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Adoptive transfer of unresponsiveness to allogeneic skin grafts with hepatic $\gamma\delta^+$ T cells

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SUMMARY

C3H/HEJ mice injected with irradiated multiple minor incompatible B10.BR lymphoid cells via the portal vein showed delayed rejection of subsequent B10.BR skin grafts. Similar delayed rejection was produced by lateral tail vein injection of B10.BR hepatic mononuclear cells or H-2^k cells pulsed *in vivo* with B10 minor histocompatibility antigens. Inhibition of C3H anti-B10.BR immunity *in vivo* (assessed by delayed graft rejection) and *in vitro* (assessed by B10.BR-induced lymphokine production) can be transferred by radioresistant, plastic-adherent F4/80$^+$33D1$^-$CD4$^-$CD8$^-$ $\alpha\beta$TcR$^-$$\gamma\delta$TcR$^-$ mononuclear hepatic cells from (C3H/HEJ × C3H.SW)F_1 mice injected 36 hr earlier with 100×10^6 irradiated spleen cells. By 10 days post-injection, cells transferring delayed rejection are radiosensitive, plastic non-adherent, F4/80$^-$33D1$^-$CD4$^-$CD8$^-$$\alpha\beta$TcR$^-$$\gamma\delta$TcR$^+$ cells. Injection of interleukin-2 (IL-2) *in vivo* into mice receiving pretreatment with B10.BR cells via the portal vein, or adoptive transfer into such mice of immune anti-B10.BR lymphoid cells, abolished delayed rejection on subsequent skin grafting. Delayed rejection or modulation of lymphokine production was associated in all cases with suppression of IL-2 production and preferential retention of IL-4 production from cells stimulated *in vitro*.

INTRODUCTION

Development of techniques to produce allo-specific tolerance remains the goal of transplant biologists. To date clinical transplantation depends upon treatment with a variety of non-specific immunosuppressive regimes. Where described in a number of model systems, allospecific tolerance seems to be associated with specific deletion of reactive clones (most applicable in early ontogeny),[1] induction of anergy[2,3] or production of ill-defined 'suppression'.[4,5]

We have examined in some detail unresponsiveness induced in mice to multiple minor histocompatibility antigens (B10.BR skin grafted to C3H/HEJ mice), and have suggested that immunosuppression produced by pre-challenge with B10.BR antigen via the portal vein[6,7] is strongly correlated with decreased IL-2 production on stimulation with B10.BR antigens, without concomitant decreases in IL-4 production.[8] We have presented data indicating that mRNA for IL-2 is similarly decreased in such pretreated mice,[9] that analogous data are seen after lateral tail vein pre-immunization in the presence of monoclonal antibodies to the complementary adhesion molecules ICAM-1 and LFA-1,[10] and that unresponsiveness can be induced directly *in vivo* by transfer of antigen-pulsed hepatic APC.

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In the experiments described below we have examined the possibility that cellular adoptive transfer of unresponsiveness from animals given B10.BR antigen via the portal vein is a function of a macrophage-like cell type at early times post-antigen challenge, but associated with a $\gamma\delta$TcR$^+$ T-lymphocyte population at later times (greater than 10 days post-antigen challenge). In addition, experiments were performed to show that unresponsiveness in these mice can be prevented or 'broken' by simultaneous administration of high doses of interleukin-2 (IL-2) or injection of hyperimmune lymphocytes, respectively.

MATERIALS AND METHODS

Mice

C3H/HEJ, B10.Sgn, C3H.SW, BALB/C, BALB/c nu/nu and B10.BR mice were purchased from the Jackson laboratories, Bar Harbour, ME. (C3H/HEJ × C3H.SW)F_1 mice were bred in our laboratory facility. Mice were housed five/cage and allowed food and water *ad libitum*. All mice were used at 8–12 weeks of age.

Monoclonal antibodies

The following monoclonal antibodies (mAb) were used: anti-L3T4 [GK-1.5; American Type Culture Collection (ATCC), Rockville, MD]; anti-Lyt-2 (2.43; ATCC); anti-IL-2 (S4B6; ATCC); anti-IL-4 (11B11; ATCC); anti-mouse macrophage (F4/80; ATCC); anti-mouse dendritic cells (33D1; ATCC); anti-IL-10 (SXC2; ATCC); anti-$\alpha\beta$TcR$^+$ (H57-597; ATCC); anti-

$\gamma\delta$TcR$^+$ (UC713D5; a kind gift from Dr J. Bluestone, University of Chicago). All mAb were grown on ascites in BALB/c nu/nu mice and purified by ammonium sulphate precipitation and resuspension in phosphate-buffered solution (PBS) before use. In control groups a crude preparation of rat or hamster immunoglobulin (30% saturated ammonium sulphate preparation) was used. When injected intraperitoneally (i.p.) 100 μg of protein was used for each recipient; *in vitro* assays used 5 μg/ml of the immunoglobulin (Ig) preparations (lymphokine assays) or 50 μg/ml (cell binding to surface-activated flasks).

Portal vein immunization

All animals were anaesthetized with nembutal. A midline abdominal incision was made and the viscera exposed. Cells were injected in 0·1 ml through a superior mesenteric vein using a 30-gauge needle as described. After injection the needle was rapidly withdrawn and haemostasis secured without haematoma formation by gentle pressure using a 2 mm^3 gel-foam. Complications were seen in less than 10% of mice and these were excluded from analysis because of haemorrhage post-injection.

Cell enrichment on antibody-coated surfaces

Cell subsets from liver mononuclear cell preparations were prepared using AIS MicroCELLector T-25 flasks and different monoclonal antibodies as per the manufacturer's instructions (Applied Immune Sciences Inc., Menlo Park, CA). Control flasks were T-25 flasks coated with normal rat or hamster IgG or were normal tissue culture flasks preincubated with culture medium only. $6{\cdot}5 \times 10^6$ cells were adhered in duplicate to the flasks for 60 min at 37°. After washing gently three times non-adherent cells were pooled from duplicate flasks, concentrated and counted (depletion in all cases ranged from 5·0 to 12·5%). For culture assays using non-adherent cells 5×10^4 cells were incubated in triplicate in microtitre plates with responder immune cells for 24 hr, stimulated with irradiated cells, and supernatants harvested at 60 hr for lymphokine assays. For assays using adherent cells 3×10^6 immune cells in 5 ml medium were added directly to the flasks for 24 hr, 3×10^6 irradiated stimulator cells added, and again supernatants tested at 60 hr. Control groups were treated in identical fashion except that no liver mononuclear cells were used for binding. *In vivo* assays using these cells involved testing only the non-adherent cells for depletion of the activity detected in unfractionated cells. In our hands hepatic mononuclear cell binding to various mAb-treated flasks produced at maximum a 1·5-fold increase in cell recovery on the T-25 flask compared with control flasks (normal tissue culture flasks or MicroCELLector T-25 coated with normal IgG: approximately 5–7% binding). Further depletion of non-adherent cells for a second treatment on the same flask as used for the first round of adherence depletion produced no decrease (0–5%) in recovery of non-adherent cells, and no functionally detectable enrichment/depletion as assayed (see individual experiments). FACS analysis of non-adherent cells after depletion with mAb showed, in all cases, $\geqslant$90% loss of specific fluorescent cells compared with non-separated control populations.

