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Deoxyspergualin delays xenograft rejection in the guinea pig-to-C6-deficient rat heart transplantation model

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Abstract This study aimed to investigate the effects of 15-deoxyspergualin (DSG), tacrolimus (FK 506) and cyclosporin A (CyA), alone or in combination, on delayed xenograft rejection (DXR). We used the guinea-pig-to-C6-deficient ($C6^-$)-PVG-rat heart transplantation model, since in this strain combination, hyperacute rejection is avoided. In $C6^-$ control rats, the guinea pig xenografts survived for 39.2 ± 6.3 h (mean ± SD). Splenectomy alone resulted in a xenograft survival of 71.8 ± 7.8 h, but the addition of CyA or FK 506 did not further improve graft survival (73.6 ± 3.0 h and 72.0 ± 17.6 h, respectively). In contrast, DSG treatment increased graft survival to a mean of 99.8 ± 9.2 h. When CyA or FK 506 was combined with DSG, no additional effects were observed (105 ± 24.3 h and 95.1 ± 5.6 h, respectively). DSG alone or in combination with FK 506 or CyA resulted in a significant reduction in the serum IgM levels and reduced the deposits of IgM and IgG in rejected grafts. However, all xenografts were still heavily infiltrated by ED1 + macrophages, regardless of the treatment used. Thus, DSG treatment resulted in moderate prolongation of xenograft survival in $C6^-$ rats. The effect seems to be related to suppression of xenoreactive antibody production. To prolong xenograft survival further, strategies that inhibit macrophage infiltration seem required.

Key words Xenotransplantation · 15-deoxyspergualin · Guinea pig · Rat

Introduction

The first barrier to successful transplantation of vascularized organs between discordant species is hyperacute rejection, a rapid and violent reaction, leading to destruction of the graft within minutes or hours [10]. Significant progress in controlling hyperacute rejection has recently been achieved through the generation of transgenic pig donors expressing human complement regulators – e.g., decay-accelerating factor (hDAF) [5]. When such transgenic pig hearts were transplanted to nonimmunosuppressed cynomolgus monkeys, hyperacute rejection did not develop and the grafts survived for 3–4 days. Nevertheless, even when hyperacute rejection has been overcome, a xenograft will be rejected by what has been termed acute vascular rejection or delayed xenograft rejection (DXR), characterized by mononuclear cell infiltration, endothelial cell activation, and thrombosis [2, 13]. Accumulating evidence indicates the important role of xenoreactive antibodies in the initiation of DXR. The binding of antibodies to xenograft endothelial cells leads to upregulation of genes encoding for adhesion molecules, procoagulant factors and cytokines. The activated endothelium promotes inflammation and thrombosis [19]. DXR is now recognized as a major block in the development and clinical application of xenotransplantation.

Transplantation of guinea pig hearts to rats is a small animal model commonly used in studies on discordant xenograft rejection. Recently, a subpopulation of PVG

rats has been reported to have a complement factor 6 (C6) deficiency [11]. When guinea pig hearts were transplanted to such rats, hyperacute rejection did not occur and the grafts survived for 1–2 days [13]. The use of C6-deficient ($C6^-$) rats thus offers a possibility to study the later phases of the immune response to vascularized discordant xenografts.

Our group has previously identified several immunosuppressive protocols with efficacy in the pig-to-rat islet transplantation model [24, 26]. In the present study, the efficacy of two of these immunosuppressive protocols, 15-deoxyspergualin (DSG) combined with cyclosporin A (CyA) and tacrolimus (FK 506) as single therapy, are evaluated in the guinea pig-to-$C6^-$-rat heart transplantation model.

Materials and methods

Animals

Outbred Hartley guinea pigs (ALAB, Sollentuna, Sweden) weighing 150–250 g were used as donors. C6-sufficient ($C6^+$) PVG rats (Möllegaard, Skensved, Denmark) and $C6^-$ PVG rats (Bantin & Kingman, Freemont, Calif.) weighing 200–250 g were the recipients. Animals were given unlimited access to water and laboratory chow. All protocols were approved by the Animal Ethics Committee of Karolinska Institute, Stockholm, Sweden.

