

Xenogeneic Proliferation and Lymphokine Production Are Dependent on CD4⁺ Helper T Cells and Self Antigen-presenting Cells in the Mouse

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Summary

We studied proliferation and interleukin 2 production by B6 mouse spleen cells in response to stimulation by irradiated cynomolgus monkey spleen cells and compared the results with responses against whole MHC-disparate allogeneic controls (BALB/c). We found that (a) primary xenogeneic helper responses were absent, whereas primary allogeneic responses were brisk, (b) secondary xenogeneic helper responses were dependent on CD4⁺ T cells and responder antigen-presenting cells (APCs), whereas allogeneic responses could be mediated by either CD4⁺ or CD8⁺ T cells independently and were primarily dependent on the presence of stimulator APCs, and (c) secondary xenogeneic helper responses were blocked by an antibody directed against responder class II MHC molecules. These results suggest that mouse helper T cells recognize disparate xenoantigens as processed peptides in association with self class II MHC molecules, similar to the recognition of nominal antigens and unlike direct allo-recognition.

Less is known about cell-mediated immunity to xenoantigens than to alloantigens (1). The greater disparity of xenogeneic compared with allogeneic MHC antigens might suggest more vigorous immune responses to the former, as appears to be the case for humoral immunity (2). On the other hand, the strong cellular responses to MHC alloantigens depend in part on their similarity to self MHC molecules (3). Therefore, cellular immunity against more disparate xenoantigens might actually be weaker than that against alloantigens. A number of studies on in vitro cell-mediated cytotoxicity have been reported (reviewed in reference 1), some of which revealed diminished precursor frequencies of xeno-reactive compared with allo-reactive cytotoxic effector cells. Fewer reports have been published concerning xenogeneic helper T cell responses, and the available data are conflicting. Several groups have described primary xenogeneic helper responses of equal (2, 4–15) or even greater magnitude (16) compared with allo-responses. Others have reported weak or no (17–25) responses. Limited data are available to shed light on the reasons underlying this discrepancy. Relatively few studies have examined the cellular requirements of the xenogeneic helper immune response (4, 5, 15, 25–27), particularly for murine xeno-responses (4, 25). Only one study, describing human anti-mouse responses, measured IL-2 production rather than the potentially less T cell-specific proliferation response (5).

In the present report, we describe in vitro studies of the

cellular requirements for primary and secondary proliferation and IL-2 production by mouse spleen cells stimulated by cynomolgus monkey spleen cells. Our results show that mice have substantially diminished helper T cell responses to xeno-compared with alloantigens and that they use different pathways for xenogeneic and allogeneic immunity. Specifically, the data suggest that mouse helper T cells recognize disparate xenoantigens as nominal antigens presented in association with self MHC molecules rather than directly as in alloantigen recognition.

Materials and Methods

Animals. 6–30-wk-old C57BL/6J (B6, H-2^b) male, BALB/cByJ (BALB/c, H-2^d) female, and C3H/HeJ (C3H, H-2^k) male mice (The Jackson Laboratory, Bar Harbor, ME) served as spleen donors. Adult male cynomolgus monkeys (*Macaca fascicularis*) from the Transplantation Unit's large animal research facility served as skin graft and spleen donors. B6 mice primed against xenoantigens were obtained by grafting cynomolgus monkey skin 4–19 wk before in vitro assays according to the method of Billingham and Medawar (28).

Culture Medium. Tissue culture medium (TCM)¹ consisted of RPMI 1640 supplemented with 20 mM Hepes, 2 mM L-glutamine, 0.1 mM nonessential amino acids, gentamicin (100 µg/ml), 0.1% FCS (all from Gibco Laboratories, Grand Island, NY), and 0.05

¹ Abbreviation used in this paper: TCM, tissue culture medium.

mM 2-ME (Sigma Chemical Co., St. Louis, MO). The fetal calf serum was screened for support of allo-stimulated proliferation (Gibco Laboratories, lot no. 33P4292) and was heat-inactivated. TCM was supplemented with 10% FCS for in vitro assays.

Preparation of Spleen Cell Suspensions. Mice were killed by cervical dislocation and their spleens were removed aseptically. The spleens were processed into single-cell suspensions in TCM by mechanical disruption and passage through a 21-gauge needle. The cells were filtered (110- μ m nylon mesh), ACK-treated to remove erythrocytes, washed, and resuspended in TCM for use in in vitro assays. Cynomolgus monkey spleens were obtained aseptically from electively killed animals. Cell suspensions were prepared as in the mouse, cryopreserved in RPMI 1640 containing 10% DMSO (Aldrich Chemical Co., Milwaukee, WI), gentamicin (100 μ g/ml), and 30% FCS, and stored at -80°C . Just before use, the cryopreserved cells were rapidly thawed, washed, and resuspended in TCM for use in in vitro assays.