Priming against multiple minor (B10.BR) or major (BALB/c) histocompatibility antigens

This was essentially as described elsewhere.[7] C3H/HEJ mice received two skin grafts from incompatible donors at 21-day intervals, on each occasion with irradiated incompatible spleen cells (100×10^6 i.p. mixed with 100 μg poly A:U as adjuvant). Spleen cells were used from these mice 21 days after the last challenge as a source of immune cells.

Velocity cell sedimentation

Hepatic cells were prepared as described elsewhere,[8] by enzyme digestion (collagenase and DNAse) for 45 min at 37°. 50×10^6 hepatic mononuclear cells pooled from five F_1 mice each injected 36 hr or 10 days earlier with 100×10^6 irradiated B10 spleen cells were sedimented for 4 hr at 4° under unit gravity, as described elsewhere.[11] Fractions corresponding to cells of different size, differing in sedimentation velocity by 1 mm/hr, were collected, centrifuged at 200 *g* at 4° for 8 min, resuspended in culture medium, and counted. Total recovery of input cells was always in the range 80–90%.

For culture studies, cells representing 0·4% of each fraction (the equivalent of 5×10^4 unfractionated cells) were added in triplicate to B10.BR or BALB/c immune cells in 250 μl medium, and wells were stimulated with the appropriate irradiated cells 24 hr later. Lymphokine production was assessed as described below.

For *in vivo* graft studies, cells representing 16% of each fraction (essentially the equivalent of 2×10^6 unfractionated cells) were injected into six/group C3H/HEJ recipients and these mice were grafted 48 hr later with B10.BR skin grafts.

Lymphokines

A transfected cell line producing recombinant murine IL-2 was a kind gift of Dr F. Melchers (Basel, Switzerland).[12] Unconcentrated supernatants of this cell line produced over 5000 units IL-2/ml.

Preparation of cells and lymphokine assays

Immune spleen cell suspensions were prepared aseptically from pools of three mice for each experiment. $2{\cdot}5 \times 10^5$ responder cells were subsequently stimulated with $2{\cdot}5 \times 10^5$ irradiated (2000 rads) spleen stimulator cells in triplicate in α-minimal essential medium supplemented with 2-mercaptoethanol and 10% fetal calf serum (αF_{10}). In some cases responder cells were precultured, before stimulation, with 5×10^4 hepatic mononuclear cells. Supernatants were pooled at 60 hr from replicate wells and assayed in triplicate for lymphokine production as described below. In some experiments the culture wells then received 1 μCi/well of [^{3}H]thymidine ([^{3}H]TdR) and proliferation was measured by harvesting the contents of the well 14 hr later and counting in a well-type β-counter.

IL-2 activity was assayed using the IL-2-dependent cell line, CTLL-2. Recombinant IL-2 for standardization of assays was purchased from Genzyme (Cambridge, MA). All assays were set up in the presence of 11B11 to block potential stimulation of CTLL-2 with IL-4. IL-4 activity was assayed with the IL-4 responsive line CT4.S. Recombinant IL-4 for standardization of IL-4 assays was purchased from Pharmingen (San Diego, CA). All assays for IL-4 were set up in the presence of S4B6 to block IL-2-mediated stimulation. Both the IL-2 and IL-4 assays reproducibly detected 0·2 units of recombinant lymphokine added to cultures. The concentration of mAb used blocked 10 units of lymphokine activity.

Varying volumes (10–100 μl) of supernatant to be tested for activity were transferred to flat-bottomed microtitre plates and

the plates frozen (−20°) to kill any cells carried over. Each well then received 5×10^3 CTLL-2 or CT4.S with the appropriate mAb (11B11 or S4B6, respectively). At 42 hr, 1 μCi [^{3}H]TdR was added to each well and the proliferation was assessed 6 hr later. All studies included a standard IL-2/IL-4 titration curve so that the activity in the supernatants could be expressed as U/ml. In control studies, 11B11 had no effect on the stimulation of CTLL-2 in the presence of 10 U rIL-2, while the addition of S4B6 caused $\geqslant 95\%$ inhibition of the response. Similarly, 11B11 blocked ($\geqslant 95\%$ inhibition) the proliferation of CT4.S cells in the presence of rIL-4.

IL-10 was assayed using inhibition of IL-2 production from cultures of a BALB/c, keyhole limpet haemocyanin (KLH)-specific, IL-2-producing, Th1 cell line (2×10^5 cells) incubated with 24 hr KLH-pulsed, irradiated, plastic-adherent, splenic antigen-presenting cells (APC) (BALB/c).[13,14] The latter APC were precultured in triplicate (5×10^4/well) in 200 μl final volume with KLH (10^{-1} μg/ml) and 100 μl of supernatant putatively containing IL-10 with/without anti-IL-10 mAb (30 μl SXC-2). After 24 hr APC were washed three times with warm αF_{10} and indicator KLH-specific T cells were added to each well. IL-10 supernatant (with/without anti-IL-10) was added back to these cultures, supernatants harvested at 48 hr and assayed in standard fashion for IL-2. One unit of IL-10 was defined as that needed to give 50% inhibition of IL-2 release from the KLH line incubated in the presence of 24-hr KLH-pulsed APC (no added IL-10). A crude supernatant of D10 cells (ATCC) restimulated with conalbumin (48 hr) was used as a standard IL-10 preparation. The SXC-2 used inhibited 10 units of IL-10 as defined by this assay.

RESULTS

Inhibition of graft rejection *in vivo* and IL-2 production *in vitro* with hepatic mononuclear cells isolated from B10.BR mice or (C3H/HEJ × C3H.SW)F_1 mice prechallenged *in vivo* with irradiated B10 spleen cells

We reported earlier that injection of irradiated multiple-minor incompatible (B10.BR) spleen cells via the portal vein (p.v.), or of hepatic mononuclear cells via the lateral tail vein (i.v.), into C3H/HEJ recipients led to prolonged survival of B10.BR skin allografts. This was associated with a diminished production of IL-2, but not IL-4, on restimulation *in vitro* of spleen cells taken from pretreated mice.[8] We hypothesized that hepatic APC were less able to stimulate Th1 compared with Th2 cells. As a more direct test of the role of hepatic mononuclear cells on graft prolongation (*in vivo*) and failure to restimulate Th1 cells for IL-2 production *in vitro*, we analysed the effect of hepatic mononuclear cells isolated from mice pulsed with antigen *in vivo*. (C3H/HEJ × C3H.SW)F_1 mice were immunized with 10×10^7 irradiated B10 spleen cells via the portal vein and at 36 hr and 10 days thereafter hepatic cells were transferred either intravenously (i.v.) into C3H mice which were subsequently grafted with B10.BR or BALB/c skin, or into cultures of C3H anti-B10.BR or anti-BALB/c spleen cells. In some cases hepatic mononuclear cells were irradiated before use (Table 1). Data in Fig. 1 and Table 1 represent typical data for one of three such experiments.

