Immunology 1998 **94** 101–108

Inhibition of cytotoxic alloreactivity by human allogeneic mononuclear cells: evidence for veto function of CD2$^+$ cells

G. RADDATZ, A. DEIWICK, T. SATO & H. J. SCHLITT *Klinik für Abdominal und Transplantationschirurgie, Medizinische Hochschule Hannover, Hannover, Germany*

SUMMARY

In animal models of organ transplantation, infusion of donor-derived leucocytes or bone marrow cells can support tolerance induction. To date, little is known about the suppressive effects of human allogeneic mononuclear cells on alloreactivity in the human system. To study this, mixed leucocyte cultures (MLC) were incubated in the presence and absence of viable allogeneic mononuclear cells (MNC) (modulator cells) of stimulator/donor origin, and the cytotoxic and proliferative potential of the resulting effector cells was determined. The experiments showed that: viable allogeneic MNC from bone marrow and from lymph nodes and peripheral blood (PBMC) were able to suppress allospecific cytotoxicity by an average of 60%; that allospecific as well as non-specific inhibitory effects could be observed with unseparated PBMC; that CD2$^+$ PMNC showed predominantly allospecific inhibition of cytotoxicity with little effect on proliferation whereas CD2$^-$ PBMC showed non-specific inhibitory effects (both for cytotoxicity and proliferation), which could be eliminated by indomethacin; that addition of interleukin-2 (IL-2) up to 50 U/ml to the MLC could not reverse the inhibitory effect; and that selective removal of CD8$^+$ cells from the CD2$^+$ modulator population diminished the specific inhibitory effect only partially. These findings demonstrate that viable human MNC from different compartments can have a marked suppressive effect on alloreactivity *in vitro*. For peripheral blood mononuclear cells (PBMC) the data suggest that various mechanisms can contribute to allosuppression, including specific suppressive veto effects by CD2$^+$ cells. Such inhibitory effects might be applicable *in vivo* for down-regulating allospecific cytotoxicity and to facilitate the acceptance of allografts.

INTRODUCTION

In vivo data from animal models of organ transplantation suggest that application of allogeneic leucocyte cells may – under certain conditions – lead to a down-regulation of the alloresponse and may contribute to tolerance induction.[1–4] The immunological mechanisms by which these allogeneic cells down-regulate the alloresponse, however, are not entirely clear. The relevant mechanisms seem to vary considerably between different models, and allospecific as well as non-specific suppressive effects have been observed. Non-specific mechanisms include the induction of suppressive cytokines, such as interleukin-4 (IL-4), interleukin-10 (IL-10) or transforming growth factor-β (TGF-β)[5,6] or prostaglandin production,[7,8] natural suppressor effects,[9,10] or even limited graft-versus-host reactivity. Specific effects can be caused by anergy induction as a result of inadequate co-stimulation,[11] by activation of regulatory cells in the recipient's immune system,[12,13] or by veto effects of the donor cells.[1]

Veto effects are of particular interest for clinical transplantation because they can lead to an effective and specific down-regulation of alloreactive cytotoxic cells within a few days. Therefore, immunomodulatory concepts based on veto effects do not necessarily require pretreatment of the graft recipient but could be applied at the time of transplantation. Veto effects of allogeneic mononuclear cells and their suppressive effect on alloreactivity have been well characterized in several animal models *in vitro* and *in vivo*. After the initial description of this effect by Miller,[2] it was extensively studied in rodent models.[3,4,14,15] Thomas *et al.*[16] later established the concept in a kidney transplant model in outbred rhesus monkeys using donor bone marrow cells. Bone marrow-treated but not control animals showed long-term survival of kidney allografts. *In vitro*, a veto effect could clearly be demonstrated, and CD2$^+$, CD3$^-$, CD8$^+$, CD16$^+$, CD38$^-$, DR^{dim+} cells from donor bone marrow were defined as the most effective cell population in this model *in vitro* and *in vivo*.[16]

Although this concept holds much promise for clinical application, there is little published data on immunosuppressive or immunomodulatory effects of allogeneic leucocytes in

Received 23 October 1997; revised 22 January 1998; accepted 27 January 1998.

Correspondence: Dr H. J. Schlitt, Klinik für Abdominal und Transplantationschirurgie, Medizinische Hochschule Hannover, D-30623 Hannover, Germany.

the human system.[17–19] In particular, it is not clear whether human leucocytes also can exert veto functions and, if they can, which cell populations are important. This knowledge would be critical for designing innovative clinical treatment strategies. Therefore, it was the aim of this study to investigate the potential of viable human mononuclear cells (MNC) to inhibit alloreactivity *in vitro* and to characterize the relevant cell populations and the underlying mechanisms in more detail. To this end, viable MNC populations from blood, lymph nodes, or bone marrow were added to mixed leucocyte cultures (MLC) under various conditions, and their effect on the generation of a proliferative response and, in particular, on the development of allospecific cytotoxic effector cells was analysed. Specific immunosuppressive effects of allogeneic leucocytes might be a basis for selective immunosuppression after clinical organ transplantation in the future.

MATERIALS AND METHODS

Culture conditions, monoclonal antibodies and cytokines

Cells were cultured in RPMI-1640 supplemented with 200 mM L-glutamine, 50 mM 2-mercaptoethanol (2-ME), 20 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS) at 37° in a humidified chamber containing 5% CO_2. TC199 was used for washing and cell handling at room temperature. Mouse monoclonal antibodies (mAb) for phenotyping of bone marrow cells (BMC), lymph node cells (LNC) and peripheral blood mononuclear cells (PBMC) were purchased from Becton Dickinson (Heidelberg, Germany; CD2, 3, 4, 8, 16, 19/20, 33, 34, 38 and human leucocyte antigen (HLA)-DR) or from Serotec (Camon, Wiesbaden, Germany; CD14, 56 and 25). For HLA class I staining and panning, mouse mAbs were produced by hybridomas HLA-A2 (HB54), HLA-A3 (HB122) and HLA-B7 (HB59) obtained from the American Type Culture Collection (ATCC, Rockville, MD). Interleukin-2 (IL-2) was obtained from Biochrom (Berlin, Germany) and indomethacin from Sigma (Deisenhofen, Germany).

