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Porcine Fetal Ventral Mesencephalic Cells Are Targets
for Primed Xenoreactive Human T Cells

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Xenotransplantation of porcine fetal ventral mesencephalic (pfVM) cells to overcome the dopamine shortage in the striatum of patients with Parkinson's disease seems a viable alternative to allotransplantion of human fetal donor tissue, especially because the latter is complicated by both practical and ethical issues. There is, however, little known about the xenospecific immune responses involved in such an intracerebral xenotransplantation. The aim of our study was to investigate whether 1) naive human peripheral blood mononuclear cells (PMBC) display cytotoxicity against pfVM cells of E28 pig fetuses, and 2) priming of human PBMC by xenogeneic antigen presenting cells (APC) modulates pfVM-directed cellular cytotoxicity. For this purpose fresh PMBC from nine individual donors were primed by incubation with either irradiated pfVM cells or porcine spleen cells (PSC) as APC in the presence of IL-2 for 1 week before assessing cytotoxicity in a ^{51}Cr release assay. Also, direct NK reactivity and antibody-dependent cellular cytotoxicity (ADCC) of fresh PMBC against pfVM cells was assessed. No direct cytotoxicity of naive cells (either NK reactivity or ADCC) against pfVM cells could be determined. Only PMBC primed with PSC were capable of lysing pfVM cells. PBMC primed with pfVM cells did not show cytolytic capacity towards pfVM. Interestingly, large differences in xenospecific T-cell responses exist between individual donor PBMC. Thus, human T cells are capable of killing pfVM cells in a xenoreactive response, but only after priming by donor APC. The large interindividual differences between human donors in their xenoreactive response may influence patient selection for xenotransplantation and chances of graft survival for individual patients.

Key words: Xenotransplantation; Ventral mesencephalon; Parkinson's disease; T cells;
Antibody-dependent cellular cytotoxicity (ADCC); Porcine

INTRODUCTION

A transplantation therapy using human fetal dopaminergic cells to substitute the shortage of dopamine in the striatum of patients suffering from Parkinson's disease has been the subject of both experimental and clinical investigation (6,17,26,35,36,42,50). Xenotransplantation using porcine fetal ventral mesencephalic (pfVM) tissue is considered a viable alternative that addresses shortage of human fetal tissue as well as a number of practical and ethical issues involved in the use of human fetal tissue (18,20,21,33). Although clinical trials using pfVM as a donor source have already taken place (10,

14,44), little is known about immunological mechanisms involved in intracerebral xenotransplantation.

Naturally occurring xenoreactive antibodies and complement play an important role in the rejection of transplanted vascularized solid organs by initiating hyperacute rejection (1,37); in a later stage, these factors may play a central role in delayed xenograft rejection (2,38). Although hyperacute rejection of porcine cell suspension grafts after transplantation is unlikely, in vivo studies have shown that there is an important role for antibodies and complement in the rejection of such intracerebral xenografts (4,5,24,28,29,48). Studies concerning the transplantation of vascularized organs or pancreatic islets be-

yond species borders have shown that if hyperacute rejection has been overcome, mechanisms involving the cellular compartment of the immune system are still capable of destroying the graft (9,23,27,43,51). The question is whether these findings can be extrapolated to transplantation of fetal xenogeneic brain cells into a supposedly immunoprivileged organ such as the brain. In vitro there is evidence of human T-cell reactivity (7) and human NK reactivity (47) against pf brain cells. Also, a role for host T cells in the rejection of intracerebral xenografts can be deducted from the beneficial effect of cyclosporin A (8) or blocking of swine leucocyte antigen (SLA) class I (11,34) on the survival of such a graft in a xenogeneic animal model. Other in vivo studies provide further evidence of cellular mechanisms playing an important role in the rejection of intracerebral xenotransplantation (13,39).