No treatment used affected the immune response to BALB/c cells, measured *in vivo* or *in vitro* (data not shown, but see ref. 8). However, graft rejection *in vivo* and IL-2 production *in vitro* (but not IL-4 production) were inhibited at both time-points tested by antigen-pulsed cells from F_1 animals and by B10.BR hepatic mononuclear cells (as reported elsewhere). Inhibition of IL-2 production was correlated with detectable increases in *in vitro* production of IL-10 from restimulated cells (Table 1). Interestingly, while inhibition by B10.BR cells was radio-resistant (2000rads), that produced by antigen-pulsed hepatic mononuclear cells was radio-resistant early (36 hr) but not at 10 days. While data in Fig. 1 pertain only to cells obtained from F_1 animals at 10 days post-injection with antigen (B10), in other experiments (not shown here) we have demonstrated *radioresistance of 36-hr pulsed* F_1 hepatic cells in prolonging graft rejection *in vivo*, analagous to the radio-resistance of their function *in vitro* at this time (Table 1).

Characterization of hepatic cells transferring inhibition of IL-2 production and graft rejection

While hepatic mononuclear cells from antigen-pulsed animals were able to transfer inhibition of anti-B10.BR reactivity *in vivo* and *in vitro* at 36 hr and 10 days following antigen exposure, the difference in radiosensitivity at these times implied there might be different cells involved in these situations. Accordingly, experiments were designed to characterize those cells by biophysical means (unit gravity sedimentation, Fig. 2, or plastic adherence, Fig. 3) and using a variety of mAb (Figs 3 and 4).

Analysis of Fig. 2a shows, as indicated earlier, minimal effects on IL-4 production *in vitro* using the various cell populations. However, a biphasic inhibition curve for IL-2 production is seen with both B10.BR hepatic cells and 10-day pulsed cells, with peak inhibition present in the cell fractions sedimenting in the region 2·5–4 mm/hr and 5·3–6·5 mm/hr. In contrast, inhibition from 36-hr pulsed hepatic cells was found concentrated in cells sedimenting with a velocity of 5·3–6·5 mm/hr, and essentially no-where else. There was no inhibition of lymphokine production from anti-BALB/c immune cells (data not shown, but see Table 1).

Figure 2b shows the ability of the same fractions obtained with velocity sedimentation to prolong skin graft survival when adoptively transferred into naive C3H/HEJ recipients. Day 10-pulsed cells sedimenting with velocities of 2·5–4 mm/hr and 5·3–6·5 mm/hr could cause significant graft prolongation, equal to that produced by 36-hr pulsed cells sedimenting with a velocity of 5·3–6·5 mm/hr. There was an inconsistent, generally insignificant, graft prolongation effect occasionally seen with faster sedimenting cells also from 10-day pulsed populations (■). While not shown in this figure, where tested both *in vitro* and *in vivo*, inhibition was antigen specific, as already documented for unfractionated cells (Table 1).

To characterize further the hepatic inhibitory cells, we tested their ability to adhere to standard plastic tissue culture flasks, and to MicroCELLector flasks coated with mAb against murine dendritic cells/macrophages. These data are shown in Fig. 3. Inhibition (of IL-2 production) using F_1 hepatic mononuclear cells from 36-hr but not 10-day *in vivo*-pulsed animals was due to plastic-adherent, radio-resistant cells. In addition, at 36 hr, but again not at 10 days, post-*in vivo* pulsing, these inhibitory cells adhered to flasks coated with mAb to murine tissue macrophages (F4/80) but not dendritic cells (33D1). This conclusion was based on analysis of both the enriched, adherent, populations and the depleted, non-adherent, cells. Panel (a) shows data from *in vitro* assays to measure inhibition of IL-2 production

Table 1. Sensitivity to irradiation of inhibition of IL-2 production by *in vivo* antigen-pulsed hepatic monocytes at different times after antigen challenge

	Anti-B10.BR			Anti-BALB/c	
	Lymphokine in culture medium (units/ml)‡				
Source of F_1 cells*	IL-2	IL-4	IL-10	IL-2	IL-4
None	8·4±1·9	2·7±0·6	⩽1·5	7·9±1·5	2·8±0·5
36 hr after in vivo *injection*					
Non-pulsed	8·0±1·6	2·5±0·8	⩽1·5	8·6±2·0	2·6±0·6
Pulsed	2·3±0·9§	3·9±1·1	8·9±2·5	8·2±2·1	2·9±0·8
Pulsed†	2·9±0·8§	3·2±1·3	9·0±2·3	6·9±2·0	2·5±0·9
B10.BR cells	1·9±0·5§	3·1±1·1	11±3·0	8·1±2·2	2·7±0·8
10 days after in vivo *injection*					
Non-pulsed	7·9±2·3	2·3±0·7	⩽1·5	7·3±2·1	2·5±0·6
Pulsed	3·1±1·3§	3·1±0·9	9·5±2·6	8·5±1·8	3·0±0·8
Pulsed†	6·8±2·2	2·5±1·0	⩽1·5	7·9±2·4	2·4±0·9
B10.BR cells	2·2±0·9§	2·8±0·8	7·5±2·7	8·4±2·0	2·6±0·8
B10.BR cells†	2·3±0·6§	2·7±0·8	8·9±2·5	8·3±2·3	2·6±0·7

* Six (C3H/HEJ × C3H.SW)F_1 mice were injected with 0·5 ml PBS (non-pulsed) or with 100×10^6 irradiated (2000 rads) B10 spleen cells (in 0·5 ml PBS) via the portal vein (pulsed). 36 hr (rows 2–4) or 10 days (rows 6–8) later hepatic mononuclear cells were prepared from three mice/group and from three age-matched B10.BR mice (rows 5, 9, 10). 5×10^4 cells were incubated with $1{\cdot}5 \times 10^5$ cells/well C3H anti-B10.BR or anti-BALB/c immune T cells. These cells were stimulated 24 hr later with the appropriate irradiated spleen stimulator cells, supernatants pooled within groups at 60 hr, and assayed for IL-2, IL-4 or IL-10 production.