Mononuclear cell preparation from bone marrow, lymph nodes and peripheral blood

Unless otherwise indicated, PBMC were obtained from buffy coats of healthy blood donors. BMC were obtained from vertebral bodies of cadaveric organ donors (with appropriate consent) by carefully pressing the bone stroma with the stamp of a syringe into petri dishes filled with TC199. Cells from lymph node (LN) were also obtained from cadaveric organ donors and harvested by cutting lymph nodes into small pieces before pressing them through a metal net (40 μm). For comparative studies, peripheral venous blood from the same donor was also used. After isolation, cells from each compartment were separated by Ficoll-Hypaque (1·077 g/l, Pharmacia, Freiburg, Germany) and washed three times in TC199. Viability when tested with trypan-blue staining was >95%.

Mixed leucocyte culture (MLC)

MLCs were performed by culturing 20×10^6 responder cells (PBMC from individual A) with 20×10^6 irradiated (50 Gy) stimulator cells (PBMC from an allogeneic individual B) in 250-ml cell culture flasks in a final volume of 40 ml of supplemented RPMI medium. For some cultures, IL-2 at various concentrations was added to the culture at the start. After 6 days the cells were harvested, washed twice, separated as described below, and tested for cytotoxicity against autologous, allogeneic and third-party targets.

Modulator-cell assays

To estimate the potential of viable allogeneic mononuclear cells for suppression of MLC-induced cytoxic T-lymphocyte (CTL) activity, 10×10^6 viable BMC, LNC or PBMC of stimulator origin were added to the MLC at the time of culture initiation (modulator cells); control cultures were also set up with an identical number of irradiated modulator cells. Allospecificity of the suppressive effect was tested by using third party stimulator and target cells. To avoid cold-target cell inhibition, viable cells of the modulator population were removed prior to the CTL-assay by antibody-mediated plastic adhesion (panning). To achieve this, at the end of the 6-day culture, cells were washed twice and 30×10^6 cells were incubated with polymorphic anti-HLA class I mouse mAb (10 μg/10^7 cells/ml), specific for the modulator population, on ice for 30 min. After washing twice, cells were incubated in goat anti-mouse (GAM)-coated petri dishes (10 μg GAM/ml phosphate-buffered saline (PBS), 32 ml/dish, incubated overnight and washed twice with PBS containing 1% FCS) at 4° for 2 hr. Non-binding cells were removed by shaking the dishes gently. The resulting cell suspension was washed twice and screened by flow cytometry using directly labelled polymorphic anti-HLA class I antibodies; contamination by viable modulator cells was less than 3%. Control cultures were treated accordingly.

^{51}Chromium-release assay

CTL activity was measured by a 4-hr ^{51}Cr-release assay in V-shaped microtitre plates in a final volume of 200 μl. Effector cells were MLC-generated (primed) responder cells. Target cells were 6-day cultured concanavalin A (Con A)-induced blasts (10^4/well) labelled with 150 μCi Na_2CrO_4 ^{51}Cr for 2 hr at 37°. Prior to labelling, blasts were incubated for 30 min at 37° with 10 mg/ml α-methyl-mannoside (Sigma) and washed twice to remove Con A from the cell surface. Effector/target cell ratios were 3:1, 10:1, 30:1 and 70:1. Data were based on the mean of triplicates, and the level of cytotoxicity was calculated by the formula:

$$\text{Specific target cell lysis (\%)} = \frac{(\text{Maximum release} - \text{Spontaneous release})}{(\text{Test release} - \text{Spontaneous release})} \times 100$$

Proliferation assays

Aliquots of effector cells after panning and counting were transferred into 96-well round-bottom microtitre plates (10^4 cells/well). After addition of [^{3}H]thymidine (0·5 μCi/well, 5 Ci/mmol; NEN, Dreieich, Germany) plates were incubated for 8 hr under standard conditions in a final volume of 200 μl/well. Cells were than harvested with an automatic cell harvester (Pharmacia LKB, Uppsala, Sweden). Activity was measured in a liquid scintillation counter (LKB Wallac, Turku, Finland). The data represent the mean of triplicates ± standard deviation (SD).

Flow cytometric analyses

Cells at a concentration of 5×10^5 per well (round-bottom microtitre plates), were washed twice with buffer solution

(PBS, 0·5% bovine seum albumin (BSA), 0·1% NaN_3). After incubation with Intraglobin (Serapharm, Münster, Germany) cells were washed twice and then stained with specific mouse mAb against cell surface markers. In the case of unconjugated mAb, an additional incubation step with fluorescein isothiocyanate (FITC)-conjugated goat $F(ab')_2$ anti-mouse immunoglobulin G (IgG) reagents (Medac, Hamburg, Germany) followed. All incubations were performed at 4° for 30 min. Fluorescence analyses were performed by using a fluorescence-activated cell sorter (FACScan) flow cytometer (Becton-Dickinson, Heidelberg, Germany). Parameters of 20 000 events were accumulated into list mode file with a live gate set to include all viable cells. Unstained cells and cells stained with an isotype-matched irrelevant control mAb served as controls.

