radioresistant Asialo-GM1$^+$ cells were depleted from the recipients with a single injection of anti-Asialo-GM1 antibody 2 days before irradiation and transplantation, mortality decreased significantly (to 11%). During the GVHD, anti-host specific cytotoxic T cell (CTL) activity could be shown *in vitro* in the spleens of mice suffering from the GVHD if suppressor activity was first abolished by *in vitro* culture procedures. This CTL activity, however, was not detectable in the spleens of anti-ASGM1 antibody pretreated hosts. The results indicate that radioresistant ASGM1$^+$ cells of host origin are necessary for the induction of both anti-host CTL and lethal GVHD.

Keywords graft-versus-host disease anti-asialo-GM1 Asialo-GM1 cytotoxic T cells

INTRODUCTION

Graft-versus-host disease (GVHD) has remained a serious problem after bone marrow transplants in man (van Bekkum, 1985). The cellular interactions leading to this undesired event have been under intense investigation in recent years. Donor-derived mature T cells have been implicated as the effectors of the disease (van Bekkum, 1985), but the exact cellular interactions taking place during the induction phase of GVHD remain unknown. It has been postulated that during acute GVHD cytotoxic T cells are the most important effector cells (Hamilton, Bevan & Parkman, 1981; Kubota, Ishikawa & Saito, 1983), while helper T cells are the effectors of chronic GVHD (Gleichman *et al.*, 1984). Interest has also been focused on the role of natural killer (NK) cells in GVHD. Lopez *et al.* (1980) reported that the natural killer (NK) cell status of the bone marrow recipient before transplant is predictive of post-transplant GVHD occurrence, since patients with normal or high NK activity suffered from GVHD statistically more often than patients with low NK activity. Murine models have also emphasized the importance of NK cells in the pathogenesis of GVHD; Charley *et al.* (1983) reported that depletion of Asialo-GM1$^+$ (ASGM1$^+$) cells (mostly NK cells in mice (Kasai *et al.*, 1980; Young *et al.*, 1980)) from the recipient before and after haemopoietic cell transplant across minor H-2 barrier prevented mortality caused by GVHD. Also accumulation of NK cells in the tissues affected by GVHD (Guillen *et al.*, 1986) and increased NK

activity during GVHD (Roy *et al.*, 1982; Dokhelar *et al.*, 1981; Varkila & Hurme, 1985) have been observed.

To further analyse the role of NK cells in GVHD, the NK active cells from F_1 hybrid mice were removed with anti-ASGM1 antibody treatment and their regeneration inhibited with gamma irradiation. When these NK cell-depleted F_1 mice were inoculated with parental spleen cells their mortality to GVHD was significantly lower than among untreated F_1 hybrids. In this report I also show that specific anti-host CTL can be demonstrated *in vitro* from the spleens of anti-ASGM1 recipients if suppressor cells are depleted or if spleen cells taken from GVHD mice are restimulated *in vitro* with recipient type cells in the presence of exogenous IL-2. This anti-host CTL activity was absent from the spleens of the recipients that were treated with anti-ASGM1 antibody before parental cell transplant.

MATERIALS AND METHODS

Mice. CBA/Ca mice and (CBA/Ca × C57BL/6)F_1 hybrids, which had been reared in conventional conditions, were obtained from the breeding unit of this department and used at the age of 12–16 weeks.

Rabbit anti-Asialo-GM1 antiserum. Rabbit anti-Asialo-GM1 (ganglio-N-tetraosylceramide) antibody, which is highly specific for murine NK cells (Kasai *et al.*, 1980; Young, *et al.*, 1980), was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Three different batches of antiserum were used in these studies and when tested they had the same titres in reducing NK activity *in vitro* and *in vivo*.

In the experiments, 20 μl of antiserum was injected i.v. at the times indicated. This treatment effectively abolished NK activity as described in the text. Also the percentage of ASGM1$^+$ spleen cells measured using a FACS analyser (FACS-IV, Beckton-Dickinson, Sunnyvale, Ca) was less than 1% 2 days after antibody injection compared to 16–20% before.