† Pulsed hepatic mononuclear cells were irradiated (2000 rads) before culture.

‡ Arithmetic mean (±SEM) units lymphokine/ml at 60 hr. See the Materials and Methods for more detail. Immune cells in row 1 were stimulated in the absence of any hepatic mononuclear cells. Proliferation in control (unstimulated/lymphokine stimulated) cultures was: CTLL-2, 545±105, 28,540±4240; CT4.S, 320±75, 23,455±3250, respectively. Proliferation of CTLL-2 in the presence of supernatants from KLH-immune cells stimulated in the presence/absence of D10 supernatant (crude IL-10) was 3015 and 18,655, respectively. No lymphokines were detected in supernatants of unstimulated immune cells. IL-10 data are shown only for B10.BR-stimulated cells. Production in BALB/c-stimulated cells was below the level of detection for this assay (⩽1·5 units/ml).

§ $P \leqslant 0{\cdot}05$ compared with row 1.

with adherent cells. Essentially no perturbation of IL-4 synthesis was seen with any cell population tested. When non-adherent cells from 36-hr pulsed hepatic mononuclear cells, depleted on mAb-coated flasks or by plastic adherence alone, with/without irradiation, were assessed for inhibition of skin graft rejection *in vivo*, comparable data were seen (panel b). F4/80 and plastic adherence all decreased functional inhibition of graft rejection *in vivo*, when compared with untreated cells or irradiation treatment only. Inhibition from these 36-hr pulsed hepatic cells was thus associated with $F4/80^+$, $33D1^-$, plastic-adherent, radio-resistant cells. Data with hepatic mononuclear cells from 10-day pulsed mice (not shown) showed that only irradiation caused any decrease in activity in the hepatic cell preparation. Adherence to plastic, or to F4/80- or 33D1-coated flasks, produced no observable depletion of activity.

Data in Fig. 4 show *in vitro* (panel a) and *in vivo* (panel b) analyses of cells after adherence to flasks coated with mAb to various T-cell-specific antigens, namely CD4, CD8 or anti-$\alpha\beta/\gamma\delta$ TcR. Again, while only data using B10.BR immune cells are shown, no significant reproducible change in lymphokine production from BALB/C immune cells was ever observed in these studies (see also Table 1). Only plastic adherence caused significant enrichment for inhibitory activity from 36-hr pulsed hepatic cells, with no enrichment seen on anti-L3T4, -Lyt-2, -$\alpha\beta$TcR or -$\gamma\delta$TcR mAb-coated flasks (Fig. 4a). All activity in these latter cases remained in the non-adherent cells (Fig. 4b). There was an irreproducible tendency for augmentation of IL-2 production using $\alpha\beta TcR^+$ cells from these mice (Fig. 4a); this phenomenon has not yet been investigated further. By contrast, using hepatic mononuclear cells from 10-day pulsed mice, adherence to anti-$\gamma\delta$TcR mAb-coated flasks caused marked enrichment of inhibitory activity (panel a) with corresponding loss of activity in the non-adherent population (panel b). By these criteria, inhibition of IL-2 production from 10-day pulsed hepatic cells was due to radio-sensitive, $L3T4^-$, $Lyt\text{-}2^-$, $\alpha\beta TcR^-$, $\gamma\delta TcR^+$ cells. Again, using either preparation of hepatic cells, no significant perturbation of IL-4 synthesis was seen.

Comparison of the *in vitro* data with the effects of adherence-depleted cells on skin graft rejection (Fig. 4b) confirmed that only irradiation or adherence to $\gamma\delta$TcR mAb-coated flasks caused significant loss of activity from 10-day pulsed hepatic cells. Data for 36-hr cells are not shown in this figure but, as expected (see Fig. 3), no mAb depletion produced any notice-

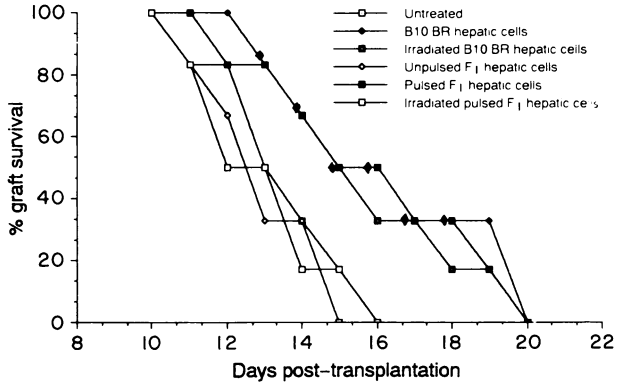

Figure 1. Inhibition of rejection of B10.BR skin grafts in C3H/HEJ mice given 10-day B10-pulsed (C3H/HEJ × C3H.SW)F_1 hepatic mononuclear cells is abolished after irradiation of hepatic cells. Groups of six mice received i.v. injections, 48 hr before skin grafting, of 2×10^6 hepatic cells from (C3H/HEJ × C3H.SW)F_1 given 100×10^6 irradiated B10 spleen cells via the portal vein (p.v.) 10 days earlier or from unpulsed F_1 mice. Some groups received irradiated hepatic cells. Graft survival was prolonged relative to untreated controls only for groups receiving irradiated/unirradiated B10.BR hepatic cells, or unirradiated B10-pulsed F_1 cells ■, ◆, or ■, respectively): Mann–Whitney *U*-test, $P \leqslant 0{\cdot}05$.

