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Lymphokine activity production in graft-*versus*-host reactions across minor histocompatibility antigen barriers

M. HIROKAWA, H. TAKATSU, A. OHSHIMA, A. CHUBACHI, K. KUDO, H. NIITSU, T. TAKAHASHI, K. YOSHIDA & A. B. MIURA *The Third Department of Internal Medicine, Akita University School of Medicine, Akita, Japan*

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SUMMARY

Activated T cells responding to murine minor histocompatibility antigens (HA) were characterized according to the patterns of lymphokine activity production. Although B10.D2/nSN and BALB/c are mutually non-reactive in mixed lymphocyte reaction (MLR), graft-*versus*-host reaction (GVHR) can be induced by the injection of a large amount of B10.D2/nSN lymphoid cells into irradiated BALB/c recipient mice. Spleen cells from such GVHR mice spontaneously produced interleukin 3 (IL-3)-depedent cell-stimulating activity in cultures, but did not produce interleukin 2 (IL-2). Normal B10.D2/nSN spleen cells also produced IL-3-like activity, but not IL-2 in MLR supernatants, in response to irradiated BALB/c splenocytes. In addition, B-cell stimulatory factor-1 (BSF-1)/interleukin 4 (IL-4) and colony-stimulating factor (CSF) activity were detected in MLR supernatants. The properties of the produced lymphokine activities were similar to those produced in syngeneic transplant mice and syngeneic MLR, but a difference in the time course of lymphokine production existed between GVHR and syngeneic transplant mice. These results indicate that T cells may be activated *in vivo* in allogeneic transplantation when the donor and the recipient are matched for major HA, and are non-reactive in MLR. Also, the character of lymphokine-producing T cells activated by minor HA may not be qualitatively different from those responding to irradiated syngeneic cells.

Keywords graft-*versus*-host reactions minor histocompatibility antigens T cell-derived lymphokines mixed lymphocyte reaction

INTRODUCTION

Graft-*versus*-host disease (GVHD) is one of the major obstacles to successful human allogeneic bone marrow transplantation (BMT) (Sullivan, 1983). Severe acute GVHD is often fatal. Immunodeficiency and autoimmune disorder-like syndromes associated with chronic GVHD occasionally disable post-transplant patients. However, frequency of leukemia relapse after BMT is lower in patients with chronic GVHD than in either those without chronic GVHD or those who had been transplanted from genetically identical twins (graft-versus-leukemia effect) (Gale & Champlin, 1986). In order to find the means to manipulate the immunological effects of the allogeneic marrow graft, it is very important to elucidate the cells and mechanisms involved in GVHD.

Most studies on graft-*versus*-host-reactions (GVHR) have been performed using the combinations of crossing major histocompatibility antigens (HA) in mice. It is well known that GVHD can occur in human allogeneic BMT when the donor and recipient are HLA-identical and their cells are non-reactive in mixed lymphocyte culture (MLC) (Sullivan, 1983). In order to explore the mechanism of GVHR induced by crossing minor HA barriers, we used B10.D2/nSN and BALB/c mice, which are H-2- and mls-identical, and differ only with regard to minor HA. Although they are mutually non-reactive in MLC, GVHD can be induced by the injection of large numbers of B10.D2/nSN lymphoid cells into irradiated BALB/c recipients (Hamilton, Bevan & Parkman, 1981; Holda, Maire & Claman, 1985). This model system is very similar to human allogeneic BMT. Recently, it has been demonstrated that L3T4-positive T cells participate in the initiation of GVHD in this combination (Hamilton, 1987). To further characterize the T cells activated by minor HA, we first examined whether T cell-derived lymphokines were produced in GVHR mice. Next, we investigated whether the production of lymphokine activities could be detected in the supernatants of mixed lymphocyte reaction (MLR) across minor HA barriers, and compared the patterns of lymphokine production with those in syngeneic and fully

Correspondence: Makoto Hirokawa, M.D., The Third Department of Internal Medicine, Akita University School of Medicine, 1-1-1 Hondo Akita 010, Japan.