Fractionation and depletion of modulator cells

To separate PBMC into $CD2^+$ and $CD2^-$ subpopulations, $6{\cdot}6 \times 10^6$ MNC/ml (in TC199 + 10% FCS) were mixed with 10^9 cells/ml neuraminidase (Behringwerke AG, Marburg, Germany)-treated (20 U/10^9 cells/ml in TC199, 30 min, 37°, 2×) sheep red blood cells (SRBC) (BAG, Lich, Germany) at a ratio of 10:1 and in a final volume of 10 ml of TC199. After incubation at 37° for 10 min, cells were centrifuged (15 min, 200 ***g***) and the cell sediment was incubated on ice for a further 30 min. The cells were then resuspended in TC199, underlayed with Ficoll-Hypaque (Pharmacia) and centrifuged at 400 ***g*** for 30 min. Following centrifugation, erythrocytes and $CD2^+$ PBMC were located in the sediment, and $CD2^-$ cells in the interphase. SRBC were removed by incubation with 7 ml of lysis reagent 0·14 M NH_4Cl, 0·25 mM EDTA, 5 mM $KHCO_3$, pH 7·5 at 37° for 5–10 min. Depletion of $CD8^+$ cells was performed by panning with anti-CD8 mAbs (Clone AICD8·1 (Eri 8), kindly provided by S. C. Meuer, Heidelberg, Germany) using a technique identical to that described above. After panning, fewer than 3% of $CD2^+$ cells were $CD8^+$.

RESULTS

Can viable MNC from blood, lymph nodes and bone marrow suppress an alloresponse?

To study whether human MNC can interfere with the generation of MLC-induced alloreactive CTL, responder cells were cultured in the presence or absence of viable MNC from different compartments (peripheral blood, lymph nodes, and bone marrow) (modulator cells) and then their cytolytic capacity was analysed. The composition of cell populations in these compartments showed considerable differences. In lymph nodes and blood, lymphocytes were the dominant population. In contrast to blood, where T cells were predominant, lymph nodes also contained considerable numbers of B cells. On the other hand, 13 ± 6% $CD16^+$ cells were detectable in PBL, whereas these cells were almost entirely absent in lymph nodes. The composition of subpopulations in bone marrow was completely different: lymphocytes were rare (≈8%) and considerable numbers of precursor cells expressing the differentiation markers CD33 (without monocytes: ≈16%) and CD34 (4%) could be found in bone marrow (Table 1).

In spite of these marked differences, mononuclear cells from peripheral blood, as well as from lymph nodes and bone marrow, had a clear suppressive effect on the cytotoxic alloresponse (Fig. 1). The extent of inhibition varied between 20 and 85% with a median inhibition of cytotoxicity of 60%. Cold-target cell inhibition was excluded by removing viable modulator cells from the effector cell population prior to the ^{51}Cr-release assay. Irradiation of the modulator cells completely abolished their suppressive effect (data not shown).

Is the inhibitory effect of PBMC allospecific?

With unseparated PBMC as modulator cells, allospecific suppression could be observed in four out of nine allogeneic combinations (Table 2, experiments 2 and 4), while non-specific suppression was found in five cases (Table 2, experiments 1, 3 and 5). In contrast to cytotoxicity, the proliferative capacity was generally not decreased by the addition of modulator cells. Moreover, no correlation was found between the specificity of the effect and the degree of major histocompatibility complex (MHC) class I and class II matching between stimulator and modulator cells (Table 2).

Which cell populations contribute to the inhibitory effect of PBMC?

To analyse the contribution of different subpopulations to the suppressive effect, PBMC were separated into $CD2^+$ and $CD2^-$ cells (Table 3). When $CD2^-$ cells were used as modulator cells, predominantly non-specific suppression of cytotoxicity could be observed. Moreover, the corresponding assays also showed a clear decrease in the proliferative capacity. In contrast, $CD2^+$ cells showed an allospecific response (Fig. 2), although in some cases also a weak non-specific inhibitory effect was seen. With $CD2^+$ modulator cells a slight decrease in the proliferative response occurred only in those cases in which a non-specific suppressive component was observed for cytotoxicity (data not shown).

Are prostaglandins involved in the suppressive effect?

Monocytes/macrophages are known to be strong inhibitors of *in vitro* responses, which are mediated by their production of prostaglandins. To evaluate the contribution of prostaglandins for the suppressive effects in our system, indomethacin, a cyclooxygenase inhibitor that prevents synthesis of prostaglandins, was added to the MLC. Under these conditions, the suppressive potential of $CD2^-$ modulator cells could be abolished completely. The effect was dose dependent (data not shown), and the highest test concentrations used (10^{-6} M) completely blocked non-specific suppression of cytotoxicity (Fig. 3) as well as of proliferation (data not shown). In contrast, indomethacin had no effect on the specific and non-specific suppressive potential of $CD2^+$ modulator cells (Fig. 3).

Can the specific suppressive effect of $CD2^+$ cells be reversed by IL-2?

To study whether a competition for cytokines in the cultures was the mechanism of suppression, excess IL-2 was added at initiation of the MLC at concentrations between 10 and 300 U/ml. In the [^{3}H]thymidine assay, low amounts of exogenous IL-2 had already led to a marked increase in proliferation in the MLC (data not shown). In contrast to the increase in

Table 1. Phenotypic characterization of MNC from different human compartments

Surface marker	% Positive cells (mean ± SD)		
	Bone marrow ($n=5$)	Lymph node ($n=5$)	PBMC ($n=10$)
CD2	4·4±3·0	40·8±17·1	30·8±7·1
CD3	5·1±2·1	49·9±8·8	37·8±7·8
CD4	7·3±1·7	38·2±5·0	32·0±9·1
CD8	1·9±2·4	13·7±4·2	10·8±1·9
CD14	2·4±1·3	<1	15·5±6·3
CD16	12·6±7·6	<1	13·2±5·6
CD19/20	2·4±1·3	41·4±9·7	5·8±3·5
CD33	18·6±6·1	ND	17·3±5·1
CD34	3·7±2·2	<1	<1
CD38	33·4±8·3	7·4±5·4	41·0±8·7
CD56	<1	2·1±1·1	6·2±2·1
CD25	<1	3·5±2·2	2·3±1·6
HLA-DR	13·7±6·8	47·6±11·3	24·3±5·1

ND, not determined; PMBC, peripheral blood mononuclear cells.