APC Depletion. Responder and stimulator spleen cell populations were depleted of APCs by passage over Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, NJ) columns according to a published method (29). Briefly, columns containing 13–17 ml of packed Sephadex G-10 were equilibrated with 50 ml of depletion medium (RPMI 1640 containing gentamicin, 100 μ g/ml, and 2% FCS). Spleen cells ($40\text{--}100 \times 10^6$ in 0.8–1.6 ml of TCM) were loaded into the columns, eluted with 5–10 ml of depletion medium (51–90% cell yield, 2.2–4.1 ml collected volume), and used directly in in vitro assays after dilution with TCM to the appropriate cell concentration.

mAb Blocking of Proliferation. MLR cultures were prepared as described below in the presence or absence of dilutions of 25-9-3s ascites (hybridoma kindly provided by D. Sachs, National Cancer Institute, Bethesda, MD), an anti-I-A^b mAb directed against the class II MHC molecules of the B6 mouse.

T Cell Subset Depletions. mAbs used in the subset depletions included anti-L3T4 (GK1.5), anti-Lyt2.2 (2.43), and anti-Thy-1.2 (HO-13-4), all prepared into ascites in our Unit from hybridomas obtained from the American Type Culture Collection (Rockville, MD). Processed rabbit serum (Low-Tox-M, lot no. 4378; Cedarlane Laboratories, Hornby, Ontario) served as a source of complement. Reagents were used at titers on the plateau of cytotoxicity based on the results of preliminary chromium-release assays (anti-L3T4 and anti-Lyt-2.2 at 1/500, anti-Thy-1.2 at 1/1,000, and complement at 1/8–1/10). Full-strength antibody was added to pelleted responder spleen cells ($30\text{--}45 \times 10^6$) and the mixture was incubated for 5–20 min at 4°C . After this, complement diluted in TCM was added (final cell concentration $40\text{--}50 \times 10^6$ /ml, final antibody and complement titers as noted above), the incubation was continued for 30 min at 37°C , and the cells were washed in RPMI 1640. The entire treatment was repeated once. Before the final wash, the suspensions were filtered (110- μ m nylon mesh) to remove dead cells and debris. The cells were resuspended in TCM at the appropriate cell concentration and used directly in in vitro assays.

Proliferation Assay (MLR). Culture conditions were chosen based on the results of preliminary optimization experiments (described in Results). Untreated or treated mouse responder spleen cells (4×10^5) and either monkey (2×10^5) or mouse (4×10^5) irradiated (2,000 cGy) stimulator spleen cells were added in a final volume of 200 μ l of TCM containing 10% FCS (final concentration) to triplicate wells of 96-well flat-bottomed microculture plates (No. 3072; Falcon Labware, Oxnard, CA). The cultures were incubated at 37°C in humidified air containing 5% CO_2 for varying lengths of time (see Results). [^3H]TdR (1 μCi /well; New England Nu-

clear, Boston, MA) was added 11–24 h before the end of culture. The samples were harvested onto glass fiber filters, and [^3H]TdR uptake was measured by β scintillation counting (30 s/sample) on a 1209 RackBeta counter (Pharmacia-LKB, Gaithersburg, MD). Results are expressed as mean counts per minute for triplicate samples.

IL-2 Production Assay. Preliminary experiments demonstrated no increased sensitivity of detecting IL-2 with the addition of the anti-IL-2 receptor antibody 7D4 to the MLR cultures; therefore, this reagent was not used. Supernatants (50 μ l) from the MLR cultures were transferred to equivalent wells of new flat-bottomed plates after varying durations of incubation (see Results) and stored at -20°C . The MLR wells were replenished with an equal volume of TCM containing 10% FCS and the plates were returned to the incubator. For determination of IL-2 content, IL-2-dependent CTLL tumor cells (kindly provided by T. Munitz, National Institutes of Health, Bethesda, MD) from partially exhausted cultures were washed four times in TCM, resuspended in TCM containing 10% FCS, and added (150 μ l) to the thawed supernatants at 4,000 cells/well. The final supernatant dilution was 1:4. Incubation and measurement of proliferation of CTLL were performed as described for the MLR assay, except for a 24–32-h incubation period and an 8–9-h [^3H]TdR label period. To correct for the nonlinear proliferation response of CTLL to IL-2, counts were converted to equivalent units/milliliter of IL-2 using a formula derived from a three-parameter nonlinear regression of control CTLL proliferation on graded doses of a murine IL-2 standard. Separate controls for calculating the conversion formula were set up for each experiment. The term “IL-2 production” is used throughout the text for simplicity, although the possibility that the present assay might be measuring the production of other lymphokines in addition to IL-2 cannot be rigorously excluded. It should be noted that the xeno-stimulated supernatants supported the proliferation not only CTLL but also of the HT2 IL-2 indicator line (data not shown).

Data Analysis. Data are expressed as the mean of triplicate samples \pm SEM. Each experiment shown was repeated at least three times with virtually identical results.

