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The avidity of allospecific cytotoxic T lymphocytes (CTL) determines their cytokine production profile

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SUMMARY

Donor-specific CTL present within the cardiac allograft during a rejection episode are distinct from those that populate the cardiac allograft in the absence of rejection. Whereas the former generally have a high avidity for donor cells, the latter mainly have a low avidity for donor cells. This observation made us reason that high-avidity CTL are implicated in transplant rejection, whereas low-avidity CTL are not. In the present study, we analyse whether both CTL subsets were distinct with respect to their IL-2, IL-4, IL-6 and interferon-gamma (IFN-γ) secretion pattern. CTL clones with either a high or a low avidity for donor antigens were stimulated with donor cells, third party cells, or immobilized anti-CD3 MoAb and the amount of cytokine released was measured. High- and low-avidity CTL clones were found to differ with respect to their IFN-γ production profile. Stimulation with donor cells resulted in IFN-γ secretion by high-avidity CTL clones, but not by low-avidity CTL clones. CD3 stimulation, in contrast, led to secretion of equivalent amounts of IFN-γ by both CTL subsets. These observations indicate that low-avidity CTL are fully capable of producing IFN-γ, but, in contrast to high avidity CTL, fail to do so when they encounter donor cells. As IFN-γ favours the occurrence of transplant rejection, this observation emphasizes the relevance of high-avidity CTL in the rejection process. Additionally, the data show that the cytokine production profile of CTL depends on the nature of the stimulus.

Keywords avidity transplantation interferon-gamma cytokine

INTRODUCTION

The relevance of donor-specific CTL in acute allograft rejection is still a matter of debate. Support for the involvement of CTL as terminal effector cells is provided by experiments showing the exquisite antigen specificity of the alloresponse [1,2] and the ability of CTL clones grown from rejecting allografts to destroy allogenic tissue when injected into appropriate hosts [3]. In contrast, experiments showing the occurrence of graft rejection in $CD8^+$ lymphocyte-depleted mice have been interpreted as evidence against a critical role for CTL in transplant rejection [4]. In such modified animals, eosinophils were assumed to be responsible for the observed graft damage [5]. This recruitment of eosinophils into the graft was ascribed to a decrease in the Th1:Th2 cytokine ratio, which in turn was assigned to the depletion of Th1-promoting, Th2-suppressing donor-reactive $CD8^+$ CTL. Although this model demonstrates that other cells besides donor-specific CTL can function as terminal effector cells, these cells may only become relevant when the immune system is aberrant.

Also, the presence of donor-specific CTL within stable transplants [6,7] argues against a critical role of CTL within the rejection process. However, we found that donor-specific CTL propagated from rejecting and stable allografts were distinct [8–10]. As assessed by their sensitivity to CD8 or CD4 blocking, the former CTL mainly had a high avidity for donor cells, whereas the latter CTL generally had a low avidity for donor cells [11–14]. The avidity of a T lymphocyte is a term that reflects the relative strength with which this T cell binds an antigen-bearing cell. According to current concepts, the avidity of a T cell portrays the overall strength of T cell receptor (TCR)–MHC/peptide interactions and hence depends on the intrinsic affinity of the TCR for its ligand and the number of TCR–MHC/peptide complexes that associate [15–18].

The selective presence of high-avidity, donor-specific CTL within rejecting allografts made us reason that this particular CTL subset is important in acute rejection. A relationship between the avidity of donor-specific CTL and the rejection status of the

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allograft was indeed found by subsequent longitudinal studies within individual heart transplant recipients. It appeared that high-avidity CTL populated the cardiac allograft immediately preceding and during acute rejection, whereas low-avidity CTL populated the cardiac allograft outside a rejection episode [19]. In the same period, the *in vivo* efficiency of virus-specific CTL was reported to depend on their avidity. Despite the fact that both high- and low-avidity CTL effectively killed virus-infected cells *in vitro*, only high-avidity CTL could do so *in vivo* [20]. Yet, in the transplant model, only CTL with a high avidity for donor cells might exhibit their effector function *in vivo*.

In the present study, we further characterize the response of high- and low-avidity CTL towards donor cells. Since increasing evidence has indicated that T lymphocytes producing different cytokine profiles have different cytolytic potentials [21], we assumed that it might be informative to analyse both high- and low-avidity CTL clones for their cytokine secretion pattern. Previous analysis of solid endomyocardial biopsies (EMB) obtained from heart transplant recipients showed that EMB with and without histological signs of clinical cardiac rejection differed with respect to their cytokine gene expression [22]. The IL-2, IL-4 and IL-6 genes were considerably more often expressed in the former EMB than in the latter EMB. However, the source as well as the specificity of the observed response remained undefined. In the present study, we evaluate the ability of high- and low-avidity CTL clones to release these cytokines after donor, third party, and polyclonal (CD3) stimulation. Additionally, we analyse their ability to release interferon-gamma (IFN-γ), as the gene expression and production of this cytokine within the allograft [23–25] and the draining lymph nodes [26] repeatedly have been found associated with rejection.

