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$CD3^+CD57^+$ lymphocytes are not likely to be involved in antigen-specific rejection processes in long-term allograft recipients

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SUMMARY

Cytofluorometric investigation of peripheral blood lymphocytes in 380 long-term (>1 year post-transplantation) allograft recipients showed a significant increase in the proportion of $CD3^+57^+$ lymphocytes (>20%) in 20% of patients with renal allografts, 66% of patients with cardiac allografts and 44% of patients with liver allografts. Most of these $CD3^+57^+$ cells expressed the CD8 antigen and a variable proportion the HLA-DR antigen. A retrospective analysis showed a poorer prognosis for the clinical outcome in those patients with elevated numbers of $CD3^+57^+$ cells in peripheral blood. However, $CD57^+$ lymphocytes could rarely be detected in renal infiltrates by immunohistology. Using the Southern blot technique to analyse the T cell receptor rearrangement of separated $CD57^+$ cells, no clonal or oligoclonal expansion of T cell clones could be detected. Nevertheless, there might be a bias towards the use of particular TCR-Vβ gene families in at least some patients, as shown by analysis with monoclonal antibodies. In summary, $CD57^+$ T cells are not likely to be directly involved in the rejection process. The data support the idea of a polyclonal and/or superantigen-driven expansion, but not of an antigen-driven expansion of these cells.

Keywords $CD57^+$ cells allotransplantation T cell receptor rearrangement flow cytometry immunohistology

INTRODUCTION

$CD3^+57^+$ cells are a minor lymphocyte subset in healthy individuals [1]. Although several studies have shown a significant increase in $CD3^+57^+$ lymphocytes in the peripheral blood of patients with renal allografts [2,3], the biological function of these cells has not been elucidated. It has been reported that high levels of $CD3^+57^+$ cells suppress the T cell-dependent immunoglobulin synthesis in B lymphocytes [4]. Furthermore, $CD3^+57^+$ lymphocytes have been regarded as primed cytotoxic cells or resting pre-cytotoxic cells [5]. However, no data appear to be available on the antigen specificity of $CD57^+$ T cells.

We report here the results of cytofluorometric investigation of peripheral blood lymphocytes in more than 400 allograft recipients (renal, cardiac, liver). To clarify further the biological significance of $CD3^+57^+$ lymphocytes in patients with allotransplants, $CD57^+$ cells were characterized by two-colour immunofluorescence and investigated for their clonality and their usage of TCR-Vβ gene families. Further, graft biopsies were taken from patients with a deteriorated kidney transplant function due to rejection processes. These graft biopsies of patients with or without increased proportions of $CD3^+57^+$ cells in peripheral blood were immunohistologically investigated for the presence of $CD57^+$ cells within lymphocyte infiltrates to determine whether these cells might be directly involved in the rejection process.

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PATIENTS AND METHODS

Patients

Between 1985 and 1991, about 380 long-term allograft recipients (>1 year post-transplantation (Tx) underwent an immune monitoring program. The frequency of immunological analysis of long-term allograft recipients, who were hospitalized because of various complications, was dependent on both the clinical course and therapeutic approach (3–20 investigations per patient). The patients studied included 302 long-term renal allograft recipients (1–18 years post-Tx) hospitalized because of deterioration in graft function. In 246 of these 302 cases, the diagnosis was verified by histologic analysis of core biopsies (152 acute rejections, 132 chronic rejections of mixed forms, five cyclosporin toxicities). In addition, 64 patients with long-term cardiac allografts and 14 long-term liver allograft recipients with or without graft complications were studied.

The immunosuppressive therapy of long-term allograft recipients consisted of cyclosporin in combination with corticosteroid ($n=85$), azathioprine/corticosteroid ($n=15$), or triple drug regimen ($n=280$). Rejection therapy consisted commonly of methylprednisolone bolus treatment (3–5 times 5 mg/kg body wt daily or 1 g/day for 3 consecutive days). Steroid-resistant rejections were treated with anti-thymocyte globulin or repeated steroid bolus.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of heparinized peripheral blood over Ficoll–Hypaque as described [6].

Phenotypic analysis

PBMC were analysed after double-fluorescence labelling with MoAbs, using an EPICS-Profile I cytofluorometer (Coulter Electronics).