Results

B6 Mice Do Not Generate Primary In Vitro Helper T Cell Responses to Xenoantigens. Xenogeneic MLR and IL-2 production assays were performed using mouse responder cells and irradiated monkey stimulator cells. The results of a representative experiment are shown in Figs. 1 and 2, *a* and *b*. The conditions used in this experiment were based on the results of preliminary optimization experiments. Strong primary allogeneic proliferation (Fig. 1 *a*) and IL-2 production (Fig. 2 *a*) were observed. Peak values, based on stimulation index, occurred at 4 and 1.5 d of culture for primary allo-MLR and IL-2 production, respectively. In contrast to these brisk responses, primary xenogeneic proliferation (Fig. 1 *b*) and IL-2 production (Fig. 2 *b*) were barely detectable or absent at all time points tested (5–7 d for MLR and 0.5–4 d for IL-2 production).

In additional experiments, culture parameters were varied widely in order to increase the chances of detecting a primary xenogeneic response. Variations included screening of several lots of FCS from different commercial sources, titration of serum concentration, titration of responder and stimulator numbers in culture, an extensive kinetics study, and,

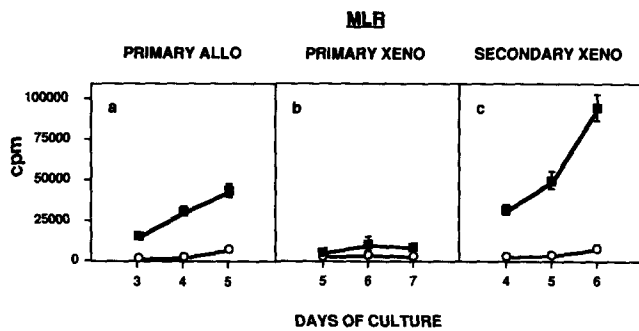


Figure 1. Kinetics of the proliferation response (MLR) of naive (*a* and *b*) and xeno-primed (*c*) B6 spleen cells to irradiated allogeneic (BALB/c, *a*) and xenogeneic (cynomolgus monkey, *b* and *c*) splenocyte stimulators, showing minimal primary xenogeneic proliferation. Allo- or xeno-stimulated (■) and self-stimulated (O) proliferation were measured. Responder xeno-priming was achieved by grafting of cynomolgus monkey skin 4 wk before in vitro testing. Optimized culture conditions were used (see Materials and Methods) based on the results of preliminary screening experiments. Proliferation was measured by [³H]TdR uptake.

in the case of IL-2 production, titration of anti-IL-2 receptor mAb 7D4. Minimal primary xenogeneic proliferation and IL-2 production were detected in these experiments (data not shown).

B6 Mice Generate Brisk Secondary In Vitro Helper T Cell Responses to Xenoantigens. A trivial explanation for the absence of primary in vitro xeno-responses is an inhibitory effect of the monkey stimulator cells on the mouse responder cells. Partly to test this possibility, secondary in vitro responses to xenoantigens were examined. The results of a representative experiment are shown in Figs. 1 *c* and 2 *c*. B6 mice that had received skin grafts from cynomolgus monkeys served as spleen donors. The conditions utilized in this experiment were based on the results of preliminary optimization ex-

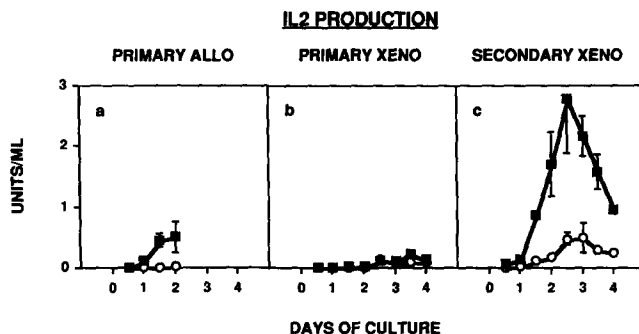


Figure 2. Kinetics of IL-2 production by naive (*a* and *b*) and xeno-primed (*c*) B6 spleen cells in response to irradiated allogeneic (BALB/c, *a*) and xenogeneic (cynomolgus monkey, *b* and *c*) splenocyte stimulators, showing minimal primary xenogeneic IL-2 production. Allo- or xeno-stimulated (■) and self-stimulated (O) IL-2 production were measured. Responder xeno-priming was achieved by grafting of cynomolgus monkey skin 4 wk before in vitro testing. Supernatants were harvested at the times shown from MLR cultures similar to those described in Fig. 1 and tested for stimulation of CTLL proliferation. Proliferation data were converted to IL-2 concentration based on the response of CTLL to graded doses of an IL-2 standard, as described in Materials and Methods.

periments. Strong proliferation (Fig. 1 *c*) and IL-2 production (Fig. 2 *c*) were observed. Peak values, based on stimulation index, occurred at 6 and 2 d of culture for secondary xeno-MLR and IL-2 production, respectively. These brisk secondary xenogeneic helper responses have been observed consistently in repeated experiments. It should be noted that the self-stimulated IL-2 production evident in Fig. 2 *c* was consistently observed in experiments involving xeno-primed mice and was apparently related to the effects of priming with monkey skin grafts.

