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Demonstration of direct xenorecognition of porcine cells by human cytotoxic T lymphocytes

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SUMMARY

It is not known whether human cytotoxic T cells can recognize porcine major histocompatibility antigens directly, or whether recognition occurs by co-operation with syngeneic human antigen-presenting cells (APC). Limiting dilution assays were used to quantify human anti-pig precursor cytotoxic T-cell (CTLp) frequencies and to analyse the 'kinetics' of the interaction between human lymphoid cells and porcine splenic cells. Single-hit kinetics are demonstrative of direct recognition, as only one cell type, the CTLp, is diluted out, whereas multi-hit kinetics indicate that more than one cell is limiting and provide evidence for co-operative recognition of xenoantigens. Initial assays indicated that the frequency of CTLp reactive with alloantigens on human splenic targets (mean 1/1845; $n=3$) was approximately sixfold greater than the frequency of CTLp reactive with porcine splenic cells (1/12,082; $n=3$). However, not all of the assays performed using the xenogeneic combination produced single-hit kinetics. Subsequent assays were performed by mixing limiting numbers of human peripheral blood mononuclear cells (PBMC) or APC-depleted PBMC preparations with porcine splenocytes. There was a significant difference in the frequency of xenospecific CTLp between PBMC and APC-depleted preparations ($P=0{\cdot}034$). The overall frequency increased in the APC-depleted group. Variation between the seven human donors was also significant ($P=0{\cdot}006$). There was no significant difference in frequency between the two cell preparations after correction for the proportion of $CD3^+$ cells ($P=0{\cdot}13$). There was, however, a significant departure from single-hit kinetics in the PBMC group ($P=0{\cdot}004$) which was not observed in the APC-depleted group ($P=0{\cdot}052$). It is concluded that human cytotoxic T cells can be activated by porcine xenoantigens directly. However, the direct recognition mechanism can be altered in the presence of human APC.

INTRODUCTION

The main barrier to xenotransplantation between phylogenetically discordant species is hyperacute rejection due to the presence of naturally occurring antibodies.[1] The cell-mediated response to a xenograft is less well understood. *In vivo* models of cell-mediated xenograft rejection have been reviewed by Auchincloss.[2] The role of T cells was demonstrated by the fact that only T-cell-deficient nude animals accept xenografts indefinitely. In addition, immunosuppressive agents designed to inhibit T-cell function prolonged xenograft survival, but not as effectively as they prolonged allograft function. In general, xenograft survival was more difficult to prolong than allograft survival in these models. The pertinent question raised is whether T-cell-mediated recognition of xenoantigens is quantitatively or qualitatively different from allorecognition?

The mixed leucocyte culture has been used *in vitro* to model the transplantation response *in vivo*. It is apparent that xenogeneic mixed leucocyte reactions (MLR) in the murine anti-human combination are weaker than the allogeneic murine anti-murine response.[3] Xenogeneic MLR were found to be similar to allogeneic MLR in that the stimulated cells were T lymphocytes with specificity for xenogeneic class II major histocompatibility complex (MHC) molecules. However, it was found that the responding lymphocyte population required the presence of both T cells and 'adherent' cells for generation of a response.[4] Subsequent studies have demonstrated that autologous APC are required for mouse T-cell recognition of human xenoantigens.[5,6]

The weak response to xenoantigens *in vitro* is not necessarily due to an inability of the T cells to recognize xenogeneic MHC directly, but may be attributed to species specificity of accessory molecule interactions. T cell–MHC binding is stabilized by accessory molecules such as CD4 binding to a monomorphic domain of class II molecules, and CD8 to the $\alpha 3$ domain of class I molecules. The association of adhesion molecules further stabilizes intercellular interactions [e.g. lymphocyte function-associated antigen-1 (LFA-1)→intercellular adhesion molecule-1 (ICAM-1), CD2→LFA-3]. Greenstein *et al.*[7] demonstrated that human cells could interact with murine LFA-1 but not with murine CD4 or CD8. Expression of human CD4 transgenes on