Cytotoxicity assays. Spleens from mice were removed and teased apart, and the resulting cell suspension was washed once in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland). For natural killer cell assays cells were then resuspended in Eagle's minimum essential medium (Orion Diagnostica, Helsinki, Finland) with 10% FCS and 10 mM Hepes. The cell concentration was adjusted and three doubling dilutions were made. The killer: target ratios used were 80:1, 40:1, 20:1, and 10:1. The various concentrations of killer cells (in 100 μl) were plated in triplicate in round-bottom wells of a microtitre plate and 1×10^4 ^{51}Cr-labelled target cells (in 100 μl) were added per well. The target cells were NK-sensitive lymphoma (YAC-1) cells (Kiessling, Klein & Wigzell, 1975), which were labelled for 60 min with 100 μCi of sodium ^{51}Cr-chromate ($Na_2{}^{51}CrO_4$, 1 mCi/ml; The Radiochemical Centre, Amersham, UK) and then washed twice. The plates were incubated at 37°C in a 5% CO_2 atmosphere for 4 h before harvesting for gamma counting. Maximum (max.) was the amount of ^{51}Cr released from Triton-treated (Triton X-100, 5% v/v, Rohm & Haas, Philadelphia, PA) target cells. Spontaneous release (spont.) was that released by target cells incubated in medium alone. The percentage specific lysis was calculated using the formula:

$$(\text{experimental} - \text{spont.})/(\text{max.} - \text{spont}) \times 100.$$

For assaying the CTL activity in the spleens of experimental mice, cells were collected as described above and then assays were performed using one of the following methods: (1) 5×10^6 spleen cells were cocultured with 2×10^6 (CBA × C57BL/6)F1 spleen cells in 2 ml of RPMI-1640 medium containing 10% FCS with 10 mM Hepes, glutamine, antibiotics and 5×10^{-5} M 2-mercaptoethanol in 24-well tissue culture plates (Tissue culture Multi-well plate, Flow Laboratories). The stimulator cells were irradiated (2000 rad) in order to avoid any possible influence of F_1 anti-parent reactivity (Shearer & Cudkowicz, 1975). After 5 days incubation in a humidified 5% CO_2 atmosphere, the cells were harvested, washed once in RPMI-1640 medium and then resuspended in Eagle's minimal essential medium with 10% FCS and 10 mM Hepes. Various concentrations of attacking cells were then plated in the wells of a microtitre plate, and 2×10^4 ^{51}Cr labelled target cells were added per well. The target cells were (CBA × C57BL/6)F_1 spleen cells that had been cultured 48 h in the

presence of 4 μg/ml concanavalin A (Con A), labelled with ^{51}Na chromate, and then washed twice. The attacker: target cell ratios used were 5:1, 2·5:1, 1·25:1 and 0·6:1. The plates were then spun briefly and incubated at 37°C in a 5% CO_2 atmosphere for 3 h before the supernatants were harvested for gamma counting. The percentage lysis was then calculated as described above. (2) The other CTL assay was a modification of a method described by Wagner *et al.* (1976). Briefly, spleen cells of experimental mice were cultured at a concentration of 4×10^6 cells/ml in RPMI 1640 complete medium in 96-well microtitre plates. Two hundred microlitres were put in each well and 12–24 wells per group were used. Cells were cultured at 37°C in a 5% CO_2 atmosphere without stimulator cells. After 48 h cells were harvested and assayed for CTL activity as described above.

Inducing graft-versus-host disease. (CBA × C57BL/6)F_1 cells were lethally irradiated (750 rad) and allowed to rest for 4 h and then reconstituted with an i.v. injection of 30×10^6 CBA spleen cells and 5×10^6 F_1 bone marrow cells in RPMI-1640. Mice were then maintained under standard conditions with no isolation and observed daily during the observation period.

Production of ConA supernatant. Spleen cells from SDK rats were stimulated *in vitro* for 48 h with 4 μg/ml of Con A (5×10^6 cells/ml in RPMI-1640 medium supplemented as described above). Cells and debris were then removed by centrifugation, and the supernatant was stored at −20°C. Each batch was tested before use for its ability to sustain the growth of the IL-2-dependent T cell line (CTLL-2) (Gillis *et al.*, 1978).

Statistics. Life table analysis was performed as described earlier (Peto *et al.*, 1977). Logrank test was used to test the significance of difference between the survivals of experimental mice.