The MoAbs used were: anti-CD3-PE, anti-CD3-FITC, anti-CD4-FITC, anti-CD4-PE, anti-CD8-FITC, anti-CD8-PE, anti-HLA-DR-PE (Dianova, Hamburg, Germany) and anti-CD57-FITC (Becton-Dickinson, Heidelberg, Germany).

The $CD57^+$ lymphocyte population was regarded as increased if its proportion was higher than 20% of the population of lymphocytes.

For double-flourescence labelling using MoAbs against TCR-V-families, a four-step procedure was used. First, cells were incubated with a MoAb against a particular TCR-V-family (Diversi-T^R, Biermann, Bad Nauheim, Germany), then with a goat $F(ab')_2$ anti-mouse immunoglobulin (Medac, Hamburg, Germany). To saturate the free binding sites of the secondary antibody, a mouse control antibody was used that did not react with the cells. Finally, PBMC were labelled with a second MoAb directly linked with PE.

Separation of $CD57^+$ lymphocytes

$CD57^+$ cells were separated using an immunomagnetic system [7]. The immunobeads were purchased from Dynal (Oslo, Norway). Beads coupled to goat-anti-mouse IgG were used together with the MoAb anti-CD57 (anti-Leu-7) from Becton-Dickinson.

Separated cells were either directly taken for DNA isolation or stored at $-20°C$ before DNA preparation.

DNA extraction and Southern blot analysis

DNA analysis was performed using a modification of the method originally described by Southern [8].

Aliquots of 10 μg of DNA were digested with an appropriate restriction enzyme (*Eco*R1, *Hind*III, *Bam*H1), run on a 0·7% agarose gel, denaturated, neutralized, and transferred to nylon filters (Hybond N; Amersham International, Amersham, UK). Filters were hybridized with a probe labelled with ^{32}P-dCTP (Amersham) using a random oligonucleotide priming kit (Boehringer, Mannheim, Germany). Hybridization was performed overnight at 68°C.

The sensitivity of this procedure was found to be about 2%, i.e. any T cell clone would need to represent at least 2% of the $CD57^+$ PBMC to be detected by this procedure (data not shown).

Table 1. Number of patients with an increased proportion of $CD57^+$ cells (> 20%) in peripheral blood in comparison with healthy controls

Patients	Number of patients with an increase in $CD57^+$ PBMC/total number of patients investigated
Healthy controls	5/102 (5%)
Long-term allograft recipients	
Renal	61/302 (20%)*
Liver	6/14 (44%)*
Heart	43/64 (66%)*
Short-term allograft recipients (< 3 months post-Tx)	
with acute CMV-infection	38/43 (88%)*
without CMV-infection	10/125 (8%)

* $P<0·01$.

PBMC, Peripheral blood mononuclear cells; CMV, cytomegalovirus.

Altogether, PBMC of 15 patients carrying an allotransplant were investigated. All patients were characterized by an increase in peripheral $CD3^+57^+$ lymphocytes.

Probes

Hyrbridizations were performed using the following probes. Probe pB400, a 400 bp *Eco*R1 fragment recognizing the $C_\beta 1$ region of the human TCR-β gene, which also cross-hybridizes with the $C_\beta 2$ region [9], was the gift of Dr M. J. Owen (ICRF, London, UK). Probe pH60, a 700 bp *Hind*III-*Eco*R1 fragment recognizing the human TCR-$J_\gamma 1$ and -$J_\gamma 2$ regions [10], was the gift of Dr T. H. Rabbits (MRC Laboratory of Molecular Biology, Cambridge, UK).

Immunohistology

Sections (5 μm thick) from paraffin embedded material were dewaxed, rehydrated and incubated with pronase (1 mg/ml; Boehringer) for 10 min at 30°C. Immunohistological staining was performed using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique [11].

The MoAbs used were anti-CD43 (MT1, kindly provided by Dr M. Ernst, Biotest, Frankfurt, Germany), recognizing T cells and granulocytes, and anti-CD57 (Leu-7, Becton-Dickinson, Heidelberg, Germany). Both MoAbs recognize paraffin-resistant epitopes.