Secondary In Vitro Helper T Cell Responses to Xenoantigens Depend Upon the Presence of Responder (B6) APCs. The absence of primary in vitro helper T cell responses to xenogeneic stimulators could result from one of two general mechanisms. On the one hand, helper T cells capable of recognizing xenoantigens directly on xenogeneic APCs might exist but at too low a precursor frequency to be detected in primary in vitro assays. These low-frequency cells should be expanded following in vivo skin grafting and therefore be detectable in secondary in vitro cultures. On the other hand, there might be an absence of cells capable of responding directly to xenoantigens, in which case the secondary responses found in vitro would require that peptides of xenoantigens be presented in association with self MHC molecules, in the manner used to recognize nominal antigens.

To distinguish between these two possibilities, secondary xenogeneic proliferation and IL-2 production were measured from cultures in which responder and/or stimulator populations were depleted of APCs by passage over Sephadex G-10 columns at the start of culture. Primary allogeneic cultures served as controls, and primary xenogeneic responses were also measured. The results of a representative experiment are shown in Figs. 3 and 4. Brisk allogeneic proliferation was observed in untreated cultures (Fig. 3, *a* and *c*). Depletion of responder APCs alone had a minimal effect on allogeneic proliferation, depletion of stimulator APCs alone diminished but did not eliminate the allo-MLR, and depletion of both populations of APCs eliminated this response (Fig. 3, *a* and *c*). These results show that direct helper T cell-allogeneic APC interaction is the predominant pathway of alloantigen recognition. As before, brisk secondary xenogeneic proliferation was observed in an untreated culture (Figs. 3 *d*). In contrast with the allo-MLR, depletion of responder APCs alone virtually eliminated the secondary xeno-MLR, whereas depletion of stimulator APCs alone had no effect (Fig. 3 *d*). Depletion of both populations of APCs reduced this response to background levels (Fig. 3 *d*). Primary xenogeneic proliferation was not detected (Fig. 3 *b*).

A similar pattern was observed for IL-2 production (Fig. 4). Brisk IL-2 production was observed in untreated allo-stimulated cultures (Figs. 4, *a* and *c*). Responder APC depletion alone partially diminished allo-stimulated IL-2 production, stimulator APC depletion alone had a somewhat greater negative effect, and depletion of both populations of APCs reduced allo-IL-2 production to background levels (Fig. 4 *a* and *c*). Brisk IL-2 production was observed in an untreated secondary xeno-stimulated culture (Fig. 4 *d*). In contrast with allogeneic IL-2 production, responder APC depletion alone

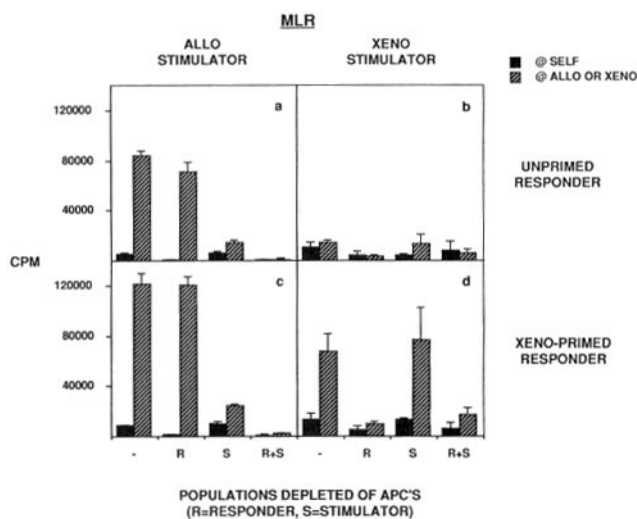


Figure 3. Responder and/or stimulator APC-depleted primary allogeneic (a), primary xenogenic (b), xeno-primed allogeneic (c), and secondary xenogenic (d) proliferation (MLR) in B6 mice, showing that secondary xenogenic proliferation is dependent on responder APCs. Naive (a and b) and xeno-primed (c and d) B6 spleen cells were used as responders, and irradiated BALB/c (a and c) and cynomolgus monkey (b and d) spleen cells were used as allogeneic and xenogenic stimulators, respectively. Allogeneic (a and c) and xenogenic (b and d) responses were measured at 4 and 6 d of culture, respectively. Responder xeno-priming was achieved by grafting of cynomolgus monkey skin 4 wk before in vitro testing. APC depletion was achieved by passing cell populations over Sephadex G-10 columns. Proliferation was measured by [³H]TdR uptake.