RESULTS

Protection from lethal GVHD. Graft-versus-host disease was induced by injecting lethally irradiated (750 rad) (CBA × C57BL/6)F_1 mice with 30×10^6 spleen cells together with 5×10^6 syngeneic F_1 bone marrow (BM) cells. This procedure led to a rapid GVH-reaction resulting in 80% mortality within 9 days (Fig. 1). Autopsy revealed intestinal haemorrhage, and blood cultures taken just before death showed Gram-negative bacteraemia (data not shown), which are the main causes of death in GVHD (Rolink *et al.*, 1982). The irradiated, nontransplanted control mice died 12–16 days after irradiation and 100% of the irradiated controls that received only syngeneic BM cells

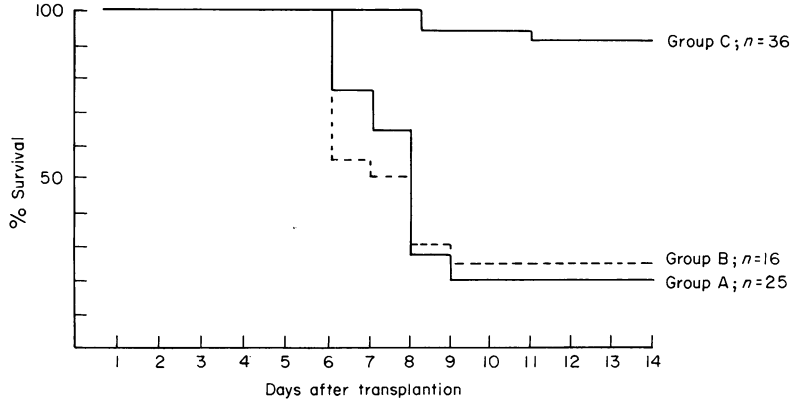

Fig. 1. The lifetable. Experimental mice were divided into three groups. In each group recipient (CBA × C57BL/6)F_1 mice were irradiated (750 rad; day 0). In group A F_1 mice were transplanted with 30×10^6 CBA spleen cells 4 h after irradiation. In group B F_1 mice were transplanted with 30×10^6 spleen cells taken from donors which had been pretreated with anti-ASGM1 antibody (20 μl, on day −2). In group C F_1 recipient mice were pretreated with 20 μl of anti-ASGM1 antibody on day −2 and thereafter they were treated as mice in group A. In each group recipients also received 5×10^6 protective syngeneic bone marrow cells together with parental spleen cells. The data shown in the lifetable is pooled from four different transplantation experiments.

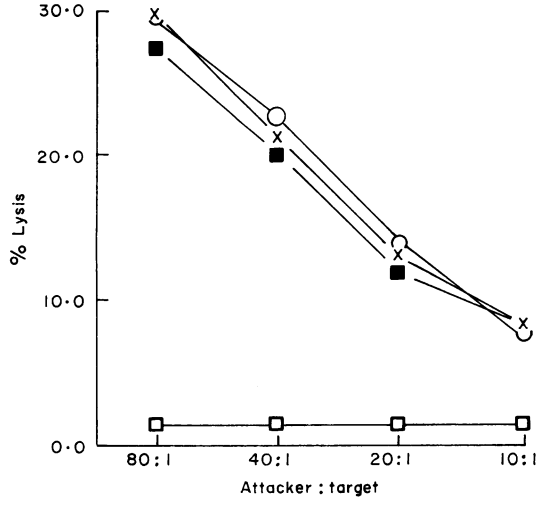

Fig. 2. Natural killer (NK) cell activity against YAC-1 targets in the spleens of the experimental mice. (×) Normal CBA mice; (■) (CBA × C57BL/6)F_1 mice that were irradiated on day 0 (750 rad) and transplanted with 30×10^6 CBA spleen cells; (○) (CBA × C57BL/6)F_1 mice that were irradiated on day 0 and transplanted with 30×10^6 CBA spleen cells taken from donors which had been pretreated with anti-ASGM1 antibody (20 μl, on day −2). (□) (CBA × C57BL/6)F_1 mice that were pretreated with anti-ASGM1 antibody (20 μl, on day −2), and irradiated on day 0 (750 rad) and transplanted with 30×10^6 normal CBA spleen cells. All the recipients received 5×10^6 protective syngeneic F_1 BM cells together with parental spleen cells.