Table 1. Spleen weight and spleen index

Day	Spleen weight* (mg, mean ± s.e.) GVHR	Spleen weight* (mg, mean ± s.e.) Syngeneic	Spleen index GVHR	Spleen index Syngeneic
14	247 ± 48†	130 ± 3	11·9	6·3
21	145 ± 6‡	109 ± 5	7·5	5·4
28	168 ± 12‡	103 ± 4	9·0	4·9
42	114 ± 3‡	149 ± 6	6·8	7·3
56	ND	145 ± 10	ND	7·3
Normal BALB/c	121 ± 6		6·3	

Spleen index was calculated thus: $\frac{\text{spleen weight (mg)}}{\text{body weight (g)}}$

* Of at least three mice. Statistical comparisons were made with respective syngeneic transplant groups: † $P < 0{\cdot}01$; ‡ $P < 0{\cdot}05$.

GVHR, graft-*versus*-host reaction; ND, not determined.

allogeneic MLR. We found that T cell-derived lymphokines are produced *in vivo* and *in vitro* in response to minor HA, and that the properties of produced activities are similar to those that respond to irradiated syngeneic cells. However, there was a difference in the time course of the lymphokine production between GVHR and syngeneic transplant mice.

MATERIALS AND METHODS

Mice

Female B10.D2/nSN (H-2^d, mls b), BALB/c (H-2^d, mls b) and C3H/He (H-2^k, mls c) mice were purchased from the Shizuoka Laboratory Animal Center (Shizuioka, Japan). All mice used were from 7 to 12 weeks of age.

Induction of GVHR

Spleens were pooled from donor B10.D2/nSN or BALB/c mice. Erythrocyte-free cell suspensions were prepared by passing the spleens through a stainless steel mesh screen and treatment with hypotonic shock followed by washing three times in Hanks' balanced salt solution (HBSS). Recipient BALB/c mice were irradiated with 600 rad from a ^{60}Co source at a dose rate of 15 rad/min. Spleen cells (4×10^7) were injected intravenously in a volume of 0·2 ml of HBSS into recipient BALB/c mice within 2 h or irradiation. BALB/c recipients of B10.D2/nSN cells are referred to as GVHR mice, and BALB/c recipients of BALB/c cells as syngeneic controls. They were given tetracycline in their drinking water (250 mg/*l*, Boehringer, Mannheim, FRG) for the first 14 days.

Preparation of spleen cell conditioned medium (SCM)

At various times after transplantation, at least three mice from each experimental group were killed, and the spleens were pooled. Erythrocyte-free cell suspensions were prepared as described above; 5×10^6 spleen cells were cultured in 1·0 ml of culture medium in the absence of mitogen in 24-well tissue culture plates (Falcon no. 3047, Becton Dickinson Labware, Oxnard, CA) at 37°C. As a culture medium, RPMI 1640 (Nissui Seiyaku Co. Tokyo, Japan) supplemented with 1% fresh normal

