

## Interactions of allogeneic human mononuclear cells in the two-way mixed leucocyte culture (MLC): influence of cell numbers, subpopulations and cyclosporin

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### SUMMARY

With organ allografts considerable numbers of donor-type mononuclear cells are transferred to the recipient, leading to bilateral immunological interactions between donor and recipient lymphocytes. To study such bilateral immune reactions in detail, human two-way MLC were performed. In this model proliferation kinetics, patterns of activation, and survival of the two populations were analysed, and the relevance of initial cell subset composition, relative cell numbers, and the effect of immunosuppression on this co-culture were evaluated. It could be demonstrated that with an initial 50:50 ratio of two populations of allogeneic cells one population dominated after 21 days of co-culture in 78 out of 80 combinations (97%) tested; the other population decreased markedly after an initially stable phase of 6–7 days. With unequal starting conditions the larger population dominated when resting cells were used, but small populations of preactivated cells or separated CD8<sup>+</sup> cells could also dominate. Depletion of CD16<sup>+</sup> natural killer (NK) cells and of CD2<sup>-</sup> cells (B cell and monocytes) had no effect on domination. Addition of cyclosporin delayed or blocked the domination process while addition of IL-2 accelerated it. Disappearance of one population was associated with detection of apoptotic cells. The findings indicate that co-cultures of allogeneic mononuclear cells are generally not stable for more than 1 week, but lead to active elimination of one population. CD8<sup>+</sup> cells and particularly preactivated cells seem to play the most important role in that process, while NK cells are of less importance. Cyclosporin can prolong survival of allogeneic cells in co-culture. These observations suggest that under the conditions of clinical organ transplantation even small amounts of immunocompetent donor cells transferred by the graft may persist for some time and may, thereby, have the chance to exert immunomodulatory functions.

**Keywords** mixed leucocyte culture two-way MLR

### INTRODUCTION

Two-way MLC were first described in 1964 by Bain *et al.* [1] and by Bach & Hirschhorn [2] for measuring histocompatibility between two different individuals. Two years later the one-way MLC was introduced [3] and it has predominantly been used afterwards since it allows more detailed studies of immune responses against allogeneic and xenogeneic cells. In the one-way MLC proliferation of stimulator cells is typically inhibited either by irradiation or by pretreatment with anti-proliferative drugs like mitomycin C. These inactivated cells can still present alloantigens and produce cytokines, but proliferation is not possible, and these cells usually die after a few days in culture. Thus, by

measuring proliferation following several days of co-culture, the response of only one population ('responder cells') is measured. In this one-way model, the stimulator cells are usually considered to represent the graft, i.e. the more or less passive target of the recipient's immunocompetent cells.

In contrast to this view, it could be demonstrated that donor organs contain considerable numbers not only of antigen-presenting leucocytes, but also of immunocompetent cells like T, B and natural killer (NK) lymphocytes, which are transferred to the recipient's body by organ transplantation [4,5]. Many of these cells leave the graft and can be detected in the peripheral circulation for several days, but they can also persist in tissues for longer periods [6,7]. The immunocompetence of these cells could be demonstrated *in vitro* and is also evident from a number of cases of graft-versus-host disease following clinical organ transplantation *in vivo* [8–10].

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These observations demonstrate that the immune reactions occurring particularly in the early phase after organ transplantation are basically bilateral and are therefore not adequately represented by a standard one-way MLR. Thus, it was the aim of this study to analyse the course of bilateral immune reactions of allogeneic mononuclear cells using a two-way MLC *in vitro* system. Very early studies have already suggested that the two-way MLR may be qualitatively different from the corresponding one-way MLR [11]. In the present study it was therefore analysed how two populations of allogeneic cells interact during co-culture, how these cell populations develop over time, what initial conditions determine the outcome of the culture, and what the effect of immunosuppression on this interaction is. These studies could be a basis for further understanding of the complex immunological interactions occurring after organ transplantation.

## MATERIALS AND METHODS

### *Cell preparations, antibodies, and culture media*

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll–Paque density gradient centrifugation from HLA-typed buffy coats obtained from healthy blood donors at the blood bank of the Medizinische Hochschule Hannover. Combinations of allogeneic cells used for the two-way MLC were selected on the basis of an HLA class I mismatch detectable by one of the available MoAbs; apart from that the combinations represented random matches of their HLA pattern.

For staining and cell separation various MoAbs were used. Antibodies against CD2 (Leu-5b), CD3 (Leu-4), CD4 (Leu-3a+3b), CD8 (Leu-2a), CD20 (Leu-16), and HLA-DR were obtained from Becton Dickinson (Heidelberg, Germany); against CD45RA, CD14 and CD56 from Serotec (Oxford, UK); and against CD45RO/UCHL1 and CD25/ACT-1 from Dako (Glostrup, Denmark). All antibodies were labelled either with FITC or PE. MoAbs against polymorphic HLA class I antigens were purified from culture supernatant of the hybridomas HB54, HB59, and HB122 recognizing HLA A2 and B17, B7 and B40, and A3, respectively. Hybridomas had been obtained from the American Type Culture Collection (ATCC, Rockville, MD). Antibodies against CD16 for panning of NK cells were obtained from culture supernatant of hybridoma 368H4SN kindly provided by R. E. Schmidt (Hannover, Germany).