survived over the observation period. In the other experimental group, F_1 recipient mice received 20 μl of anti-ASGM1 antibody intravenously 2 days before the irradiation and transplantation described above. Eighty-nine percent of mice treated with anti-ASGM1 antibody survived the 2 weeks follow-up period (highly significantly better survival than in the untreated group, $P < 0{\cdot}001$, logrank test) (Fig. 1). When the spleen cell donor mice (CBA) were pretreated with anti-ASGM1 antibody 2 days before transplant there was no noticeable improvement of survival after transplant (Fig. 1).

NK activity in the spleens of the experimental mice. Results from the mortality experiments described above indicated that a radioresistant host ASGM1$^+$ cell is important to the induction of lethal GVHR. The anti-ASGM1 antibody treatment used in the experiments abolished the NK activity very efficiently. When the NK activity in the spleens of experimental mice was measured against YAC-1 targets a considerable activity was seen after the transplant in control mice while antibody treated mice did not show any NK activity during the period investigated (days 4–6 after transplant) (Fig. 2). This NK activity is probably of recipient origin, since cells taken from anti-ASGM1 antibody treated and transplanted mice were not NK active although transplant donor mice were not ASGM1$^+$ cell depleted. In addition, the appearance of donor type NK cells from their precursors in irradiated mice takes 7–9 days (Hurme & Sihvola, 1984).

ASGM1 antigen has been described as being expressed on all of the active murine NK cells (Kasai *et al.*, 1980), but it may also be expressed on activated cytotoxic T cells (Stitz *et al.*, 1986). The depletion protocol of ASGM1$^+$ cells used in our experiments was unlikely to effect any donor-derived GVH-reactive cytotoxic T cells since only a single dose of antibody, given 2 days before transplant was needed to prevent GVHD mortality. Also, treatment of the donor animals only, had no beneficial effect on the survival of the experimental mice. To ascertain that circulating anti-ASGM1 antibody was absent at the time of transplant, we tested the ability of F_1 recipient serum to inhibit CBA spleen cell NK activity 40 h after i.v. injection of 20 μl of anti-ASGM1 antibody. As Table 1 shows the tested serum did not have any residual anti-NK cell activity.

Anti-host reactive cytotoxic T cells. It has been shown earlier that anti-host reactive cytotoxic T cells from mice exhibiting GVHD cannot be demonstrated without boosting with recipient type

Table 1. Evidence for the lack of residual anti-ASGM1 antibody activity in the recipients at the time of transplantation (NK activity against YAC-1 target (% lysis))

		Attacker:target ratio		
		80:1	40:1	20:1
(a)	Normal CBA spleen cells incubated in culture media	20·7	16·8	10·1
(b)	CBA spleen cells incubated in normal F_1 recipient serum	22·5	16·2	12·5
(c)	CBA spleen cells incubated in anti-ASGM1 antibody treated F_1 recipient serum	23·0	19·1	10·9
(d)	anti-ASGM1 antibody treated F_1 recipient spleen cells	0·8	0·7	0·7

Freshly collected spleen cells were incubated for 40 min at 10×10^6 cells/ml at 4°C in: (a) RPMI-1640 medium alone; (b) Normal (CBA × C57BL/6)F_1 serum; (c) (CBA × C57BL/6)F_1 serum drawn from the mice injected i.v. 40 h previously with 20 μl of anti-ASGM1 antibody; (d) Group d gives the NK activity in the spleens of the mice injected i.v. 40 h previously with 20 μl of anti-ASGM1 antibody. NK activity of the spleen cells was measured, against ^{51}Cr-labelled YAC-1 cells.