With respect to this cell-mediated xenoreactivity, human lymphocytes may have the capacity to directly recognize and lyse pfVM cells through direct activity of NK cells, or antibody-dependent NK cell activity (ADCC), as previously reported (47). Alternatively, xenograft cells could activate or "prime" human T cells. Priming human lymphocytes for xenospecific cytolytic activity is most effectively mediated by "professional" antigen presenting cells (APC), such as macrophages or dendritic cells. pfVM explants have been shown to contain APC, which may lead to priming of human T cells as was shown with macrophages that were selected by in vitro culturing and were capable of inducing human T-cell proliferation (7). These data raise the question if freshly isolated pfVM cells themselves are capable of priming human lymphocytes of different individuals for xenospecific cytolytic activity and to what degree pfVM cells are susceptible to cytolytic activity by human T cells that were primed by "professional" xenogeneic APC, such as spleen cells.

In this study we investigated direct in vitro reactivity of human peripheral blood mononuclear cells (PBMC) of different donors against VM cells of E28 pig fetuses. Furthermore, we analyzed whether priming of human PBMC with pfVM induces xenospecific cytolytic activity and whether xenogeneic APC can enhance the ability of PBMC to kill pfVM cells.

MATERIALS AND METHODS

Reagents

Hibernation Medium. The hibernation medium used was 2.236 g/l KCl, 0.48 g/L $MgCl_2 \times 6H_2O$, 0.9 g/L glucose, 0.89 g/L $Na_2HPO_4 \times 2H_2O$, 1.511 g/L $NaH_2PO_4 \times H_2O$, 6 ml/L lactic acid (30%, Sigma). The pH was set at 7.2 by adding KOH, and osmolarity was adjusted to 300 mM by adding sorbitol (22).

Trypsin Medium. The trypsin medium used was Ca^{2+} and Mg^{2+}-free Hanks's buffer, 0.6% glucose, 100 U/100 mg per ml penicillin/streptomycin, 0.3 mM/ml HEPES, 500 mg/ml DNAse, and 0.1% Worthington's trypsin.

Trypsin Inhibiting Medium. The trypsin inhibiting medium used was Ca^{2+} and Mg^{2+}-free Hank's buffer solution, 500 mg/ml DNAse, 100 U/100 mg per ml penicillin/streptomycin, 20% fetal calf serum (FCS), and 100 mg/ml soybean trypsin inhibitor.

Washing Medium. The washing medium used was Ca^{2+} and Mg^{2+}-free Hank's buffer solution, 500 mg/ml DNAse, 0.6% glucose, 100 U/100 mg per ml penicillin/ streptomycin, 0.3 mM/ml HEPES with 10% or 20% FCS.

Cryopreservation Medium. The cryopreservation medium used was 0.9% saline, 0.6% glucose, and 10% dimethyl sulfoxide (DMSO) (AIM-V medium, Gibco).

Preparation of pfVM and Porcine Spleen Cells

Fetuses from 28 day pregnant cross-bred Dutch Yorkshire Landrace pigs were obtained by hysterotomy and the VM was microscopically isolated and cut in 6–8 pieces (19,31,49). Half of the tissue fragments were stored in 0.9% saline at 4°C for 2 h before being processed into a single cell suspension; the other half was stored in a hibernation medium at 4°C for 44 h before being cryopreserved (25).

A single cell suspension was made incubating VM tissue fragments in a trypsin medium for 15 min at 37°C and subsequent incubation in a trypsin inhibiting medium for 5 min at room temperature. Subsequently, the tissue was rinsed three times using a 10% FCS-containing washing medium and gently dissociated using fire-polished constricted Pasteur's pipettes with decreasing bore diameters. The number of viable cells was determined by the trypan blue dye exclusion test, and viability was expressed as percentage of viable cells with respect to the total number of cells. Viability in this study was used as quality control only; cell suspensions with viability lower than 90% were not used.

For cryopreservation, the hibernated tissue fragments were transferred to a 1-ml cryotube containing 1 ml of cryopreservation medium and stored on melting ice before being frozen in a programmed freezer using a freezing program described previously (25,41) and stored in liquid nitrogen (−196°C) afterwards. To thaw the frozen tissue fragments, the cryotube was placed in 37°C water for 2 min. Subsequently the supernatant was slowly diluted with washing medium containing 20% FCS before replacing the supernatant with washing medium to wash out DMSO before processing into a single cell suspension (25).