Separation of PBMC into CD2<sup>+</sup> and CD2<sup>-</sup> cells was performed by E-rosette formation with sheep erythrocytes. In brief, PBMC were mixed with neuraminidase (Behringwerke AG, Marburg, Germany)-treated sheep erythrocytes (BAG, Lich, Germany) and incubated at 37°C for 10 min, centrifuged (15 min, 200 g) and the cell sediment incubated on ice for another 30 min. Afterwards cells were resuspended in TC199 medium, underlaid with Ficoll–Hypaque (Pharmacia, Freiburg, Germany) and centrifuged at 400 g for 30 min. To obtain CD2<sup>+</sup> cells sheep erythrocytes were removed by incubation with 7 ml lysis reagent (0.14 M NH<sub>4</sub>Cl, 0.25 mM EDTA, 5 mM KHCO<sub>3</sub>, pH 7.5) at 37°C for 5–10 min and subsequent washing; CD2<sup>-</sup> cells were harvested from the interface and washed.

Further depletion of cell populations was performed by antibody-mediated plastic adhesion (panning). To this end, 30 × 10<sup>6</sup> CD2<sup>+</sup> cells were incubated with anti-CD4 or anti-CD8 MoAbs (at concentrations of 10 µg/ml) on ice for 30 min. Anti-CD16 antibody for panning proved to be optimal at a final 1:10 dilution of the culture supernatant and was used for the experiments at this

dilution. After washing, 30 × 10<sup>6</sup> cells were incubated in goat anti-mouse (GAM)-coated Petri dishes at 4°C for 2 h. Non-binding cells were removed after shaking the dishes carefully. Control staining after panning of CD4<sup>+</sup> cells showed that 80–85% were CD8<sup>+</sup>, after panning of CD8<sup>+</sup> cells 90–95% were CD4<sup>+</sup>. Efficacy of depletion of NK cells by panning of CD16<sup>+</sup> cells was controlled by staining for CD56; the content of these cells was reduced from a mean of 9.5% before panning to a mean of 0.8% after panning.

All cell cultures were performed 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°C in a humidified chamber containing 5% CO<sub>2</sub>. For some experiments, PBMC were prestimulated by 15 ng/ml anti-CD3 MoAb OKT3 (Muromonab-CD3; Ortho, Raritan, NJ). IL-2 (Biotest, Dreieich, Germany) was added to the medium of some cultures at a final concentration of 20 U/ml. Cyclosporin was provided by Novartis (Nürnberg, Germany); it was dissolved in ethanol, diluted with culture medium, and used at final concentrations between 50 and 400 ng/ml. Cyclosporin-containing medium was removed on day 2 and exchanged for fresh culture medium without cyclosporin.

### *Two-way MLC*

Two-way MLC were set up using various types of cells, i.e. whole blood mononuclear cells, CD2<sup>+</sup> cells, CD2<sup>+</sup>CD16<sup>-</sup> cells, CD2<sup>+</sup>CD4<sup>-</sup> cells, CD2<sup>+</sup>CD8<sup>-</sup> cells, or cells preactivated by OKT3 stimulation. A total number of 2 × 10<sup>7</sup> cells was cultivated in cell culture flasks in 20 ml of culture medium with or without 20 U/ml IL-2. Medium was exchanged when its colour changed to yellow, usually once or twice per week. Initial cell ratios between the two populations of allogeneic cells were variable, as indicated in the respective experiments. Cell counting error in the 50:50 setup was < 3%. Cell cultures were analysed for composition of cell populations and subsets at various time points using 21 days as end point.

For proliferation assays and determination of cell numbers after different periods of culture, 2 × 10<sup>5</sup> cells/well were cultured in 200 µl culture medium in triplicates using 96-well U-shaped microtitre plates. In the one-way MLC, 10<sup>5</sup> cells, 'responder' cells, were mixed with 10<sup>5</sup> irradiated (30 Gy) allogeneic 'stimulator' cells; in the two-way MLC, 10<sup>5</sup> cells of each of the two allogeneic populations were mixed. Following 2, 4, 6, 8, 10 and 14 days of culture <sup>3</sup>H-thymidine (0.5 µCi/well, 5 Ci/mmol; NEN, Dreieich, Germany) was added and plates were incubated for 8 h. Cells were then harvested with an automatic cell harvester (Pharmacia LKB, Uppsala, Sweden). Activity was measured in a liquid scintillation counter (LKB Wallac, Turku, Finland). The data are given as mean ct/min of triplicates ± s.d.