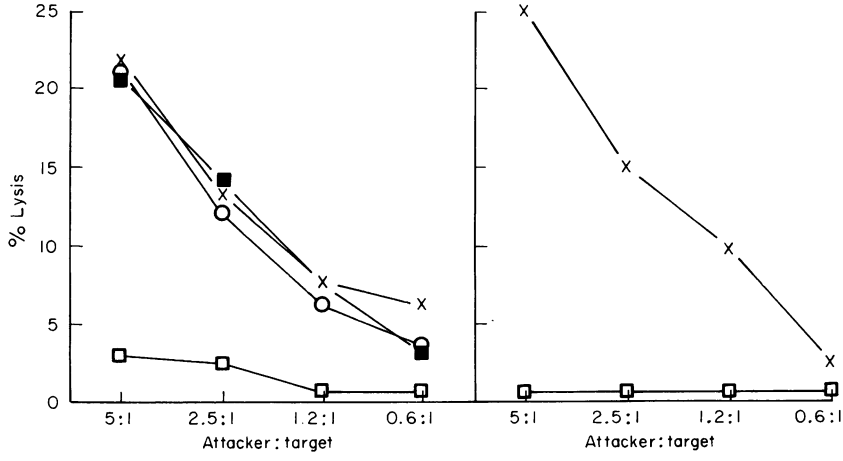

Fig. 3. Cytotoxic T cell activity in the spleens of the experimental mice against F_1 Con A blasts. (a) 5×10^6 spleen cells of the experimental mice were cultured with 2×10^6 irradiated F_1 spleen cells in the presence of conA supernatant as a source of IL-2 for 5 days and then assayed against ^{51}Cr labelled F_1 Con A blasts. (■) Normal CBA mice. (×) (CBA × C57BL/6)F_1 mice that were irradiated on day 0 (750 rad) and transplanted with 30×10^6 CBA spleen cells. (○) (CBA × C57BL/6)F_1 mice that were irradiated on day 0 and transplanted with 30×10^6 CBA spleen cells taken from donors which had been pretreated with anti-ASGM1 antibody (20 μl, on day −2). (□) (CBA × C57BL/6)F_1 mice that were pretreated with anti-ASGM1 antibody (20 μl, on day −2), and irradiated on day 0 (750 rad) and transplanted with 30×10^6 normal CBA spleen cells. All the recipients received 5×10^6 protective syngeneic F_1 Bm cells together with parental spleen cells. (b) spleen cells of the experimental mice were cultured without stimulator cells for 72 h and then assayed against ^{51}Cr labelled F_1 con A blasts. (×) and (□) as in Fig. 3a.

stimulators *in vitro* (Hamilton *et al.*, 1981). In our GVHD model we also could not show direct anti-host cytotoxicity from spleen cells of experimental mice taken on day 5 after transplant (1–2 days before the peak of deaths among GVHD mice), but when cells from GVHD mice were restimulated with irradiated F_1 cells for 5 days in culture in the presence of exogenous Con A supernatant as a source of IL-2, cytotoxicity against F_1 Con A blasts could be demonstrated (Fig. 3A). Cells taken from anti-ASGM1 antibody pretreated mice could not be restimulated into active CTL even in the presence of IL-2. Later experiments showed that the failure to demonstrate direct anti-host CTL activity was not due to lack of IL-2, but rather was caused by suppressor cells present in mice with GVHD. In those experiments spleen cells from experimental mice were cultured for 72 h without adding any exogenous Con A supernatant or stimulator cells and then measured cytotoxicity against F_1 Con A blasts. Cells taken from the GVHD mice showed clear CTL activity, but cells from anti-ASGM1 antibody pretreated mice were not cytotoxic (Fig. 3B). The method used has previously been shown to be effective in depleting suppressor cells generated during *in vivo* allostimulation (Wagner *et al.*, 1976).

Recently it was reported that ASGM1$^-$ natural suppressor (NS) cells are generated in the early phase of GVHD (Maier, Holda & Claman, 1985). Whether they are responsible for the T cell suppression seen in our model remains to be seen.

Our results described above indicate that ASGM1$^+$ cells are needed in the recipient for induction of GVH-reactive T cells after allogeneic transplantation. This cytotoxic activity can be shown *in vitro* if suppressor activity is abolished.

DISCUSSION

The cellular interactions involved in graft-versus-host disease have proved to be very complex. There is general agreement about the role of donor-derived T cells as the effectors of GVHD (van Bekkum, 1985), but a number of reports have indicated that host lymphocytes also participate in GVHD.