MATERIALS AND METHODS

All experiments described in this study were performed with donor-specific CTL clones established from graft-infiltrating lymphocyte (GIL) cultures or peripheral blood of six heart transplant patients (KU, BE, MI, ZA, PO, FO).

Generation of CTL clones

GIL and peripheral blood mononuclear cells (PBMC) were obtained from EMB and blood samples of heart transplant recipients, respectively, as described before [19]. Donor-specific T cell clones were generated from GIL or PBMC by limiting dilution at 0·3 cells/well in culture medium expanded with 0·5–1% phytohaemagglutinin (PHA; Difco, Detroit, MI). Culture medium consisted of RPMI 1640 Dutch Modification (GIBCO, Paisley, UK) supplemented with 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% pooled human serum, and 10% lectin-free lymphocult-T-LF (Biotest GmbH, Dreieich, Germany) as a source of IL-2. Irradiated (30 Gy) donor B-lymphoblastoid cell lines (B-LCL; 5×10^4 cells/ml) and PBMC of healthy blood donors (5×10^5 cells/ml) were added as feeder cells. After 2 weeks, wells with visible cell growth were restimulated with irradiated donor B-LCL and PBMC of healthy blood donors. After 7–14 days, clones were phenotyped as described below and assayed for donor-specific cytotoxicity. Clones used in the present study consisted entirely of either $CD4^+WT31^+$ cells or $CD8^+WT31^+$ cells and exhibited cytotoxicity towards donor cells but not towards third party cells or the natural killer (NK)-sensitive cell line, K562.

Target cells

B-LCL originated from infection of fresh PBMC or spleen cells with Epstein–Barr virus (EBV) obtained from the marmoset cell line B95-8. These cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, UT).

Phenotypic analysis

T cell clones were analysed for WT31, CD8 (Becton Dickinson, San Jose, CA), and CD4 (Immunotech, Marseilles, France) expression by three-colour flow cytometry on a FACScan after staining with MoAb directly conjugated for fluorescein, peridinin chlorophyll protein (PerCP), and PE, respectively.

Cell-mediated lympholysis

The cytotoxic capacity of T cell clones was tested against donor B-LCL, third party B-LCL (B-LCL that do not share MHC antigens with the donor or with the acceptor), and the K562 cell line. Briefly, effector cells (cloned cells) were incubated with 2500 ^{51}Cr-labelled target cells at different effector:target (E:T) ratios in 0·2 ml culture medium without Lymphocult-T-LF. After 4 h of incubation (37°C, 5% CO_2), supernatants were harvested using a Skatron harvesting system (Skatron-AS, Lier, Norway) and the release of ^{51}Cr was assayed in a Packard gamma-counter (Packard, Downers Grove, IL). Spontaneous and maximum release were defined by incubation of target cells with culture medium (without Lymphocult-T-LF) in the absence or presence of Triton X-100 detergent (5% v/v solution in 0·01 Tris buffer), respectively. Microcultures were considered cytolytic when the experimental lysis percentages exceeded 10%. The cytotoxicity of all clones was directed towards MHC molecules of the donor.

CTL avidity

As CD8 and CD4 molecules interact with the same molecule (MHC class I and II molecules on antigen-bearing cells, respectively) as the TCR, it is believed that both CD8 and CD4 molecules serve to enhance the overall strength of TCR–MHC/peptide interactions. Accordingly, by blocking the CD8 or CD4 molecules on a T cell, one can define whether the avidity of this T cell for an antigen-bearing cell is sufficiently high to overcome the need for these molecules. To block CD8 molecules a 1:500 dilution of FK18 (2 μg/ml), a mouse anti-human CD8 MoAb of the IgG3 subclass, was used (a kind gift of Dr F. Koning, Department of Immunohematology and Bloodbank, University Hospital Leiden, The Netherlands). To block CD4 molecules a 1:250 dilution of RIV6 (2 μg/ml), a mouse anti-human CD4 MoAb of the IgG2a subclass, was used (a kind gift of Dr M. F. Leerling, RIVM, Bilthoven, The Netherlands).

