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Frequencies of Cytotoxic T Lymphocyte Precursor Estimate in Three Different Populations

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SUMMARY There is speculation that high cytotoxic T lymphocyte precursor frequencies (CTLpf) correlate with poor clinical outcome of bone marrow/organ transplantation. It is also believed that human umbilical cord blood is immunologically naive, and, therefore cord blood T cells may be less able to mediate graft versus host disease than marrow-derived T cells. CTLpf were determined in peripheral blood mononuclear cells collected from healthy adults, human umbilical cord blood and renal dialysis patients who were randomly selected and entered into this study. A highly sensitive non-radioactive Europium release cytotoxicity assay was optimized and modified to carry out the CTLpf estimation by using the principle of limiting dilution analysis. The results of CTLpf in healthy adults ranged from 1/694 to 1/66,666, median 1/7,339 (n=10); cord blood ranged from 1/1,562 to 1/35,714, median 1/10,162 (n=6) and dialysis patients ranged from 1/1,054 to 1/17,857 median 1/5,208 (n=9). The results demonstrated that there is little difference of CTLpf median values between the groups, but there is a wide variation of CTLpf between individuals within a population. It suggests that this variation should be taken into account when considering CTLpf assay as pre-transplantation cross-match procedure.

Allogeneic graft rejection is T cell dependent and thus T cells play a central role in immunological graft rejection. The reactivity of the T cells is directed against major histocompatibility complex (MHC) and/or minor histocompatibility complex (mHC).[1] Naive or memory T cells are incapable of killing target cells directly and are therefore referred to as CTLp to denote their inactive state. The naive T cells are present at orders of magnitude higher than other sub-populations of T cells, those directed to nominal antigens directly recognize MHC alloantigens and are responsible for the extraordinary strength of allograft responses.[1,2] An alloreactive CTLp has the potential to differentiate into a clone of activated CTL after engaging the specific alloantigen recognized by its T cell receptor.[2,3]

The existence of differences in CTLpf against different HLA class-I antigens has been demonstrated among unrelated healthy individuals.[4] *In vitro* CTLp responses to mHC antigen have been detected after *in vivo* sensitisation by multiple blood transfusions in patients with severe aplastic anemia.[5] Kaminski and co-workers also showed a significant correlation between high CTLpf against the recipient and the severity of acute graft versus host disease (GVHD) after 'matched' unrelated bone marrow transplantation (BMT). Recent reports supported the above findings and showed that major variants exist in alloreactive CTLpf against HLA class I antigens in humans.[6]

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Reviewing the above research reports, it may be suggested that cytotoxic T cells play an important role in allograft rejection, particularly when CTLpf estimates seem to correlate well with the clinical course after transplantation. Limiting dilution analysis (LDA) is used for determining the frequency of defined cells and the *in vitro* CTLpf assay utilizes the principle of LDA.[7] Currently several protocols are in use for CTLpf estimation, the one we describe here was modified and performed in peripheral blood mononuclear cells (PBMC) collected from healthy adults, cord blood and patients with chronic renal failure on dialysis awaiting transplantation.[8,9]

MATERIALS AND METHODS

Informed consent

All protocols used for the recruitment of subjects/patients and the collection of cord blood were reviewed and approved by the appropriate ethics committee.

Study populations

Healthy donors

Thirty blood samples were collected from healthy volunteers of the cell donor panel of the Division of Transplantation Sciences, University of Bristol. Ten of them were used as responder and the remaining twenty were used as stimulator targets.

Renal dialysis patients

Nine patients with end-stage renal disease on hemodialysis/continuous ambulatory peritoneal dialysis were entered in this study. Patients having malignancy, autoimmune disorders or on immunosuppressive drug therapy were excluded from the study.

Cord blood

Six cord blood samples were collected randomly after normal full-term deliveries. Maternal consent was obtained in all cases. All aspects of cord blood clamping and placental delivery were at the midwives' discretion, based on standard local practice.

Isolation and storage of cells

Forty milliliters of blood was collected in a sterile tube containing preservative-free sodium heparin (25 units/ml of blood). PBMC were isolated within twelve hours of collection by density gradient centrifugation (Lymphoprep of specific gravity 1077, Nycomed, Norway) and were cryopreserved in liquid nitrogen until required.