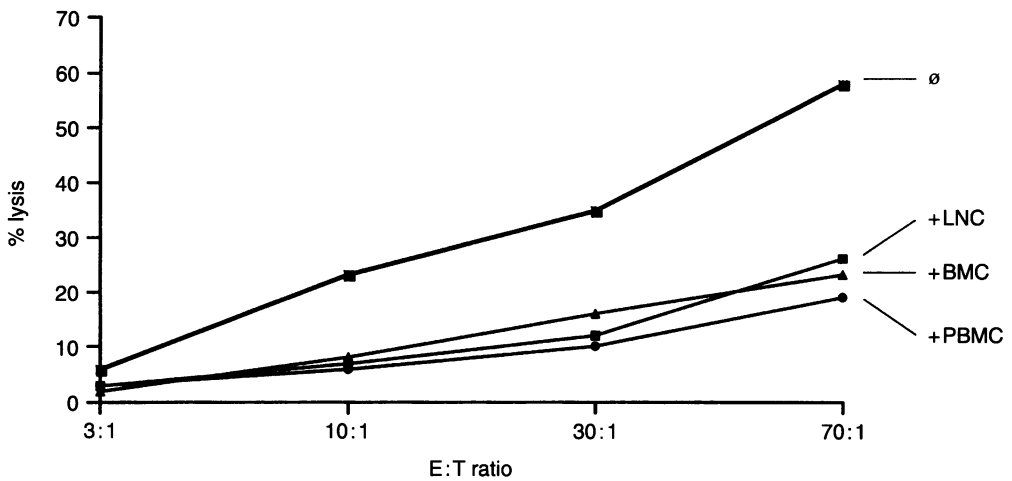

Figure 1. Suppression of cytotoxic alloreactivity by viable stimulator-type cells from bone marrow (BMC), lymph nodes (LNC) and peripheral blood (PBMC). Mixed leucocyte cultures were set up with responder and irradiated stimulator cells and vital lymph node cells (LNC), bone marrow cells (BMC), or peripheral blood mononuclear cells (PBMC) were added on day 0. After 5 days of culture, cytotoxicity of the resulting effector cells against cells of stimulator origin was assayed at different effector/target (E/T) cell ratios (results are given of one representative experiment out of five performed). ϕ, without modulator cells added.

the proliferative response, concentrations of IL-2 of up to 50 U/ml could not reverse the suppressive effect of $CD2^+$ modulator cells in any of three experiments (Fig. 4). Excessive amounts of IL-2 (300 U/ml), however, were able to reverse the suppressive effect, but the cytotoxic response against autologous and third party target cells was also clearly increased under these conditions (data not shown).

What is the contribution of $CD8^+$ cells to the suppressive effect of the $CD2^+$ population?

In rhesus monkeys and several other model for veto function, the subpopulation responsible for the self-directed allosuppression belonged to the population of $CD8^+$ cells. Therefore, the relevance of $CD8^+$ cells for suppression observed in our experiments was analysed. To this end, $CD8^+$ cells were depleted from the $CD2^+$ fraction of PBMC by panning. After removal of $CD8^+$ cells, the allospecific inhibitory effect of $CD2^+$ PBMC was only partially abolished (Fig. 5). In contrast, the non-specific suppressive effect disappeared almost completely.

DISCUSSION

The study has shown that freshly isolated, viable allogeneic mononuclear cells can effectively suppress a cytotoxic alloresponse in a human *in vitro* model. This observation is in concordance with data of Thomas *et al.*[16,20] who have described a similar phenomenon in a rhesus monkey *in vitro* model. Apart from allogeneic bone marrow cells – which they have studied extensively in their model – suppression in our human model could also be achieved by PBMC and by LNC. These findings are remarkable because the composition of (vertebral body) BMC, LNC and PBMC is considerably different. Basically, this observation indicates that different types of leucocytes may be capable of suppressing an allo-

Table 2. Specificity of the suppressive effect of unseparated allogeneic peripheral blood mononuclear cells

Expt. no.	MLC setup	Proliferation* (c.p.m.)	Cytotoxicity† (% lysis)	Suppression‡ (%)	HLA-match (modulator/stimulator cells) A and B locus	HLA-match (modulator/stimulator cells) DR locus
1	A→B$\frac{}{}$	5559	37			
	A→B$\frac{}{}$+B	3657	3	92	–	–
	A→C$\frac{}{}$	5695	34			
	A→C$\frac{}{}$+B	3703	16	53	1	1
	A→D$\frac{}{}$	6401	27			
	A→D$\frac{}{}$+B	3508	8	70	0	2
2	A→B$\frac{}{}$	ND	68			
	A→B$\frac{}{}$+B	ND	45	34	–	–
	A→C$\frac{}{}$	ND	41			
	A→C$\frac{}{}$+B	ND	49	No	1	1
	A→D$\frac{}{}$	ND	35			
	A→D$\frac{}{}$+B	ND	39	No	0	2
3	A→B$\frac{}{}$	4644	48			
	A→B$\frac{}{}$+B	4955	5	91		
	A→C$\frac{}{}$	4598	44			
	A→C$\frac{}{}$+B	3837	21	52	1	1
	A→D$\frac{}{}$	4107	52			
	A→D$\frac{}{}$+B	4557	19	66	1	0
4	A→B$\frac{}{}$	6623	64			
	A→B$\frac{}{}$+B	3170	31	52		
	A→C$\frac{}{}$	5632	78			
	A→C$\frac{}{}$+B	7644	75	4	0	1
	A→D$\frac{}{}$	6298	50			
	A→D$\frac{}{}$+B	7877	58	No	1	0
5	A→B$\frac{}{}$	26204	59			
	A→B$\frac{}{}$+B	15916	5	92		
	A→C$\frac{}{}$	10393	45			
	A→C$\frac{}{}$+B	10264	13	71	1	0

Mixed leucocyte cultures (MLC) were set up either with stimulator and modulator cells of same origin (B) or with MHC-different third-party stimulator cells (C or D). Predominant suppression of cytotoxicity against B indicates allospecific suppression, while suppression of cytotoxicity also against third-party cells (C or D) indicates non-specific suppression. Five representative experiments (out of 10) are shown. ND, not determined.