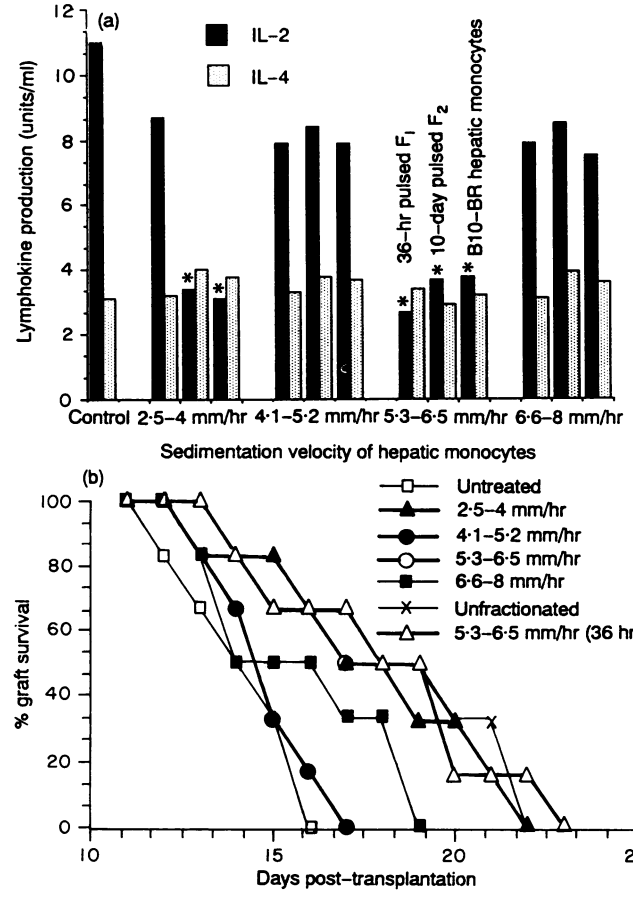

Figure 2. Inhibition of lymphokine production *in vitro* from B10.BR-stimulated C3H anti-B10.BR immune cells (a), or B10.BR skin graft rejection in C3H/HEJ mice (b), with hepatic cells derived from pools of five/group B10.BR or 36-hr/10-day B10-pulsed (C3H/HEJ × C3H.SW)F_1 mice. Hepatic cells were fractionated into populations of different size based on velocity sedimentation before use. See the Materials and Methods for more detail. In (a), IL-2 and IL-4 were assayed at 60 hr in culture supernatants, pooled from replicate cultures, using CTLL-2 and CT4.S targets. Data shown are arithmetic means for triplicate cultures (SEM, not shown to retain clarity, ⩽ 10% in all cases). * Represents values significantly different from control cultures (no added hepatic cells), $P \leqslant 0{\cdot}05$. Data from one of three studies. In (b), a control group (—) received no hepatic cells. For simplicity only the data for that one sedimented fraction of cells from 36-hr B10-pulsed mice able to prolong graft survival (sedimentation velocity 5·3–6·5 mm/hr) are shown (△——△), though all fractions for 10-day-pulsed mice are included. Data for (×), (○), (▲) and (△) are significantly different from untreated mice: Mann–Whitney *U*-test, $P \leqslant 0{\cdot}05$; for (■), $P \leqslant 0{\cdot}10$.

able loss of inhibition of skin graft rejection compared with untreated cells. These data are consistent with a model in which inhibition of IL-2 production *in vivo* (perhaps a function of enhanced IL-10 production) occurs after portal venous immunization, and this in turn promotes skin graft survival. In addition, there seems to be a production of inhibitory hepatic cells capable of adoptively transferring this decreased reactivity to the antigens infused via the portal vein. Early (36-hr) post-immunization inhibition is associated with plastic-adherent, 5·3–6·5 mm/hr sedimenting, radio-resistant, F4/80$^+$,33D1$^-$ cells, while at later times (day 10 and following) radio-sensitive, $\gamma\delta$TcR$^+$ cells are implicated.

Co-injection of IL-2, or adoptive transfer of immune cells, breaks inhibition of skin graft rejection produced by portal venous immunization

We next investigated whether induction of graft prolongation in this model (using portal venous pre-immunization) could be abolished by simultaneous administration of exogenous IL-2, or by co-injection of anti-B10.BR immune cells. Data in Fig. 5 are from one of two such studies: only B10.BR graft survival is shown for clarity (there was no perturbation of BALB/c skin graft survival).

As noted before, pretreatment via the portal vein, but not the lateral tail vein, produces graft-specific enhancement of B10.BR grafts. This effect was abolished by adoptive transfer of high (50×10^6) but not low (10×10^6) numbers of anti-B10.BR immune cells, or by ongoing injection of IL-2 lymphokine (5000 units in 0·5 ml medium). When lymphokine production from antigen-stimulated spleen cells of treated mice was assayed in separate studies (12 days post-skin grafts), portal vein immunized mice which also received immune cells or IL-2 did not show the expected reversal in IL-2/IL-4 ratio and enhancement of IL-10 production (Table 2, data from one of three studies).

DISCUSSION

There still exists considerable controversy regarding the possible mechanism(s) responsible for induction and/or maintenance of unresponsiveness in experimental and clinical transplant situations. Previous data from this laboratory have documented that C3H mice grafted with tail skin from multiple minor incompatible B10.BR donors show specific delayed graft rejection if they are first pretreated via the portal vein with irradiated B10.BR cells. It has been suggested that this inhibition is associated with a preferential stimulation of recipient Th2-type anti-B10.BR T cells by antigen presented by host hepatic mononuclear cells, and indeed, when tested at early times (days 10–20) post-