Porcine spleen was obtained from the same sow do-

nating the fetuses to guarantee at least semi-identity in MHC make up between spleen cells and pfVM. After mechanical dissociation of pieces of porcine spleen tissue, lymphocytes were derived using Lymfoprep (Axis-Shield PoC As) gradient centrifugation. Cells were either used fresh or after cryopreservation using a standard lymphocyte freezing program in a programmable freezer and storing in liquid nitrogen until further use.

Isolation and Preparation of Human Serum and Peripheral Blood Mononuclear Cells (PBMC)

Human PBMC from healthy volunteers were obtained by Ficoll-Hyperque (Nycomed) gradient centrifugation. Human serum was obtained from healthy donors and heat inactivated before use.

To prime PBMC, fresh cells were incubated (1:1) with 30 Gy irradiated pfVM cells or porcine spleen cells as APC in serum-free AIM-V medium to which 15 U/ml IL-2 was added (37°C, 5% CO_2). After 1 week cells were harvested and used in a cytotoxicity assay.

Determination of Cytotoxicity

For measurement of PBMC activation and subsequent lysis of pfVM and porcine spleen cells we followed the protocol described by Yi et al. (51). PBMC from nine individuals, either stimulated with adult porcine spleen cells, pfVM cells, or medium were incubated at 37°C with thawed porcine spleen cells or thawed pfVM cells that were labeled with ^{51}Cr (200 µCi). After 4 h, ^{51}Cr release was measured using a Packard gamma counter.

To determine direct NK cell-mediated cytotoxicity, freshly isolated human PBMC from six individuals were incubated at 37°C with ^{51}Cr (200 µCi) labeled fresh pfVM cells for 4 h with or without IL-2, before ^{51}Cr release was measured using a Packard gamma counter.

To determine ADCC, fresh pfVM cells were labeled with ^{51}Cr (200 µCi) and subsequently incubated at 37°C for 30 min with heat-inactivated human serum. Serum was obtained from two donors that were known to possess high levels of antibodies directed against pfVM. After rinsing three times with AIM-V medium and 2% human serum albumin, cells were incubated at 37°C with PBMC from six human donors for 4 h. Subsequently, ^{51}Cr release was measured using a Packard gamma counter.

In all experiments Triton-X100 was added to determine maximum release and AIM-V medium to determine background release. The percentage specific lysis was calculated as the measured minus background release divided by the difference between maximum and background. All measurements were performed in triplets. Data are expressed as mean values.

Statistics

Statistical analyses were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA). To assess differences, the Wilcoxon matched pairs test was performed.

RESULTS

pfVM Cells Are Lysed by Primed Human T Cells

Human PMBC primed with porcine spleen cells showed a significant xenoreactive response against porcine VM cells in half of the individuals tested (Fig. 1B). In contrast, cells primed by pfVM cells did not show a xenospecific response to pfVM (Fig. 1A). Differences were significant comparing human PMBC primed using PSC with human PMBC primed with pfVM or medium control in their ability to lyse pfVM at an E:T ratio of 100:1 ($p = 0.0117$), 50:1 ($p = 0.0117$), and 12.5:1 ($p = 0.0039$) (Wilcoxon matched pairs test).

All donors lysed porcine spleen cells when primed by xenogeneic APC (spleen cells) (Fig. 1D), but not after priming by pfVM cells (Fig. 1C). Differences were significant comparing human PMBC primed using PSC with human PMBC primed with pfVM or medium control in their ability to lyse porcine spleen cells for all E:T ratios ($p = 0.0039$, Wilcoxon matched pairs test).

Although PMBC primed by xenogeneic APC lysed pfVM, porcine spleen cells were lysed more efficiently, indicating that pfVM are poorly recognized ($p = 0.0039$ for all E:T ratios, Wilcoxon matched pairs test).

Loading of pfVM and spleen cells with ^{51}Cr was comparable as demonstrated by comparable ^{51}Cr release after addition of Triton 1% and background release. Interestingly, large differences were observed in the degree of xenospecific lysis between PBMC of the different individuals tested (Fig. 1B, D).