### *Flow cytometry*

The two allogeneic populations in each culture were differentiated by two-colour flow cytometry using MoAbs to polymorphic HLA class I epitopes and directly labelled MoAbs to various differentiation antigens. In brief, after incubation with a human immunoglobulin preparation for Fc receptor blocking, cell suspensions were first incubated with the respective anti-HLA class I antibodies at previously determined optimal staining concentrations at 4°C for 30 min. Secondary antibodies were FITC-coupled goat anti-mouse IgG. A second round of staining was then performed with directly labelled PE-coupled antibodies to various cell differentiation antigens at optimal staining concentrations or by propidium

iodide (Sigma Chemicals, Deisenhofen, Germany). Cells incubated with irrelevant isotype-identical MoAbs were used as negative controls. For flow cytometric analysis, a gate was set to include all lymphocytes including blast cells based on forward and side scatter characteristics, and parameters of 20 000 events were accumulated into a list mode file.

To determine the degree of apoptosis in the culture, terminal deoxynucleotidyl transferase (TUNEL) staining was carried out. In brief,  $2 \times 10^6$  cells were transferred into microtitre plates at different times after culture. After 30 min of fixation with 4% paraformaldehyde solution, cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Freshly prepared TUNEL reaction mixture (Boehringer, Mannheim, Germany) was added and incubation for 60 min at 37°C in a humidified atmosphere followed. Cells incubated with a reaction solution without terminal transferase were used as negative controls, and permeabilized cells in the presence of DNase I (Sigma) served as positive controls. By flow cytometry, a total number of 20 000 lymphocytes was accumulated into a list mode file, and the ratio of apoptotic cells was determined.

#### Statistical analysis

Statistical analysis was performed by Student's *t*-test.  $P < 0.05$  was regarded as significant.

## RESULTS

#### Functional characteristics of two-way MLC

The kinetics of cell proliferation in the two-way MLC compared with the one-way MLC were similar. Maximum proliferation usually occurred on day 7–8 of the culture and decreased thereafter both in the absence and in the presence of exogenous IL-2. The extent of proliferation was usually comparable to that in each of the corresponding one-way MLC. There was no additive proliferative response in the two-way MLC, although the initial number of

viable cells was twice as high as in the two one-way MLC. Representative examples of the proliferative responses in three combinations of two-way MLC ( $2 \times 10^5$  viable MNC) in comparison with the corresponding one-way MLC ( $10^5$  viable MNC) are shown in Fig. 1.

#### Population dynamics in long-term two-way MLC

All of the allogeneic cell populations used had a random HLA match, the only bias being that combinations were selected that could be differentiated by one of the available MoAbs against HLA class I antigens. Starting with a 50:50 ratio of unseparated allogeneic mononuclear cells a major shift in the two cell populations could be observed after 3 weeks of culture in almost all cases. One of the two populations dominated while the other was strongly diminished or had disappeared in 78 out of 80 cultures studied (97.5%), particularly in the presence of IL-2 (Fig. 2). Only in two cases did the proportion of the two populations stay in equal balance for the entire co-cultivation period. Repeat assays with the cells from the same individuals gave identical results in seven out of seven combinations tested.

Following the development of the two allogeneic cell populations over time, stable conditions were observed during the first 5–7 days of culture. Only after this period did the decrease of one population and the (relative and absolute) increase in the other begin. Numbers of viable cells in the two-way MLC over the 3 weeks of culture changed from initially  $10 \times 10^6$  ( $= 2 \times 10^5$ ) cells to a total mean of  $9.5 \pm 0.9 \times 10^6$  cells under standard culture conditions and to a mean of  $17.5 \pm 1.8 \times 10^6$  cells after addition of exogenous IL-2 (Fig. 2).

When mononuclear cells from three to five different individuals (e.g. A, B, C and D) were used to analyse the outcome in the various combinations of co-cultures (e.g. A + B, A + C, A + D, B + C, B + D, and C + D), a hierarchy of domination could be recognized in most cases (e.g.  $C > A \geq D > B$ ).