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murine T cells augments xenorecognition of a human B-cell line; however, proliferation is still threefold lower than that produced by stimulation with an allogeneic murine B-cell line.[8] Murine cytotoxic T lymphocytes (CTL) preferentially recognize L cells transfected with chimeric class I MHC molecules expressing human $\alpha 1$ and $\alpha 2$ domains, and the murine $\alpha 3$ domain.[9,10] Human recombinant cytokines have been shown to reconstitute the ability of purified T cells to respond to murine xenoantigens in mixed leucocyte cultures indicating the xenospecificity of cytokines.[11]

The requirement for autologous antigen-presenting cells (APC) for the recognition of xenoantigens is not absolute. There is evidence that T cells can recognize some xenogeneic MHC antigens directly. Kievits *et al.*[12] showed that mouse CTL recognition of xenogeneic human MHC molecules was not restricted by self MHC. Incubation with HLA positive 'cold targets' also inhibited the response. Experiments with transgenic mice have shown that xenogeneic HLA expressed on murine cells can be recognized directly as allelic variants of H2 molecules.[13,14]

The shortage of human organ donors has stimulated investigation of possible xenotransplantation with the pig being chosen as a potential organ donor. However, the majority of *in vitro* investigations of T-cell xenoreactivity have been performed using murine systems. It is unlikely that these will be representative of human anti-porcine xenoreactivity. The limited evidence available indicates that the human anti-porcine reaction is less dependent on APC than is the human anti-murine response.[5]

In this study, we hypothesize that human cytotoxic T cells can recognize porcine xenogeneic MHC antigens directly. To test this, human anti-porcine cytotoxic T-lymphocyte precursor (CTLp) frequencies were measured by limiting dilution analysis of peripheral blood mononuclear cells (PBMC) and of APC-depleted PBMC populations. This enabled one facet of human anti-porcine xenoreactivity to be quantified and the mechanism of the interaction to be investigated.[15]

MATERIALS AND METHODS

Isolation of responder cell populations

Blood was obtained from normal healthy volunteers. Twenty-millilitre samples were collected by venepuncture into sterile universal tubes containing 100 U of preservative-free heparin (Sigma Chemical Co., Poole, U.K.). PBMC were prepared by centrifugation (400 ***g***, 25 min) over a Ficoll–Metrizoate (Lymphoprep; Nycomed, Birmingham, U.K.) density gradient. The interfacial cells were recovered, washed in RPMI-1640 medium (Northumbria Biologicals, Cramlington, U.K.), and resuspended in complete medium consisting of RPMI-1640 supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS), 1×10^{-2} M HEPES, 10^5 U/l penicillin (Sigma) and 100 mg/l streptomycin (Sigma). APC were depleted from some aliquots of cells by serial plastic adherence (45 min, 37°) and passage through nylon wool (Cellselect leucocyte filter, NPBI, Emmer-Compascuum, The Netherlands) columns.[16]

The resultant cell populations were assessed for the presence of T cells and residual APC by immunofluorescence staining and flow microfluorimetry (FACScan, Becton Dickinson, Cowley, U.K.). PBMC and APC-depleted cell populations were incubated with optimum dilutions of murine monoclonal antibodies specific for human CD3 (*Rhodaminia* phycoerythrin conjugated; Serotec, Oxford, U.K.) and human HLA-DR (CR43, fluorescein isothiocyanate-conjugated; Dakopatts A/S, Glostrup, Denmark) for 30 min at 4° prior to washing and analysis.

Isolation of stimulator populations

Porcine splenic cells were prepared by gently teasing a pig spleen (Whitley Bay Meat Company, Newcastle, U.K.) apart into RPMI-1640 medium. The resultant cell suspension was washed in RPMI-1640 by centrifugation (400 ***g***, 10 min). The mononuclear cells were prepared by density gradient centrifugation as above. The interfacial cells were recovered, washed and cryopreserved in liquid nitrogen using 10% (v/v) dimethylsulphoxide (Sigma) as a cryoprotectant. Human splenic cells were prepared and stored using similar methodology.

Limiting dilution analysis

Replicate cultures ($n = 12$–20 per dilution) across a range of limiting numbers of PBMC or APC-depleted cell populations were mixed with 1×10^5 irradiated (25 Gy) splenic cells (allogeneic human or porcine) and cultured in complete medium containing an optimal concentration of human recombinant interleukin-2 (rIL-2) (5–10 U/ml; Boerhinger Mannheim, Lewes, U.K.) in round-bottomed microtitre plates (Nunc, Roskilde, Denmark) for 11 days at 37° in a humid atmosphere containing 5% CO_2.

