Abstract

Although the involvement of complement in hyperacute rejection of xenotransplants is well recognized, its role in rejection of devascularized xenografts, such as pancreatic islets, is not completely understood. In this study, we investigated whether complement participates in the immunopathology of xeno-islet transplantation in a concordant rat to mouse model. Rat pancreatic islets were implanted under the kidney capsule of normal and cobra venom factor (CVF)-decomplementized diabetic C57BL/6 mice. Graft survival was monitored by blood glucose levels. Deposition of IgM and C3 on grafted islets in vivo or on isolated islets in vitro (after incubation with normal and decomplementized mouse serum), as well as CD4- and CD8-positive leucocyte infiltration of grafts, was checked by immunohistochemistry. In addition, complement-mediated cytotoxicity on rat islet cells was evaluated by a 3-(4, 5-dimethylthiazolyl)-2.5-diphenyl-2H-tetrazolium-bromide (MTT) assay. A significant C3 deposition was found on grafted islets from the first day after transplantation in vivo, as well as on isolated islets after incubation with mouse serum [...]
Decomplementation with cobra venom factor prolongs survival of xenografted islets in a rat to mouse model

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SUMMARY

Although the involvement of complement in hyperacute rejection of xenotransplants is well recognized, its role in rejection of devascularized xenografts, such as pancreatic islets, is not completely understood. In this study, we investigated whether complement participates in the immunopathology of xeno-islet transplantation in a concordant rat to mouse model. Rat pancreatic islets were implanted under the kidney capsule of normal and cobra venom factor (CVF)-decomplemented diabetic C57BL/6 mice. Graft survival was monitored by blood glucose levels. Deposition of IgM and C3 on grafted islets in vivo or on isolated islets in vitro (after incubation with normal and decomplemented mouse serum), as well as CD4- and CD8-positive leucocyte infiltration of grafts, was checked by immunohistochemistry. In addition, complement-mediated cytotoxicity on rat islet cells was evaluated by a 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide (MTT) assay. A significant C3 deposition was found on grafted islets from the first day after transplantation in vivo, as well as on isolated islets after incubation with mouse serum in vitro. By MTT assay, complement-mediated cytotoxicity for islet cells was found. Decomplementation by CVF decreased C3 deposition on either isolated or grafted islets, delayed CD4- and CD8-positive leucocyte infiltration, led to significant inhibition of complement-mediated cytotoxicity for islet cells, and prolonged graft survival (mean survival time 21.3 versus 8.5 days; \( P < 0.01 \)). Our results indicate that decomplementation can prolong the survival time of devascularized xenografts across concordant species. The deposition of complement on transplanted islets may contribute to xenograft rejection by direct cytotoxicity and by promoting leucocyte infiltration.

INTRODUCTION

Allotransplantation of pancreatic islets is making the step from an experimental procedure to an accepted alternative to pancreas transplantation. While recent results are encouraging, it is evident that this therapeutic approach will be limited by organ shortage. Therefore, xeno-islet transplantation (XIT) has been considered as a possible solution. In discordant species combinations, whole organ xenografts trigger a hyperacute rejection (HAR). It has been recognized that the humoral immune response plays a crucial role in HAR. Xenoreactive natural antibodies and complement pre-existing in recipients induce donor endothelial cell activation and damage, which recruit other effector mechanisms, leading ultimately to graft destruction. However, in devascularized xenografts, such as pancreatic islets, HAR does not occur and the term concordant and discordant can not be applied with the same meaning. Although the T-cell response has been recognized as playing a major role in xeno-islet rejection, XIT in B-cell-deficient mice exhibits a significantly prolonged survival time, suggesting that some humoral factors may also participate in the rejection. In previous studies, a significant deposition of IgM and C3 was found on grafted islets in a rat to mouse model, while this deposition was not seen in islet allotransplantation. Although a direct complement-mediated cytotoxicity on xenogeneic islet cells has been shown in vitro, the effect of complement on grafted islets in vivo remains to be investigated.

In order to evaluate the impact of complement on xenogeneic islet rejection in a concordant model (as related to organ xenotransplantation), rat islets of Langerhans' were transplanted into mice decomplemented by cobra venom factor.
C3 levels in serum were measured by radial immunodiffusion using a method as described previously. Briefly, nephelometry was established by 5% isoflurane (Forene®; Abbott, Cham, Switzerland). After midline incision and exposure of the pancreas, 10 ml Hank’s balanced saline solution (HBSS) with 2 mg/ml collagenase type XI (Sigma, St Louis, MO) was injected into the pancreatic duct. After pancreatectomy, the pancreas was digested in a 37°C water bath with gentle shaking for 19 min. The isolated islets were purified further on a EuroFicoll (Sigma) gradient centrifugation. The purified islets were washed three times and resuspended in HBSS solution for islet transplantation.