Co-cultivating two populations of unseparated mononuclear

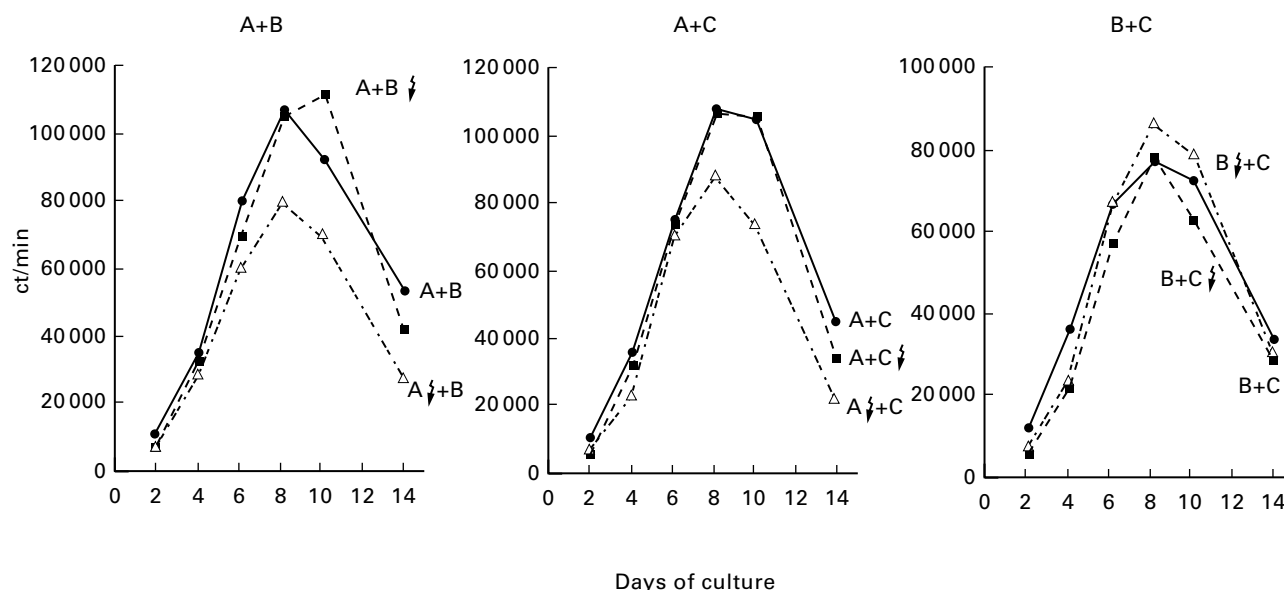
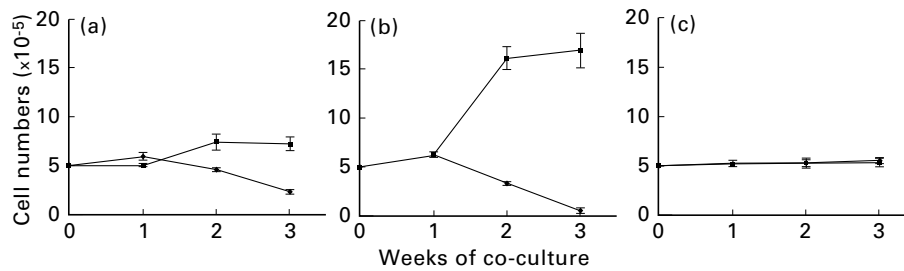


Fig. 1. Kinetics of proliferative response in two-way versus the corresponding one-way MLC in the presence of IL-2. Representative results from three different combinations of allogeneic cells are shown. Results are shown as mean ct/min of triplicates for different periods of culture.



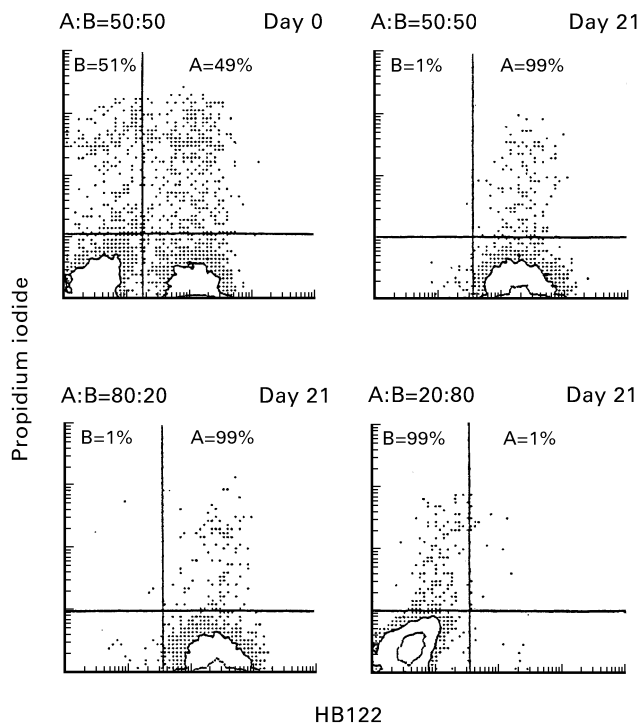
**Fig. 2.** Development of cell numbers in two allogeneic populations during two-way MLR (a) with normal cell culture medium, (b) in the presence of 20 U/ml IL-2, and (c) in the presence of 400 ng/ml cyclosporin plus 20 U/ml IL-2. Cell populations had an initial ratio of 50:50 and were analysed by flow cytometry using MoAbs to polymorphic HLA class I antigens differing between the two populations.

cells at a starting ratio of 20:80, the larger population always dominated (10 out of 10 experiments) irrespective of which population had dominated when identical (i.e. 50:50) starting conditions were used. Figure 3 shows a representative example of the outcome of two-way MLC after 3 weeks using different starting conditions. With an initial cell ratio (A:B) of either 50:50 or 80:20 population A dominated over B completely after 3 weeks of culture; with a starting ratio of 20:80, however, B dominated after that period.