RESULTS

Phenotypic analysis of patients' PBMC

A significant increase in the proportion and number of $CD57^+$ cells, in particular of $CD3^+57^+$ cells, was found in 20% of patients with renal allografts, in 44% of patients with liver allografts and in 66% of patients with cardiac allografts (Table 1). Follow-up studies have shown that the increase in $CD3^+57^+$ cells first appeared about 6–9 months post-Tx and turned out to be fairly stable over the follow-up period (up to 3 years). In most patients, the increase in $CD3^+57^+$ PBMC was associated with a rise of $CD8^+$ T cells resulting in a decrease of the proportion of $CD4^+$ to $CD8^+$ cells to less than 1.

Table 2. Proportion of CD3⁺ and CD57⁺ cells in graft-infiltrating lymphocytes found in patients with normal or increased proportion of CD3⁺57⁺ cells in peripheral blood

Proportion of CD3⁺CD57⁺ cells in peripheral blood	Proportion of CD3⁺ and CD57⁺ cells in graft-infiltrating lymphocytes*	
	CD3⁺ cells	CD57⁺ cells
Normal (<20%) $n=24$	57±17·2%	0±0%
Increased (>20%) $n=10$	50±11·5%	0·05±0·1%

*Mean ±s.d.

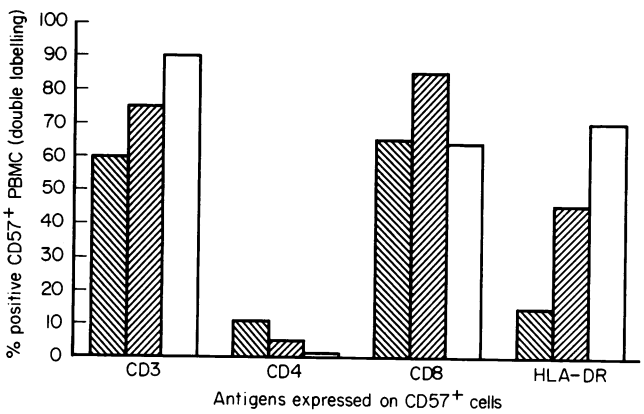

Fig. 1. Expression of CD3, CD4, CD8 and HLA-DR on CD57⁺ peripheral blood mononuclear cells (PBMC) in three patients representative of the whole population of patients investigated. ▧, Patient 1; ■, patient 2; □, patient 3.

The changes in phenotype of PBMC were also found in allograft recipients with acute cytomegalovirus (CMV) infection during the first weeks post-Tx (Table 1). However, in most patients with acute CMV infection these phenotypic changes disappeared some weeks later following virus clearing. Furthermore, most of the long-term allograft recipients carrying an increased proportion of CD57⁺ PBMC did not have any clinical signs of post-transplantational CMV infection.

Retrospective study in patients with renal allografts

The retrospective analysis showed a poorer prognosis for the clinical outcome in those patients with elevated numbers of CD57⁺ T cells in peripheral blood. Whereas 42 of 52 patients (81%) with an increased proportion of CD57⁺ PBMC lost their graft within 1 year after hospitalization, only 73 of 248 patients (20%) with a normal proportion of CD57⁺ cells developed an irreversible deterioration of graft function in the same time. There were no differences in either the histology or the therapeutic approach for these two groups of patients.

Phenotypic characterization of CD57⁺ PBMC

Results in three patients representative of the population of patients investigated are given in Fig. 1. More than about 65%

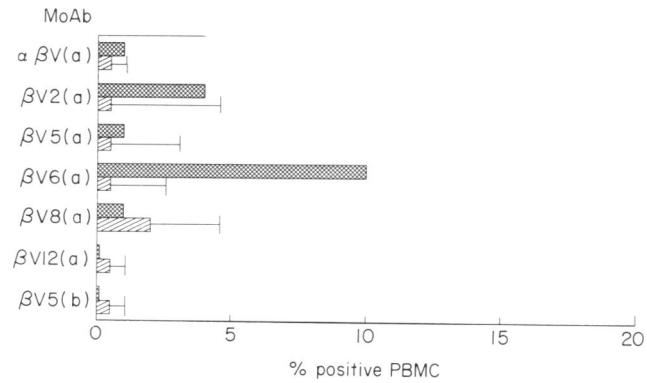

Fig. 2. Reactivity of MoAb against Vβ families of the T cell receptor with peripheral blood mononuclear cells (PBMC) of a long-term allograft recipient. Results demonstrate a significant increase in *βV6(a)*⁺ PBMC in comparison with healthy controls. ▧, Patient; ■, healthy controls ($n=20$).