HLA typing

The normal healthy adults and renal dialysis patients were typed for HLA. Cord blood was not typed for HLA and no attempt was made to HLA match responders and stimulators. In those cases where HLA typing was performed, pairs were found to be mismatched at HLA loci tested.

Culture medium

The culture medium was referred to as the complete culture medium (CCM), made up with RPMI 1640 (Sigma-Aldrich, UK) supplemented with 10% heat inactivated (56°C for 30 minutes) human AB serum from normal healthy male donors (Sigma, USA), streptomycin (100 μg/ml, Calbiochem, USA), penicillin (100 units/ml, Calbiochem, USA) and 3mM L-glutamine (Sigma-Aldrich, UK).

CTLpf assay by LDA

On day 0 (zero) cryopreserved PBMC were thawed and the responder cells titrated (two-fold dilution) in CCM starting with a concentration of 4×10^4 down to a concentration of 0.0625×10^4 cells/100μl/well (4×10^4, 2×10^4, 1×10^4, 0.5×10^4, 0.25×10^4, 0.125×10^4, 0.0625×10^4). Then constant numbers of stimulator cells (5×10^4/well) were irradiated at 30 Gy (BL 437 Irradiator, CIS Bio International, France) added to diluted responder cells in 96-wells round bottomed tissue culture plates (Falcon 3077, USA). The stimulator cells (100μl) were plated out from lower concentrations to higher concentrations to avoid cross-over contamination. For each concentration and baseline control, 16 or 24 replicates were cultured in CCM, supplemented with 25 units/ml Cetus rIL-2 (Proleukin, Eurocetus, the Netherlands). The baseline control was irradiated stimulator cells only, cultured without responder cells. Cultures were incubated at 37°C, 5% CO_2 in humidified atmosphere. On day 5, half of the culture media from each well of the assay plates was replaced with freshly prepared CCM containing 25 units/ml rIL-2. The wells were then cultured for another 2 of a total of 7 complete days.

Targets set up

At the same time as setting up a CTLpf assay, non irradiated

PBMC of original stimulators were adjusted to 1 x 10^6 cells/ml and set up as targets in CCM containing 20 units/ml Cetus rIL-2 and 2 μg/ml PHA (Phytohaemagglutinin, Murex Diag, UK), in a 24 well flat bottomed tissue culture plates (Costar, USA) and incubated for 7 days at 37°C, 5% CO_2 in humidified atmosphere. On day 3 and day 5, each well of plates containing target cells was divided and fed with freshly prepared culture medium containing rIL-2 to give a concentration of 20 units/ml.

Europium (Eu) labelling procedures

Cellular cytotoxicity assay was performed by a modified Eu-release CTLpf method.[9,10] Briefly, at the end of 7 days culture, PHA stimulated targets were harvested and washed twice with phosphate buffer saline centrifuged at 400 x *g* for 10 minutes at 4°C. In the meantime, labelling buffer was prepared by adding 50 μl diethylene triaminopentaacetate (1,250 μM stock, Sigma, USA) and 50 μl $EuCl_3$ (500 μM stock, Fluka, Switzerland) to 1 ml HEPES buffer (Sigma, USA). After the second wash 1 ml of labelling buffer to each 10 x 10^6 target cells and then 35 μl of 10 mg/ml stock dextran sulphate (Fluka, Switzerland) were added. The cell suspension was mixed gently and incubated for 15 minutes at 4°C. The labelling reaction was stopped by adding 30 μl of 100 mM $CaCl_2$ per 1 ml labelling buffer/10 x 10^6 cell suspension and incubated for a further 10 minutes at 4°C. Then wash buffer was made up by adding 790 μl of 1 M $CaCl_2$ per 500 ml of RPMI 1640 and 3 mM L-glutamine to give a final concentration of 2 mM $CaCl_2$. After a total of 25 minutes incubation, the cells were washed four times with wash buffer at 300 x *g* for 6 minutes at 4°C. Then the cells were washed two more times at 400 x *g* for 6 minutes at 4°C with test medium (RPMI 1640 supplemented with 10% AB sera and 3 mM L-glutamine). Finally, the cells were resuspended in test medium and the temperature gradually increased from 4°C to room temperature (RT, 18-22°C).