Decomplementation

C57BL/6 male mice were divided into four groups (control, CVF1, CVF2 and CVF3). In the CVF1 group, 500 mg/kg CVF (Imutran, Cambridge, UK) was injected intraperitoneally (i.p.) into mice 24 hr prior to islet transplantation. The mice of the CVF2 group received 500 mg/kg CVF i.p. 24 hr prior to and 3 days after islet transplantation. For the CVF3 group a single injection of CVF (500 mg/kg) was given to mice on day 4 after transplantation. The mice of the control group were injected with the matching volumes of phosphate-buffered saline (PBS).

In order to evaluate the efficiency of decomplementation by CVF, serum was collected daily and pooled from four non-transplanted mice of the control group, CVF1, CVF2 and CVF3. The C3 levels in serum were measured by radial immunodiffusion using a method as described previously. Briefly, 1% agar was stabilized at 45°C in a water bath, and goat anti-mouse C3 (29·5 mg/ml; courtesy of Dr I. Shozo, Geneva, Switzerland) added at 1:150. Gel was poured on a glass plate and circular wells were punched out in the gel. Approximately 7 µl of diluted samples and standards, respectively, was added to the wells. The plates were incubated at room temperature in a moist box for 48 hr, washed in 0·9% NaCl overnight, and coloured with 1% tannic acid (Sigma, Stenheim, Germany). Ring-shaped precipitates of samples were measured and compared to a standard curve obtained by plotting the square of diameters of standards against dilutions.

Immunohistochemistry

Kidneys were taken from control and CVF-treated mice on days 1, 4 and 7 after islet transplantation (n=3 for each group and day) and snap-frozen in Tissue Tek (Miles, Elkhart, Indiana, USA). The samples were soaked in liquid nitrogen and then stored at −80°C until immunohistochemical testing. Tissue sections were incubated with PBS plus 0·5% bovine serum albumin (BSA; Sigma) for 15 min to block non-specific binding, and then stained with guinea-pig anti-porcine insulin

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(Dako, Zug, Switzerland), goat anti-mouse IgM (Sigma, Buchs, Switzerland), sheep anti-mouse C3 (The Binding Site, Birmingham, UK), rat anti-mouse CD4 and rat anti-mouse CD8 antibody (Serotec, Oxford, UK), respectively. After a 30-min incubation, the sections were washed and further stained with corresponding fluorescein isothiocyanate (FITC)- conjugated second antibodies for 45 min. The results were checked under a fluorescence microscope (Zeiss, Jena, Germany) by three different examiners. Immunostaining was evaluated semi-quantitatively with a scoring system (0, –, +, ++, +++).

The freshly isolated and purified rat islets were fixed with –20°C methanol for 10 min, washed twice with PBS, and then incubated with PBS containing 0.1% BSA to block non-specific binding. Islets were incubated with normal or complement-antibodies mouse serum, respectively, for 1 hr at 4°C. After three washings, islets were incubated with a goat anti-mouse IgM or sheep anti-mouse C3 antibody for 1 hr. Islets were washed and further stained by a diluted rabbit anti-goat (Sigma) or rabbit anti-sheep FITC-conjugated antibody (Sigma) for 30 min. The staining was checked under a fluorescence microscope. The experiment was repeated with three different islet isolations.

Cytotoxicity assay
Normal or CVF-decomplemented serum was serially diluted with 10% DMEM in a 96-well plate. The isolated islet cell suspension with 2 μg/ml actinomycin D was added to each well (10^3 cells/well). Rat islet cells were incubated with the serum for 20 hr at 37°C and then 50 μl of 5% MTT was added to each well. After a 4-hr incubation, the plate was centrifuged at 500 g for 10 min and the supernatant was discarded. The cells in each well were lysed with 100 μl/well isopropanol–HCl in a VARI shaker for 5 min. The optical density was measured at 570 nm with an ELISA reader. The results are expressed as a percentage of cell survival. The experiment was repeated with three different islet isolations.

RESULTS
Evaluation of decomposition by CVF
After the administration of a single dose of CVF (500 μg/kg i.p.), the C3 concentration in the serum had decreased to below 10% of normal levels and persisted for 3 days, as shown in Fig. 1.

A second administration of CVF 4 days after the first could prolong decomposition to another 3 days (data not shown). The purified CVF, administered at 500 μg/kg i.p., did not have any obvious toxic effect on treated animals.

Normal mouse serum at a concentration of 25% induced 71% cytotoxicity on HB-MVEC, compared with heat-inactivated FCS. This value was used as a positive control (= 100% cytotoxicity), as shown in Fig. 1. With a single administration of CVF (500 μg/kg i.p.) there was a drop in complement-mediated cytotoxicity to below 20% of the positive control. This persisted for 4 days after CVF administration.