To analyse the avidity of a CTL clone, cell-mediated lympholysis (CML) experiments were performed in the absence and presence of FK18 or RIV6. For this, clones were preincubated with FK18 or RIV6 for 30 min at 37°C before ^{51}Cr-labelled target cells were added. As a control for TCR-mediated lysis, CML experiments were performed in the presence of 1 μg/ml anti-CD3 MoAb (RIV9, RIVM).

Cytokine production

Clones to be tested for cytokine release were used at least 1 week after the last restimulation. The day before the cytokine-release assay, the clones were washed and seeded (5×10^4 cells/well) in V-bottomed 96-well plates (Greiner, Alphen aan de Rijn, The Nether-

lands) in culture medium without Lymphocult-T-LF. The next day, cells were incubated with irradiated (60 Gy) donor B-LCL or irradiated third party B-LCL (5×10^4 cells/well) in a final volume of 0·2 ml culture medium without Lymphocult-T-LF. For polyclonal stimulation by immobilized anti-CD3 MoAb, cells were transferred to 96-well round-bottomed culture plates (Costar, Cambridge, MA) that had been preincubated with 5 μg/ml RIV9 (90 min, 37°C). Controls were wells containing clones incubated with medium alone and wells containing stimulator cells incubated with medium alone. Plates were centrifuged for 5 min at 400 ***g*** and incubated for the indicated time at 37°C. After incubation, supernatants were removed and analysed for the amount of cytokine released. IL-2, IL-4 and IL-6 release was assessed using commercially available ELISA (IL-2, Immunotech; IL-4, IL-6, CLB, Amsterdam, The Netherlands).

IFN-γ release was determined according to the following protocol. IFN-γ-binding MoAb 350B10G6 (Medgenix, Fleurus, Belgium) was covalently coupled to microtitre plates with a hydrazide surface (carbohydrate binding plates; Costar) as described by Brillhart & Ngo [27]. *In situ* oxidation and coupling were performed by addition of 50 μl of MoAb 350B10G6 (2 μg/ml) in 50 mM acetate buffer pH 5·0 to each well. After 30 min of incubation at room temperature, 50 μl of 5 mM $NaIO_4$ in acetate buffer were added to each well. The plates were incubated again for 30 min, washed with washing buffer (Tris-buffered saline + 0·05% Tween 20, pH 7·4), and blocked with 100 mM Tris + 0·05% Tween-20 + 1% bovine serum albumin (BSA) (pH 7·5). After 1 h, plates were decanted and stored at −20°C until use.

Plates were washed and standards (3000–4·1 pg/ml; diluted in cell culture medium without Lymphocult-T-LF) prepared from a stock solution of recombinant human IFN-γ (R&D Systems, Minneapolis, MN) or cell culture supernatants were added to the wells (100 μl/well). After 2 h, wells were washed and incubated for another 2 h with 100 μl (0·25 μg/ml) of biotin-conjugated anti-human IFN-γ (Medgenix; clone 67F12A8) diluted in High Performance ELISA buffer (CLB). Plates were washed and 100 μl poly horseradish peroxidase (HRP)-labelled streptavidin (1:10 000; CLB) were added to each well. After 30 min, wells were washed again and 100 μl substrate solution (3,5,3′,5′,tetramethylbenzidine, 0·1 mg/ml in 0·11 M acetate buffer, pH 5·5 + 0·003% H_2O_2) were added. The reaction was stopped after 15 min by addition of 50 μl/well of 2 N H_2SO_4 and the optical density (OD) was measured at 450 nm.

Statistical analysis

Differences between groups were analysed using the Mann–Whitney *U*-test of the statistical program INSTAT (Graphpad Software, San Diego, CA).