Cytotoxicity assay

The contents of each well of the original assay plate were mixed and two identical aliquots were transferred to new round-bottomed 96 well plates and tested against Eu-labelled PHA stimulated targets. The Eu-labelled targets were added to each well at a concentration of 5,000 cells/well. The targets were original stimulator and control. The lysis of Eu-labelled targets by effector cells (responders) releases the Eu-diethylene triaminopentaacetate (Eu-DTPA) complex in culture medium. This is a highly fluorescent substance that can be measured in a time resolved fluorometer. To determine the spontaneous release of Eu-DTPA complex from lysed targets, test medium was used instead of effector cells during the test time. Maximum release, the total releasable fluorescence from target cells was obtained by incubation with 1% Triton X-100 (Fluka, Switzerland). The assay plates were incubated for 3 hours at 37°C in 5% CO_2 in humidified atmosphere. After incubation plates were centrifuged again at 600 x *g* for 5 minutes at RT to pellet the cells. Then 20 μl aliquots of supernatant were carefully transferred to the flat-bottomed 96 well low autofluoroscent reader plates (Nunc, Denmark), each well was prefilled with 200 μl of enhancement solution (Delfia, Finland). Cytotoxicity was measured by detecting Eu-release in the supernatant using a time resolved fluorometer (1234 Arcus, Delfia, Finland).

Statistical analysis

Calculation of baseline

The mean counts per second (fluorometer reading) and the standard deviation (SD) between replicate wells of the baseline culture were calculated. Coefficient variation (CV) should not exceed 10%.

Scoring of wells

Wells were scored positive when counts exceeded the baseline +3 x SD.

Calculation of CTLpf

The population (%) of negative wells at each responder cell dilution were calculated and plotted against the responder cell concentration per well. When 37% of the wells are negative, it can be assumed that there is an average of one precursor cell per well and the frequency of the CTLp can be calculated.[8]

Computation of CTLp

CTLpf were calculated by the Jackknife method using a computer program developed by Strijbosch and colleagues.[11] This analysis provides a frequency estimate (f) with 95% confidence intervals and *p* values for *single Hit Kinetics.*

RESULTS

Tables 1, 2 and 3 show the CTLp frequencies and 95% confidence intervals as calculated by the Jackknife method using Strijbosch's program[11] in three groups of individuals. The responder PBMC from individual healthy adults, human umbilical cord blood and renal dialysis patients were tested against stimulator PBMC from different normal healthy adults. Fig. 1 is a representative experiment showing 37% negative wells when PBMC of a renal patient as responder were tested against a normal healthy individual as shown in Table 3 (number 9). Fig. 2 is a summary of the median CTLpf values for all three groups.

DISCUSSION

The CTLpf assay utilizes the principle of LDA for determining the frequency of defined cells.[7] The CTLpf assay can be used to quantitate the frequency of cytotoxic T cell precursors present either in the patient or the donor. There is a wide variation of CTLpf between individuals within the same group: CTLpf ranged 1:694 to 1:66,666 in healthy adults; 1:1,562 to 1:35,714 in human umbilical cord blood and 1:1,054 to 1,17,857 in renal patients as shown in tables 1, 2 and 3. There is no significant difference in CTLp frequency amongst the three tested groups. These findings confirmed the high value of

Table 1 CTLpf in healthy adults (n = 10) tested against different healthy adults. CTLpf median value in normal healthy adults was 1:7,339 and ranged from 1:694 to 1: 66,666

Experiment	Responder/stimulator	CTLpf	95% confidence interval	
			Upper limit	Lower limit
1	DH/CP	1:694	1:486	1:1,212
2	CL/ID	1:1,124	1:844	1;1,683
3	JK/DS	1:3,058	1:2,202	1:4,975
4	PS/DG	1:5,917	1:4,608	1:8,474
5	EM/CR	1:5,988	1:4,115	1:11,111
6	PL/PC	1:8,690	1:6,896	1:11,904
7	JW/RL	1:9,615	1:5,882	1:27,027
8	PC/PL	1:19,230	1:14,492	1:29,411
9	AH/PM	1:28,571	1:21,276	1:43,478
10	RW/JBH	1:66,666	1:47,619	1:111,111

