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Quantitation of cyclosporine-sensitive and -resistant allospecific cytotoxic cells at birth

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Abstract In the absence of clinically relevant models of acute rejection we have attempted to develop an assay to measure cyclosporine-resistant allospecific cytotoxic cells in vitro, beginning at birth. The principle of limiting dilution analysis was applied to investigate umbilical cord bloods as responders. Responders were incubated for 1 h in different concentrations of cyclosporine and irradiated HLA mismatched stimulator cells from healthy adults added, followed by recombinant IL-2. After 7 days, responders were tested against three europium-labelled PHA blasts: stimulator, responder and third party. A significant number of cyclosporine-resistant allospecific cytotoxic cell precursors were found in cord blood indicating prior activation. They may have been primed in utero against non-inherited maternal HLA antigens. Cyclosporine-resistant allospecific cytotoxic cell precursors were demonstrated in human umbilical cord blood using a quantitative assay. These cells may influence the reaction to subsequent transplants.

Key words Cyclosporine · Cytotoxic cells · Limiting dilution analysis · Europium-release assay · Cord blood

Introduction

Cyclosporine (CyA) inhibits transcription of interleukin-2 (IL-2) and the IL-2 receptor (CD25) in naïve human T-cells [2, 3, 4]. CyA also modulates other cytokines including IL-3, IL-4, IL-5, TNF-α, IFN-γ, GM-CSF and CD40L [5]. Despite HLA matching, up to 50% of patients develop CyA-resistant acute rejection in the early posttransplant period. The frequency of CyA-resistant anti-donor cells is completely unknown. We therefore attempted to develop an assay to quantitate these cells in vitro beginning with 'naive' cord blood (CB) cells as a baseline.

Materials and methods

Preparation of cells

Human umbilical CB mononuclear cells (CBMC) and peripheral blood mononuclear cells (PBMC) from healthy adults were collected into preservative-free sodium heparin, separated by density gradient centrifugation and cryopreserved.

Culture reagents

Complete culture medium (CCM) included RPMI 1640, 10% human AB serum, L-glutamine and no antibiotics. Powdered CyA (Novartis) was dissolved in absolute alcohol to give a stock solution of 1 mg/ml. The working solution was diluted 1:100 in CCM with vigorous agitation. Specific radioimmunoassay confirmed the CyA concentration in CCM.

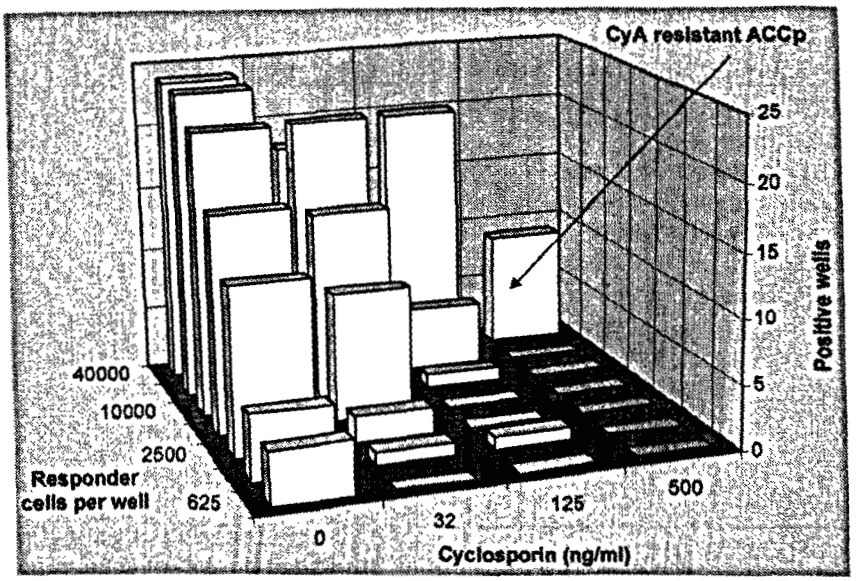

Fig. 1 This three-dimensional graph shows results from a representative allospecific cytotoxic cell precursor frequency (ACCpf) assay. The y-axis shows seven dilutions of responders from 40000 down to 625 cells/well. The x-axis shows four different concentrations of cyclosporine A (CyA) from 0 to 500 ng/ml. The z-axis shows the proportion of positive wells amongst the 24 replicates. ACCpf is calculated for each concentration of CyA. *Arrow* indicates CyA-resistant cells at high concentration (500 ng/ml)

Allo-specific cytotoxic cell precursor frequency (ACCpf) assay [1, 6]

This involved the following steps:

1. CBMC were thawed and titrated from 4×10^4 through seven dilutions down to 0.0625×10^4 cells per 100 µl per well in U-bottomed 96-well plates (24 replicates per dilution). CyA was added to responder CBMC and incubated for 1 h to allow the drug to enter the cells. CyA remained present throughout the culture period. Next, irradiated stimulator cells (30 Gy) were added in a single concentration of 5×10^4 cells per 100 µl per well followed by Cetus recombinant IL-2 (rIL-2) at 25 Cetus units per ml. Cultures were fed on day 5 by replacing half of the medium with freshly prepared CCM containing rIL-2 and CyA. Assays were cultured for another 2 days. At day 7 the contents of each well of the assay plates were resuspended and two identical aliquots transferred to new round-bottomed 96-well plates.