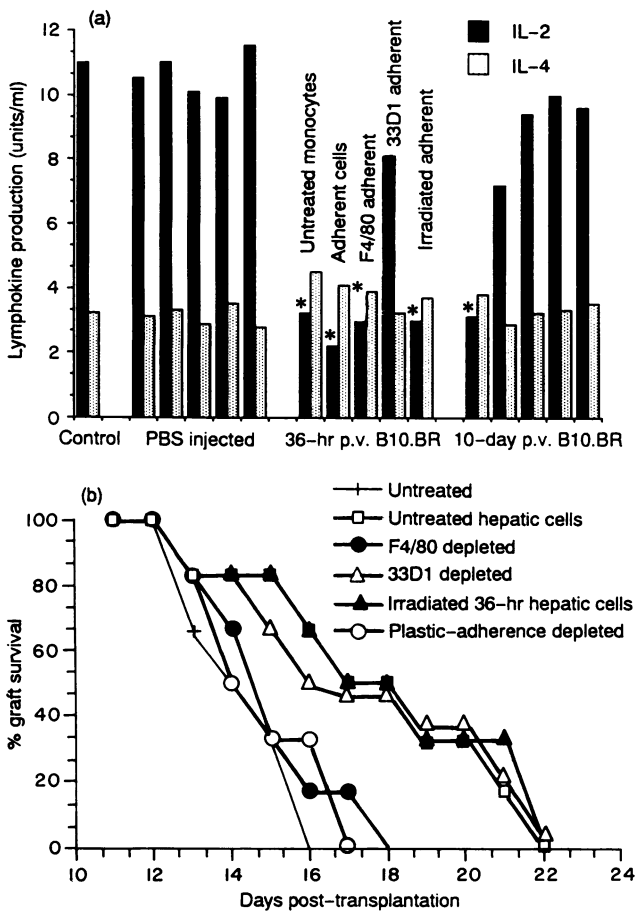

Figure 3. (a) Inhibition of IL-2 production *in vitro* from restimulated anti-B10.BR immune spleen cells precultured for 24 hr with adherent-enriched hepatic cells from (C3H/HEJ × C3H.SW)F_1 mice treated with PBS or pulsed 36 hr/10 days earlier with 100×10^6 irradiated B10 spleen cells via the p.v. Adherent cells were prepared as described in the Materials and Methods. In some groups cells adherent to normal culture flasks were irradiated (2000 rads) before addition of immune cells. Non-adherent cells (depletion in all flasks ranged from 5 to 12·5%) were assayed in separate experiments (see b). 3×10^6 immune cells were used per flask (in 5 ml medium) and at 24 hr 3×10^6 irradiated stimulator spleen cells added. Supernatants pooled from replicate cultures were tested at 60 hr using CTLL-2 or CT4.S for IL-2/IL-4 assays, respectively. All data represent arithmetic means of triplicate cultures (SEM, not shown to retain clarity, $\leqslant 10\%$ in all cases). * Represents groups significantly different from control cultures (no added hepatic cells), $P \leqslant 0{\cdot}05$. (b) Inhibition of B10.BR skin graft rejection using non-adherent hepatic cells, obtained from 36-hr B10-pulsed F_1 mice and isolated from the flasks described in (a). Cells recovered from duplicate flasks were pooled and 2×10^6 cells injected i.v. into six/group C3H/HEJ mice. Control groups received no treatment or unfractionated hepatic mononuclear cells. All groups received B10.BR skin grafts 48 hr later. Groups (▲), (△) and (□) show graft survival significantly different from control mice: Mann–Whitney *U*-test, $P \leqslant 0{\cdot}05$.

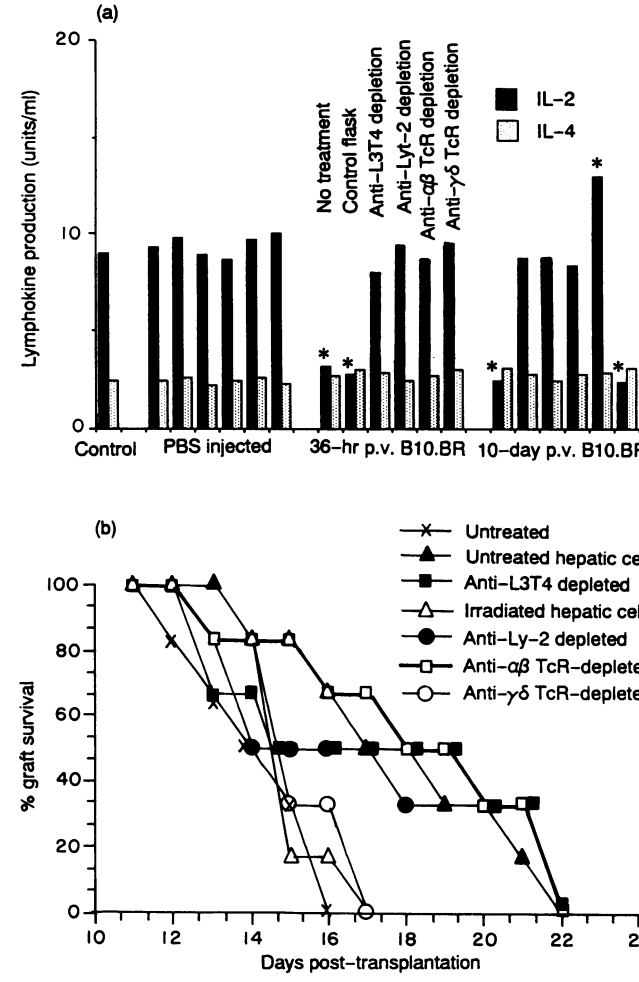

Figure 4. (a) Inhibition of IL-2 production *in vitro* from restimulated anti-B10.BR immune spleen cells precultured for 24 hr with hepatic mononuclear cells from F_1 mice (PBS treated or pulsed 36 hr/10 days earlier with irradiated B10 spleen cells via the p.v.) enriched on duplicate T-25 MicroCELLector flasks precoated with monoclonal antibodies to different T-cell surface molecules. See the Materials and Methods and legend to Fig. 3 for more details. Control flasks were standard tissue culture flasks. Non-adherent cells (depletion in all cases ranged from 5 to 12·5%) were assayed in separate experiments (see b). Supernatants were pooled from duplicate flasks at 60 hr and assayed with CTLL-2 or CT4.S, respectively. Data points represent arithmetic means of triplicate determinations (SEM, not shown to retain clarity, $\leqslant 10\%$ in all cases). * Groups significantly different from control (no added hepatic cells), $P \leqslant 0{\cdot}05$. (b) Inhibition of rejection of B10.BR skin grafts using non-adherent hepatic cells, isolated from 10-day B10-pulsed F_1 mice, and pooled from duplicate flasks described in (a). See also the Materials and Methods and legend to Fig. 3 for more details. A control group (×) received no hepatic cells. Data shown are for groups of six mice. (▲), (■), (□) and (●) are significantly different from control: Mann–Whitney *U*-test, $P \leqslant 0{\cdot}05$.

treatment, spleen cells from such pretreated skin grafted mice show enhanced IL-4/IL-2 production ratios relative to cells from non-portal vein immunized animals.[8,9] Blocking early signalling events necessary for effective Th1 activation using mAb to the adhesion molecules ICAM-1/LFA-1 also leads to delayed rejection, analagous to results seen in other graft models.[15] More recent data show that antibodies to IL-10 can apparently reverse this delayed rejection, which may imply that ongoing inhibition of rejection (and suppression of Th1 cells) is dependent upon lymphokines released by activated Th2 cells.[13]

If indeed portal venous immunization delays rejection because antigen is taken up by hepatic APC which are unable to trigger effective rejection responses and instead anergize Th1

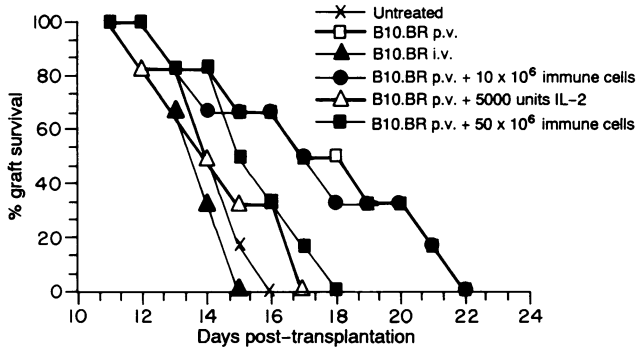

Figure 5. Inhibition of graft rejection after p.v. preimmunization is abolished by adoptive transfer of immune cells, or by alternate day treatment with exogenous IL-2. Groups of six mice received no pretreatment (×) or 100×10^6 irradiated spleen cells i.v. or p.v. 36 hr later different subgroups of p.v. prechallenged mice were also injected i.v. with 10×10^6 or 50×10^6 cells from a pool of three anti-B10.BR immune mice, or began injections intraperitoneally with 5000 units recombinant IL-2 in 0·5 ml culture medium. This latter group continued to receive IL-2 on alternate days for the duration of the experiment. All mice received B10.BR skin grafts on this same day and graft survival followed. Only groups (●) and (□) showed enhanced survival relative to control mice: Mann–Whitney *U*-test, $P \leqslant 0{\cdot}05$.

cells, then it should prove possible to transfer delayed rejection with *in vivo*-pulsed hepatic APC. Data presented in Fig. 1 indicate that *in vivo* B10-minor antigen-pulsed F_1 hepatic mononuclear cells can transfer/induce B10 minor-specific non-responsiveness to B10.BR skin grafts when injected intravenously. This function is apparently radio-sensitive. *In vitro* studies of lymphocytes obtained from these C3H mice given such hepatic mononuclear cells suggest that the non-responsive state is associated with inactivation of Th1 cells and stimulation of Th2 cells to secrete elevated amounts of IL-10 (Table 1).