*Effect of subpopulations and preactivation on population dynamics*  
 Analysis of the subset composition of the populations in the two-way MLR revealed an association of initially higher numbers of

CD8<sup>+</sup> cells and of CD45RO<sup>+</sup> cells (difference ≥ 5%) with dominance after co-culture. This association was observed in 10 out of 14 combinations studied for this parameter. However, in two out of the 14 cases domination also occurred with identical numbers of these cell subsets in both allogeneic populations. In another two out of the 14 combinations the population containing lower numbers of CD8<sup>+</sup> cells dominated. The findings suggest that these cell populations may play an active role in the domination process, but that excess of these cell populations is not an absolute prerequisite for domination.

To study the role of the subpopulations in the domination process in more detail, depletion of various subpopulations from mononuclear cells was performed, and the effect of this depletion on the domination/elimination process was studied. Depletion of CD2<sup>-</sup> cells, i.e. mainly of B cells and monocytes/macrophages, by E-rosette formation with sheep erythrocytes did not influence the population changes at all. Moreover, depletion of NK cells from CD2<sup>+</sup> cells by panning with an anti-CD16 antibody did not have any detectable effect. In contrast, depletion of either CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes from CD2<sup>+</sup> cells was very critical. In these analyses, separated populations were not completely pure, but CD2<sup>+</sup>CD4<sup>-</sup> cells contained 80–85% CD8<sup>+</sup> cells and CD2<sup>+</sup>CD8<sup>-</sup> cells were 90–95% CD4<sup>+</sup>. As shown in Table 1, population A dominated over B when CD2<sup>+</sup> cells were used on

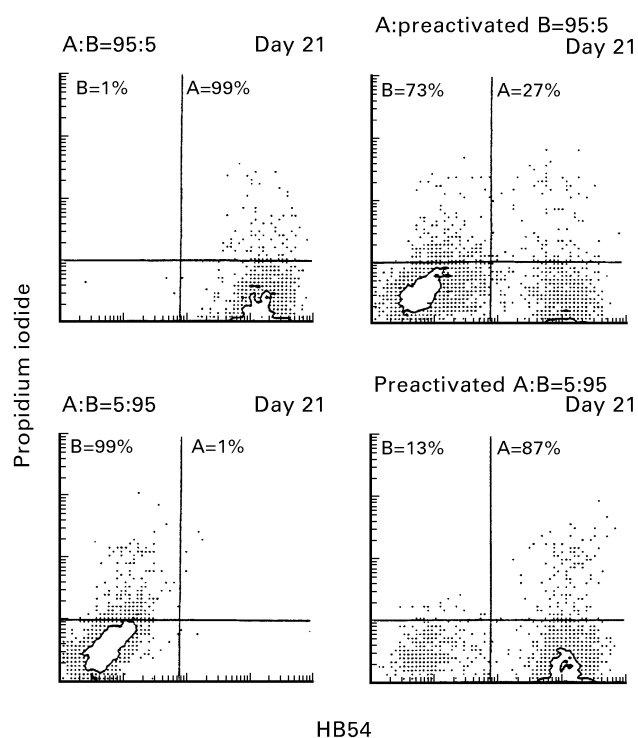


**Fig. 3.** Effect of initial ratio of the two allogeneic populations (A, HLA-A3<sup>+</sup>; B, HLA-A3<sup>-</sup>) of CD2<sup>+</sup> cells on population dynamics after 3 weeks. With an initial ratio on day 0 of 50:50 only population A survived after 3 weeks, while B disappeared. An initial ratio of 80:20 also led to domination of A, while an initial ratio of 20:80 led to a domination of B and disappearance of A after 3 weeks. Two-colour flow cytometric analysis was performed by staining with anti-HLA-A3-specific antibody H122 (abscissa) and propidium iodide (ordinate).

**Table 1.** Domination/elimination effects in two-way MLC over 21 days for various cell populations and starting conditions (relative cell numbers at days 0 and 21 for populations A and B, representative experiment)

Population	Cell type	Percentage day 0 → day 21		Percentage day 0 → day 21	
		day 0	day 21	day 0	day 21
A	CD2 <sup>+</sup>	50	99	20	1
B	CD2 <sup>+</sup>	50	1	80	99
A	CD2 <sup>+</sup>	50	99	ND	ND
B	CD2 <sup>+</sup> CD8 <sup>-</sup>	50	1		
A	CD2 <sup>+</sup>	50	3	80	99
B	CD2 <sup>+</sup> CD4 <sup>-</sup>	50	97	20	1
A	CD2 <sup>+</sup> CD8 <sup>-</sup>	50	2	80	7
B	CD2 <sup>+</sup>	50	98	20	93
A	CD2 <sup>+</sup> CD4 <sup>-</sup>	50	98	ND	ND
B	CD2 <sup>+</sup>	50	2		

ND, Not done.