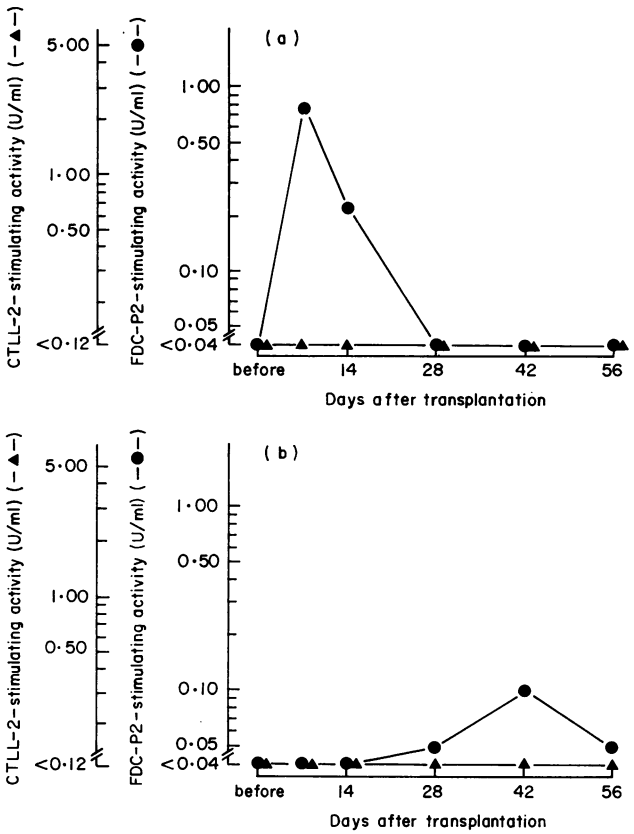

Fig. 1. Spontaneous production of IL-3-dependent FDC-P2 cell-stimulating activity by unstimulated spleen cells from GVHR (a) and syngeneic transplant mice (b). Each point shows the activity produced by the pooled splenocytes from at least three mice.

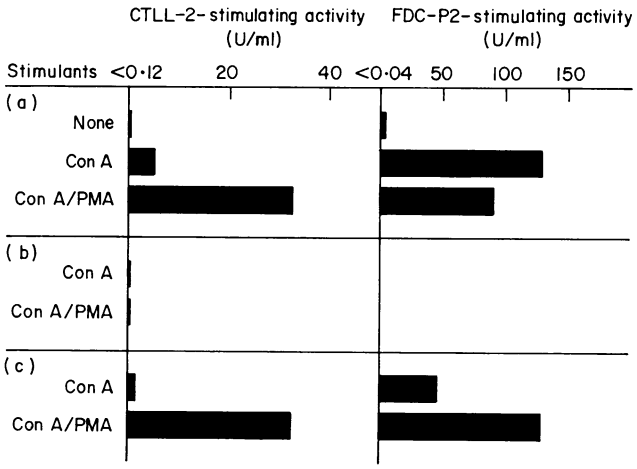

Fig. 2. Effect of metabolic inhibitors on lymphokine production by mitogen-stimulated murine splenocytes. Normal BALB/c splenocytes were cultured in the presence of Con A and/or PMA without metabolic inhibitors (a) or with 1 μg/ml actinomycin D (b) or with 10 μg/ml cytosine arabinoside (c). Con A = concanavalin A; PMA = phorbol myristate acetate.

mouse serum (NMS) derived from BALB/c mice, 5×10^{-5} M 2-mercaptoethanol (2-ME; Sigma Chemical Co., St. Louis, MO), 50 U/ml penicillin, and 50 μg/ml streptomycin were used (RPMI/NMS). The supernatants were harvested after 72 h of incubation. They were stocked at −80°C until the assay for FDC-P2- and CTLL-2-stimulating activities.

Preparation of lymphokine-containing supernatants from mitogen-stimulated murine splenocytes and effects of metabolic inhibitors

Spleen cells (5×10^6) from normal BALB/c mice were cultured for 24 h in 1 ml of RPMI 1640, supplemented with 10% fetal calf serum (FCS) in the presence of 5 μg/ml concanavalin A (Con A) (Sigma) and/or 10 ng/ml phorbol myristate acetate (PMA; Sigma) with and without metabolic inhibitors at desired concentrations. Actinomycin D and cytosine arabinoside were purchased from Sigma. Supernatants were tested for CTLL-2-and FDC-P2-stimulating activities. In order to determine DNA replication, 200 μl of the cell suspensions were cultured in 96-well plates for 24 h. The cultures were than pulsed with ^{3}H-thymidine (^{3}H-TdR; 0·5 μCi/well) and further incubated for 16 h. ^{3}H-TdR incorporation was measured by the liquid scintillation counting method.

MLC

Spleen mononuclear cells, as a source of responder and stimulator cells, were obtained by Ficoll-Isopaque density gradient centrifugation; 1×10^7 responder spleen cells and 1×10^7 irradiated (3300 R) spleen cells were suspended in 2 ml of RPMI/NMS medium, and were cultured in 24-well plates for 1 to 9 days at 37°C in 5% CO_2 in air with 100% humidity. The supernatants were tested for lymphokine activities.