(65–88%) of CD57⁺ PBMC expressed CD8, but less than 10% CD4. Only one patient was found to express CD4 at up to 50% of CD57⁺ PBMC. The proportion of CD3⁺ cells varied from about 40% to 90%, indicating a variation in the proportion of CD3⁺57⁺ to CD3⁻57⁺ PBMC. The expression of HLA-DR on CD57⁺ PBMC varied from patient to patient and in different investigations of PBMC from the same patient at different times, ranging from 2 to 80%.

Patients with acute CMV infection were found to carry a similar phenotype of CD57⁺ cells.

Immunohistological investigation of graft-infiltrating lymphocytes

Only one out of 34 grafts investigated could be demonstrated to contain detectable levels of CD57⁺ cells (Table 2). There was no significant difference between grafts of patients with an increase in peripheral CD3⁺57⁺ cells and patients with a normal proportion of these cells. Furthermore, no significant difference between patients with or without clinical evidence for CMV infection was observed.

Southern blot analysis

All DNA samples of CD57⁺, CD57⁻ and whole PBMC showed a germ-line configuration of TCRβ genes when tested using three different restriction enzymes. Thus, all samples tested had 4·0 and 12·0 kb bands with *Eco*R1 ($C_\beta 1$ germ line), 3·5 and 8·0 kb bands with *Hind*III ($C_\beta 2$ germ line) and 24·0 kb band with *Bam*HI ($C_\beta 1$ and $C_\beta 2$ germ line). Figure 3 shows a representative example of CD57⁺, CD57⁻ and whole PBMC of one patient. When the same DNA samples were tested for rearrangements of the TCRγ genes, they too failed to show detectable rearrangements.

Reactivity of PBMC with MoAb against TCR-Vβ families

PBMC of one of 20 patients investigated demonstrated an increase in the Vβ6 family of up to more than 10% of total PBMC (Fig. 2). In double-labelling studies, all βV6(a)⁺ cells were found to co-express CD8. About 58% co-expressed CD57 and about 15% HLA-DR.

PBMC of a further five patients were characterized by an increase of one Vβ family up to more than 5% of total PBMC.

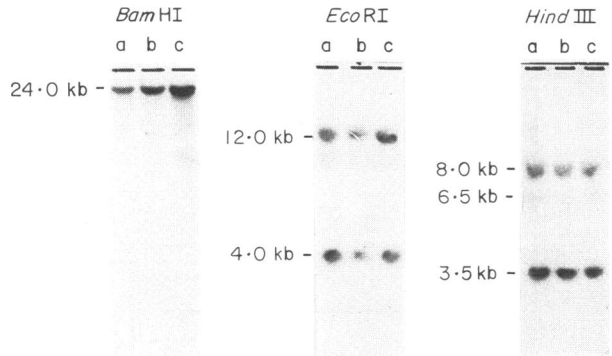

Fig. 3. Southern blot analysis using the TCRβ-probe pB400. a, peripheral blood mononuclear cells (PBMC); b, CD57$^+$ PBMC; c, CD57$^-$ PBMC.

Indeed, PBMC of two patients were found to show an increase in βV8(a)$^+$ cells, PBMC of two other patients an increase in βV5(a)$^+$ cells and PBMC of another patient an increase in βV6(a)$^+$ cells.