No NK Cell-Mediated Cytotoxicity or ADCC to pfVM

Human PBMC from six different individuals were tested for NK reactivity against pfVM cells with and without IL-2 as additive to the medium. No NK cell-mediated cytotoxicity could be determined in any of six individuals tested, neither with nor without addition of IL-2 (Fig. 2; data not shown for testing without IL-2). In addition to direct NK cell activity, ADCC was assayed using serum from two individuals with high levels of pfVM-specific antibodies and PBMC from six individuals. No ADCC reactivity could be determined (Fig. 3). Although cytotoxicity levels seemed to decrease with a lower E:T ratio, percentages of specific lysis were very low and should be considered negative.

DISCUSSION

In this study we show that porcine VM cells can be targets for cytolytic activity by human xenoreactive T
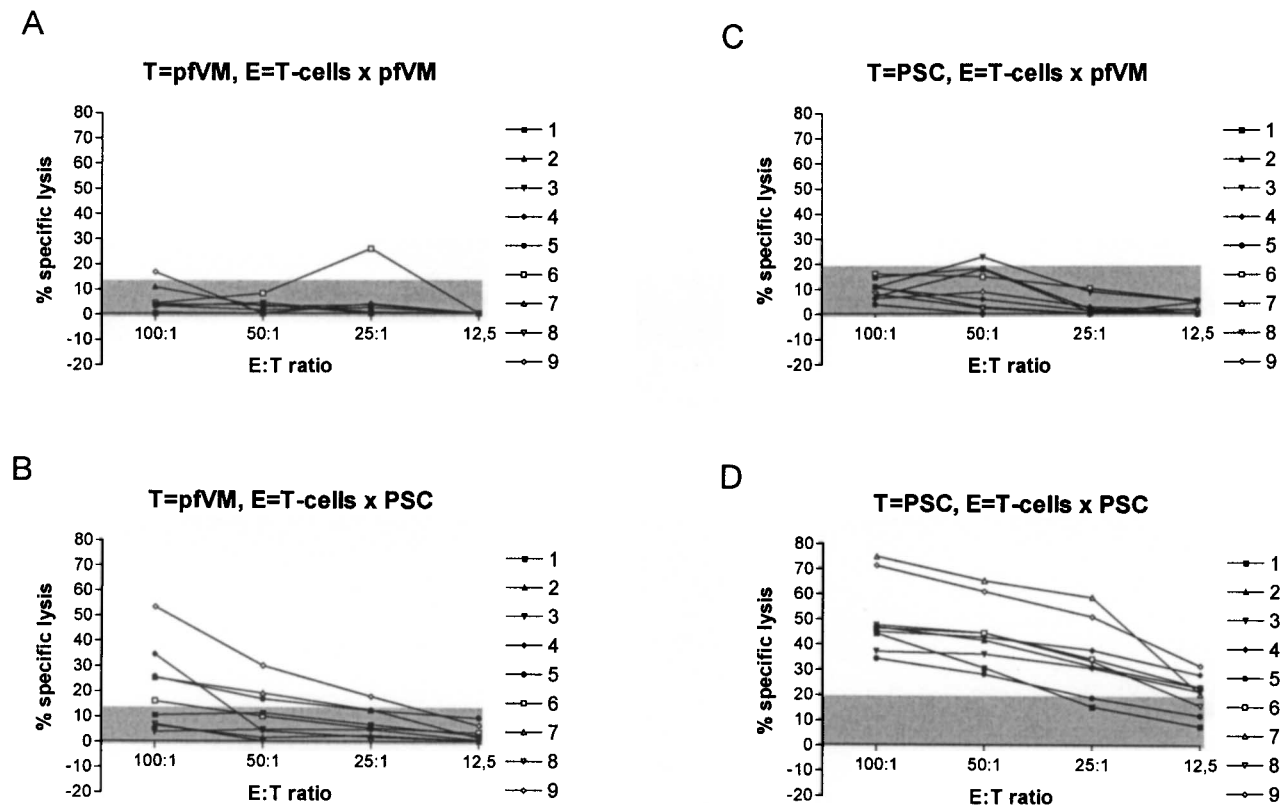
Figure 1. Xenoreactive cytotoxicity of PBMC from nine individuals, stimulated by pfVM or PSC for 1 week, to either pfVM or PSC. T = target cells and E = effector cells (human PMBC stimulated with either pfVM or PSC). The shaded areas represent the control: PMBC that were cultured for 1 week without exposure to either pfVM or PSC and tested for specific lysis of pfVM (A, B) or PSC (C, D). The height of the shaded area is defined by the individual with the highest specific lysis of either pfVM or PSC. All measurements were performed in triplets and expressed as mean values. Intra-assay variability between triplets expressed as standard deviation of the mean ranged from 0 to maximum 6.3% SD but was typically in the range of 1–2% SD.

