*Short communication***Intensity and mechanisms of in vitro xenorecognition of adult pig pancreatic islet cells by CD₄⁺ and CD₈⁺ lymphocytes from Type I diabetic or healthy subjects****S. Lalain, L. Chaillous, E. Gouin, P. Saï**

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Summary The intensity and mechanisms of cell-mediated rejection of pig islet cells were studied in 49 Type I diabetic and 34 healthy subjects. Human peripheral mononuclear cells proliferated strongly in response to pig islet cells ($p < 0.001$), though with notable interindividual variations (stimulation index 2 to 215). The variance of stimulation index was higher in diabetic than healthy subjects ($p < 0.0001$). The response to islet cells was stronger ($p < 0.01$) than that to pig splenocytes. Proliferation in response to islet cells was strongly decreased ($p < 0.01$) when CD₄⁺ T cells were blocked with monoclonal antibodies, whereas the blocking of CD₈⁺ cells or NK cells gave less pronounced effects. The response to islet cells was decreased ($p < 0.01$), but not abolished, after antigen-presenting cells were removed. Purified CD₄⁺ cells alone did not proliferate in response to islet cells but recovered their proliferative ability when mixed with antigen-presenting cells, whereas CD₈⁺ cells alone proliferated in the presence of interleukin-2 in

response to islet cells. Proliferation was blocked ($p < 0.01$) by anti-DR monoclonal antibodies. During proliferation in response to islet cells, interleukin-10 increased 43-fold ($p < 0.01$) but interferon- γ increased only slightly. No statistical differences were detected between diabetic and control subjects with respect to lymphocyte subsets and the recognition mechanisms or to interferon- γ / interleukin-10 production in response to islet cells. These results provide the first detailed information on human cell-mediated xenoreaction to pig islet cells. This situation involves a dominant CD₄ class II-restricted Th2 response, with an indirect recognition pathway, as well as a CD₈ T-cell response resulting from direct recognition. This strong reaction constitutes a serious obstacle which may vary in degree among subjects. [Diabetologia (1999) 42: 330–335]

Keywords Islets, pig, xenograft, human, lymphocytes.

Type I diabetes could be treatable by a graft of pig islets [1]. Rejection of this discordant xenograft remains, however, a major problem. Despite advances

in the understanding of hyperacute rejection that have made short-term engraftment of porcine tissue a feasible objective, cell-mediated rejection can occur. This cellular rejection could be a serious problem in the case of islets, which are possibly less susceptible to hyperacute rejection [2, 3].

In vitro human cellular recognition in the discordant human-pig situation has been mainly investigated with stimulator pig lymphocytes or endothelial cells [4–6], i.e. cells which are not intended to be grafted in diabetes. In terms of the disease which islet grafts are intended to combat, it is thus important to characterise human anti-pig islet response since islets have particularities different to lymphocytes, e.g. they do not constitutively express class II molecules

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Abbreviations: PIC, Pig islet-cells; PBMC, peripheral blood mononuclear cells; SI, stimulation index; APC, antigen-presenting cells; MHC, major histocompatibility complex; MoAbs, monoclonal antibodies; SPF, specific pathogen-free; IFN- γ , interferon- γ IL, interleukin; ICA, islet cell autoantibodies; mUW, modified University of Wisconsin.

of the major histocompatibility complex (MHC) which represent targets for xenogeneic cellular recognition [5–7], even though the islet preparations could be contaminated by non-endocrine class II-positive cells (dendritic cells, endothelial cells). Furthermore, because the immunogenetic context of diabetes could influence xenogeneic islet rejection through recurrence of autoimmunity, it is important to characterise human anti-pig islet response in diabetic patients and not only in healthy subjects as done in preliminary studies [8, 9]. The aim of this work was to investigate in vitro this cell-mediated barrier to prolonged islet engraftment. The intensity and mechanisms of human lymphocyte proliferation were studied in response to islet cells from adult pigs and comparisons made between Type I diabetic and healthy subjects.