In a murine GVHD model, Norin, Emeson & Veith (1981) reported that anti-lymphocyte serum treatment of the recipient before transplant prevented GVHD. It has been shown that depleting the recipient of L3T4$^+$ cells is effective in preventing GVHD (Cobbold *et al.*, 1986), and NK cells may also be involved in the induction of GVHD (Lopez *et al.*, 1980; Charley *et al.*, 1983; Guillen *et al.*, 1986).

In the parent to non-irradiated F_1 ($P \rightarrow F_1$) GVHD model, cytotoxic T cells have been shown to be the effectors of acute GVHD (Kubota *et al.*, 1983). In the same model, helper T cells were shown to be the most important effectors in chronic GVHD (Gleichman et al., 1984). Our earlier studies have shown that in the $P \rightarrow F_1$ GVHD model neither depletion of ASGM1$^+$ NK cells with antibody nor enhancement of their activity with an interferon inducer, polyinosinic: polycytidylic acid (poly I:C), effects the appearance of GVHD, although elevated NK activity is seen during the disease (Varkila & Hurme, 1985).

When spleen cell transplants have been performed across the minor H-2 barrier in mice, T cells have been shown to be the effectors of the resulting GVHD (Korngold & Sprent, 1978), but also NK cells may have an important role, since depletion of ASGM1$^+$ cells abolishes GVHD mortality (Charley *et al.*, 1983). NK cells were recently postulated to be effector cells of cutaneous GVHD in the same experimental model (Guillen *et al.*, 1986). Whether the NK cells described above were of recipient or donor origin was not shown in these reports.

In the GVHD model used in this study recipient F_1 mice are first immunosuppressed with lethal X-ray irradiation and then transfused with parental spleen cells capable of inducing GVHD. In this model host-versus-graft reaction plays no apparent role. Also in this model anti-host reactive cytotoxic cells are generated *in vivo* as the data show in Fig. 3. However, ASGM1$^+$ cells of recipient origin are needed for the induction of these cytotoxic T cells, which when activated, are able to cause lethal GVHD (Fig. 1). For *in vitro* demonstration of this CTL activity two requirements exist in our GVHD model: (1) During the *in vitro* restimulation an exogenous source of IL-2 is needed; or (2) direct CTL can be shown if GVHD-related suppressor cells, which are shortlived, are first depleted

by culturing them *in vitro* for 72 h. The ASGM1$^+$ GVHD inducer cells seen in our model may be NK cells, which, being radioresistant (Hochman, Cudkowicz & Dausset, 1978) have survived the conditioning irradiation. Recent data have indicated that NK cells can work as accessory cells in CTL induction (Scala *et al.*, 1985). Using another approach (host-versus-graft reaction *in vivo*) we have shown that ASGM1$^+$ cells are needed for the induction *in vivo* of alloreactive cytotoxic T cells (manuscript in preparation). The results shown in the present report are in accordance with above described findings.

It was recently postulated (Stitz *et al.*, 1986) that activated T cells possess ASGM1, since it was shown that generation of virus specific CTL activity in mice could be prevented by treatment of mice *in vivo* with the same anti-ASGM1 antibody as in our study, if antibody was given during the immunization. However, pretreatment given 1 day before immunization with 30 μl of antibody (20 μl of antibody on day −2 in our study) did not effect the generation of CTL. Also we confirmed that there was no anti-ASGM1 antibody activity in the recipient serum at the time of transplantation (Table 1). Taken together it seems that the antibody pretreatment used in our protocol does not directly effect the donor derived anti-host CTL responsible for GVHD.

In both clinical medicine and experimental models GVHD has been prevented by depletion of mature T cells from the transplanted cell population (Korngold & Sprent, 1978; Prentice, Bladlock & Janossy, 1984). However, this protocol may have disadvantages, since increased graft rejection and higher relapse rates of the malignant disease (Gale & Reisner, 1986) have been reported. In experimental models, bone marrow cells have been shown to be rejected by NK cells (Kiessling *et al.*, 1977) and it has been shown that rejection of transplanted allogeneic bone marrow can be prevented by depleting recipient NK activity by NK 1·1 antiserum (Lotzova, Savary & Pollack, 1983). Based on the results of the present report depletion of recipient NK cell is also beneficial in attempting to prevent GVHD.

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