Surgical procedures

The animals were anesthetized with Hyponorm (1 mg/ml fluonisonum with 0.2 mg/ml fentanylum, Janssen Pharmaceutica, Beersee, Belgium), 1 ml/kg B.W. i.m.. Heterotopic heart transplantation was performed with the microsurgical technique described by Ono and Lindsey [17]. In brief, the donor aorta was anastomosed to the recipient's infrarenal aorta and the donor pulmonary artery to the recipient's infrarenal vena cava. Heart xenografts were evaluated visually during the first 30 min after reperfusion and then three times daily, by abdominal palpation. Rejection was defined as the total cessation of heart contractions and was confirmed by visual inspection during laparotomy.

Experimental design

The nonimmunosuppressed groups included guinea pig heart transplantation to $C6^+$, $C6^-$ and splenectomized $C6^-$ rats. These animals were given saline i.m.. In the immunosuppressed groups, all animals were $C6^-$ and were splenectomized immediately before heart transplantation. CyA (15 mg/kg B.W. per day p.o.), FK 506 (1 mg/kg B.W. per day i.m.), DSG (10 mg/kg B.W. per day i.m.), or DSG plus CyA or FK 506 (same dosages as used individually) were administered from day –2 until rejection. Each group contained eight rats. CyA was purchased from Sandoz Pharma (Basel, Switzerland). FK 506 was obtained from Fujisawa Corporation (Osaka, Japan) and DSG was provided by Nippon-Kayaku (Tokyo, Japan). The xenografts were harvested at rejection. In a longitudinal study of the rejection process, xenografts from splenectomized $C6^-$ rats treated with DSG plus CyA were harvested at 36 ($n = 6$) and 72 h ($n = 6$) following transplantation.

Table 1 Monoclonal antibodies used for the immunohistochemical stainings

Antigen	Code	Clone	Specificity
TCR	MCA453	R75	Rat α/β TCR
CD4	MCA55P	W3/25	T helper cells, macrophages
CD8	MCA48	Ox-8	T suppressor, T cytotoxic cells, NK cells and granular intraepithelial leukocytes in small bowel
ED1	MCA341	ED1	Macrophages, monocytes and dendritic cells, but not granulocytes
ED2	MCA342	ED2	Resident macrophages, but not monocytes, dendritic cells, or granulocytes

Rat anti-guinea-pig antibodies in serum

To assess the effects of immunosuppressive agents on xenoreactive antibody production, blood was collected from the ophthalmic venous plexus on day –2 and at rejection. Samples were allowed to clot for 30 min at room temperature and then centrifuged. Serum was aspirated and stored in aliquots at –70 °C pending use.

Quantitation of rat anti-guinea-pig antibodies was performed by ELISA [20]. Briefly, guinea pig platelets were prepared as the target antigens. Enzyme-linked immunosorbent assay plates were coated with the solubilized antigen solution and left at 4 °C overnight. After blocking of nonspecific binding and washing, the various rat sera were applied in a dilution of 1:50 and incubated for 3 h at room temperature. After another washing, the secondary antibody, goat anti-rat IgG, conjugated to alkaline phosphatase, was appplied for 2 h at room temperature. The wells were washed and substrate solution was added. The optical density was read by a microplate autoreader at a wavelength of 405 nm.

Immunohistochemistry

Heart samples were snap-frozen in liquid nitrogen and subsequently stored at –70 °C. Serial sections, 6 µm thick, were cut in a cryostat (–20 °C), air-dried, and then stored at –70 °C. The sections were fixed in cold acetone, diluted 1:2 in distilled water for 30 s, and then subjected to a final fixation in cold acetone (100 %) for 5 min. Several monoclonal antibodies were used for immunohistochemical stainings (Table 1). Incubations with these antibodies were followed by incubations with rabbit anti-mouse IgG antibodies and 1 % normal rat serum. After a final incubation with a monoclonal mouse peroxidase-anti-peroxidase reagent, the peroxidase reaction was developed, using a carbazole-containing buffer for 15 min. The slides were then counterstained with hematoxylin and mounted in glycerin gelatin. Control experiments were performed by omitting the primary antibody.