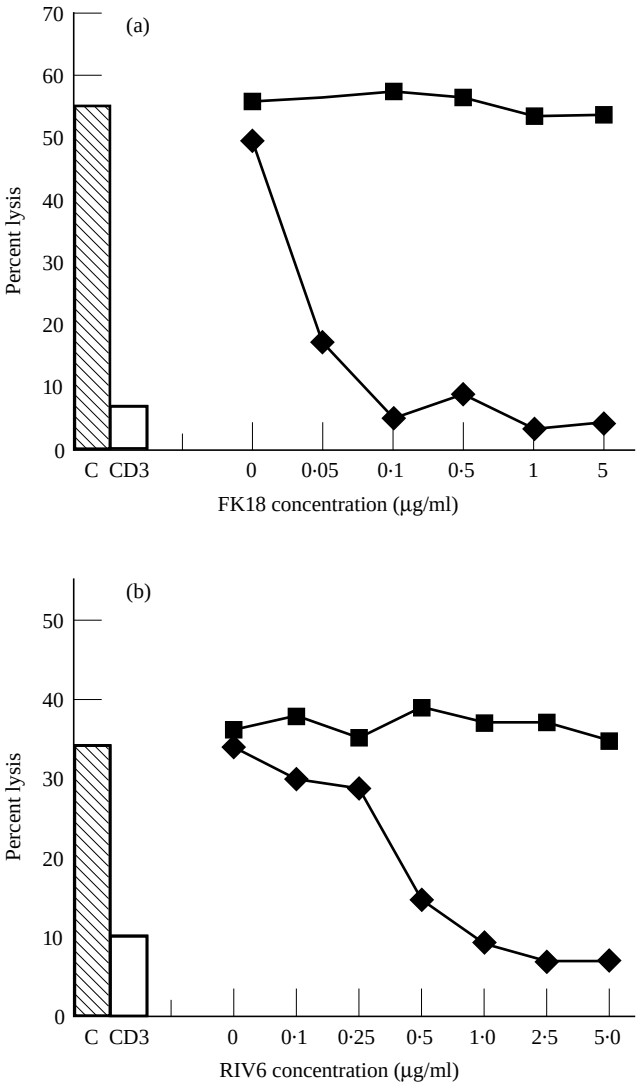

Fig. 1. The susceptibility of $CD8^+$ CTL (a) and $CD4^+$ CTL (b) clones to inhibition by different doses of anti-CD8 MoAb (FK18) and anti-CD4 MoAb (RIV6), respectively. Clones whose cytotoxic capacity is not affected by FK18 or RIV6 (■) are considered to bind their target cells with a high avidity. Clones that fail to lyse their target cells in the presence of FK18 or RIV6 (◆; lysis < 10%) are considered to bind these cells with a low avidity. Alternatively, FK18 was added to CD4-bearing cells and RIV6 to CD8-bearing cells to control for the specificity of the response (C). Anti-CD3 MoAb was added as a control for T cell receptor (TCR)-mediated lysis.

RESULTS

Cytotoxic capacity and avidity of T cell clones

T cell clones obtained from peripheral blood or GIL cultures of heart transplant recipients were tested for their cytotoxic capacity in a standard 4-h ^{51}Cr-release assay. Clones that specifically lysed B-LCL of donor origin were selected and subsequently examined for their avidity for donor antigen. For this analysis, $CD8^+$ CTL clones were tested for their capacity to lyse donor B-LCL in the absence or presence of anti-CD8 MoAb (Fig. 1a). Likewise, $CD4^+$ CTL were tested for donor-directed cytotoxicity in the absence or presence of anti-CD4 MoAb (Fig. 1b). As depicted in Fig. 1, 2 μg/ml of anti-CD8 or anti-CD4 MoAb were sufficient to distinguish between clones that bind donor cells with a high (■) or a low (◆) avidity. As a control for TCR-mediated cytotoxicity, blocking experiments were performed with anti-CD3 MoAb. All clones used in the present study showed a significant reduction of donor target cell lysis after preincubation with anti-CD3 MoAb.

Cytokine profile of high- and low-avidity CTL clones

Fifteen donor-specific CTL clones obtained from peripheral blood samples or GIL cultures of heart transplant patients were characterized for their IL-2, IL-4, IL-6 and IFN-γ release after donor, third party, or CD3 triggering. The results are shown in Table 1.

Table 1. Cytokine profile of CTL clones that have either a low or a high avidity for donor antigen

				IL-4						IL-6					
Clone	Type	% lysis* −MoAb	% lysis† +MoAb	do‡ 6 h	do 20 h	3P§ 6 h	3P 20 h	CD3¶ 6 h	CD3 20 h	do 6 h	do 20 h	3P 6 h	3P 20 h	CD3 6 h	CD3 20 h
1 KU7PBL	CD4	19	0	21	46	–	–	19	118	–	20	–	–	–	–
2 BE3GIL	CD8	51	0	ND	–	ND	–	ND	80	ND	–	ND	–	ND	–
3 KU5PBL	CD8	44	3	–	–	–	–	41	265	–	–	–	–	–	–
4 MI6PBL	CD4	23	5	–	58	–	–	–	50	–	19	–	–	–	–
5 ZA21GIL	CD4	29	9	–	–	–	–	>450	>450	–	–	–	20	–	25
6 PO9PBL	CD4	29	9	–	44	–	–	–	69	–	35	–	–	–	22
7 ZA26GIL	CD4	20	7	–	50	–	–	>450	>450	–	–	–	19	–	26
8 PO27PBL	CD4	61	40	69	280	–	–	>300	>300	–	–	–	–	–	–
9 KU11PBL	CD8	69	46	–	30	–	–	–	80	–	21	–	–	–	–
10 FO4GIL	CD8	90	65	–	–	–	–	40	42	–	–	–	–	–	–
11 BE32GIL	CD8	63	47	ND	365	ND	–	ND	412	ND	–	ND	–	ND	–
12 FO110GIL	CD4	55	53	–	–	–	–	–	–	90	269	–	33	–	–
13 ZA12GIL	CD8	16	18	–	–	–	–	–	–	–	–	–	–	–	–
14 ZA1GIL	CD8	44	52	–	–	–	–	–	–	23	26	30	27	–	–
15 FO6GIL	CD8	71	78	54	57	–	–	52	50	–	–	–	26	–	–