Cell proliferation assay

FDC-P2 cell line (Dexter *et al.*, 1980) was used for the detection of interleukin (IL)-3-like activity (Ihle *et al.*, 1982), and was maintained in RPMI 1640, supplemented with 10% FCS and 10% WEHI-3 conditioned medium. CTLL-2, and IL-2-dependent murine T cell line (Gillis & Smith, 1977), was maintained in RPMI 1640, supplemented with 10% FCS and 10% Con A-stimulated rat SCM. Either FDC-P2 or CTLL-2 cells were incubated (1×10^4 cells/well) with two-fold diluted test samples in 96-well flat-bottomed plates (Nunc, Roskilde, Denmark) for 24 h at 37°C. As standard preparations, purified murine IL-3 (purchased from Genzyme, Boston, MA) and human recombinant IL-2 (Shionogi Pharmaceutical Co., Osaka, Japan) were used for FDC-P2 and CTLL-2 assays, respectively. Proliferation was determined either by the incorporation of ^{3}H-TdR or by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983). One unit of activity is defined as the amount of factor required to induce 50% of the maximal proliferation of FDC-P2 or CTLL-2 cells. According to the probit analysis (Gillis *et al.*, 1978), FDC-P2-stimulating activity can be significantly assessed as low as 0·04 U/m, and CTLL-2-stimulating activity as low as 0·12 U/ml.

Assay for BSF-1/IL-4

BSF-1 activity was detected by its ability to stimulate the proliferation of purified splenic B cells in the presence of anti-IgM antibody (Howard *et al.*, 1982). Splenic B cells were enriched by the following method: spleen cells from BALB/c mice were treated with an optimum dose of monoclonal anti-Thy-1.2 antibody (clone F7D5; Serotec) at room temperature for 30 min, and with rabbit complement (Low-Tox-M; Cedarlane) at 37°C for 40 min. The T cell-depleted spleen cells were then passed through a Sephadex G-10 column to remove adherent cells, and were suspended in RPMI 1640, supplemented with 10% FCS and 5×10^{-5} M 2-ME; 1×10^5 purified B cells were cultured in 0·2 ml containing 10 μg/ml of affinity-purified goat anti-mouse IgM (Cooper Biomedical, Malvern, PA) and two-fold dilutions of test materials. As a positive control, we used PMA-stimulated EL-4 supernatant (Howard *et al.*, 1982). After 56 h, each well was pulsed with 0·5 μCi of ^{3}H-TdR and harvested 16 h later. ^{3}H-TdR uptake was estimated by liquid scintillation counting. One unit of BSF-1 activity was defined as the amount to induce 50% of the maximal proliferation. According to the probit analysis, BSF-1 activity is significantly assessed as low as 0·08 U/ml.

Table 2. Inhibition of mitogen-induced DNA synthesis by cytosine arabinoside

Metabolic inhibitors	Stimulants	^{3}H-thymidine incorporation (ct/min)
None	None	9562 ± 258
	Con A	179 402 ± 8692
	Con A/PMA	191 078 ± 6870
Cytosine arabinoside (10 μg/ml)	Con A	5997 ± 922
	Con A/PMA	9850 ± 204

Results are expressed as the mean ± s.d. of triplicate cultures. In the presence of cytosine arabinoside, mitogens were added to the cultures after a delay of 2 h.

Con A, concanavalin A; PMA, phorbol myristate acetate.

Assay for colony-stimulating factor (CSF)

Assays for CSF activity were carried out by using a modification of the methylcellulose culture method described by Iscove & Sieber (1975). Briefly, 1×10^5 BALB/c bone marrow cells in 1·0 ml of Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY) supplemented with 30% FCS, 0·96% methylcellulose and 10% (v/v) test materials were incubated in 35 mm plastic Petri dishes (Lux, Miles Laboratories, Naperville, IL) for 7 days at 37°C in 5% CO_2 in air. Aggregates containing more than 50 cells were scored as colonies. In the absence of exogenous CSF, 2 ± 0 (mean ± s.d.) colonies were formed.