**Fig. 4.** Population dynamics in two-way MLC using a population of preactivated cells. When resting CD2<sup>+</sup> cells of A (HLA-A2<sup>+</sup>) and B (HLA-A2<sup>-</sup>) were used at relative ratios of 5:95 or 95:5 the larger population always dominated over the smaller one. In contrast, when cells preactivated by 4-day stimulation with 15 ng/ml OKT3 were used at a 5:95 ratio, those cells always dominated the larger population in both directions. Two-colour flow cytometric analysis was performed by staining with anti-HLA-A3-specific antibody H54 (abscissa) and propidium iodide (ordinate).

both sides. Depletion of CD4<sup>+</sup> cells from the 'weaker' population B as well as depletion of CD8<sup>+</sup> cells from the 'stronger' population A switched the direction of population change, and cells from B finally dominated over A. Nine additional experiments gave identical results, indicating that the population containing markedly higher numbers of CD8<sup>+</sup> cells eliminates the other. Using unequal starting conditions of 20:80 in the same allogeneic combination a depletion of CD8<sup>+</sup> cells from the larger population led to domination of the smaller population (Table 1); this again demonstrates the functional importance of CD8<sup>+</sup> cells for the domination/elimination effect in the co-culture.

Incubation of mononuclear cells with 15 ng/ml OKT3 for 4 days prior to the two-way MLC caused a relative increase in the number of CD8<sup>+</sup> cells to 41.0 ± 4.2%, expression of CD45RO to 73.5 ± 13.4%, of CD25 to 74.5 ± 9.1%, and of HLA-DR to 43.0 ± 4.2%, demonstrating marked T cell activation. These preactivated cells dominated freshly isolated mononuclear cells even when they made up only 5% of cells at the start of the two-way MLC (Fig. 4). This effect occurred irrespective of the direction of population change that was observed in the co-culture using resting cells and 50:50 starting conditions.

#### Influence of cyclosporin and IL-2 in the two-way MLC

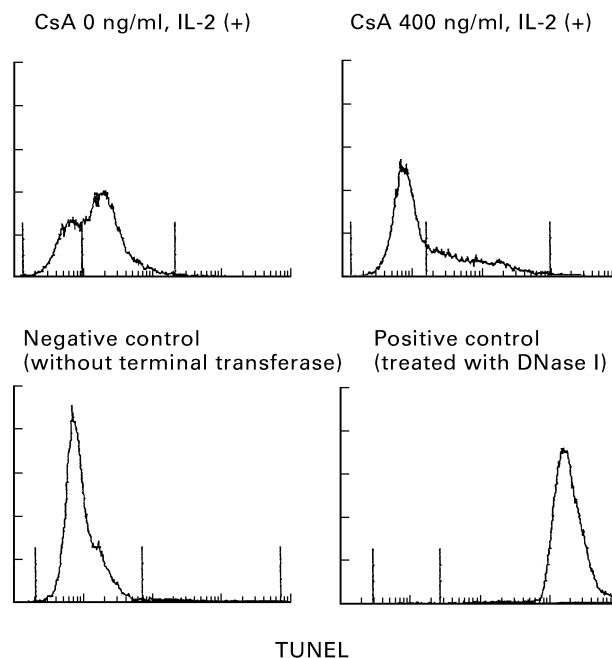
Addition of cyclosporin at concentrations between 50 and 400 ng/ml could block the domination/elimination process in the two-way

**Table 2.** Effect of cyclosporin on lymphocyte subpopulations in the two-way MLC after 21 days (+ 20 U/ml IL-2) (mean ± s.d.)

Surface markers	Cyclosporin (%)	
	0	400 ng/ml
CD3 <sup>+</sup>	96.0 ± 1.5	94.8 ± 1.6
CD4 <sup>+</sup>	46.5 ± 16.7	67.8 ± 7.0*
CD8 <sup>+</sup>	50.6 ± 13.9	31.3 ± 7.1*
CD3 <sup>-</sup> CD56 <sup>+</sup>	3.4 ± 1.9	3.3 ± 1.3
CD45RO <sup>+</sup>	92.9 ± 2.7	72.2 ± 7.6
CD25 <sup>+</sup>	59.0 ± 21.7	58.0 ± 16.7
HLA-DR <sup>+</sup> CD3 <sup>+</sup>	53.6 ± 14.8	50.2 ± 12.0

\*  $P < 0.01$ .