Immunofluorescence

Unfixed tissue sections were examined for deposits of IgG, IgM and C3 with immunofluorescence. Fluorescein-labeled rabbit anti-rat IgG and IgM were used for direct immunofluorescence staining. Goat anti-rat C3, followed by fluorescein-labeled anti-goat immunoglobulin, was used for indirect staining. All incuba-

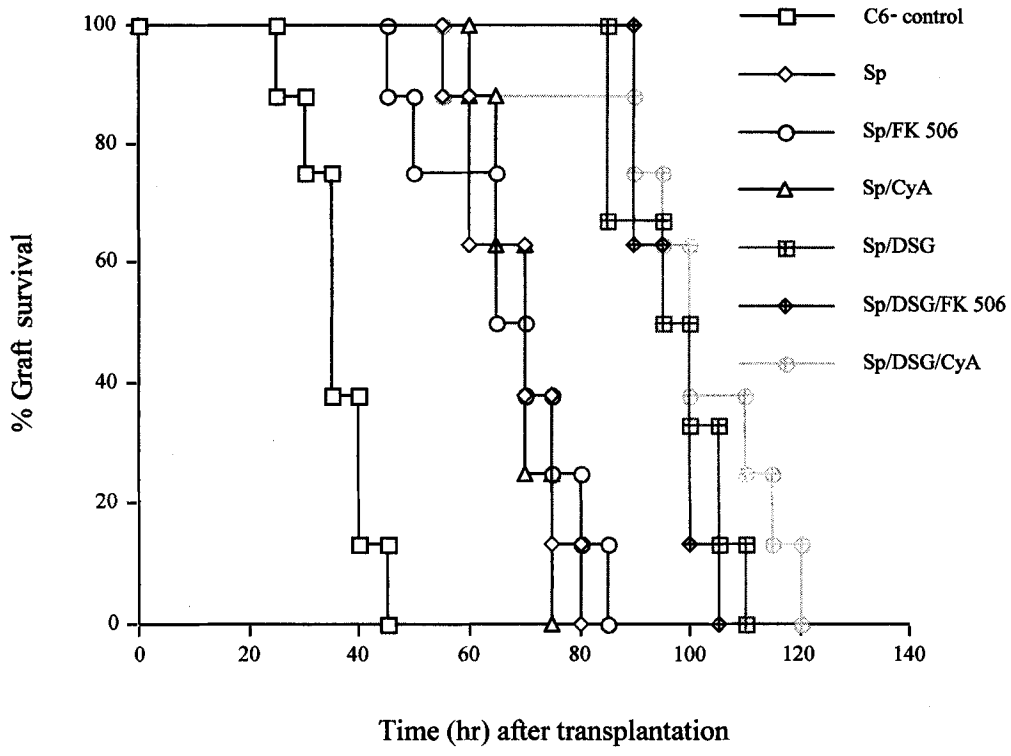

Fig. 1 Guinea pig heart survival in C6⁻ PVG rats. Graft survival was markedly prolonged in rats receiving DSG (*Sp* splenectomy)

tions were carried out for 30 min. The slides were mounted in phosphate-buffered saline and glycerol and analyzed for green fluorescence with a UV microscope (Zeiss, Welwyn Garden City, UK).

Statistics

Data are given as means ± SD. The differences in survival times were analyzed with the log rank test. The numbers of infiltrating cells in the various treatment groups were compared using ANOVA. Differences in serum antibody levels between pretreatment and rejection samples were analyzed using the paired Student's *t*-test with Bonferroni's correction. For analyses of associations between survival times and the study parameters (cell types and serum antibody levels), linear regression analysis was performed using the whole material, regardless of treatment, as one group. A P value of < 0.05 was considered significant.

Results

Xenograft survival

Effect of C6 deficiency

In C6⁻ control rats, the xenografts survived for 39.2 ± 6.3 h, while C6⁺ rats rejected such grafts in 9 ± 2 min.

Effect of splenectomy in C6⁻ rats

Splenectomy alone resulted in a significant delay in rejection compared to C6⁻ control rats (71.8 ± 7.8 h vs 39.2 ± 6.3 h, $P < 0.01$).