Materials and methods

Peripheral blood mononuclear cell and subset preparations. We studied 49 recent-onset Type I diabetic patients (25 men and 24 women; mean age $\pm$ SD: 24 ± 6 years) within 2 weeks of diagnosis. Healthy volunteers (32 ± 9 years; 10 men and 24 women) with no personal or familial history of diabetes or autoimmune disease and no islet cell autoantibodies (ICA) were also studied. Peripheral blood mononuclear cells (PBMC) were obtained from venous blood by Hypaque-Ficoll centrifugation and suspended in RPMI 1640 (Gibco, Grand Island, N.Y., USA). Human T cells were enriched by removal of antigen-presenting cells (APC) by negative selection, using incubation with magnetic microspheres (Dynabeads, Dynal, Oslo, Norway) coated with anti-MHC class II monoclonal antibodies (MoAbs). The number of residual class II-positive cells in the negative fraction was less than 0.5 % as estimated by flow cytometry analysis (FACScan analyser, Becton-Dickinson, San Jose, Calif., USA), and the effectiveness of APC depletion was confirmed by the abolition of proliferation during 3-day cocubation of PBMC with 5 μ g/ml PHA [5]. Human CD₄⁺ and CD₈⁺ T-cell fractions were enriched by positive selections, using microspheres (Dynabeads) coated with anti-CD₄ or anti-CD₈ MoAbs. Antibody-bound cells were removed using a magnet. The number of residual negative cells in the positive fraction were maintained below 0.5 %, as estimated by flow cytometry analysis.

Specific pathogen-free pigs: islet and splenocyte isolations. One of the difficulties to be solved before pig islet grafts can be used in humans is the risk of transmitting infectious agents. This led us to isolate islets from pigs raised in specific pathogen-free (SPF) conditions. This strategy minimises microbial contamination and provides islets capable of reacting to nutrients, hormones and neuromediators [10]. We used 23 Large-White SPF pigs (80–120 kg), aged 20–30 weeks. The 23 pigs were from the same nucleus since they were all products of the same boar mated with 4 sows. Pancreata were removed, soaked in betadine solution, inflated with 100 ml ice-cold modified University of Wisconsin (mUW) solution, inflated again with 0.5 ml/g of mUW solution containing 3.22 U/ml collagenase P (act. 1.61 U/mg, Boehringer Mannheim, Meylan, France), and placed in a digestion chamber filled with mUW solution kept at 36 °C. The flow rate through the chamber was 40 ml/min. Crude islets were pelleted by centrifugation, suspended in mUW and purified on continuous Optiprep gradient (Life International Technology,

Cergy-Pontoise, France) with a COBE 2991 processor. Islet purity, as assessed by dithizone staining, exceeded 90 %. Pig islet cells (PIC) were suspended in Ham's F10 medium (5.5 mmol/l glucose; Biomédia, Boussens, France) supplemented with 2 % Ultrosor (BioSeptra SA, Villeneuve la Garenne, France), antibiotics (penicillin, streptomycin; Seromed, Berlin, Germany) and Fungizone, and cultured for 1 week before being used in the experiments. Spleens were removed in Hank's balanced salt solution (HBSS) (Seromed), and splenocytes were flushed out. Erythrocytes were lysed by ammonium chloride and the cells were resuspended in RPMI.

Proliferation and cytokine production of PBMC in response to pig cells. Human PBMC were aliquoted into flat-bottomed 96-well microtitre plates (Costar, Cambridge, Mass., USA) at 5×10^5 /well with 60 000 PIC or splenocytes, in 0.3 ml culture medium containing 10 % autologous decomplexed human serum. For all experiments, these pig stimulator cells were prepared as live cells, and in some instances the same experiments were also done with pig cells prepared as lysates by freezing and thawing. Plates were incubated at 37 °C in 5 % CO₂ for 7 days and pulsed by adding 3.7×10^4 Bq [³H] thymidine (sp. act. 7.4×10^8 Bq/mmol; Amersham, Les Ulis, France) for the final 18 h. Cells were then harvested and filters counted. Data were obtained from the mean values of quadruplicate wells and the results were also expressed by a stimulation index (SI), i.e. the ratio between the count with stimulator pig cells and the basal count. The interassay coefficient of variation (SD/mean $\times$ 100) of the SI ranged from 5 to 10 %.