MLC. These cyclosporin concentrations are in the range of the drug levels achieved *in vivo* after clinical organ transplantation; they are known to inhibit T cell activation and proliferation as well as development of cytotoxic cells markedly. Interestingly, the blocking effect of cyclosporin was also maintained in the presence of exogenous IL-2. In these cultures the 50:50 ratio of allogeneic cells remained stable over 3 weeks in eight out of nine combinations where domination was found in the absence of cyclosporin. Subset compositions after 3 weeks of co-culture in two-way MLC with and without cyclosporin and IL-2 are shown in Table 2. This shows that considerable cell activation had occurred under these conditions in terms of IL-2 receptor (CD25) expression, HLA-DR expression on T cells and expression of CD45RO. One major difference that could be observed in the presence of cyclosporin



**Fig. 5.** Apoptosis in two-way MLC in the presence and absence of cyclosporin. In the standard two-way MLC supplemented with IL-2 a considerable number of cells showing terminal deoxynucleotidyl transferase (TUNEL) staining could be detected on day 7 of culture; this staining was markedly diminished in the culture containing cyclosporin (CsA).

was a predominance of CD4 cells, whereas CD8 dominated in the absence of the drug.

#### *Cell death and apoptosis in the two-way MLC*

In the standard two-way MLC with a 50:50 ratio of allogeneic cells, staining by propidium iodide revealed a mean of 1.4% positive cells at the beginning of the culture. This number then increased to 25% on day 7, to 32% on day 14, and to 43% on day 21.

Analysis of apoptosis by TUNEL staining revealed a peak on day 7 (68% of all lymphocytes). In the presence of cyclosporin this peak disappeared (Fig. 5), suggesting marked inhibition of apoptosis under these conditions.

### DISCUSSION

In contrast to the straightforward setup of a one-way MLR with one (rather passive) stimulator and one active responder cell population, the interactions occurring in a two-way setting are much more complex. This is already suggested by the observation that the proliferative response in the two-way MLR is not just a summation of two one-way MLR. Although the number of viable immunocompetent cells in the two-way culture was twice as high as in the corresponding one-way cultures, the proliferative response was similar to that of the corresponding one-way MLR. This suggests that the bilateral interactions interfere with activation of the two populations. This might be due to cytokine effects, to cytotoxic effector functions of alloreactive T cells and NK cells on both sides, to a different extent of apoptosis in the two populations, or to a combination of these mechanisms.

A central finding of the study was that under standard conditions human two-way mixed leucocyte cultures are unstable over time. With very few exceptions, one of the two populations markedly decreased or disappeared during 3–4 weeks of co-culture, while the other population survived and proliferated strongly. A similar observation has recently been described for two-way MLR of cattle PBMC; cells resulting from the culture after 3 weeks were predominantly of one animal [12]. Although details of the domination/elimination process were not analysed in that study, these findings suggest that considerable population shifts may be a general phenomenon under the conditions of the two-way MLC.

Reciprocal studies of one-way MLR have shown that the immune reactions occurring in the two directions usually differ in strength [3]. Therefore, it is very likely that the bilateral interactions in the two-way culture are also unequal in their relative intensity, which could explain the development of a disequilibrium. Unequal strength of the proliferative response in the corresponding one-way MLC was also observed in our experiments, but the findings were not generally predictive of the outcome of the cultures observed under two-way conditions. An inferior extent of proliferative response, however, does not preclude a dominant cytotoxic response. Since the relative numbers of cytotoxic precursor cells for the respective alloantigens in the two populations have not been studied, it could well be that there would have been a better correlation for this parameter.

Domination of one population and elimination of the other was markedly accelerated by addition of exogenous IL-2 to the two-way culture. In contrast, cultures remained in equilibrium for the whole period when cyclosporin was added. Since cyclosporin is known to interfere with T cell activation, particularly by blocking transcription of the gene for IL-2 [13], this cytokine seems to be

critical for the mechanisms associated with the domination/elimination process. Remarkably, the stabilizing effect of cyclosporin also persisted in the presence of exogenous IL-2, suggesting that a cyclosporin effect separate from blocking transcription and production of IL-2 seems to be involved.

Stabilization of the co-cultures in the presence of cyclosporin could be explained by two mechanisms. One could be an interference with the development of cytotoxic cells in the dominating population; this is suggested by a predominant activation and expansion of CD4<sup>+</sup> lymphocytes in both cultures in the presence of cyclosporin. The other mechanism could be inhibition of apoptosis in the population that would have been eliminated. Cyclosporin is able to block activation-associated apoptosis of lymphocytes [14] and markedly decreased apoptosis could also be found in the two-way cultures in the presence of cyclosporin. Apoptosis, therefore, appears to be an important mechanism for the development of imbalances between the two populations under two-way MLR conditions.