*[^{3}]Thymidine-uptake after 7 days of MLC.

†Against stimulator-type target cells.

‡Compared to culture without modulator cells.

Table 3. Phenotypic characterization of sheep red blood cell-separated peripheral blood mononuclear cells

Surface marker	% Positive cells (mean ± SD, $n=8$) Unseparated	$CD2^+$	$CD2^-$
CD2	28·4 ± 6·3	97·3 ± 8·3	4·4 ± 2·5
CD3	41·2 ± 8·2	86·9 ± 7·2	3·2 ± 1·1
CD14	16·4 ± 4·8	1·4 ± 0·9	46·7 ± 9·7
CD20	6·6 ± 2·4	0·9 ± 0·5	22·7 ± 5·5
CD56	5·1 ± 3·7	13·9 ± 9·3	2·8 ± 1·7
CD33	21·0 ± 6·7	1·5 ± 1·2	37·8 ± 13·7

response and suggests that different mechanisms might be involved.

To analyse the mechanism of suppression in our model in more detail, methodological problems of the selected assay system first had to be excluded. As allogeneic modulator cells added to a mixed leucocyte culture are still viable at the end of the culture – in contrast to irradiated stimulator cells which have all died by then – they can directly interfere with the cytotoxic assay in several ways. On one hand, their presence can cause a relative reduction in the number of effector cells counted prior to the cytotoxic test. On the other hand, these cells can compete with the ^{51}Cr-labelled target cells for lysis. The ensuing reduction in ^{51}Cr-release could then be misinterpreted as suppression of cytotoxicity (cold-target inhibition). In our study these obstacles were avoided by removal of the viable modulator cells prior to the cytotoxic assay so that these methodological problems could be excluded.

The mechanism of suppression was studied in detail only for PBMC. Separation of these cells according to their CD2 expression could break down the specificity of the suppressive effect in a non-specific and a specific component. $CD2^-$

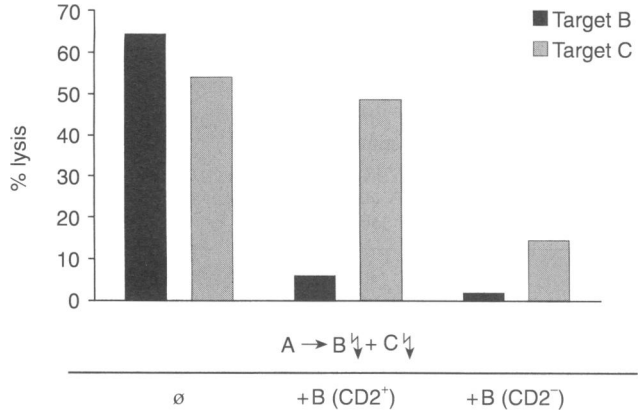

Figure 2. Different patterns of suppression by $CD2^+$ and $CD2^-$ peripheral blood mononuclear cells. Mixed leucocyte cultures with responder cells (A), and irradiated allogeneic stimulator cells (B and C) were set up and vital $CD2^+$ or $CD2^-$ mononuclear cells from B were added at initiation of culture. After 5 days, the cytotoxic function of effector cells (A) against stimulator-type target cells (B or C) was analysed. The figure gives the results for one representative experiment out of six performed.

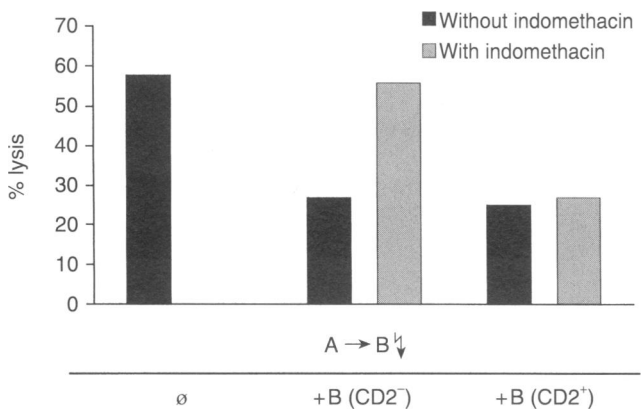

Figure 3. Influence of prostaglandins on allosuppression by $CD2^+$ and $CD2^-$ PBMC. Mixed leucocyte cultures with responder cells (A), and irradiated allogeneic stimulator cells (B) were set up in the presence of $CD2^+$ or $CD2^-$ vital PBMC of stimulator origin as modulator cells with or without 10^{-6} mol/l of the prostaglandin-synthetase inhibitor indomethacin (one representative of three experiments is shown).

modulator cells caused non-specific suppression of cytotoxicity as well as of proliferation. As this effect could be inhibited by indomethacin it is probably mediated by prostaglandins. Prostaglandin synthesis predominantly by monocytes/macrophages is known to be a non-specific inhibitor in cell cultures containing considerable amounts of those cells.[7,8] Non-specific suppression of cytotoxic as well as proliferative alloresponses by allogeneic bone marrow cells, particularly after depletion of T cells, was also described by Mathews *et al.*[19] and may be based on a similar mechanism. In contrast, the inhibitory effect of $CD2^+$ cell observed in our study was predominantly specific. A non-specific component was also observed in some experiments but was much weaker than the specific suppression. The suppressive effect of $CD2^+$ cells was not inhibited by indomethacin and became even more prominent because of an increased lysis in the control cultures. Therefore, it is