Similar proliferation assays in response to PIC were done while PBMC subsets were blocked, different MoAbs being introduced (10 μ g/ml) for the entire co-incubation period. Rat IgG MoAbs directed against human CD₄ (RPA-T4) or CD₈ (OKT8), or against CD16 from NK cells (B73–1), were used. To determine whether the recognition involved MHC class II-restricted mechanisms, similar proliferation assays in response to pig cells were done while PBMC were blocked with MoAbs directed against HLA class II DR (L243) or DQ (SPVL3), and which did not cross-react with pig cells, as determined by flow cytometry analysis. Antibodies of similar isotypes were used as controls. To determine whether human T cells interact directly or indirectly with PIC, T cells were enriched by removal of APCs by negative selection, as described above, before being introduced into the proliferation assay. In addition, enriched CD₄⁺ or CD₈⁺ cells were tested alone or mixed with APCs, for proliferation against PIC. CD₈⁺ cells were tested in the absence or presence of 5 U/ml interleukin (IL)-2.

Under conditions similar to those described for proliferation, 100 μ l of supernatant were removed from each well after 2 days of co-incubation for human interferon- γ (INF γ), interleukin (IL)-10 and IL-4 assays (Biotrak ELISA system, Amersham Life Science) using a solid phase involving a bound capture antibody and a horseradish peroxidase-conjugated antibody for detection. Standard curves were generated using recombinant interleukins.

Data are presented as mean values $\pm$ SEM. The statistical significance of differences was evaluated using Student's *t*-test, analysis of variance, Chi-square analysis or Fisher test, *p* < 0.05 being considered as significant.

Results

Human PBMC recognise live pig islet cells (PIC) (Fig. 1A): strong proliferations were detected with both Type I diabetic (*p* < 0.001) or control (*p* < 0.001)

Fig. 1A,B In vitro human lymphocyte (5×10^5) responsiveness of Type I diabetic ($n = 49$) and healthy ($n = 34$) subjects to 60 000 live pig islet (A) or spleen (B) cells. Vertical bars indicate the means $\pm$ SEM of the stimulation index (SI) of lymphocytes co-incubated for 7 days with pig cells. SI were calculated as the ratios of the mean ^3H -thymidine uptakes with stimulator cells to the mean basal counts. Each individual point represents the mean of the SI for a given subject tested during three separate experiments with different pig islet cells (PIC) preparations. All SIs were above 2 (a value defined as four SD above the mean syngeneic proliferation of pig splenocytes in response to PIC) and thus considered as significant. The variance of SI in response to PIC was higher in diabetic than healthy subjects ($p < 0.0001$). Five diabetic patients displayed a response above the highest control proliferation (NS $p = 0.07$, Fisher test). Absolute values of basal ^3H -thymidine uptake by lymphocytes in the absence of PIC were not different between diabetic and healthy subjects (3991 ± 102 cpm vs 4823 ± 133 cpm, respectively). * ($p < 0.01$) and ** ($p < 0.001$) indicate significant differences between basal and ^3H -thymidine uptakes stimulated by pig spleen cells or PIC, respectively

subjects (SI: 37.6 ± 6.3 and 23.9 ± 3.3 for diabetic and control subjects, respectively; Fig. 1A). Pig splenocytes did not proliferate in response to syngeneic PIC. Strong proliferations were also observed when human PBMC were co-cultured with dead PIC prepared by freezing and thawing (data not shown).