Lymphoblasts were prepared from splenic cells by incubation for 3 days in complete medium containing 5 μg/ml phytohaemagglutinin (PHA) (Sigma). These cells were recovered by centrifugation and 1×10^6 were labelled with 200 μCi $Na_2[^{51}Cr]O_4$ (ICN Flow, High Wycombe, U.K.) for 90 min at 37°. The labelled cells were washed twice with RPMI-1640 and once in complete medium by centrifugation at 400 ***g***. The cells were then adjusted to a concentration of 2×10^4 cells/ml in the complete medium.

Cytotoxic activity in the microcultures was detected after incubation of each culture for 4 hr with 2×10^3 ^{51}Cr-labelled donor lymphoblasts. Assay supernatants (100 μl) were harvested after the plates had been centrifuged at 70 ***g*** for 5 min and each sample was analysed by γ-spectrometry (1272 Clinnigamma, Wallac LKB, Milton Keynes, U.K.). Cultures were considered positive when their ^{51}Cr release exceeded the mean +3 SD of the spontaneous release from control cultures containing only irradiated splenic cells. A proportion of the responding cultures were incubated with ^{51}Cr-labelled third-party splenic blasts as a specificity control.

Statistical analyses

CTLp frequencies were estimated as a proportion of the total responder cell population or as a proportion of the number of $CD3^+$ cells by use of maximum likelihoods methods in GLIM for overall analysis of grouped data,[17] or by the method of Strijbosch *et al.*[18] for individual analyses. The GLIM analysis is essentially a generalization of the Strijbosch method and allows analysis of several individuals and treatments within grouped data. The assumption of single-hit kinetics was made throughout the mathematical modelling; χ^2 goodness of fit tests were

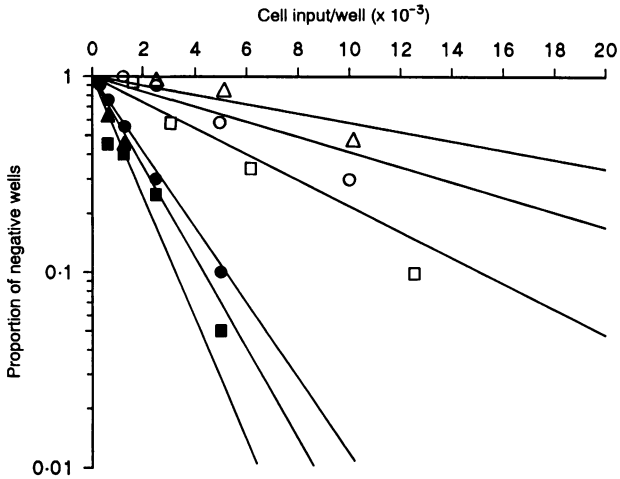

Figure 1. Limiting dilution titrations. Titration of human PBMC stimulated by allogeneic splenic cells (closed symbols). Titration of human PBMC stimulated by porcine xenogeneic splenic cells (open symbols).

Table 1. Proportion of CD3 positive cells and class II MHC antigen positive cells in PBMC and APC-depleted PBMC populations

	PBMC		APC-depleted PBMC	
Donor	% CD3$^+$	% class II$^+$	% CD3$^+$	% class II$^+$
1	58·8	9·5	62·9	1·9
2	67·6	6·0	79·9	0·4
3	52·3	15·2	67·6	2·2
4	72·5	7·4	79·3	1·1
5	60·7	13·7	73·2	3·4
6	51·3	13·4	55·2	0·8
7	70·0	14·6	80·6	1·8

carried out on individual assays and on the grouped data to test the validity of this assumption.

RESULTS

Human allospecific and anti-pig xenospecific CTLp frequencies were initially quantified in this study. Blood from a further series of individuals was assayed to investigate the response to porcine xenoantigens before and after the depletion of class II MHC positive APC. Each cytotoxicity assay developed specificity for the original stimulator cells as no significant cytolysis of third-party lymphoblasts was observed.

The frequency of CTLp reactive with allogeneic human splenic cells was approximately sixfold higher (mean 1/1,845) than the frequency of CTLp reactive with xenogeneic splenic cells (mean 1/12,082, $n=3$; Fig. 1). One of four initial assays of xenoreactivity did not yield data that was consistent with single-hit kinetics. In this case, the recognition of the porcine splenic cells was relatively enhanced with increasing responder cell numbers producing rapid saturation of the titration.