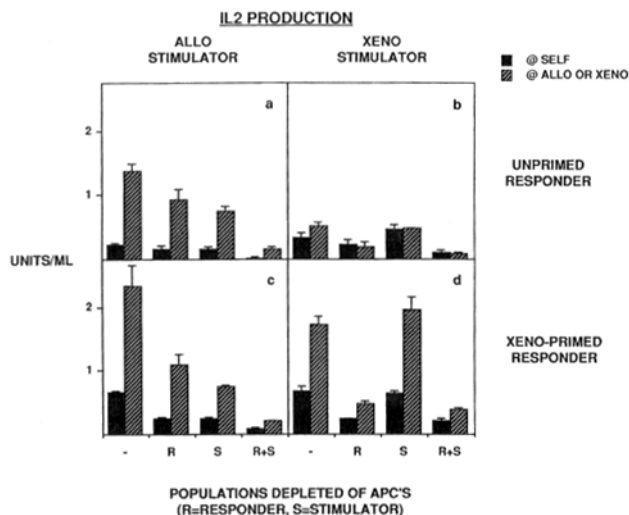


Figure 4. Responder and/or stimulator APC-depleted primary allogeneic (a), primary xenogenic (b), xeno-primed allogeneic (c), and secondary xenogenic (d) IL-2 production in B6 mice, showing that secondary xenogenic IL-2 production is dependent on responder APCs. Responder xeno-priming was achieved by grafting of cynomolgus monkey skin 6 wk before in vitro testing. Supernatants from allogeneic (a and c), primary xenogenic (b), and secondary xenogenic (d) MLR cultures similar to those described in Fig. 3 were harvested at 1.5, 4, and 2 d of culture, respectively, and tested for stimulation of CTLL proliferation. Proliferation data were converted to IL-2 concentration based on the response of CTLL to graded doses of an IL-2 standard, as described in Materials and Methods.

markedly reduced secondary xenogenic IL-2 production, whereas stimulator APC depletion alone had no effect (Fig. 4 d). Depletion of both responder and stimulator APCs reduced this response to background levels (Fig. 4 d). No primary xeno-IL-2 production above background was detectable with or without APC depletion (Fig. 4 b). As noted above, self-stimulated IL-2 production was evident in the untreated xeno-primed samples (Fig. 4, c and d).

These findings demonstrate that secondary xenogenic helper T cell responses of B6 mice are dependent on the presence of self APCs. This suggests that B6 helper T cells respond to xenoantigens after processing and presentation of their peptides in association with self MHC molecules on self APCs, similar to responses to nominal antigens. To investigate further the self APC requirement, an I-A^b-specific mAb 25-9-3s directed against all class II MHC molecules expressed by B6 mice was added to secondary xenogenic and control allogeneic cultures (Table 1). The anti-I-A^b mAb strongly inhibited secondary B6 xenogenic proliferation (Table 1) but not monkey allogeneic proliferation in response to the same stimulators (data not shown). Control studies using mouse allogeneic cultures showed that the activity of the mAb was specific for the H-2^b haplotype (Table 1). These results suggest that xenogenic peptides were recognized in association with class II MHC molecules on the B6 APCs.

Secondary In Vitro Helper T Cell Responses to Xenoantigens are Dependent Upon B6 CD4⁺ Helper T Cells. The demonstration that the B6 mouse responds to xenoantigens in association with self class II MHC molecules suggests that the response is mediated by CD4⁺ helper cells. To demonstrate this directly and to exclude any possible role of CD8⁺ helper T cells, secondary in vitro proliferation and IL-2 production in response to monkey spleen cell stimulation were measured using responders depleted of T cell subsets by antibody plus complement. As before, monkey skin grafts were used to prime the responders. Results were compared with those for allogeneic and primary xenogenic cultures.

The results of a representative experiment are shown in Fig. 5. Brisk proliferation was observed in untreated primary allogeneic cultures (Fig. 5, a and c). Treatment of the responders with complement alone had a mild variable effect of allogeneic proliferation (Fig. 5, a and c). Depletion of either the CD4⁺ or the CD8⁺ subset alone partially diminished the allo-MLR relative to complement-treated control (Fig. 5, a and c), as expected from the ability of either subset to recognize alloantigens directly. Depletion of both subsets (CD4⁺ plus CD8⁺, or Thy-1⁺) virtually eliminated the allo-MLR (Fig. 5, a and c). Similar to previous results, brisk proliferation was observed in an untreated secondary xenogenic culture (Fig. 5 d), and this was mildly augmented by treatment of responders with complement alone (Fig. 5 d). In contrast with the allo-MLR, depletion of CD4⁺ cells alone completely abrogated the secondary xeno-MLR, whereas depletion of CD8⁺ cells alone had no effect relative to complement-treated control (Fig. 5 d). Depletion of both subsets (CD4⁺ plus CD8⁺, or Thy-1⁺) abrogated secondary xenogenic proliferation (Fig. 5 d). Proliferation was absent in the primary xenogenic cultures (Fig. 5 b). Similar to these results,

Table 1. Effect of Anti-I-A^b mAb 25-9-3s on Mouse Allogeneic and Secondary Xenogeneic Proliferation