In addition, the C3 levels were also checked and were below 10% of the controls in all grafted mice on the day of transplantation (the CVF1 and CVF2 groups) and the day after the second CVF administration (the CVF2 group) or the first CVF administration on day 4 (the CVF3 group) (data not shown).

Prolonged graft survival in complement-depleted recipients
All diabetic mice became normoglycemic within 3 days after islet transplantation. Compared with control mice, the survival time of grafted islets was significantly prolonged in CVF1 and CVF2 mice (19.7 and 21.3 days versus 8.5 days; each P < 0.01). However, no difference was found between the complement-depleted groups, CVF1 and CVF2, as shown in Fig. 2. In contrast, a single CVF administration at day 4 after islet transplantation (the CVF3 group) did not prolong the survival.
time of grafted islets, suggesting an involvement of complement at early, but not late, stages after XIT.

**Immunohistological changes after decomplementation**

Immunohistochimistry was performed on kidneys taken on days 1, 4 and 7 after islet transplantation. In the control group, IgM deposition was found on grafted islets from day 1 and persisted weakly until day 7, while a more marked C3 deposition was seen on grafted islets at days 1 and 4, and had decreased on day 7 after transplantation. In the CVF1 and CVF2 groups, C3 deposition was significantly reduced (Fig. 3a–d) while the IgM deposition was not affected. Moreover, the difference of C3 deposition between control and CVF1/CVF2 groups became less obvious at day 7 after transplantation, but the insulin staining showed a different pattern. Insulin staining in the control group presented a scattered pattern, while that in the CVF groups was relatively intact (Fig. 4a,b). There was no obvious difference in immunohistology between the CVF1 and CVF2 groups, and between the control and CVF3 groups.

Further immunohistological analysis of grafted islets revealed a CD4- and CD8-positive leucocyte infiltration beginning at day 4 and becoming more evident on day 7 after transplantation in the control group (Fig. 4c,e) as well as in the CVF3 group. In contrast, CD4- and CD8-positive leucocyte infiltration was absent in the CVF1 and CVF2 groups on day 4 and very weak on day 7 after transplantation (Fig. 4d,f), suggesting an involvement of complement in leucocyte infiltration. The results of immunohistology are summarized in Table 1.

**Complement and IgM binding on isolated xeno-islets**

In order to confirm C3 deposition seen in vivo, isolated rat islets were incubated with normal or decomplemented mouse serum, and complement and IgM binding was investigated further in vivo. By immunofluorescence staining, IgM binding was found only on microvessel-like structures of rat islets (Fig. 5a). In contrast, C3 binding was homogeneously distributed on the islet surface (Fig. 5c), suggesting that C3 deposition on xenoislets might be IgM independent. After incubation of rat islets with serum from CVF-treated mice, C3 binding on islets was significantly decreased while IgM deposition was unchanged (Fig. 5b,d).

**Complement-mediated cytotoxicity of mouse serum on rat islet cells**

Intact serum of untreated mice exerted a strong complement-mediated cytotoxicity on rat islet cells incubated at higher serum concentrations, as measured by the MTT assay. This cytotoxicity was serum-concentration dependent. In vivo decomplementation by CVF could abrogate completely the cytotoxicity of low concentrations of mouse serum (12.5%) against rat islet cells (97.7 ± 6.8 SD versus 78.7 ± 6.5 SD percentage islet cell survival; P < 0.05). Using higher

![Control](a) ![CVF](b)

![Control](c) ![CVF](d)

**Figure 3.** Effect of decomplementation on C3 deposition on grafted islets. Immunofluorescence staining on xeno-islets at day 1 after transplantation. Insulin and C3 staining in control (a, c) and CVF-treated recipients (b, d) (Original magnification ×150).
Figure 4. Effect of decomplementation on leucocyte infiltration. Immunofluorescence staining on xenografted islets at day 7 after transplantation. Staining for insulin, CD4 and CD8 in control (a, c, e) and CVF1 and CVF2 recipients (b, d, f). Original magnification ×150.

Table 1. Summary of the immunohistology of xenografted islet transplantation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=3)</td>
<td>CVF1 +2 (n=6)</td>
<td>Control (n=3)</td>
</tr>
<tr>
<td>IgM</td>
<td>+</td>
<td>+</td>
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<tr>
<td>C3</td>
<td>++</td>
<td>–</td>
<td>+</td>
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<tr>
<td>CD4</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>CD8</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
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</table>

Symbols: –, negative; ±, weak; +, positive; ++, strongly positive.

Figure 5. Effect of decomplementation on IgM and C3 binding on isolated rat islets in vitro. Immunofluorescence staining for IgM and C3 on freshly isolated rat islets after incubation with intact (a, c) or complement-depleted mouse serum (b, d). Original magnification ×400.