cells, but only after they have been primed by xenogeneic APC. Without priming, human PBMC do not appear to show cytolytic activity towards xenogeneic pfVM cells.

Both direct and indirect routes have been described in which T cells can react with tissue antigens. After xenotransplantation, xenoantigens have been found in cervical lymph nodes of the recipient, suggesting indirect antigen presentation (32). Also there is in vivo evidence for a possible antigen presenting role of host microglia (15,16). Such an indirect route of T-cell activation may well be very important in intracerebral pfVM transplantation, because over 90% of the transplanted pfVM cells die within the first weeks of transplantation (3,46, 52). These dying pfVM cells could provide a substantial antigenic load to host APC. Although transplanted pfVM cells appear to die predominantly from apoptosis (30), and apoptotic cell clearance usually does not trigger an immune response (40), Shrestha et al. have shown that apoptotic cells can evoke both humoral and cellular responses after transplantation (45).

In the experiments we describe in this article, T-cell activation is only possible via a direct route where donor APC activate human T cells. Cells that could function as APC after intracerebral xenotransplantation and directly activate host T cells are microglia, macrophages, and possibly also astrocytes. Indeed, Brevig et al. have shown that macrophages isolated from pfVM and cultured in vitro upregulate MHC and are able to stimulate T cells, as do isolated astrocytes, although not as efficiently as do macrophages (7). However, in our experiments pfVM appears to be a poor source of APC, because pfVM cells were not able to prime human PBMC during in vitro coculture for a week. Furthermore, Duan et al. have shown in an in vivo allogeneic transplant model that cotransplantation of spleen cells caused rejection of a neural graft (12). The above findings show that stimulation of allogeneic and xenogeneic immune responses is promoted by the presence of "professional" donor APC (expressing SLA class I and II). These data also support our findings that a pfVM cell suspension (with a low content of APC that could be considered
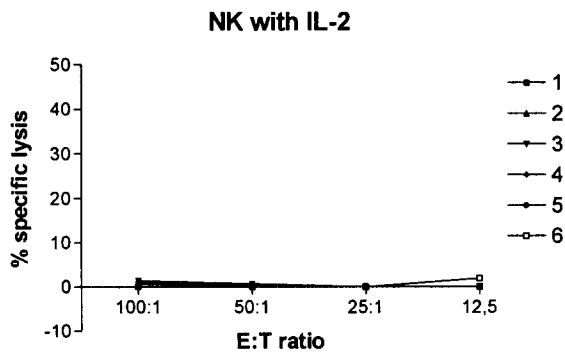
NK with IL-2

Figure 2. NK cell-mediated cytotoxicity of PMBC from six individuals to pfVM cells in the presence of IL-2 as was analyzed in a ^{51}Cr release assay. All measurements were performed in triplets and data are expressed as mean values. Intraassay variability between triplets expressed as standard deviation of the mean ranged from 0 to maximum 13.3% SD.

professional APC) is a rather poor stimulator of a xenospecific T-cell response. This is in contrast to porcine spleen cells, which were able to stimulate T cells from different human donors quite effectively. Once stimulated by porcine spleen cells, human T cells do recognize and lyse pfVM cells. Interestingly, marked differences were observed between cytotoxicity of stimulated T cells from different human donors to both pfVM and porcine spleen cells. Because pfVM and PSC cells were isolated from non-inbred pigs, we used the sow that donated the fetuses as a source of spleen cells and used cryopreserved pfVM and PSC from the same harvest as fresh stimulator cells as target cells. These measures were made to limit differences in MHC make up between pfVM and PSC, but also between stimulator cells and target cells. Therefore, the differences we observed

with respect to individual cytotoxicity of human T cells to pfVM and PSC may well reflect significant variations in interindividual xenogeneic response patterns, possibly genetically determined. Although speculative, these findings could have significance for the clinical application of xenotransplantation in Parkinson's disease in terms of patient selection and graft survival.