Effects of immunosuppression in C6⁻ splenectomized rats

Treatment with CyA or FK 506 did not further improve graft survival (73.6 ± 3.0 h and 72.0 ± 17.6 h, respectively). When DSG was combined with splenectomy, xenograft survival increased to 99.8 ± 9.2 h ($P < 0.01$ vs splenectomy alone). CyA or FK 506 combined with DSG gave no additional effects (105 ± 24.3 h and 95.1 ± 5.6 h, respectively) (Fig. 1).

Rat anti-guinea pig antibodies in serum

In C6⁻ control rats, the serum levels of anti-guinea-pig IgM xenoantibodies at rejection did not differ from the pretreatment levels. In rats treated with splenectomy alone or in combination with CyA or FK 506, the levels of IgM xenoantibodies showed a tendency to decrease, but this trend did not reach statistical significance. However, DSG treatment resulted in a significant decrease in IgM xenoantibodies (Fig. 2). When all rats were ana-

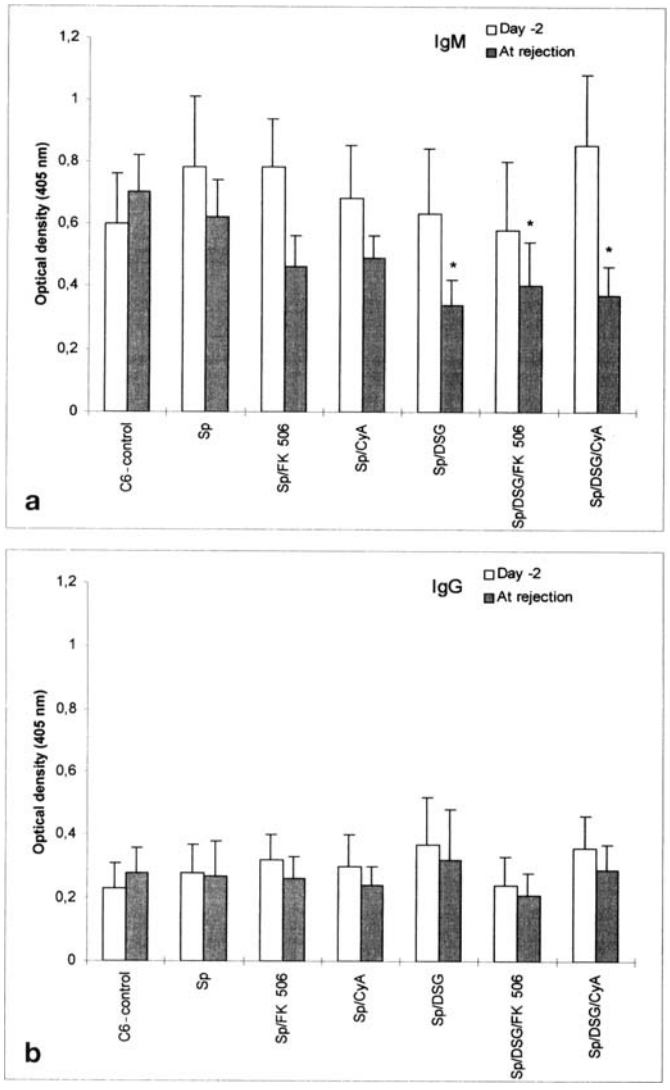

Fig. 2 Rat anti-guinea-pig IgM (**a**) and IgG (**b**) antibody levels determined with platelet membrane ELISA on day –2 and at rejection. *$P < 0.05$ vs the levels on day –2 (*Sp* splenectomy)

lyzed as one group, the levels of IgM xenoantibodies at rejection were strongly related to survival time ($P < 0.01$) (Fig. 3). The serum levels of IgG xenoantibodies were low both before treatment and at rejection. No significant changes were observed, regardless of the treatment.