Table 2 CTLpf in human umbilical cord blood (n = 6) tested against healthy adults. CTLpf median value was 1:10162 and ranged from 1 :1,562 to 1 :35,714

Experiment	Responder/stimulator	CTLpf	95% confidence interval	
			Upper limit	Lower limit
1	CB2/NB	1:1,562	1:1,145	1:2,757
2	CB2/GK	1:5,580	1:4,201	1:8,310
3	CB501/AJ	1:8,130	1:6410	1:11,111
4	CBB/ML	1:12,195	1:8,620	1:20,833
5	CBG/DB	1:12,500	1:8,695	1:21,739
6	CB3/SM	1:35,714	1:25,000	1:58,714

Table 3 CTLpf in renal patients (n = 9) tested against randomly selected healthy donors. CTLpf median value in renal patients was 1:5,208 and ranged from 1:1,054 from 1:17,857

Experiment	Responder/stimulator	CTLpf	95% confidence interval	
			Upper limit	Lower limit
1	TS/WF	1:1,054	1:736	1:1,851
2	BW/MG	1:1,102	1:846	1:1,582
3	SK/RM	1:1,209	1:874	1:1,960
4	MG/JTD	1:4,385	1:3,690	1:5,435
5	SK/DH	1:5,208	1:3,773	1:8,403
6	DH/RM	1:5,714	1:4,484	1:7,874
7	PL/NB	1:6,178	1:4,716	1:8,928
8	SK/TD	1:6,849	1:5,681	1:8,771
9	DH/JBH	1:17,857	1:14,285	1:24,390

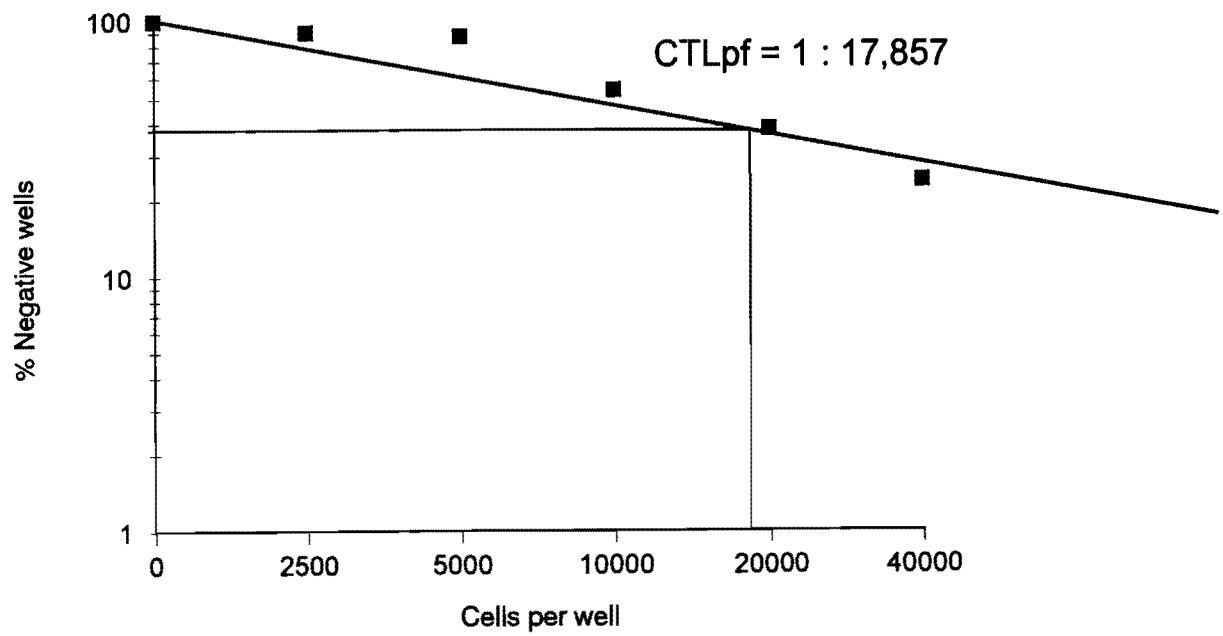

Fig. 1 A straight line passing through the origin in the responder/stimulator pair. This represents a single-hit curve, which means a single cell type is limiting. By interpolating at % negative wells = 37% the frequency of responder can be estimated.