DISCUSSION

Based on the results of our immune-monitoring programme that involved 380 long-term allograft recipients (renal, cardiac, liver), we selected 300 patients with renal allografts for a retrospective analysis. Remarkably, the graft survival in patients with deteriorated graft function was found to be poorer in the group of patients with an expansion of peripheral CD57$^+$ T cells. These findings raised the question of whether CD3$^+$57$^+$ cells might be directly involved in the rejection process. In an attempt to answer this question we investigated graft biopsies from 34 patients with histologically proven rejection for the presence of CD3$^+$57$^+$ cells within the organ-infiltrating lymphocytes. There was no significant difference between transplants of patients with an increase in peripheral CD3$^+$57$^+$ lymphocytes and patients with a normal proportion of CD3$^+$57$^+$ PBMC (Table 2). This was found for patients both with and without clinical evidence of CMV infection. Only one transplant contained detectable levels of CD57$^+$ cells. The patient who was carrying this transplant had significant clinical signs of a CMV infection before the rejection process started. Furthermore, he showed an increase in CD3$^+$57$^+$ cells in peripheral blood. However, the proportion of CD57$^+$ cells in the graft was only about 1% of the whole population of graft-infiltrating cells. These findings make it unlikely that CD3$^+$57$^+$ lymphocytes are directly involved in the rejection process.

Several authors have demonstrated that CD3$^+$57$^+$ lymphocytes are responsible for lectin-dependent and anti-CD3 induced cytotoxicity in peripheral blood and therefore suggested that CD3$^+$57$^+$ cells might be primed cytotoxic cells or resting precytotoxic cells [3,5]. So far, no data have been available regarding antigen specificities of CD57$^+$ T cells. Therefore, we decided to study the pattern of T cell receptor gene rearrangements of these cells to provide further information on their possible representation of an oligoclonal expansion which would reflect a specific response to a defined antigen. For this purpose total DNA of CD57$^+$ PBMC was investigated by Southern blot analysis using specific gene probes for TCRβ and TCRγ. So far we have not been able to demonstrate any oligoclonality in the CD3$^+$57$^+$ PBMC of our patients (Fig. 3). However, as the sensitivity of the procedure used was about 2% in our hands, only T cell clones representing at least about 2% of the whole population of CD57$^+$ PBMC would be detectable. Nevertheless, from our results it seems likely that the population of CD3$^+$57$^+$ cells might be a polyclonal population without any expansion of particular cell clones.

Another approach to provide further information on the usage of TCR genes in CD3$^+$57$^+$ cells is the use of MoAbs directed against specific TCR-V families. In our experiments we used the MoAbs $\alpha\beta$V(a), βV2(a), βV5(a), βV6(a), βV8(a), βV12(a) and βV5(b) which cover about 20% of the whole range of TCR-Vβ regions. As shown in Fig. 2, in one out of 20 patients investigated there was an increase in Vβ6(a)$^+$ cells of up to more than 10% of total PBMC. All Vβ6(a)$^+$ cells co-expressed CD8 and about 58% CD57. In other words, about 20% of all CD3$^+$57$^+$ cells used TCR-β genes belonging to the Vβ6 family. The PBMC of five other patients were characterized by an increase of one Vβ family of up to more than 5%. Possibly there is a bias towards the use of specific TCR-Vβ gene families, at least in some patients. The usage of different Vβ-families in different patients might be due to different MHC backgrounds in these patients and does not necessarily reflect different antigen specificities. There are examples of bias towards TCR-Vβ-chain usage correlating with MHC background [12–14]. Alternatively, a bias towards the use of specific TCR-Vβ gene families at least in some patients might suggest that the expansion of CD57$^+$ PBMC could be the result of the interaction with superantigen-like structures. Superantigens have been described in bacteria, bacterial toxins and viruses.

In interpretating our results, two different explanations for the increase in CD3$^+$57$^+$ lymphocytes would seem to be quite reasonable. On the one hand the increase in CD3$^+$57$^+$ lymphocytes could simply reflect an expression of the CD57 antigen in former CD3$^+$57$^-$ cells. The CD57 antigen is known to be an epitope on the carbohydrate moiety of several neural cell adhesion molecules [15]. Therefore, one could speculate that CD57 is an adhesion molecule that is upregulated in T lymphocytes under certain conditions of immune stimulation.

On the other hand, the increase might reflect a polyclonal expansion of this particular cell population due to certain immune disbalances. To consider this possibility we have started *in vitro* investigation of PBMC of healthy donors to address the question of whether there are any conditions of immune disbalance that selectively stimulate the proliferation of CD3$^+$57$^+$ cells within the bulk population of PBMC. So far we have not been able to find any such conditions.

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