Studies attempting to characterize the cell type responsible for transferring the inhibition of IL-2 production and delayed graft rejection showed a more complex situation existed. There is heterogeneity in the hepatic mononuclear cell population producing these phenomena, as assessed by both biophysical criteria (cell size determined by velocity sedimentation; Fig. 2) and cell-surface molecule expression (using mAb; Figs 3 and 4). Soon after antigen is presented to the system, adoptive transfer of inhibition of rejection (and of activation of Th2 cells) is associated with medium-size (*s* value 5·3–6·5 mm/hr; Fig. 2), plastic-adherent, radio-resistant cells, which can be depleted by binding to mAb directed to cells of the macrophage lineage (F4/80), and not to dendritic cells (33D1) (Fig. 3).

Velocity sedimentation studies of hepatic cells to 10 days post-pulsing *in vivo* with B10-minor antigens indicated that there are two different sized (2·5–4 mm/hr and 5·3–6·5 mm/hr) cell populations responsible for transferring inhibition. Both populations induce graft prolongation *in vivo* on adoptive transfer (Fig. 2) and inhibit IL-2 production *in vitro* with no apparent effect on IL-4 production (Fig. 2). In contrast to earlier times (36 hr post-*in vivo* pulse with antigen), the cell types responsible for transferring inhibition of graft rejection are radio-sensitive, non-adherent to plastic, and do not bind to mAb to macrophages (F4/80) or dendritic cells (33D1) but do to mAb directed at the $\gamma\delta$TcR (Figs 3 and 4). Data presented in Fig. 4 show that the inhibitory activity for depressing IL-2

Table 2. Lymphokine production from portal vein pretreated mice given immune cells or IL-2 before B10.BR skin grafts

	Lymphokine in culture medium (units/ml)†		
Source of cells*	IL-2	IL-4	IL-10
Untreated	9·5±2·1	2·4±0·6	≤1·5
B10.BR i.v.	8·9±2·4	2·6±0·5	≤1·5
B10.BR p.v.	2·6±1·0	3·5±0·8	6·9±2·0
B10.BR p.v. + 50×10^6 immune cells	7·9±1·9	2·7±0·5	≤1·5
B10.BR p.v. + 5000 units IL-2	8·8±2·1	2·8±0·6	≤1·5

* Spleen cells were pooled from groups of three mice 12 days after grafting with B10.BR tail skin. 36 hr prior to skin grafting mice received either PBS (0·15 ml) or 100×10^6 irradiated B10.BR spleen cells via the lateral tail vein (i.v.) or portal vein (p.v.). Separate groups of mice received 50×10^6 C3H anti-B10.BR immune spleen cells i.v. immediately before grafting (immune cells were pooled from three mice 14 days after rejection of their second B10.BR skin graft), or were injected i.p. with 5000 units recombinant IL-2 on alternate days (0, 2, 4, etc.), beginning immediately before grafting. Spleen cells from these five groups were cultured in microtitre plates with irradiated B10.BR stimulator cells for 48 hr and tested in standard fashion for lymphokine release into the supernatant (see the Materials and Methods).

† Lymphokine assays were as described in Table 1 and the Materials and Methods. Data represent arithmetic means±SEM of triplicate cultures. Control cultures of CTLL-2 and CT4.S (±lymphokine) showed the following incorporation of radioactivity: 873±109, 19,755±1760; 545±78, 17,690±2135. Proliferation of CTLL-2 in the presence of supernatants from KLH-immune cells stimulated with/without a crude D10 supernatant were 3025±675, 18,890±2255, respectively. No IL-2 production was detected from unstimulated KLH-immune cells. There was no evidence for detectable 'carry-over' of IL-2 on spleen cells from IL-2-treated mice (last row), as detected using supernatants from unstimulated (with B10.BR spleen cells *in vitro*) cultures.

production, with persistence of IL-4 production, was associated with $\gamma\delta$TcR$^+$ cells. Moreover, mAb (anti-$\gamma\delta$TcR) depletion of 10-day pulsed hepatic cells before adoptive transfer abrogated the inhibitory potential of the inoculum. Thus at later times (10 days post-pulse with minor antigens) the hepatic cells associated with inhibition of skin graft rejection as a whole did not exhibit macrophage-like characteristics, but were $\gamma\delta^+$TcR$^+$CD4$^-$CD8$^-$ cells sedimenting with two different sedimentation velocities. These differences in sedimentation velocity may reflect different activation stages of the same $\gamma\delta^+$TcR$^+$ population. While there is little data yet to indicate a physiological role for $\gamma\delta^+$ T cells, an interesting recent article describes a large increase in numbers of these cells also at the maternal-fetal interface (a 'natural' allograft situation).[16] Other studies in the literature have examined the immunoregulatory role of macrophages (e.g. ref. 17). In this particular study the alveolar macrophages were shown to regulate the APC function of dendritic cells, a suppression which could be abrogated by inhibition of the nitric oxide synthetase pathway.

In a final study we explored the role of IL-2 administration *in vivo* in the delayed graft rejection seen after portal venous immunization, and whether inhibitory cells of the type described above could act *in situ* to inhibit rejection responses mediated by

primed cells. As shown in Fig. 5, ongoing administration of IL-2 abolished the delayed rejection afforded by pretreatment with portal venous immunization. This is reminiscent of findings on the role of exogenous IL-2 in reversing tolerance to a transgenic K^b expressed in pancreatic islet cells by Miller *et al.*,[18] of transgenic expression of IL-2 in inducing diabetes in mice expressing appropriate TcR genes,[19] and in the inhibition of induction of tolerance by neonatal antigen administration.[20,21] The latter is a model apparently associated with preferential activation of IL-4-producing cells. A similar subset of $CD4^+$ T cells could apparently regulate adoptive transfer of autoimmune disease in rats.[22] Only transfer of 50×10^6 immune cells, but not 10×10^6 immune cells, caused a similar loss of the delayed rejection response in portal vein immunized mice. Since such small numbers of immune cells can transfer rapid rejection of grafts to *naive* mice (data not shown), this suggests that active inhibition of small numbers of immune cells can occur in portal vein immunized animals. Similar 'infectious tolerance' has recently been described in another model of transplant rejection by Chen *et al.*[23]

A note of interest is the transient nature of the non-responsiveness obtained following portal venous immunization as well as after adoptive transfer of the pulsed hepatic cells, despite the fact that these animals had been grafted with minor-incompatible skin grafts. Accumulating evidence in the literature suggests that the perpetuation of a tolerant state depends upon the persistence of alloantigen in the host system.[24] More recently[25] it has been reported that *in vivo*-induced anergy is reversed in the absence of antigen. Thus loss of unresponsiveness in the situations described above may indicate the loss of those particular minor alloantigens from the host microenvironment which were presented in a tolerogenic form.