Response*	Anti-I-A ^b ascites concentration	Mean cpm	
		Self-stimulated	Allo- or xeno-stimulated
BALB/c (H-2 ^d)	—	11,254 ± 873	97,765 ± 8,905
anti-B6 (H-2 ^b)	1/50	3,102 ± 203	15,642 ± 1,580
	1/100	10,023 ± 694	35,915 ± 2,351
BALB/c (H-2 ^d)	—	13,122 ± 502	70,033 ± 6,530
anti-C3H (H-2 ^k)	1/50	5,260 ± 599	80,658 ± 4,249
	1/100	9,164 ± 1,236	122,492 ± 6,771
Xeno-primed B6 (H-2 ^b)	—	18,064 ± 1,276	93,377 ± 9,017
anti-monkey	1/50	1,218 ± 163	6,175 ± 1,962
	1/100	6,051 ± 1,622	56,031 ± 14,898

* MLR cultures were prepared as described in Materials and Methods. Allogeneic proliferation was measured at 4 d and secondary xenogeneic proliferation at 5 d. The B6 responder was primed with monkey skin grafts 19 wk before the proliferation assay.

allo-IL-2 production was partially diminished by depletion of either CD4⁺ or CD8⁺ cells alone and abrogated by depletion of both subsets, and secondary xeno-IL-2 production was completely eliminated by depletion of CD4⁺ cells (with

or without depletion of CD8⁺ cells) but was not affected by depletion of CD8⁺ cells alone (data not shown).

Thus, helper responses of B6 mice to xenoantigens require CD4⁺ T cells, in contrast with allo-responses, which can be mediated by either CD4⁺ or CD8⁺ T cells independently. Together with the previous findings, this suggests that xenogeneic helper responses in B6 mice are mediated by CD4⁺ helper T cells recognizing processed peptides of xenoantigens presented in association with self class II MHC molecules on self APCs.

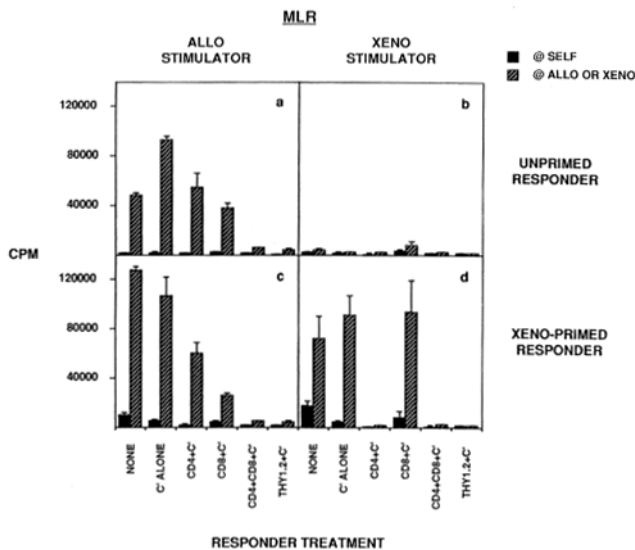


Figure 5. Responder subset-depleted primary allogeneic (a), primary xenogeneic (b), xeno-primed allogeneic (c), and secondary xenogeneic (d) proliferation (MLR) in B6 mice, showing that secondary xenogeneic proliferation is dependent on the CD4⁺ subset. Naive (a and b) and xeno-primed (c and d) B6 spleen cells were used as responders, and irradiated BALB/c (a and c) and cynomolgus monkey (b and d) spleen cells were used as allogeneic and xenogeneic stimulators, respectively. Allogeneic (a and c) and xenogeneic (b and d) responses were measured at 4 and 6 d of culture, respectively. Responder xeno-priming was achieved by grafting of cynomolgus monkey skin 9 wk before in vitro testing. Responder populations were either untreated, treated with complement alone, or depleted of T cell subsets by antibody plus complement as shown. Proliferation was measured by [³H]TdR uptake.

Discussion

We examined B6 mouse spleen cell proliferation and IL-2 production in response to stimulation by cynomolgus monkey spleen cells in order to study the xenogeneic helper T cell immune response. The findings were that (a) primary B6 spleen cell responses to xenoantigens in vitro were very weak or absent but were brisk to MHC alloantigens; (b) secondary xenogeneic responses in vitro after in vivo priming were dependent on the presence of responder APCs, whereas allo-responses were predominantly dependent on stimulator APCs; (c) secondary xenogeneic responses were blocked by an antibody directed against responder (mouse) class II MHC molecules; and (d) secondary xenogeneic responses were dependent on CD4⁺ helper T cells, whereas both CD4⁺ and CD8⁺ cells contributed to the allogeneic response. Therefore, xenogeneic helper T cell responses were weaker than allogeneic responses and involved recognition of processed antigens in association with self class II MHC molecules, in a manner analogous to the response to nominal antigens and unlike the direct recognition of allogeneic MHC molecules.