Immunohistochemistry

In $C6^+$ rats, no cellular infiltrates were detected in the rejected grafts. In $C6^-$ control rats, rejected grafts were heavily infiltrated by ED1 + macrophages. ED2 + macrophages, TCR + , CD4 + and CD8 + cells were occasionally observed, while very few NK cells were detected. Splenectomy alone or in combination with CyA, FK 506 or DSG had no significant influence on macrophage infiltration. CD4 + and CD8 + cells showed a tendency to increase in rats receiving DSG plus CyA, but no statistical difference was observed between the various treatment groups (Table 2).

When all treatment groups were analyzed together, there was a significant relation between decreasing number of intragraft ED1 + macrophages and increasing survival time ($P < 0.01$). Regarding ED2 + macrophages and CD4 + and CD8 + cells, increasing numbers of cells were instead correlated with increasing survival times ($P < 0.05$, $P < 0.001$ and $P < 0.01$, respectively) (Fig. 3).

Immunoglobulin and complement deposits

Normal, untransplanted donor hearts lacked immunoglobulin deposits. C3 deposits were very weak or absent on the endothelium, but there were diffuse deposits in the muscle. In $C6^+$ rats, hyperacutely rejected grafts showed weak deposits of IgM in small and large blood vessels. IgG deposits were predominantly located in the interstitial space. Intense C3 deposits were observed both on the endothelium and in the muscle. In untreated $C6^-$ rats, deposits of IgM, IgG and C3 were identical to those seen in $C6^+$ rats. In $C6^-$ rats treated with splenectomy, alone or in combination with CyA or FK 506, weak deposits of IgM and IgG were still detected on small capillary vessels. In DSG-treated $C6^-$ rats, there were no IgM and IgG deposits in all rats. C3 deposits were absent in the vessel and very weak in the muscle compared to untreated $C6^-$ rats.

Serial studies in xenograft recipients treated with DSG and CyA

In $C6^-$ rats receiving DSG plus CyA, marked infiltration of ED1 + macrophages, 27 ± 9.7 per field, was present already at 36 h following transplantation. Grafts harvested at 72 h and at rejection (105 h) showed a marginal further increase in numbers of ED1 + cells. ED2 + macrophages, TCR + , CD4 + and CD8 + cells also tended to increase with time after transplantation (Table 2). Focal deposits of IgM were seen in 1/8 rats at 36 and 72 h following transplantation. At 36 hours, no IgG was found in the grafts, but at 72 h, weak staining for IgG was detected in 2/8 rats. C3 deposits were noted in small capillary vessels at 36 h and at 72 h in all rats, but the staining was weak. The serum levels of IgM xenoantibodies were markedly reduced already at 36 and 72 h following transplantation, as compared to the pretreatment levels (0.362 ± 0.019 and 0.382 ± 0.013 vs 0.846 ± 0.233 and 0.674 ± 0.212 optical density units, respectively).

Table 2 Cells infiltrating xenografts. Cell counts/high-power field were done in 5 consecutive fields (*Sp* splenectomy)

Treatment	ED1	ED2	TCR	CD4	CD8	NK
$C6^-$ control	43 ± 9.3	4 ± 2.9	4 ± 2.9	2 ± 1.6	1 ± 0.8	0 ± 0.5
Sp	39 ± 7.4	6 ± 3.1	4 ± 2.7	6 ± 3.5	3 ± 1.6	2 ± 2.1
Sp/FK 506	41 ± 5.9	6 ± 3.5	3 ± 1.9	3 ± 2.3	5 ± 2.2	2 ± 1.4
Sp/CyA	38 ± 7.4	7 ± 3.3	4 ± 2.2	7 ± 2.8	4 ± 2.7	2 ± 2.1
Sp/DSG	35 ± 11.6	8 ± 3.9	5 ± 3.0	8 ± 2.7	4 ± 1.7	1 ± 0.9
Sp/DSG/FK 506	34 ± 10.6	8 ± 4.4	5 ± 2.5	5 ± 2.6	4 ± 1.3	1 ± 0.8
Sp/DSG/CyA	34 ± 8.7	9 ± 4.3	5 ± 3.1	6 ± 2.2	4 ± 1.8	1 ± 0.4
Sp/DSG/CyA 36 hr	27 ± 9.7	5 ± 2.9	0 ± 0.8	2 ± 1.7	1 ± 0.8	0 ± 0.5
Sp/DSG/CyA 72 hr	32 ± 10.6	6 ± 2.3	2 ± 1.8	5 ± 2.6	3 ± 1.8	1 ± 1.0

Discussion

In this study, DSG treatment prolonged xenograft survival in the guinea pig-to-$C6^-$-rat heart transplantation model, although to a limited extent. In DSG-treated animals, the serum levels of IgM xenoantibodies were significantly reduced and the immunoglobulin deposits in the grafts were markedly decreased. However, the xenografts were still heavily infiltrated by macrophages.