Proliferation intensity in response to PIC varied greatly among subjects (SI ranging from 2 to 215 in diabetic patients and from 2 to 97 in healthy subjects) (Fig. 1A). The variance of SI was higher in diabetic than healthy subjects ($F = 5.2$, $p < 0.0001$). Five diabetic patients had a response above the highest control proliferation (NS $p = 0.07$, Fisher test). As compared with other patients, no peculiarities were seen in these high diabetic responders with regard to clinical status, HLA haplotypes, or the presence (or titres) of ICA, glutamic acid decarboxylase (GAD) or islet cell antigen-2 (IA-2) antibodies or of a combination of these markers.

Proliferations of PBMC in response to pig splenocytes (Fig. 1B) were obtained ($p < 0.01$) and no differences were noted between diabetic and control

groups. Human responses to PIC were stronger ($p < 0.01$) than concomitant responses to splenocytes from the same pigs.

Human CD_4 T cells were mainly involved in PBMC proliferation in response to PIC, since the SI decreased sharply ($p < 0.01$) when CD_4^+ were blocked with MoAbs (Fig. 2A). The involvement of CD_8 cells was much lower, since the SI decreased less ($p < 0.01$) after blocking of CD_8^+ , than CD_4^+ cells (Fig. 2A). NK cells were only marginally involved since the SI was only slightly reduced after blocking with anti- $\text{CD}16$ MoAbs (Fig. 2A).

MHC class II-restricted mechanisms were involved in the human cell-mediated reaction against PIC (Fig. 2A) since proliferation was blocked ($p < 0.01$) by anti-DR, but not anti-DQ, MoAbs.

The proliferative responses of T cells enriched by the removal of APC were strongly decreased ($p < 0.01$) (Fig. 2A), indicating that the dominant pathway for recognition of PIC was indirect. The proliferation of human PBMC was however, not completely abolished after APC depletion, indicating that direct recognition of PIC also occurred. No difference was found between diabetic and healthy subjects with respect to the effects of blocking antibodies or APC depletion (Fig. 2A).

Purified CD_4^+ cells alone did not proliferate in response to PIC, whereas CD_4^+ mixed with APCs recovered their proliferative ability against PIC (Fig. 2B). Purified CD_8^+ cells alone proliferated in response to PIC in the presence of IL-2 (Fig. 2B), even though their response was still stronger when mixed with human APCs (Fig. 2B). No difference was found between diabetic and healthy subjects concerning the involvement of CD_4^+ and CD_8^+ cells.

Th 2 cell involvement during co-culture with PIC appeared to be much greater than that of Th 1 cells, since IL-10 increased much more than IFN in all subjects, compared with basal production in the absence of PIC, so that mean IL-10 production largely increased (43-fold, $p < 0.01$) but mean IFN increased only slightly (Fig. 3). Stimulation indexes for IL-10 increase in the presence of PIC ranged from 10 to 55 in

Fig. 2A, B The lymphocyte subsets and pathway involved in proliferative response of human PBMC to live pig islet cells (PIC). **A:** the proliferation of PBMC from healthy subjects ($n = 10$) or diabetic subjects ($n = 16$) co-cultured with 60 000 PIC for 7 days was tested in the presence of blocking anti-CD₄, anti-CD₈, or anti-CD16 MoAbs. The proliferative responses of PBMC from diabetic or healthy subjects to PIC after removal of antigen-presenting cells (APC-) are also represented. Major histocompatibility complex (MHC) restriction was tested in the presence of blocking MoAbs directed at MHC class II DR, or MHC class II DQ. Results are expressed as the mean percentage of inhibition $\pm$ SEM of the stimulation index (SI). * ($p < 0.01$) indicate significant differences with control SI during co-incubations in the presence of control MoAbs and without removal of APCs, representing the 100% values of SI for diabetic or healthy subjects (39.4 ± 9.8 and 22.6 ± 5.0 , respectively) **B** Human CD₄⁺ and CD₈⁺ T-cell subsets of healthy ($n = 4$) and diabetic ($n = 4$) subjects were enriched by selection (using MoAbs and magnetic microspheres) and tested for proliferation against PIC. Each T-cell subset was tested alone or mixed with APCs. CD₈⁺ T-cells were tested in the presence of 5 U/ml IL-2. Results indicate the means $\pm$ SEM of the SI