				IL-2						IFN-γ					
Clone	Type	% lysis −MoAb	% lysis +MoAb	do 6 h	do 20 h	3P 6 h	3P 20 h	CD3 6 h	CD3 20 h	do 6 h	do 20 h	3P 6 h	3P 20 h	CD3 6 h	CD3 20 h
1 KU7PBL	CD4	19	0	–	–	–	–	–	–	–	–	–	–	–	63
2 BE3GIL	CD8	51	0	ND	–	ND	–	ND	133	ND	–	ND	–	ND	1766
3 KU5PBL	CD8	44	3	–	–	–	–	16	185	–	–	–	–	23	144
4 MI6PBL	CD4	23	5	–	26	–	–	–	–	–	–	–	–	5	9
5 ZA21GIL	CD4	29	9	–	–	–	–	322	325	10	33	–	8	1044	>3000
6 PO9PBL	CD4	29	9	–	–	–	–	–	–	–	121	–	–	45	307
7 ZA26GIL	CD4	20	7	–	–	–	–	335	248	28	60	–	8	>1000	>1000
8 PO27PBL	CD4	61	40	–	–	–	–	74	132	192	861	–	–	1252	2657
9 KU11PBL	CD8	69	46	–	–	–	–	–	40	131	483	–	–	47	451
10 FO4GIL	CD8	90	65	–	–	–	–	189	106	590	723	–	–	>3000	>3000
11 BE32GIL	CD8	63	47	ND	59	ND	–	ND	259	ND	281	ND	–	ND	>1000
12 FO110GIL	CD4	55	53	–	–	–	–	–	–	58	290	–	–	76	122
13 ZA12GIL	CD8	16	18	–	–	–	–	26	–	214	231	–	–	>1000	897
14 ZA1GIL	CD8	44	52	–	–	–	–	–	–	110	206	–	–	860	904
15 FO6GIL	CD8	71	78	140	159	–	–	95	41	>1000	>1000	–	–	>1000	>1000

*Donor target cell lysis at an effector/target ratio of 5:1. Cultures were considered cytolytic when the experimental lysis percentage exceeded 10%.

†Effect of anti-CD8 or anti-CD4 MoAb addition on the donor-directed cytotoxicity of $CD8^+$ or $CD4^+$ CTL clones, respectively. Addition of anti-CD8 or anti-CD4 MoAb inhibited donor target cell lysis by low-avidity CTL (lysis < 10%; nos 1–7), but hardly affected cytotoxicity by high-avidity CTL clones (nos 8–15). Both high- and low-avidity CTL clones were stimulated with irradiated ‡donor B-lymphoblastoid cell lines (B-LCL), §third party B-LCL, and ¶immobilized anti-CD3 MoAb. After 6 h and 20 h, supernatants were harvested and assayed for cytokine content by ELISA. The amount of cytokine released is expressed as pg/ml. Values below the detection level of the ELISA kits (IL-2, 15 pg/ml; IL-4, 9 pg/ml; IL-6, 6 pg/ml; IFN-γ, 4 pg/ml) are depicted as negative (-). ND, Not done. Unstimulated clones did not produce detectable amounts of IL-2, IL-4, IL-6 or IFN-γ (data not shown).