Table 2. Limiting dilution assay frequency estimates of human anti-pig CTLp in human PBMC and in APC-depleted PBMC

	PBMC		APC-depleted PBMC	
Donor	Frequency	95% CI†	Frequency	95% CI
1	1/9337*	1/12,188–1/6610	1/5714	1/8273–1/3947
2	1/14,488*	1/22,169–1/9462	1/23,747	1/39,635–1/14,214
3	1/3781	1/5506–1/2596	1/1962	1/2938–1/1309
4	1/3799	1/5336–1/2706	1/4436	1/6162–1/3194
5	1/12,691*	1/18,776–1/8579	1/13,055	1/18,135–1/9394
6	1/4448	1/6217–1/3179	1/1784	1/2587–1/1230
7	1/4918	1/6863–1/3524	1/2494	1/3662–1/1698

† CI, confidence interval.

* Denotes individual analyses that deviate significantly from single-hit kinetics ($P<0·05$ by χ^2 analysis).

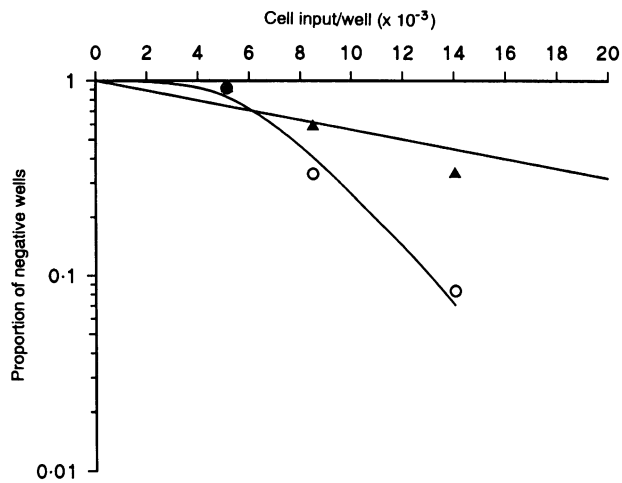

Figure 2. Example of single-hit and multi-hit kinetics. Xenospecific CTLp in PBMC (open symbols) and APC-depleted PBMC (closed symbols) respectively.

Table 3. Limiting dilution assay frequency estimates of human anti-pig CTLp for CD3$^+$ cells present in human PBMC and in APC-depleted PBMC

	PBMC		APC-depleted PBMC	
Donor	Frequency	95% CI†	Frequency	95% CI
1	1/5496*	1/7764–1/3890	1/3595	1/5207–1/2484
2	1/9788*	1/14,980–1/6393	1/18,974	1/31,681–1/11,361
3	1/1977	1/2879–1/1357	1/1326	1/1986–1/885
4	1/2756	1/3871–1/1963	1/3519	1/4887–1/2533
5	1/7711*	1/11,411–1/5214	1/9556	1/13,275–1/6876
6	1/2292	1/3205–1/1639	1/985	1/1428–1/679
7	1/3345	1/4665–1/2396	1/2010	1/2951–1/1369

† CI, confidence interval.

*Denotes individual analyses that deviate significantly from single-hit kinetics ($P<0·05$ by χ^2 analysis).

PBMC were purified from seven healthy individuals (aged = 27·6 ± 7·5 years, mean ± 95% confidence interval, range 20–44 years, five female, two male). The percentages of CD3 positive T lymphocytes and of class II MHC antigen positive cells in PBMC and APC-depleted populations were quantified. There were between 0·44% and 3·36% (mean = 1·66%, $n = 7$) class II positive cells remaining after APC depletion (Table 1).

The frequency of CTLp reactive with porcine xenoantigens in the PBMC preparations ranged from 1/3781 to 1/14,488 (Table 2, mean 1/7637, $n = 7$). There was a significant difference between the xenospecific CTLp frequency estimates in the APC-depleted populations and the values for PBMC (Table 2, $P = 0·0034$, interpersonal variation was accounted for in this calculation). The process of removing the APC had a significantly different effect on the xenospecific CTLp frequency estimates obtained in different individuals ($P = 0·006$). The overall frequency of xenogeneic CTLp increased in the APC-depleted group (Table 2). The apparent fall in the individual CTLp frequency values observed after depletion of APC from samples from donors 2, 4 and 5 was not statistically significant.