Previous studies of primary xenogeneic helper responses in vitro have yielded conflicting results. There have been reports of diminished or absent primary xenogeneic helper responses for a range of responders, including the human (18, 19), sheep

(17, 22), goat (17), guinea pig (22, 24), duck (22), goose (22), pigeon (22), rat (17, 20–23), and, in one report, the mouse (25). In contrast, several groups have described primary xenogeneic helper responses that were of a magnitude similar to or even greater than that for allogeneic responses (2, 4–16). Our results clearly show that primary B6 mouse helper responses *in vitro* to widely disparate (monkey) xenoantigens are markedly diminished compared with responses to alloantigens. This was a consistent finding over a wide range of culture conditions. A number of factors could contribute to the discrepancy in findings with other reports (2, 4–16). First, some reports of intact primary responses might reflect an unusually high precursor frequency of helper T cells recognizing xenogeneic peptides in association with self MHC molecules rather than an allo-like direct recognition mechanism. This could result from one of two possibilities: (a) foreign MHC peptides (monomorphic or polymorphic) might be unusually stimulatory, as suggested by the ability of allogeneic class I MHC peptides presented in association with self class II MHC molecules to stimulate primary helper responses *in vitro* (30); (b) responders might be immunized against xenoantigens through exposure to crossreactive environmental antigens, as suggested by the observation that germ-free rats did not generate primary proliferative xeno-responses, whereas conventional rats did (20). While others were unable to confirm the latter finding (16, 23), another study showing that primary mouse anti-human proliferation was blocked by mouse MHC class II-specific antibody (4) supports the general hypothesis. Second, the use of xenogeneic species more closely related than our mouse-monkey combination might explain some intact primary responses. Human-chimpanzee (9), sheep-goat (8, 17), duck-chicken (22) and rat-mouse (10, 11, 13) combinations might represent such close relationships. Third, the studies reporting intact primary xenogeneic responses used either non-mouse responders or mouse responder strains different from the B6 strain used in the present study (4, 11, 16, 31), raising the possibility of species or strain differences in xeno-responsiveness. With respect to the latter possibility, however, diminished primary helper xeno-responses have been found in several mouse strains (25; and Moses, R. D., and H. Auchincloss, Jr., unpublished observations). Fourth, only one other group, studying human anti-mouse responses, measured IL-2 production rather than the potentially nonspecific proliferative response as a parameter of afferent immunity (5), raising the possibility of non-T cell proliferation. Finally, it has been postulated that diminished primary xenogeneic responses might result from a failure of certain lymphokines to function across species differences, as suggested by the observation that primary xenogeneic cytotoxic responses were reconstituted by exogenous syngeneic but not xenogeneic growth factors present during the bulk MLR phase (17). Furthermore, there have been recent reports that exogenous human IL-1 can reconstitute primary human T cell proliferation in response to stimulation by mouse APCs (15, 27). Intact primary responses might then reflect nonspecific stimulation of responder lymphokine release by mitogenic serum components in the culture medium. It should be mentioned that the serum supplement used in the present study

was screened for low mitogenicity, as measured by auto-stimulated proliferation. Considering all these possibilities, we believe the differences in results for widely disparate xenogeneic combinations are best explained by an unusually high precursor frequency of helper T cells recognizing xenogeneic peptides in association with self MHC molecules, manifesting as intact primary helper responses under assay conditions in which particularly mitogenic components are present in the culture medium.

Only a limited number of studies have examined the cellular and antigenic requirements of xenogeneic helper immune responses. Studies by others suggested that secondary human anti-mouse proliferation is T cell-dependent (26) and that xenogeneic proliferation depends on *Lyt-1*⁺ (4) or *CD4*⁺ (5, 25) responder cells. Our results confirm these findings and demonstrate in the mouse the *CD4*⁺ dependency of IL-2 production as well as proliferation. There have been conflicting reports on the nature of the target antigen. Some groups have reported that xenogeneic helper responses to mouse stimulators are directed predominantly against polymorphic determinants of MHC antigens (13, 15, 18, 27), especially class II (5, 26), and to a lesser extent, class I (26) antigens, further supporting the notion of the T cell nature of these responses. Others have determined that mouse responses to xenoantigens are directed against monomorphic determinants, perhaps especially those of MHC class II molecules (4, 25). Our studies, showing similar xenogeneic responses whether the same or different donors were used for primary and secondary stimulation (unpublished observations), indicate that mouse responses to disparate xenoantigens are directed largely against monomorphic determinants. Finally, a small number of studies have explored the APC requirements of xenogeneic helper responses. There have been reports suggesting a dependency of human anti-mouse proliferation on the presence of responder (human) APCs (15, 26, 27). While a similar mechanism has been suggested in the mouse based on inhibition of mouse anti-human proliferation by responder (mouse) MHC class II-specific antibodies (4, 25) and by complement-mediated depletion of mouse MHC class II-positive cells (4), the present study demonstrates this directly for secondary mouse anti-monkey responses by G10 depletion of responder and/or stimulator APCs and for IL-2 production as well as proliferation. Taken together, the data strongly support the notion that mouse helper responses to disparate xenoantigens depend on *CD4*⁺ T cells recognizing xenogeneic peptides in association with self class II MHC molecules on responder APCs.