The number, name and source of each clone (either PBL or GIL) are depicted by the first column (Clone). The phenotype (either CD4 or CD8) of the clones is depicted by the second column (Type). The cytotoxic capacity of the clones to lyse donor target cells in the absence and presence of anti-CD4 or anti-CD8 MoAb is shown in the third (%Lysis − MoAb) and fourth (%Lysis + MoAb) column, respectively. Addition of anti-CD4 or anti-CD8 MoAb to ^{51}Cr-release assays almost completely reduced donor-directed cytotoxicity exhibited by clones 1–7 (lysis < 10%) but hardly affected donor target cell lysis by clones 8–15. Hence, the former seven clones bound donor cells with a low avidity whereas the latter eight clones bound donor cells with a high avidity. The remaining columns show the cytokine production profile of both high- and low-avidity CTL clones. For this analysis, CTL clones were stimulated for 6 h and 20 h with irradiated B-LCL of donor origin (do), with irradiated B-LCL that did not share HLA antigens

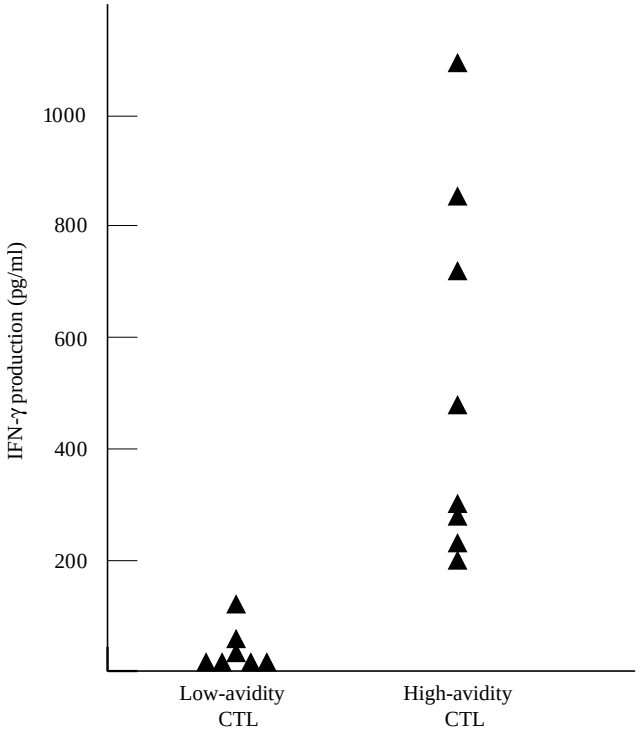

Fig. 2. The amount of IFN-γ released by low- and high-avidity CTL clones after 20 h of antigen-specific stimulation by donor B-lymphoblastoid cell line (B-LCL). Detection level is 4 pg/ml.

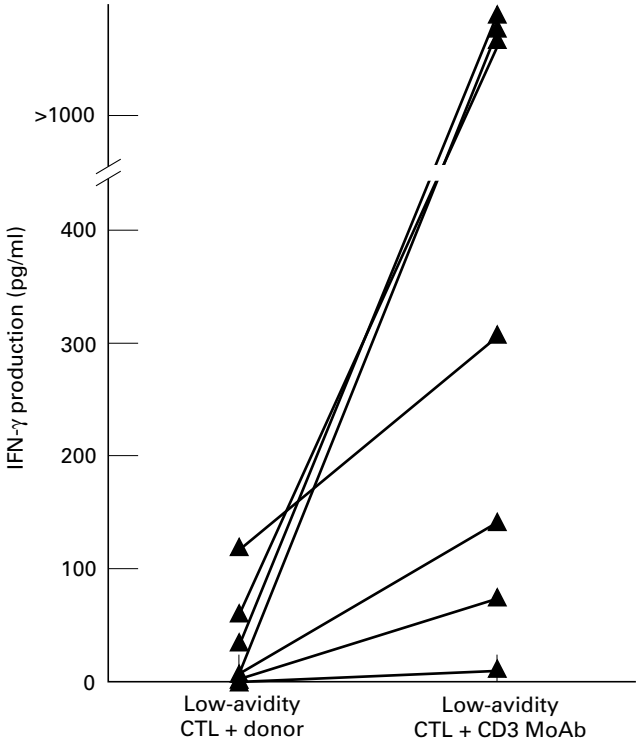

Fig. 3. The amount of IFN-γ released by low-avidity CTL after 20 h of stimulation by donor B-lymphoblastoid cell line (B-LCL) (antigen-specific; left) and immobilized anti-CD3 MoAb (right), respectively.

with the donor or acceptor (3P), and with immobilized anti-CD3 MoAb, after which the amount of cytokine released was assessed by ELISA.

As a control, unstimulated CTL clones and irradiated stimulator cells were tested for their cytokine production profile. Unstimulated clones did not secrete IL-2, IL-4, IL-6 or IFN-γ, indicating that these cytokines were not constitutively produced by CTL (data not shown). Irradiated stimulator cells, in contrast, occasionally produced IL-6 but did not produce detectable amounts of IL-2, IL-4 or IFN-γ (data not shown). This observation implies that the amount of IL-6 detected in the supernatant of stimulated CTL clones may also partly be the product of the stimulator cells added.