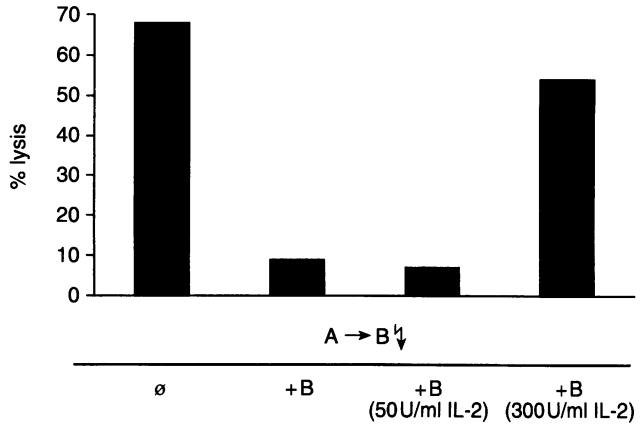

Figure 4. Effect of IL-2 on allosuppression by $CD2^+$ PBMC. Mixed leucocyte cultures were set up with vital allogeneic modulator cells of stimulator cell origin with or without different concentrations of interleukin-2, and cytotoxicity against the allogeneic target cells (B) was analysed (one representative of four experiments is shown).

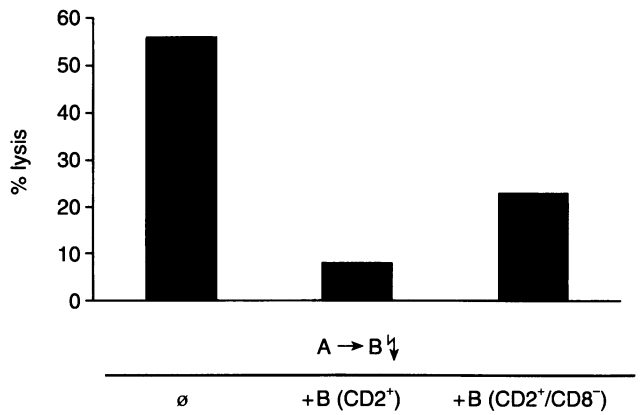

Figure 5. Suppression of alloreactivity by $CD2^+/CD8^-$ PBMC. Mixed leucocyte cultures with responder cells (A), and irradiated allogeneic stimulator cells (B) were set up in the presence of $CD2^+$ or CD8-depleted $CD2^+$ ($CD2^+/CD8^-$) PBMC of stimulator origin as modulator cells, and allogeneic cytotoxicity was measured (one representative of four experiments is shown).

unlikely that prostaglandins are involved in the suppressive effect of $CD2^+$ PBMC.

Two mechanisms might explain the specific suppressive effect of allogeneic $CD2^+$ PBMC: induction of anergy or a veto effect. Anergy is based on a diminished biological activity and/or synthesis of IL-2 after stimulation by the respective alloantigen.[21,22] In contrast to the veto effect, this non-reactivity can typically be overcome by the addition of exogenous IL-2.[17,23] In our experiments, addition of IL-2 up to concentrations of 50 U/ml had no effect on the allosuppressive potential of $CD2^+$ modulator cells and these findings indicate that anergy or competition for IL-2 are highly unlikely as explanations for their suppressive effect. Suppression could be reversed in the presence of very high doses of IL-2 (300 U/ml), but there was also a strong increase in non-specific cytotoxicity under these conditions, suggesting recruitment of lymphokine-activated killer (LAK) cells.[24]

These observations very much favour a veto effect as an explanation for the allospecific suppression caused by

allogeneic $CD2^+$ PMBC. The veto effect was first described by Miller *et al.*[2] It represents the specific suppressive effect of a cell able to block the activity of cytotoxic lymphocytes directed against a surface antigen expressed by the veto cell. Thus, the specificity of the veto effect does not depend on active recognition by the veto cell, but on the specificity of the potential cytotoxic cell. For the alloresponse this means that a cytotoxic T cell, which recognizes alloantigens on a veto cell, would be inactivated or destroyed. The veto cell therefore interferes with the development of cytotoxic cells directed against its own MHC antigens.

Several *in vivo* and *in vitro* studies in rodents suggest that the CD8 molecule plays an important role for veto function.[25–27] Moreover, in transfection experiments Sambhara & Miller observed a strong allosuppressive effect of a lymphoma T-cell line (TCR^-, $CD4^-/8^-$) after transfection with CD8 but not with CD4.[28] Therefore, mainly cytotoxic T cells as well as natural killer (NK) cells and dendritic precursor cells, which are $CD8^+$, have been discussed as veto cells.[29–32] In our human model, depletion of $CD8^+$ cells from the $CD2^+$ modulator cell fraction had two consequences: (1) the weak non-specific component of the suppressive effect was lost completely and (2) the specific suppressive effect, i.e. the supposed veto function, was retained, although at a reduced level. This demonstrates that the weak non-specific suppression of $CD2^+$ cell resides exclusively in the $CD8^+$ population. This effect is obviously different from the non-specific suppression of $CD2^-$ PBMC as it is not inhibited by indomethacin. It may reflect either a natural suppressor effect,[33,34] mediated by cytokines,[35] or cytotoxic activity of the modulator cells against the MHC antigens of the putative effector cell population, i.e. a graft-versus-host type of immunosuppression. $CD8^+$ cells have been described to play a central role for both types of effects. Concerning the specific veto effect, the results suggest that it is mediated by both $CD8^+$ and $CD8^-$ cells. Veto effects of non-CD8 cells, e.g. $CD4^+$ cells,[36] B cells,[37] cloned bone marrow cells[38] and facilitating cells[39] have been described in various models, but there are only few data available for the human system.