2. Target cell cultures were prepared as follows. PMBC and CBMC at 1×10^6 ml were cultured for 7 days in CCM containing rIL-2 and 2 µg/ml purified PHA in 24-well plates. The PHA blasts thus generated were examined microscopically, split and fed on days 3 and 5. After 7 days PHA blasts were labelled with europium-diethylenetriaminopentaacetate (Eu). Three types of Eu-PHA blasts were prepared from autologous CBMC (original responder), original stimulator PBMC and an unrelated third-party PBMC. Eu-PHA blasts were added at a concentration of 5000 cells to each split well of the assay plates. Plates were gently centrifuged at 1000 rpm for 1 min and incubated for 3 h, centrifuged and 20 µl supernatant transferred to flat-bottomed 96-well low autofluorescent reader plates prefilled with 200 µl enhancement solution. Eu release (an indicator of cytotoxicity of the PHA blasts) was measured in the supernatant using a time-resolved Fluorometer and expressed as cps.

3. For the calculation of ACCp frequency, the baseline was calculated from the mean cps ± SD of replicate wells of cultures containing Eu-PHA blasts and no responder cells. A coefficient of variation less than 10% was considered acceptable. Wells were scored positive for cytotoxicity when cps exceeded the base line by 3 times the SD The proportion (%) of negative replicate wells at each responder cell dilution was calculated and plotted against the responder cell dilution. At the point when 37% of the wells were judged negative, an average of one precursor cell per well was assumed [6]. From this reference point the frequency of the ACCp can be calculated using a computer program developed by Strijbosch [7]. This analysis provides frequency estimates expressed as ACCpf per million CBMC with 95% confidence intervals and *P* values.

Results

A typical CBMC sample primed with an unrelated stimulator (GH) and tested against Eu-PHA blasts from GH is illustrated in Fig. 1. The vertical axis (z) gives the number of positive wells out of 24 replicates at each responder dilution. The other two axes are CBMC dilution (x) and CyA concentration (y). Both increasing dilution of responder cells and increasing concentrations of CyA are associated with fewer positive wells. This was expected, but we were surprised to observe CyA-resistant ACCp at high concentrations of CyA (500 ng/ml) as indicated by the arrow. Table 1 summarises the results obtained from six CB primed and tested against GH and expressed as ACCpf per million CBMC. They show that CyA-resistant ACCp are present in all cord bloods tested except CB3 where they were undetectable. CB primed against GH and tested against a third-party Eu-PHA blast invariably gave significantly lower ACCpf values (data not shown).

Discussion

In CB the CyA-resistant ACCpf level decreases wi increasing drug concentration. Nonetheless at conce trations considered to be above the therapeutic ran (i.e. 500 ng/ml) CyA resistant ACCp were still dete

Table 1 Allospecific cytotoxic cell precursor frequency *(ACCpf)* results of six cord blood samples when tested against an unrelated stimulator (GH) in the presence of 0 or 500 ng/ml of cyclosporine A *(CyA)*. ACCpf are expressed as precursor cells per million cord blood mononuclear cells *(CBMC)* ± 95% confidence intervals *(CI)*

Responder	CyA ng/ml	ACCpf per million CBMC	
		Estimate	95% CI
1	0	255	185–326
	500	7	3–11
2	0	595	446–744
	500	20	13–27
3	0	45	28–62
	500	0	0
4	0	425	257–593
	500	29	16–42
5	0	289	45–533
	500	6	1–12
6	0	107	74–139
	500	5	0–9

ed. ACCp reacting to responder Eu-PHA blasts are thought to be mainly T-cells because they crossreact poorly with third-party Eu-PHA blasts indicating clonal specificity for HLA. But, allospecific ACCp may also include functional subsets of natural killer (NK) cells. Using different targets in a 'split-well analysis' can quantitate these different cytotoxic lineages. Whereas the ACCpf estimated with Eu-PHA blasts is attributable mainly to T-cells, ACCpf estimated with Eu-K562 cell line as target is attributable mainly to NK cells. In our experience NK cells generated in these cultures by allostimulation tend to be more resistant to CyA than T-cells. The underlying assumption in this assay is that CyA-resistant ACCp have been previously activated causing them to express CD25 and respond to exogenous rIL-2. Hitherto CBMC have been assumed to be immunologically naïve or even tolerant to non-inherited maternal HLA antigens (NIMA). Our data indicate the opposite may be true. Activated ACCp may be generated in utero in response to NIMA, a hypothesis that could be directly tested in this assay by priming with maternal stimulator cells (work in progress). On the basis of these findings we speculate that the alloimmune repertoire of an individual is established in utero. Activation against NIMA may increase the incidence of CyA-resistant acute rejection of subsequent transplants especially when they share mismatches with NIMA and particularly in paediatric recipients where short term T-cell memory could be readily reactivated.

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