In our experiments we did not see any direct human NK reactivity against pfVM cells in vitro, when testing PBMC from different donors. Also, with IL-2-stimulated human PBMC as effector cells no lysis was observed. These findings seem to be in contrast with observations reported by Sumitran et al. using isolated human NK cells as effector cells. Sumitran et al. showed low reactivity by nonstimulated NK cells, but significant lysis of pfVM by IL-2-stimulated cells (47). It should be noted, however, that both duration and concentration of the IL-2 treatment differed significantly between Sumitran's and our experiments. Moreover, Sumitran et al used a preparation of isolated human NK cells. This probably also explains why they were able to measure up to 60% lysis at an E/T ratio of 30:1, whereas in our experiments no direct cytotoxicity was found, even at an E/T ratio of 100:1, at which it is likely that a fair amount of NK cells must have been present. Nevertheless, this finding again stresses that without additional priming, existing reactivity among human PBMC against xenogeneic pfVM cells is extremely low.

As with direct NK reactivity, we also did not observe any cytotoxicity in the presence of heat-inactivated human serum. This finding seems to indicate that human NK cell-mediated ADCC to pfVM cells is also virtually absent in human PBMC and is well in line with our previous findings that only minimal amounts of pfVM-specific antibody for facilitation of ADCC (i.e., IgG subtype antibodies) are present in human serum (24).

A ADCC1

B ADCC2
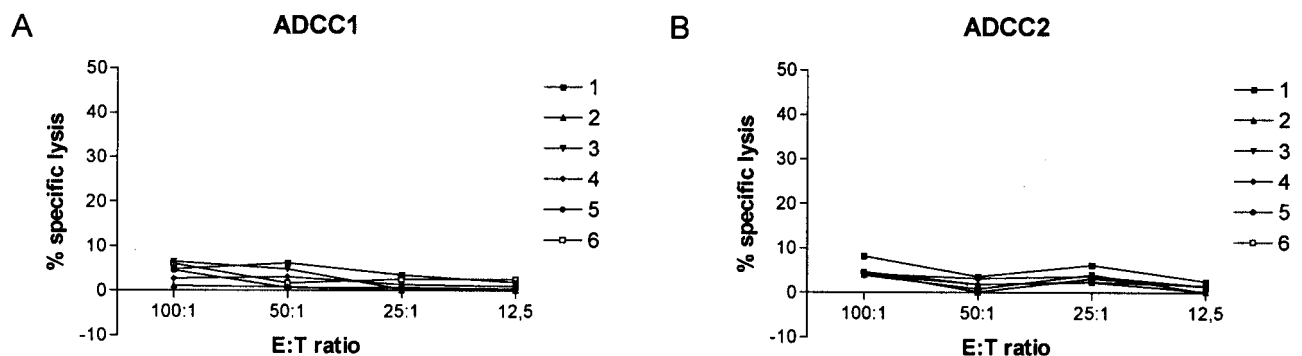
Figure 3. ADCC reactivity of PBMC from six individuals to pfVM cells that were incubated with human serum prior to a ^{51}Cr release assay. (A, B) Results after incubation with serum from the two individuals known to possess high levels of antibodies with affinity for pfVM. All measurements were performed in triplets and data are expressed as mean values. Intra-assay variability between triplets expressed as standard deviation of the mean ranged from 0 to maximum 9.6% SD.

In conclusion, human PBMC are poorly primed by pfVM cells and no direct or antibody-dependent NK reactivity was observed using pfVM cells as a target. However, human PBMC are capable of cytolytic capacity towards pfVM cells when primed by donor APC. The large interindividual differences between human donors in their xenoreactive T-cell response after direct stimulation of host T cells can in our opinion influence chances of graft survival in individual patients and warrants patient selection before xenotransplantation.

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