Lagoo-Deenadayalan *et al.*[18] have also studied suppressive effects of human PBMC and bone marrow cells obtained from organ donors. They found suppression only by bone marrow cells (and not by PBMC of the same donor). Their study, however, was based only on proliferative data whereas cytotoxicity was not analysed. As our data showed that specific allosuppression was detectable predominantly at the cytotoxic level, frequently without a change in the proliferative capacity, these findings are in accordance with our observations. In addition, the group of R. G. Miller has very recently been studying veto effects to an alloresponse in the human system.[40] In their model they have used PBMC that had been prestimulated against third-party alloantigens as modulator cells. Compared with our findings, these preactivated cells seemed to have a much stronger veto activity so that lower cell numbers were sufficient to suppress allospecific cytotoxicity. Similar to our results they found veto activity of $CD8^+$ as well as $CD8^-$ cells. These data support our observations that in the human system various cell populations may be able to act as veto cells.

For the clinical situation our findings imply that allogeneic leucocytes – and particularly lymphocytes – of donor-type might be used as a tool to suppress the immune response against the antigens of an allograft. A beneficial immunoregulatory role for donor lymphocytes has already been demonstrated *in vivo* in animal models[4] and has also been suggested for liver allografts, which contain large numbers of those cells.[41–43] Using defined populations of donor cells and, perhaps, additional strategies for enhancement of veto effects,[40,44] specific immunosuppression might be achieved and this might contribute to the induction of tolerance in organ as well as bone marrow transplantation.[45]

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 265). T. Sato was a research fellow supported by a grant from the Japanese Ministry of Education.

REFERENCES

1. Thomas J.M., Verbanac K.M., Carver F.M. *et al.* (1994) Veto cells in transplantation tolerance. *Clin Transplant* **8,** 195.
2. Miller R.G. (1980) An immunological suppressor cell inactivating cytotoxic T-lymphocyte precursor cells recognizing it. *Nature* **287,** 544.
3. Heeg K. & Wagner H. (1990) Induction of peripheral tolerance to class I major histocompatibility complex (MHC) alloantigens in adult mice: transfused class I MHC-incompatible splenocytes veto clonal responses of antigen-reactive $Lyt\text{-}2^+$ T cells. *J Exp Med* **172,** 719.
4. Martin D.R., Sheng-Tanner X. & Miller R.G. (1992) Rapid and long-term changes to host cytotoxic T lymphocyte precursors reactive to donor antigens caused by intravenous injection of histoincompatible lymphocytes. *Transplantation* **54,** 125.
5. Sher A., Fiorentino D., Caspar P., Pearce E. & Mosmann T. (1991) Production of IL-10 by $CD4^+$ lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infection. *J Immunol* **147,** 713.
6. Le Gros G., Ben-Sasson S.Z., Seder R., Finkelman F.D. & Paul W.E. (1990) Generation of interleukin 4 (IL-4)-producing cells *in vivo* and *in vitro*: IL-2 and IL-4 are required for *in vitro* generation of IL-4-producing cells. *J Exp Med* **172,** 921.
7. Bakic S.T., Vuckovic-Dekic I.J., Susnjar S. & Spuzic I. (1992) *In vitro* effect of indomethacin on mitogen-induced lymphoproliferative response in lung cancer patients. *Neoplasma* **39,** 129.
8. Endo F., Sasaki T., Sekigucci Y. *et al.* (1981) The role of monocytes and prostaglandin E in the regulation of mitogen responses *J Exp Med* **133,** 119.
9. Saffran D.C. & Singhal S.K. (1991) Suppression of mixed lymphocyte reaction by murine bone marrow-derived suppressor factor-inhibition of proliferation due to a deficit in IL-2 production. *Transplantation* 52, 685.
10. Cassell D. & Forman J. (1990) Regulation of the cytotoxic T lymphocyte response against Qa-1 alloantigens. *J Immunol* **144,** 4075.
11. Schwartz R.H. (1990) A cell culture model for T lymphocyte clonal anergy. *Science* **248,** 1349.
12. Roser B.J. (1989) Cellular mechanisms in neonatal and adult tolerance. *Immunol Rev* 107, 179.
13. Koide J. & Engleman E.G. (1990) Differences in surface phenotype and mechanism of action between alloantigen specific $CD8^+$ cytotoxic and suppressor T cell clones. *J Immunol* **144,** 32.
14. Fink P.J., Rammensee H.G. & Bevan M.J. (1984) Cloned cytolytic T cells can suppress primary cytotoxic responses directed against them. *J Immunol* **133,** 1775.
15. Miller R.G., Muraoka S., Claesson M.H., Reimann J. &