Statistical analysis

Student's *t*-test was used for the determination of statistically significant differences.

RESULTS

Spleen weight and spleen index of post-transplant mice

The spleen weight and splenic index (Simonsen, 1958) of GVHR mice markedly increased on day 14 and remained elevated until day 28, then returned to the normal level by day 42 (Table 1). Induction of GVHR was confirmed by enlargement of the spleens. In syngeneic control mice, a slight elevation of the spleen weight was observed on days 42 and 56.

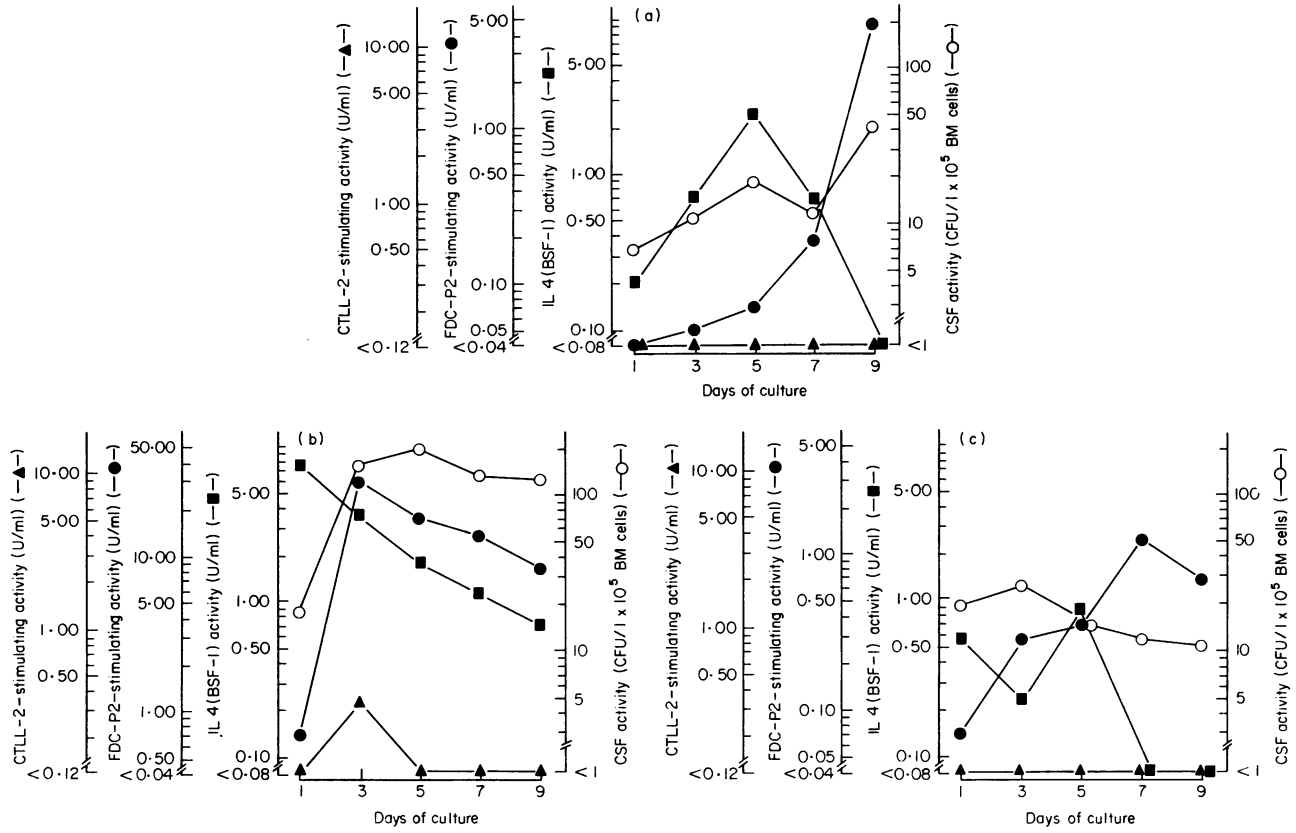

Fig. 3. Lymphokine activity production in syngeneic mixed lymphocyte reaction (MLR) (a), allogeneic MLR across major histocompatibility antigens barriers (b) and allogeneic MLR across minor HA barriers (c). Responder cells from B10.D2/nSN mice were cultured with irradiated stimulator cells from C3H/He (b) or BALB/c (c) mice. In syngeneic MLR, BALB/c responder cells were incubated with irradiated BALB/c stimulator cells. Data for one of duplicate experiments are shown.