Some general remarks can be made with respect to the data in Table 1. Comparing the cytokine profile after donor and third party stimulation reveals that the production of IL-2, IL-4 and IFN-γ was donor-specific. CTL clones fail to produce these cytokines after stimulation by third party B-LCL, but may do so when stimulated by donor B-LCL. IL-6, on the contrary, can be found in the supernatant after both donor and third party stimulation, indicating that the production of this cytokine is aspecific. Alternatively, as stated above, the IL-6 detected may be the product of the stimulator cells used.

Comparing the cytokine profile after stimulation with donor cells and immobilized RIV9 shows that the release of IL-2, IL-4 and IFN-γ, but not of IL-6, can be induced or significantly increased by CD3 stimulation. For example, only three clones (20%) produced detectable amounts of IL-2 after 20 h of donor stimulation, whereas nine clones (60%) did so after 20 h of CD3 stimulation. This observation indicates that care should be taken with ascribing functions to T cells that are simply grounded on their cytokine profile, as such a profile can easily be modulated by the stimulus chosen.

Comparing the amount of cytokines produced after 6 h and 20 h of stimulation, it can be stated that the amount of cytokine secreted and the number of clones producing detectable levels of cytokine were increased after 20 h of stimulation.

Despite these common characteristics, the data in Table 1 show that CTL clones are rather diverse in their cytokine profile. We analysed whether the various cytokine patterns observed could be explained by differences in the source (PBL *versus* GIL), phenotype (CD4 *versus* CD8), or avidity (high *versus* low) of the clones. This analysis leads to one striking observation. It appeared that low-avidity CTL and high-avidity CTL were distinct with respect to their IFN-γ cytokine profile. Low-avidity CTL barely produced IFN-γ after donor B-LCL stimulation, whereas high-avidity CTL produced significant amounts of IFN-γ both after 6 h and 20 h of donor B-LCL stimulation ($P < 0{\cdot}001$ and $P < 0{\cdot}0003$, respectively). No correlation was found between the IFN-γ profile and the phenotype or the source of the clones, demonstrating that the avidity of CTL clones is the only variable associated with the observed IFN-γ production profile. The results are shown in Fig. 2.

In contrast to donor stimulation, CD3 stimulation resulted in the production of equivalent amounts of IFN-γ by both high- and low-avidity CTL at 6 h and 20 h of stimulation ($P < 0{\cdot}10$ and $P < 0{\cdot}40$, respectively). This observation indicates that low-avidity CTL are fully capable of producing IFN-γ, but, in contrast to high-avidity CTL, fail to do so when they encounter donor cells (Fig. 3). This observation also emphasizes that the cytokine profile of cells can easily be influenced by the nature of the stimulus used.

No distinction between high- and low-avidity CTL could be

made with respect to their IL-2, IL-4 or IL-6 production pattern. Likewise, no differences in IL-2, IL-4 or IL-6 profile were found between CD4- and CD8-expressing CTL clones and between peripheral blood- and graft-derived CTL clones.

DISCUSSION

Although numerous studies have demonstrated the existence of T cells that do not require CD8 or CD4 co-receptors to exhibit their function, hardly any attention has been paid to the immunological significance of these cells. Our data in this field refer to donor-specific CTL propagated from graft biopsies or blood samples of heart transplant patients. Kinetic studies showed that CD8/CD4-dependent (low avidity) donor-specific CTL were prominent within the graft until 2 weeks preceding acute rejection. From that point onwards, CD8/CD4-independent (high avidity) donor-specific CTL rapidly accumulated within the graft and became predominant at the time of acute rejection. Successful anti-rejection therapy resulted in the destruction of these CTL and the graft was repopulated again by low-avidity donor-specific CTL [19]. As intragraft accumulation of donor-specific CTL with a high avidity precedes myocyte destruction (i.e. acute rejection), we believe that these CTL, as opposed to low-avidity donor-specific CTL, are involved in transplant rejection. In a subsequent study, the kinetics of circulating donor-specific CTL was analysed and their avidity was compared with the rejection status of the graft. Obtained data showed that donor-specific CTL with a high avidity were significantly more prominent in blood samples taken immediately preceding or during rejection (submitted for publication). In contrast to the situation in the graft, peripheral donor-specific CTL were precursor CTL and not fully mature CTL. Together these data suggest that, prior to rejection, peripheral donor-specific CTL with a high avidity migrate to the graft and differentiate into mature CTL which are involved in transplant rejection.