In summary, our data lend further support to the notion that modification of lymphokine production *in vivo* can contribute to changes in graft outcome. Furthermore, antigen-specific portal venous immunization prior to transplantation apparently produces altered lymphokine profiles because hepatic APC do not activate effectively Th1 cells, and also induce a $CD4^-CD8^-\alpha\beta TcR^-\gamma\delta TcR^+$ cell which can produce operational inhibition of Th1 activation.

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REFERENCES

1. Kappler J.W., Roehm N. & Marrack P. (1987) T-cell tolerance by clonal elimination. *Cell*, **49,** 273.
2. Jenkins M.K. & Schwartz R.H. (1987) Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *J. exp. Med.* **165,** 302.
3. Mueller D.L., Jenkins M.K. & Schwartz R.H. (1989) Clonal expansion versus clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Ann. Rev. Immunol.* **7,** 445.
4. Batchelor J.R., Lombardi G. & Lechler R.I. (1989) Speculations on the specificity of suppression. *Immunol. Today*, **10,** 37.
5. Brochu S., Roy D.C. & Perreault C. (1992) Tolerance to host minor histocompatibility antigens after allogeneic bone marrow transplantation: specific donor-host responsiveness is maintained by peripheral tolerizing cells. *J. Immunol.* **149,** 3135.
6. Qian J.H., Hashimoto T., Fujiwara H. & Hamaoka T. (1985) Studies on the induction of tolerance to alloantigens. I. The abrogation of potentials for delayed-type hypersensitivity responses to alloantigens by portal venous inoculation with allogeneic cells. *J. Immunol.* **134,** 3656.
7. Gorczynski R.M. & Holmes W. (1991) Specific manipulation of immunity to skin grafts bearing multiple minor histocompatibility differences. *Immunol. Lett.* **27,** 163.
8. Gorczynski R.M. (1992) Immunosuppression induced by hepatic portal venous immunization spares reactivity in IL-4 producing T lymphocytes. *Immunol. Lett.* **33,** 67.
9. Gorczynski R.M. & Wojcik D. (1992) Antigen presentation by murine splenic, but not hepatic, antigen-presenting cells to induce IL-2/IL-4 production from murine T cells is regulated by interactions between LFA-1/ICAM-1. *Immunol. Lett.* **34,** 177.
10. Gorczynski R.M., Gulay Z. & Wojcik D. (1993) Differential sensitivity to anti-LFA-1 inhibition of Th1 vs Th2 anti-minor histocompatibility antigen immune T cells after restimulation with antigen on hepatic or splenic APCs. *Transpl. Proc.* **25,** 807.
11. Miller R.G. & Phillips R.A. (1969) The separation of cells by velocity sedimentation. *J. Cell. Comp. Physiol.* **73,** 191.
12. Karasuyama H. & Melchers F. (1988) Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. *Eur. J. Immunol.* **18,** 97.
13. Fiorentino D.F., Zlotnik A., Vieira P., Mosmann T.R., Howard M., Moore K.W. & O'Garra A. (1991) IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* **146,** 3444.
14. Gorczynski R.M. & Wojcik D. (1994) A role for non-specific (cyclosporin A) or specific (monoclonal antibodies to ICAM-1, LFA-1 and interleukin-10) immunomodulation in the prolongation of skin allografts after antigen-specific pre-transplant immunization or transfusion. *J. Immunol.* **152** (in press).
15. Isobe M., Yagita H., Okumura K. & Ihara A. (1992) Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science*, **255,** 1125.
16. Heyborne K.D., Cranfill R.L., Carding S.R., Born W.K. & O'Brien R.L. (1992) Characterization of $\gamma\delta$ T lymphocytes at the maternal–fetal interface. *J. Immunol.* **149,** 2872.
17. Holt P.G., Oliver J., Bilyk N., McMenamin C., McMenamin P.G., Kraal G. & Thepen T. (1993) Downregulation of the antigen presenting function(s) of pulmonary dendritic cells *in vivo* by resident alveolar macrophages. *J. exp. Med.* **177,** 397.
18. Miller J.F.A.P., Morahan G., Allison J. & Hoffman M. (1991) A transgenic approach to the study of peripheral T-cell tolerance. *Immunol. Rev.* **122,** 103.
19. Heath W.R., Allison J., Hoffmann M.W., Schonrich G., Hammerling G., Arnold B. & Miller J.F.A.P. (1992) Autoimmune diabetes as a consequence of locally produced interleukin 2. *Nature*, **395,** 547.
20. Malkovsky M., Medawar P.B., Thatcher D.R., Toy J., Hunt R., Rayfield L.S. & Dore C. (1985) Acquired immunological tolerance of foreign cells is impaired by recombinant interleukin 2 or vitamin A acetate. *P.N.A.S.* **82,** 536.
21. Powell T.J. & Streilin J.W. (1990) Neonatal tolerance induction by class II alloantigens activates IL-4 secreting, tolerogen-responsive, T cells. *J. Immunol.* **144,** 854.
22. Fowell D., McKnight A.J., Powrie F., Dyke R. & Mason D.W. (1991) Subsets of $CD4^+$ T cells and their roles in the induction and prevention of autoimmunity. *Immunol. Rev.* **123,** 37.
23. Chen W., McCluskey J., Rodda S. & Carbone F.R. (1993) Changes at peptide residues buried in the major histocompatibility

complex (MHC) class I binding cleft influence T cell recognition: a possible role for indirect conformational alterations in the MHC class I or bound peptide in determining T cell recognition. *J. exp. Med.* **177,** 869.

24. STARZL T.E., DEMETRIS A.J., MURASE N., ILDSTAD S. & RICARDI C. (1992) Cell migration, chimaerism and graft acceptance. *Lancet,* **339,** 1579.
25. ROCHA B., TANCHOT C. & VON BOEHMER H. (1993) Clonal anergy blocks *in vivo* growth of mature T cells and can be reversed in the absence of antigen. *J. exp. Med.* **177,** 1517.