Table 3. Effect of pretreatment of responder spleen cells with anti-Thy-1 antibody and complement on the production of FDC-P2 cell-stimulating activity

Treatment of responder B10.D2 spleen cells	Stimulus	FD-P2-stimulating activity produced (U/ml)
—	BALB/c (3300 R)	1·27
Complement alone		1·15
Anti-Thy-1 + complement		0·08
—	Con A (5 μg/ml)*	8·00
Anti-Thy-1 + complement		0·18

* To test the efficiency of mass killing with anti-Thy-1 antibody and complement, 1×10^7 responder B10.D2 spleen cells were stimulated by Con A instead of irradiated BALB/c cells for 48 h.

Spontaneous production of IL-3-dependent FDC-P2 cell-stimulating activity by unstimulated cells from GVHR mice

Spleen cells from GVHR, syngeneic transplant and normal BALB/c mice were cultured for 72 h without exogenous stimulation. The supernatants from the cultures of normal BALB/c splenocytes exhibited no FDC-P2-stimulating activity (<0·04 U/ml). Production of FDC-P2-stimulating activity by splenocytes from GVHR mice was detected on days 7 and 14, and reached its highest level on day 7 (Fig. 1a). Spontaneous production of FDC-P2-stimulating activity by syngeneic transplant murine splenocytes was also detected on days 28, 42, and 56, as we had previously observed (Fig. 1b). IL-2 production was not detected in either GVHR or syngeneic transplant mice during the observation period.

Induction of lymphokine activity production without an increased DNA synthesis

Although B10.D2/nSN and BALB/c are non-reactive in MLR, it has been demonstrated that spleen cells from GVHR mice spontaneously produced FDC-P2-stimulating activity in cultures. To examine whether normal lymphocytes can produce lymphokine activities without an increased DNA synthesis *in vitro*, the effect of metabolic inhibitors on mitogen-induced lymphokine production was investigated (Fig. 2). Actinomycin D, which inhibits RNA synthesis, completely blocked the production of IL-2 and FDC-P2-stimulating activities. In the presence of enough cytosine arabinoside to inhibit ^{3}H-TdR incorporation by the mitogen-stimulated lymphocytes (Table 2), the production of lymphokine activities was observed. These results indicate that normal lymphocytes can produce lymphokines without an increased DNA synthesis *in vitro*. We next examined whether the production of lymphokines was induced in MLR.

Production of lymphokine activities in MLR

In the syngeneic MLR between BALB/c responder cells and irradiated BALB/c stimulator cells, production of IL-3-like activity was evident on day 7 of culture. The level of IL-3-like activity reached a maximum of 4·59 U/ml on day 9 (Fig. 3a). BSF-1/IL-4 was detected at a maximum of 2·46 U/ml on day 5, and CSF activity was also found. In contrast, IL-2 could not be detected during any of the culture periods. However, in fully allogeneic MLR across major HA barriers, production of IL-2 was observed, in addition to IL-3-like activity, BSF-1/IL-4, and CSF activity (Fig. 3b). In allogeneic MLR between B10.D2/nSN responder cells and BALB/c stimulator cells as the counterpart of the *in vivo* GVHR model, production of IL-3-like activity was evident on day 3 of the culture, and the level of IL-3-like activity reached a peak of 1·20 U/ml on day 7 (Fig. 3c). Pretreatment of responder B10.D2/nSN cells with anti-Thy-1 antibody and complement abrogated the production of IL-3-like activity (Table 3). BSF-1/IL-4 and CSF activity were produced at a maximum of 0·87 U/ml on day 5, and 26 colony-forming units (CFU) on day 3, respectively. But IL-2 was not detected during the entire period of observation. To confirm that IL-2 was not produced, MLR supernatants on days 3, 5, and 7 were concentrated ten-fold by membrane filtration having an exclusion size of mol.wt 10 kD, and were tested for IL-2 activity. No IL-2 activity was detected in any of the supernatants (data not shown).