Our finding that helper responses are dependent on associative recognition of xenoantigens on self APCs raises the question of the nature of the “defect” preventing helper T cells from recognizing xenoantigens directly on xenogeneic APCs. At least three types of interactions required for T cell stimulation could be defective. First, alloreactivity depends in part on the presence of T cells positively selected during thymic education for the expression of receptors with weak affinity for self MHC molecules (3). Defective xenoreactivity might result from a lack of positive selection of xenoreactive T cells because the antigens they recognize are too dissimilar from

self MHC antigens or, on a more fundamental level, from a complete lack of genes encoding xenoreactive T cell receptors. Second, accessory molecule interactions between responder and stimulator, including CD4 ↔ monomorphic MHC class II, CD8 ↔ monomorphic MHC class I, LFA-1 ↔ ICAM-1/ICAM-2, and/or CD2 ↔ LFA-3 interactions, might be defective across species differences. Finally, certain lymphokines involved in signal transmission between APCs and T cells might not function across species differences. Only a few studies have addressed the question of the nature of the defect in helper T cell xeno-recognition. One study showed reconstitution of primary guinea pig anti-mouse CML activity by xenogenous guinea pig but not mouse mitogen-stimulated supernatant during the induction phase (17), presumably reflecting a failure of mouse lymphokine stimulation of guinea pig helper and/or cytotoxic T cells. Two recent studies showed that proliferation of human T cells in response to mouse APCs was reconstituted by exogenous human IL-1 (15, 27), again suggesting a failure of lymphokine function across species differences as a primary xenogeneic defect. With regard to the CD4 molecule, one study demonstrated intact human CD4 interaction with mouse class II MHC molecules (32), whereas another study suggested impaired mouse CD4 interaction with human class II MHC molecules (33). The latter observation, together with other studies of the defect in cytotoxic T cell xenorecognition (34, 35) and our own preliminary studies of the defect in helper responses (Moses, R. D., H. J. Winn, and H. Auchincloss, Jr., manuscript in preparation) suggest that more than just differences in lymphokine function are involved.

Our finding of a dependence of helper responses on associative recognition of xenoantigens on self APCs also raises questions regarding the mechanisms of xenograft rejection. It has been postulated that the activation of cytotoxic T cells by helper T cells depends on intimate contact between the two cell types bound to the same APC, the so-called three-cell model (36). In the case of xenotransplantation, such three-cell conjugates would involve helper and cytotoxic T cells specific for processed xenoantigens expressed in association

with self MHC molecules. However, cytotoxic T cells activated in this way would not find modified self MHC target antigens expressed on the cells of the xenograft. Therefore, "nonclassical" mechanisms of rejection, mediated either by noncytotoxic T cells or by cytotoxic T cells specific for cells expressing processed xenoantigens in association with self MHC molecules (such as self APCs infiltrating the graft), would seem to be required for xenografts. Whether or not the three-cell hypothesis is correct, similar implications for xenograft rejection would exist if cytotoxic T cells themselves are unable to recognize xenoantigens directly on xenogeneic targets. The detection in a number of studies of directly xenoreactive cytotoxic T cells in xenogeneic cultures in vitro might at first glance argue against nonclassical mechanisms of xenograft rejection in vivo. However, these in vitro killers differ from classical cytotoxic T cells in two respects. First, an unusually high frequency of xenoreactive killers recognize class II MHC determinants as opposed to class I determinants (37-40). Second, their precursor frequencies are very low compared with levels usually observed for allogeneic killers, as demonstrated recently by limiting dilution analysis in several studies (34, 41-46). These latter observations raise the possibility that the in vitro assays are detecting very rare cytotoxic clones that may not necessarily be relevant to xenograft rejection in vivo.

Finally, helper T cells are thought to have a critical role in the induction phase of the rejection response. Our demonstration that in vitro xenogeneic (but not allogeneic) helper responses depend on CD4⁺ T cells suggests that xenograft rejection should be particularly dependent on this subset. The finding previously reported from this laboratory (47) that depletion of CD4⁺ T cells in vivo prolonged the survival of xenogeneic but not whole MHC-disparate allogeneic skin grafts in the mouse is well explained by these in vitro findings. The difference between xenogeneic and allogeneic in vitro helper responses raises the possibility that, with successful suppression of the humoral response, xenograft rejection may be controlled more easily and by more specific immunosuppression than allograft rejection.

We thank Susan Shea, Karla Stenger, and Maureen Bedle for expert technical assistance.

This work was supported in part by U.S. Public Health Service grants HL-36372 and HL-18646 and by grant 11 9C1 from the Cystic Fibrosis Foundation.

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Received for publication 13 March 1990 and in revised form 21 May 1990.

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