diabetic patients and from 11 to 60 in healthy subjects, but for IFN ranged from 1.3 to 4.5 in diabetic patients and from 1.1 to 3.7 in healthy subjects. The IL10 : IFN γ ratio stimulated by PIC was not statistically different in diabetic patients and healthy subjects. Likewise, the absolute levels of the two cytokines were not statistically different between the two groups of subjects. Interleukin-4 production from human PBMC was not detectable under basal conditions or after 24- or 48-h co-cultures with PIC.

Discussion

This study provides the first detailed information on the intensity and mechanisms of human cell-mediated rejection of adult PIC, specifically in the context of Type I diabetes (the disease which islet grafts are intended to combat) and is also the first to use SPF PIC as a strategy of sanitary control.

The proliferation of human lymphocytes in response to PIC was of high intensity and stronger than that induced by pig splenocytes, which suggests that cell-mediated rejection of PIC would be a serious obstacle. Yet, even though xenograft rejection raises general questions valid for all tissues and diseases, each disease and corresponding tissue needs to be specifically analysed.

It is noteworthy that the intensity of proliferation differed greatly among subjects, which suggests that cell-mediated rejection of PIC would not be similarly strong in all subjects. The cause of this variability was not specifically studied here. It could depend, at least in part, on the matching of human and swine leucocyte antigens [4]. This varying intensity of proliferation observed with PBMC of different subjects with the same stimulator PIC could reflect differences in the ability of individual APC to process and present xenoantigens or on individual variations in the T-cell receptor repertory.

Blocking experiments with MoAbs showed that proliferation involved mainly CD₄⁺ T lymphocytes, which is consistent with studies indicating that CD₄⁺ cells are the main cell type infiltrating xenografts [11]. Simultaneously, blocking experiments also indicated that MHC class II DR-restricted mechanisms were involved. Thus, the response of CD₄⁺ cells could have required indirect presentation of PIC antigens by class II molecules borne by human APC. This was confirmed since the proliferation of PBMC was also strongly decreased after APC depletion, indicating that the dominant pathway for recognition of porcine islet antigens was indirect, as reported for other pig targets [12, 13]. Moreover, purified CD₄⁺ cells did not proliferate alone in response to PIC but recovered their proliferative ability when mixed with APCs. Finally, assays of the IL-10 (Th₂ cytokine) and IFN γ (Th₁ cytokine) produced by human lymphocytes confronted with PIC showed that Th2 cell activation appeared to be much greater than that of Th1 cells, as preliminarily reported [14].

Despite this dominant indirect CD₄⁺ recognition, the blocking experiments suggested that CD₈⁺ could also be implicated in the proliferation of human PBMC in response to PIC, even though they were involved much less than CD₄⁺ cells. Although the proliferation of PBMC was greatly decreased after APC depletion, it was not completely abolished, indicating that direct recognition of PIC also occurred. This was confirmed because CD₈⁺ cells alone proliferated.

Fig. 3 In vitro production of cytokines by 5×10^5 PBMC from 10 diabetic or healthy subjects cultured for 48 h with 60 000 live pig islet cells (PIC). Black columns represent the mean production of IFN- γ and IL-10 in vitro challenge with PIC. Basal cytokine production in the absence of stimulator cells is also indicated (open columns). * Results were significantly increased compared to basal production ($p < 0.01$)