DISCUSSION

We have shown that spleen cells from mice with GVHR across non-H-2 and non-mls barriers spontaneously produced IL-3-dependent FDC-P2 cell-stimulating activity in cultures in the early phase of transplantation, but did not produce IL-2 during the observation periods. Syngeneic transplant murine splenocytes also produced the same activity in the later phase of transplantation, which was proven to be biochemically and functionally similar to IL-3, as we have reported elsewhere (Hirokawa *et al.*, 1989). It has been shown that FDC-P2 cells respond not only to IL-3 but to the granulocyte macrophage colony-stimulating factor (GM-CSF) and BSF-1/IL-4 (Hapel Warren & Hume, 1984; Grabstein *et al.*, 1986), and that these lymphokines are produced by mitrogen- or antigen-stimulated T cells (Ihle, Pepersack & Reber, 1981; Howard *et al.*, 1982; Mosmann *et al.*, 1986). Although further research will be necessary to characterize the FDC-P2-stimulating activity produced by spleen cells from GVHR mice, it seems likely that T cells are activated *in vivo* in response to minor HA. This idea is supported by the results reported by Hamilton, Bevan & Parkman (1981) and by Hamilton (1987); they demonstrated, by using B10.D2/nSN and BALB/c mice, that GVHD due to minor antigens can be prevented by depleting L3T4 positive T cells from the grafts.

B10.D2/nSN and BALB/c are H-2- and mls-identical, and their cells are mutually non-reactive in MLR. However, production of IL-3-like activity, BSF-1/IL-4, and CSF but not IL-2 was observed in allogeneic MLR between B10.D2/nSN responder cells and BALB/c stimulator cells. Absence of IL-2 production was confirmed by the assay for IL-2 activity with concentrated MLR supernatants. In fully allogeneic MLR across major HA barriers, IL-2 was produced in addition to IL-3-like activity, BSF-1/IL-4, and CSF. We have also demonstrated that spleen cells from normal mice were able to produce lymphokine activities in response to Con A or Con A/PMA in the presence of enough cytosine arabinoside to block ^{3}H-TdR incorporation. Yamamoto *et al.* (1985) reported that human lymphocytes can synthesize IL-2 mRNA and produce IL-2 activity against the mitogen stimulation in the presence of a DNA synthesis inhibitor. Therefore, it is considered that lymphocytes can be activated *in vitro* without an increased DNA replication, and that the assays for lymphokine activities may be useful to estimate cell activation. Furthermore, these results indicate the existence of T cells activated by minor HA *in vitro*.

In syngeneic MLR, production of IL-3-like activity, BSF-1/IL-4, and CSF but not IL-2 was observed, and these data coincide in part with the results reported by Suzuki *et al.* (1986), who demonstrated that the production of IL-3, but not IL-2 or interferon-γ, was detected in the syngeneic MLR between T cells and mitomycin C-treated non-T cells in the absence of xenogeneic protein antigens. Properties of lymphokine activities produced in allogeneic MLR across minor HA barriers were quite similar to those produced in syngeneic MLR. This indicates the possibility that the character of lymphokine-producing T cells activated by minor HA may be identical to those that respond to irradiated syngeneic cells.

We could not find the difference in the profile of lymphokine activity production between the T cells activated by minor HA and those that responded to irradiated syngeneic cells, but the time course of lymphokine production *in vivo* was different between allogeneic and syngeneic combinations. Additional bioassays may reveal functional differences between the T cells responsive to irradiated allogeneic cells and syngeneic cells.

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