Analysis of grouped data showed that there was no significant difference between the frequency of xenospecific CTLp in PBMC before and after depletion of APC provided that the data were corrected for the number of $CD3^+$ cells per culture (Table 3, $P = 0·13$). The difference in frequency between individuals remained significant ($P = 0·005$) after correction for the number of $CD3^+$ cells.

The frequency data were analysed for their conformation to single-hit kinetics by considering the χ^2 'goodness of fit' statistics. There was a significant departure from single-hit kinetics in the PBMC group ($P = 0·004$, 3/7 of the individual experiments produced multi-hit kinetics, Table 2) but not in the APC-depleted group ($P = 0·052$, all of the individual experiments produced single hit kinetics, Table 2). An example of the multi-hit kinetics obtained in PBMC analysis compared with the result obtained after APC depletion is shown in Fig. 2 (the data represent donor 2). The χ^2 statistics for goodness of fit were similar for analyses performed using total responder cell numbers and for those corrected for the number of $CD3^+$ cells.

DISCUSSION

Human anti-pig cytotoxic T-cell xenoreactivity was quantified by limiting dilution analysis. The frequency of xenospecific CTLp was found to be six times lower than the human alloreactive CTLp response. This observation is similar to those made in other investigations of xenoantigen recognition *in vitro*. For example, the frequency of murine CTLp specific for pig class I MHC transgenes expressed on mismatched murine cells was found to be 10-fold less than the frequency of murine allospecific CTLp.[14] The frequency of murine CTLp stimulated by murine splenic cells with HLA transgenes was 100-fold lower than the frequency of murine alloreactive CTLp. Split well analysis demonstrated that these responses were not restricted by self APC.[12]

There was a significant difference between xenospecific frequency estimates obtained for PBMC and APC-depleted PBMC ($P = 0·0034$). However, this was due to an increase in the number of responding T cells rather than a lack of APC as, when the data were corrected for the number of $CD3^+$ cells in each culture, the significant difference in frequency was lost ($P = 0·13$). There was also a significant variation between the response of individual donors ($P = 0·006$).

The grouped frequency estimates for PBMC were calculated after assuming that all the data conformed to single-hit kinetics. Examination of the results of individual assays (Table 2) revealed that this assumption was not true for three of the seven blood samples; in these cases the χ^2 statistics clearly showed that there was a significant variation from single-hit kinetics. It is interesting that these three samples also had the lowest frequency of xenospecific CTLp. The data tended to skew towards the y-axis with increasing responder number indicating an enhanced recognition of porcine splenic cells (Fig. 2). Limiting dilution analyses investigating titrations of human allospecific CTLp result in single-hit kinetics.[19,20]

The CTLp frequency assays for PBMC and for APC-depleted populations were carried out in exactly the same way and at the same time; therefore any experimental error would be expected to affect both groups equally. However, the result of grouped assays containing human APC significantly deviated from single-hit kinetics ($P = 0·004$), whereas the result of assays in which the majority of APC had been depleted did not ($P = 0·052$). It is most likely that the departure from single-hit kinetics is caused by the presence of human APC. These cells appear to alter the mechanism of xenospecific cytolysis in some cultures giving rise to multi-hit kinetics.

In conclusion, direct recognition of porcine cells by human cytotoxic T cells did take place in this study. However, human APC were able to contribute to the reaction. The mechanism by which APC produced this effect is unclear. It is possible that in the culture microenvironment the APC facilitated the interaction of T cells and porcine stimulators thereby augmenting productive T-cell receptor stimulation. Release of APC-derived cytokines may also augment the activation of otherwise quiescent T cells. The findings of the present study may reconcile conflicting evidence in the literature concerning the role played by self APC in the recognition of xenoantigens, as both direct and co-operative recognition appear to occur. There is therefore both a quantitative and a qualitative difference between the way a T cell recognizes a porcine xenoantigen and an alloantigen. The involvement of self APC in the generation of cytotoxicity indicates that the human immune system will respond differently to a porcine xenograft than to an allograft. This difference may explain the observation that xenograft survival is more difficult to prolong than allograft survival.[2]

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