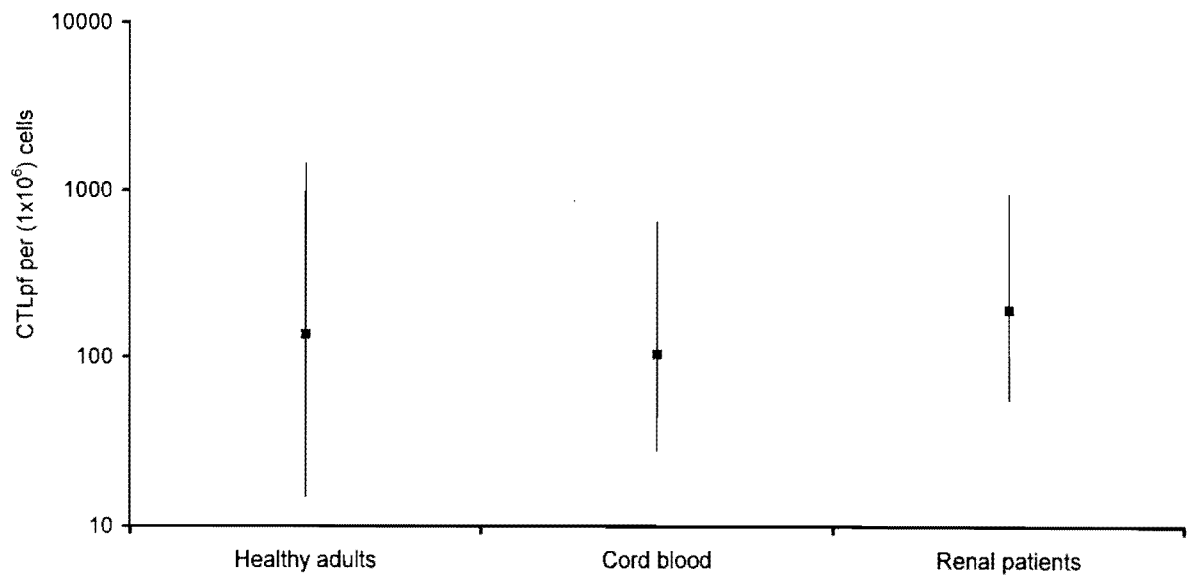

Fig. 2 Frequencies of donor reactive CTLpf in healthy adults (n=10), cord blood (n=6) and renal patients (n=9). No significant difference between the groups was observed according to the Mann-Whitney test ($p > 0.11$). The median values are indicated by a solid square in each line.

allospecific CTLpf in general, but simultaneously point to unexpected variations between individuals irrespective of age and state of health. This correlates with previous observations that there is a wide variation in the CTLpf of individuals responding to different allo-MHC antigens.[4] In humans, it was found that each individual possesses a different CTLpf against a given set of foreign determinants, like viral and bacterial antigens.[12]

Higher than expected CTLpf in cord blood may be attributable to other non T cell mediated alloreactivity, e.g. natural killer cells and antibody dependent cell mediated cytotoxicity. Autologous control confirmed that only alloreactive CTLpf were being detected in the frequency assay of cord blood. These findings indicate that cord blood is not deficient in the precursors of alloreactive cells.

CTLpf may be important for donor selection, especially in BMT. A sequential reduction of donor-specific CTLpf at post transplant in patients with a well functioning renal allograft[13] and a selective increase of donor specific CTLpf in patients with a rejecting corneal allograft were also reported.[14] As alloreactive T cells are the initiators and effectors of acute allograft rejection, these findings should be taken into account when considering CTLpf assay as a pre-transplant cross-match procedure.

Furthermore, a sensitive non-radioactive Eu-release CTLpf assay may have a value in post-transplant monitoring for quantitating T cell allo-immunity. This study should be evaluated in a large number of individuals.

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