Benivste P. (1988) The veto phenomenon in T-cell regulation. *Ann NY Acad Sci* **532,** 170.
16. Thomas J., Carver F.M., Cunningham P.R.G., Olson L.C. & Thomas F.T. (1991) Kidney allograft tolerance in primates without chronic immunosuppression – the role of veto cells. *Transplantation* **51,** 198.
17. Burlingham W.J., Grailer A.P., Fechner J.H. *et al.* (1995) Microchimerism linked to cytotoxic T lymphocyte functional unresponsiveness (clonal anergy) in a tolerant renal transplant recipient. *Transplantation* **59,** 1147.
18. Lagoo-Deenadayalan S., Lagoo A.S., Lemons J.A. *et al.* (1995) Donor specific bone marrow cells suppress lymphocyte reactivity to donor antigens and differentially modulate Th1 and Th2 cytokine gene expression in the responder cell population. *Transplant Immunol* **3,** 124.
19. Mathew J.M., Carreno M., Fuller L. *et al.* (1997) Modulatory effects of human donor bone marrow cells on allogeneic cellular immune responses. *Transplantation* **63,** 686.
20. Thomas J., Carver F.M., Kasten-Jolly J. *et al.* (1994) Further studies of veto activity in rhesus monkey bone marrow in relation to allograft tolerance and chimerism. *Transplantation* **57,** 101.
21. Rammensee H.G., Kroschewski R. & Frangoulis B. (1989) Clonal anergy induced in mature $V\beta6^+$ T lymphocytes on immunizing Mls-1^b mice with Mls-1^a expressing cells. *Nature* **339,** 541.
22. Dallman M.J., Shiho O., Page T.K., Wood K.J. & Morris P.J. (1991) Peripheral tolerance to alloantigen results from altered regulation of the interleukin 2 pathway. *J Exp Med* **173,** 79.
23. Beverly B., Kang S.M., Leonardo M.J. & Schwartz R.H. (1992) Reversal of *in vitro* T cell clonal anergy by IL-2 stimulation. *Int Immunol* **4,** 661.
24. Herberman R.B. (1987) Lymphokine-activated killer cell activity. *Immunol Today* **8,** 178.
25. Fast L.D. (1992) Generation and characterization of IL-2-activated veto cells. *J Immunol* **149,** 1510.
26. Hambor J.E., Kaplan D.R. & Tykocinski M.L. (1990) CD8 functions as an inhibitory ligand in mediating the immunoregulatory activity of $CD8^+$ cells. *J Immunol* **145,** 1646.
27. Kaplan D.R., Hambor J.E. & Tykocinski M.L. (1989) An immunoregulatory function for the CD8 moleule. *Proc Natl Acad Sci USA* **86,** 8512.
28. Sambhara S.R. & Miller R.G. (1991) Programmed cell death of T cells signaled by the T cell receptor and the γ3 domain of class I MHC. *Science* **252,** 1424.
29. Miller R.G., Zhang L., Tanner Z.F.S., Hammad N. & Radvanyi L. (1993) The veto hypothesis and transplantation tolerance. *Transplant Sci* **3,** 206.
30. Thomas J., Verbanac K.M., Smith P.J. *et al.* (1995) The facilitating effect of one-DR antigen sharing in renal allograft tolerance induced by donor bone marrow in rhesus monkeys. *Transplantation* **59,** 245.
31. Uberti J., Martilotti F., Chou T.H. & Kaplan J. (1992) Human lymphokine activated (LAK) cells suppress generation of allospecific cytotoxic T cells: implications for use to prevent graft-vs.-host disease in allogeneic bone marrow transplantation. *Blood* **79,** 261.
32. Thomson A.W., Lu L., Murase N., Demetris A.J., Rao A.S. & Starzl T.E. (1995) Microchimerism, dendritic cell precursors and transplantation tolerance. *Stem Cells Dayt* **13,** 622.
33. Holda J.H., Maier T. & Claman H.N. (1985) Murine graft-vs.-host disease across minor barriers: immunsuppressive aspects of natural suppressor cells. *Immunol Rev* **88,** 87.
34. VanVlasselaer P., Niki T. & Strober S. (1991) Identification of a factor (s) from cloned murine natural suppressor cells that inhibits IL-2 secretion during antigen-driven T-cell activation. *Cell Immunol* **138,** 326.
35. Yamamoto H., Hirayama M., Genyea C. & Kaplan J. (1994) TGF-beta mediates natural suppressor activity of IL-2 activated lymphocytes. *J Immunol* **152,** 3842.
36. Cassell D. & Forman J. (1991) Two roles for CD4 cells in the control of the generation of cytotoxic T lymphocytes. *J Immunol* **146,** 3.
37. Rammensee H.G. (1991) Maintenance of self tolerance in $CD4^+$ T lymphocytes by antigen presentation on resting B cells – a hypothesis. *Bone Marrow Transplant* **7,** 26.
38. Takahashi T. & Maki T. (1991) Prolongation of mouse skin allograft survival by cloned veto suppressor cells. *Transplant Proc* **23,** 192.
39. Kaufmann C.H., Colson Y.L., Wren S.M., Watkins S., Simmons R.L. & Ildstad S.T. (1994) Phenotypic characterization of a novel bone marrow-derived cell that facilitates engraftment of allogeneic bone marrow stem cells. *Blood* **84,** 2436.
40. Chan A.S. & Miller R.G. (1997) Activated human T lymphocytes specifically suppress CTL responses against their HLA antigens. *4th International Conference on Tolerance Induction*, p. 19. Breckenridge, CO, USA.
41. Schlitt H.J., Raddatz G., Steinhoff G., Wonigeit K. & Pichlmayr R. (1993) Passenger lymphocytes in human liver allografts and their potential role after transplantation. *Transplantation* **56,** 951.
42. Schlitt H.J., Kanehiro H., Raddatz G. *et al.* (1993) Persistence of donor lymphocytes in liver allograft recipients. *Transplantation* **56,** 1001.
43. Sun J., McCaughan G.W., Gallagher N.D., Sheil A.G.R. & Bishop G.A. (1995) Deletion of spontaneous rat liver allograft acceptance by donor irradiation. *Transplantation* **60,** 233.
44. Qi Y., Berg R., Singleton M.A., Debrick J.E. & Staerz D.U. (1996) Hybrid antibody mediated veto of cytotoxic T lymphocyte responses. *J Exp Med* **183,** 1973.
45. Martin P.J. (1996) Prevention of allogeneic marrow graft rejection by donor T cells that do not recognize recipient alloantigens: potential role of a veto mechanisms. *Blood* **88,** 962.