In the present study, donor-specific CTL clones obtained from the graft and peripheral blood of heart transplant patients were analysed for their IL-2, IL-4, IL-6 and IFN-γ production profile, and the results were compared with their avidity for donor antigen. It appeared that CTL clones that differed in the avidity with which they interacted with donor cells were distinct with respect to their IFN-γ production profile. CTL clones that interacted with donor cells with a high avidity produced significant amounts of IFN-γ after donor stimulation, while CTL clones that associated with donor cells with a low avidity did not.

IFN-γ is considered to play a potential role in allograft rejection, as it is believed to recruit macrophages into the graft, activate macrophages and lymphocytes, and increase the immunogenicity of the allograft by enhancing MHC expression on donor tissue [28]. In support of this concept, proteins and/or transcripts for intragraft IFN-γ have consistently been shown to correlate with transplant rejection [23–25]. The fact that high-avidity CTL produce IFN-γ when they encounter donor cells, whereas low-avidity CTL do not, therefore underscores our concept that high-avidity CTL are involved in allograft rejection [8–10,19,29].

Stimulation of high-avidity CTL clones with third party B-LCL did not result in IFN-γ production, demonstrating that the release of this cytokine after donor stimulation is specific and hence is based on TCR–MHC/peptide interactions. To explain a relationship between the avidity of T cells and their ability to produce IFN-γ, we should bear in mind that the avidity of a T cell as defined by CD4/CD8 inhibition studies actually reflects the overall strength of TCR–MHC/peptide interactions [17,18]. Most likely, the overall strength of TCR–MHC/peptide interactions determines the intensity of TCR triggering and consequently whether thresholds of intracellular signal pathways leading to IFN-γ production are reached. In support of this view, low-avidity CTL clones were fully capable of producing IFN-γ after stimulation by immobilized anti-CD3 MoAb, a stimulus which bypasses the requirement for TCR–ligand association.

The above theory suggests that the IFN-γ production profile of T cells is governed by the avidity with which these cells interact with antigen-bearing cells. Some other studies support this view. It has been reported that antigenic peptides that bind well to MHC molecules and/or TCR favour the generation of IFN-γ-producing cells, whereas peptides that bind less well do not [30,31]. Additionally, it has been shown that TCR transgenic $CD4^+$ T cells differentiate into IFN-γ-producing T cells only when high antigen doses are used for priming [32]. In these studies, the extent of TCR–MHC/peptide interactions is enhanced, and hence the avidity of the T cell–antigenic cell interaction.

Also the production of IL-2 by $CD8^+$ T cells [33,34] and IL-4 by $CD4^+$ T cells [30–32] has been reported to depend on the avidity with which these cells bind antigen-bearing cells. We, however, did not find any distinction between high- and low-avidity CTL with respect to their IL-2 or IL-4 production profile. The reported association between the production of IL-4 and the avidity of the interaction was found to depend upon the use of naive cells as the starting population [32]. Accordingly, the observed disparity in data might be explained by the differentiation status of the T cell population examined. While all above mentioned studies were performed on naive T cell populations, we used mature T cells. Additionally, above mentioned studies were performed on murine T cell subsets (either $CD4^+$ or $CD8^+$ T cells) with unknown cytolytic potential [30–33], whereas we used human CTL clones as the starting population. These variables (human *versus* murine T cells; CTL *versus* T cells which may not be cytolytic; and T cell clones *versus* T cell subsets) might also have contributed to the diversity in data.

An additional point we would like to emphasize is that the type of stimulus used to assess the cytokine production profile of cells has an important impact on their response. More clones produced a particular cytokine after CD3 stimulation than after antigen-specific stimulation. Low-avidity donor-specific CTL clones, generally unable to produce IFN-γ after stimulation with donor cells, did produce significant amounts of IFN-γ after CD3 stimulation. Likewise, CD3 stimulation led to IL-2 and IL-4 production by clones that failed to produce these cytokines after antigen-specific stimulation. Our data are in line with those of Maccalli *et al*. [35], who demonstrated that the cytokine pattern in response to the antigen-specific stimulus was different from the one induced by CD3 stimulation. Hence, it is clear that the cytokine profiles of T lymphocytes can be easily modulated by the type of activation signals delivered to the T cells.

In conclusion, our *in vitro* studies show that the avidity of donor-specific CTL determines their ability to produce IFN-γ when they encounter donor cells. If a similar situation exists *in vivo*, this may be the explanation for the earlier observed association between the avidity of graft-infiltrating CTL and the rejection status of the transplanted human heart.

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