erated in response to PIC, without help from human APC. Even though the xenoantigens recognised on PIC have not been studied, this direct pathway could be due to recognition of porcine MHC class I molecules by human CD $_8^+$ T cells, which has been described with other pig cell types [15]. Alternatively, this direct pathway could have involved the recognition of porcine MHC class II molecules shared by non-endocrine cells (dendritic cells, endothelial cells) which could have contaminated the islet-cell preparations [4]. This last possibility cannot be excluded since immunofluorescence with anti-MHC class II antibodies showed around 1% of class II-positive cells in one of our islet-cell preparations. This direct recognition of PIC by CD $_8^+$ T cells apparently required complementary and co-stimulatory factors to induce proliferation. In our study, CD $_8^+$ cells alone proliferated in response to PIC only in the presence of IL-2. The need for co-stimulatory factors could also explain why the response to PIC was stronger when CD $_8^+$ cells were mixed with human APC than when they were alone. This stronger proliferation could also have been due to an additional indirect mechanism by which CD $_8^+$ T cells could recognise PIC antigens after presentation by MHC class I molecules from human APC. Finally, the blocking experiments suggest that NK cells were only marginally involved in the proliferation of human PBMC in response to PIC. Since adult PIC do not bear the Gala(1,3)Gal epitope [16], our observation is contrary to reports of recognition of pig endothelial cells by human NK cells through the Gala(1,3)Gal epitope [17], even though the islet preparations could be contaminated by Gala(1,3)Gal-positive endothelial cells.

These results indicate that human cellular response to PIC differs from that of mice to the same PIC reported in our previous studies [18]. This difference has already been noted for other porcine tissues than islets [11]. In our studies, proliferation was much more intense in man than in the mouse, mainly involving CD $_4^+$ T cells of the Th2 subtype, whereas Th1 response was dominant in the mouse. Interleukin-4 production from human PBMC was undetectable after co-culture with PIC, although it was clearly stimulated from mouse splenocytes. The recognition of PIC involved only an indirect pathway in the mouse, but both indirect and direct pathways in humans. Although CD $_8^+$ T cells were involved in human direct recognition, such involvement could not be detected in the mouse. These differences indicate that the mouse is not a closely relevant model for the study of human response to adult PIC.

In addition to providing general information on human cell-mediated reactivity against adult PIC, our study suggests that the intensity of lymphocyte proliferation in response to PIC could be slightly higher in some Type I diabetic patients than in healthy subjects. Even though lymphocyte proliferation in diabetic and healthy subjects seemed to involve similar mechanisms with respect to blockage by MoAbs, responses of purified T cells and cytokine production, the system involving co-incubation with adult PIC could have induced more intense lymphocyte proliferation in some diabetic patients indicative of an "autoimmune" response. In this respect, we previously observed a higher proliferative response of splenocytes from diabetes-prone non-obese diabetic (NOD) mice than control mice to the same PIC [18]. This tendency is also consistent with the results of the only previous in vitro study in humans on this subject [19], which used fetal instead of adult pig islets. These authors also found that the lymphocytes of some diabetic patients proliferated more than those of control subjects. This is an important point since it raises the question as to whether a resurgence of the autoimmune aggression of islets occurs or not after a xenogeneic pig islet graft. If, as suggested for allogeneic islets [20], these grafted xenogeneic islets

are destroyed not only by xenogeneic aggression but also by "autoimmune" effectors, immunosuppressive strategies need to take this into account. In this study and the previous one [19], the difference in proliferation intensity in response to PIC was however, rather slight between diabetic and control subjects and could at best be seen in a few patients, so that the issue of a possible resurgence of the autoimmune process after pig islet grafts has not been settled and should be supported by additional experimental evidence.

In conclusion, human cell-mediated xenoreaction to adult PIC involves a dominant CD₄ class II-restricted Th2 response, with an indirect recognition pathway, as well as a CD₈ T-cell response resulting from direct recognition. This reaction is strong and constitutes a serious obstacle which can nevertheless differ in degree among subjects. Even though further experimental evidence is required, autoimmunity may recur in some Type I diabetic patients and complicate xenogeneic recognition, leading to more intense proliferation in response to PIC.

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