DISCUSSION

It is known that activation of complement by xenoreactive IgM is responsible for HAR of solid organs xenotransplanted across discordant species. However, little is known about the role of complement in devascularized tissue xenografts, such as pancreatic islets, where HAR does not usually occur. In this study, we show that complement also participates in the rejection of xenografted islets and that decomplementation by CVF prolongs graft survival of xeno-islets in a concordant rat to mouse model.

In our previous and present studies, complement-deposition was found on concordant and discordant xenografted pancreatic islets early after transplantation. Complement deposition was demonstrated further on isolated rat islets in vitro, and immunohistological patterns suggested an IgM-independent pathway consistent with previous reports. In order to evaluate the role of complement in xenoid rejection, rat islets were transplanted in complement-depleted mice. Decomplementation was performed by CVF, a constituent of cobra venom with extensive structural homology to mammalian C3. CVF activates complement and leads to complement consumption. Decomplementation by CVF has been used widely to investigate the role of complement in HAR.

With a single injection of CVF (500 µg/kg i.p.), a reduction of 90% in C3 levels and of 80% in bioactivity was obtained and persisted for 3–4 days. Immunohistology showed that decomplementation with CVF significantly reduced concentrations of mouse serum (50%), in vitro decomplementation by CVF could strongly reduce, but not completely block, cytotoxicity of mouse serum on rat islet cells (54.9±3.4 SD versus 24.9±1.7 SD percentage islet cell survival; P<0.01). As a control, Sprague–Dawley rat serum, even at higher concentrations, did not have any cytotoxic effect on rat islet cells (Fig. 6).

Figure 6. Complement-mediated cytotoxicity on isolated rat islet cells. Isolated rat islet cells were incubated with different concentrations of fresh serum and the cytotoxicity was measured by an MTT assay. Intact homologous rat serum was used as a negative control. Results are expressed as mean values of triplicates±SD. *P<0.05. **P<0.01 for statistical difference between normal and complement-depleted mouse serum.

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in vitro

grafted islets

in vitro

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occurred in CVF1 and CVF2 recipients with a similar pattern, increasing the sensitivity of radial-immunodi

infiltration. Nevertheless, although delayed, rejection also 11.

by CVF might be mediated by interference with leucocyte immunodi

Transplant

CD8-positive leucocyte infiltration was significantly delayed in

24

ness.

for phagocytosis, are well known but complement is also 8.

mediated or -dependent immune response plays a major role

20

been shown by immunohistological studies and flow cytometric 4.

Xenotransplantation

17

recovered cells, thereby activating complement by the classi-

ment across species is cytotoxic for isolated islet cells. 8

Although there was a significant cytotoxic effect of comple-

ment on isolated islet cells in vitro, it was not seen on whole

islets either in vitro 9 or in vivo. In fact, in spite of a marked

complement deposition on grafted islets early after transplan-

tation, insulin staining at days 1 and 4 did not show a different pattern between control and CVF groups.

IgM was only found on microvessels within whole islets, while a significant IgM binding was found on all isolated rat islets cells after incubation with mouse serum by flow cyto-

metric analysis (data not shown). These results may provide an explanation for the difference in complement-mediated cytotoxicity on the isolated islet cells in vitro and on the grafted islets in vivo, i.e. the exposure of antigens by the islet cell isolation procedure may induce IgM binding on the recovered cells, thereby activating complement by the classi-

cal pathway.

Although it has been shown that decomposition by CVF can reduce the incidence of primary non-function in a dog to rat XIT model, 17 in our model the prolongation of graft survival could not be explained solely by the abrogation of complement-mediated cytotoxicity on xeno-islets.

A significant T-cell infiltration around grafted islets has been shown by immunohistological studies and flow cytometric analysis. In our model T-cell-derived cytokines exert a cytotoxic effect on islet cells. 19 Anti-CD4 pretreatment of recipients significantly prolongs islet survival and in some models induces tolerance. 5 In T-cell-deficient nude mice, xeno-

However, although delayed, rejection also occurred in CVF1 and CVF2 recipients with a similar pattern, including CD4- and CD8-positive leukocyte infiltration (data

not shown). As no significant difference in survival time or immunohistological pattern was found between the CVF1 and CVF2 groups, persisting decomposition could not further delay or finally prevent leukocyte infiltration and rejection. Due to the redundancy of mechanisms that promote cell-attraction and activation of cellular responses, it is evident that complement is only one of many factors triggering leuco-

cyte infiltration.

The results of the present study demonstrate that comple-

ment is also involved in the immunopathology of rejection of non-primary vascularized xenografted tissue. Complement may participate in the rejection of xeno-islets by direct cyto-

toxicity and by promoting CD4- and CD8-positive leukocyte infiltration. Efficient decomposition by CVF significantly prolongs the survival of xenotransplanted islets of Langerhans' in a concordant rat to mouse model.

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