CD8<sup>+</sup> cells also seem to play an important role in the elimination process. The population containing higher numbers of CD8<sup>+</sup> cells usually dominated, and while depletion of CD8<sup>+</sup> cells frequently led to elimination of the respective population. In contrast, depletion of CD16<sup>+</sup> NK cells, like depletion of CD2<sup>-</sup> cells, from either population did not affect the domination process at all. This suggests that the domination/elimination process in the two-way MLC is mainly based on the activity of cytotoxic T lymphocytes, while NK cells and other cells in the culture seem to be of much less relevance. That NK cells play no major role in this system is also suggested by the kinetics of the elimination process. The cultures are stable for the first 5–7 days, and the domination/elimination process starts during the second week of culture. This is well compatible with the kinetics of elimination by cytotoxic T lymphocytes, but not by NK cells, which kill much more rapidly. The marked decrease of NK cells that occurred in the cultures by the second week, i.e. when elimination started, also argues strongly against a contribution of NK cells.

Thus, the mechanisms by which allogeneic mononuclear cells are eliminated in our *in vitro* culture model seem to differ from the mechanisms that have been shown to be responsible for elimination of allogeneic haematopoietic cells *in vivo*. After injection of allogeneic haematopoietic cells *in vivo*, elimination from the circulation occurs much more rapidly, i.e. usually within several hours to a few days [15,16]. Several studies, particularly in rodent models, suggest that this elimination process *in vivo* depends on NK cells. Expression of allogeneic MHC class I antigens on the allogeneic haematopoietic cells leads to activation of the recipient's NK cells by inadequate triggering of killing-inhibiting receptors [17,18]. It has also been demonstrated in rodent models, however, that survival of allogeneic haematopoietic cells can be considerably prolonged by treatment with tacrolimus, a drug which strongly interferes with IL-2 production—similar to cyclosporin [19]. Since this treatment does not lead to a marked inhibition of NK cell activity, it is very likely that cytotoxic T lymphocytes also play an important role in elimination *in vivo*. This effect is also in agreement with our *in vitro* observations where cell survival in co-culture was markedly prolonged by cyclosporin. Moreover, persistence of donor lymphocytes after liver transplantation appeared markedly enhanced in a patient shortly after bone marrow transplantation that had normal NK function but still inadequate cytotoxic T cell function at the time of liver transplantation [6,10]. Nevertheless, the much more rapid

elimination of allogeneic haematopoietic cells in the human *in vivo* suggests that different mechanisms may be involved *in vivo* compared with the *in vitro* model. It must be kept in mind, however, that disappearance of injected allogeneic cells from the circulation *in vivo* does necessarily indicate their destruction. Instead, they may just have left the blood stream and migrated into certain tissues.

The typical *in vivo* situation after injection or transfer of allogeneic cells is not adequately described by a 50:50 ratio of allogeneic cells in the two-way MLC. In the clinical situation, there is always an imbalance of cell numbers in favour of the recipient's cells. After unequal starting conditions in our *in vitro* model, the larger population dominated the smaller one—but only when resting unseparated cells or CD2<sup>+</sup> cells were used. The domination of the larger population could be reversed if the smaller population consisted predominantly of CD8<sup>+</sup> cells or—even more impressive—if the cells had been preactivated. Particularly under the latter conditions, 5% of cells in the co-culture were sufficient to dominate over 95% of (resting) allogeneic mononuclear cells. Again, this supports the hypothesis that elimination of allogeneic cells in this culture model is an active process. Furthermore, it clearly demonstrates that under certain conditions small numbers of immunocompetent cells may have a considerable functional relevance against an excessive number of allogeneic mononuclear cells.

In the clinical transplant situation where allogeneic mononuclear cells are generally transferred to the recipient by a graft, three parameters have to be considered in order to estimate potentially resulting interactions of donor- and recipient-type lymphocytes: first, the number of immunocompetent donor-type cells transferred to the recipient is usually markedly lower than the number of respective cells in the recipient; the numbers are, however, highly variable and may constitute up to 10<sup>10</sup> cells in the case of liver or lung transplantation [4,5]; second, passenger cells in donor organs are not resting blood mononuclear cells, but represent a selected population of preactivated (HLA-DR<sup>+</sup>) and mainly CD8<sup>+</sup> and CD45RO<sup>+</sup> T lymphocytes [4,5]; and third, the cellular interactions after transplantation occur in the setting of immunosuppression interfering with IL-2 production, i.e. in the presence of either cyclosporin or tacrolimus. Thus, several of the parameters present in the setting of clinical organ transplantation favour prolonged survival of the passenger cells and may thereby enable functional effects of passenger lymphocytes on the recipient's immune system [20–23]. To what extent two-way interactions between allogeneic immunocompetent cells early after organ transplantation are also important for the development of long-term microchimerism, a phenomenon frequently observed after solid organ transplantation, [24–26] remains speculative.

In conclusion, the two-way MLC represents a useful tool to evaluate bilateral interactions between allogeneic immunocompetent cells under various conditions. Although the interactions *in vivo* will be much more complex than those observed *in vitro*, the culture model can help to determine parameters that would allow donor lymphocytes to survive in an allogeneic environment and that might enable an immunoregulatory effect of such cells in allograft recipients.

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