As expected, our $C6^+$ rats rejected guinea pig heart xenografts hyperacutely. In contrast, untreated $C6^-$ rats rejected such grafts after 1–2 days. The results confirm previous reports demonstrating that modulation of the complement cascade is a very effective method for preventing hyperacute rejection [3]. In untreated $C6^-$ rats, a weak staining for C3 was still observed on the endothelium. C6 deficiency blocks a late step in complement activation that is common to the alternative and the classical pathways. Thus, in $C6^-$ rats, complement activation through C5 still occurs and the biologically active C3 a and C5 a fragments are generated. These fragments are extremely potent proinflammatory mediators which can activate host leukocytes and facilitate leukocyte binding and injury to xenogeneic endothelium [21]. Furthermore, Lin et al. [15] have shown that in the hamster-to-$C6^-$-rats heart transplantation model, the C5b-9 membrane attack complex can be restored after a few days due to C6 synthesis within the grafts.

In earlier studies in the guinea-pig-to-rat model, cobra venom factor (CVF) has often been used to avoid hyperacute rejection. Treatment with CVF alone prolongs guinea pig xenograft survival up to 3 days. There were no C3 deposits in the CVF-treated animals, and the number of infiltrating macrophages was markedly reduced, as compared to controls [14]. The longer survival may relate to the effect of CVF in reducing participation of C3 a in graft rejection. Hancock et al. [8] showed that in splenectomized rats treated with CVF and DSG, mean graft survival time was 108 h. These results did not differ from ours using $C6^-$ rats, supporting that macrophage activation may occur without C3 a or C5 a participation.

In splenectomized $C6^-$ rats, xenograft survival was twice as long as in untreated $C6^-$ rats. Splenectomy appears to play an important role in xenotransplantation. Carobbi et al. [4] reported that, in the hamster-to-rat heart transplantation model, splenectomy alone resulted in graft survival of 5.6 ± 2.7 days, compared to 3.2 ± 0.9 days in controls. The mechanisms by which splenectomy has its effect in xenotransplantation have not been fully elucidated, but seem related to inhibition of the humoral response. In the present experiment, splenectomy tended to reduce the levels of IgM xenoantibodies in serum. The combination of splenectomy and DSG resulted in a significant decrease. The spleen contains a large number of B lymphocytes and is the major source of antibody. Recent reports indicate that xenografts, compared with allografts, elicit a strong expansion of B cell compartments in the spleen, suggesting that the spleen is a major site for development of the humoral immune response in xenograft rejection [23].

The only drug affecting graft survival in this study was DSG. Previous studies in other models have demonstrated its efficacy in prolonging both allograft and xenograft survival [7, 25]. The precise mechanism of action of DSG is not entirely understood. It has been reported that DSG inhibits macrophage and T and B cell function [9, 12]. In our study, DSG treatment reduced the levels of IgM xenoreactive antibodies in serum. Intragraft deposits of IgG and IgM were also absent in all rats, while grafts from untreated rats had deposits of IgM and IgG on the vascular endothelium. Thus, our results suggest that the beneficial effect of DSG in this model is at least partly due to the suppression of xenoreactive antibodies and especially to IgM production. However, long-term graft survival was not achieved, probably because of the massive macrophage infiltration.

There is increasing evidence of the involvement of macrophages in DXR. Experimental studies have shown that macrophages can reject vascularized xenografts, even in the absence of xenoantibodies, T cells and NK cells [16, 18]. The macrophage-specific monoclonal antibodies ED1 and ED2 used in the present study enable one to study the distribution of various macrophage subpopulations. ED1 is a general macrophage marker that recognizes circulating monocytes

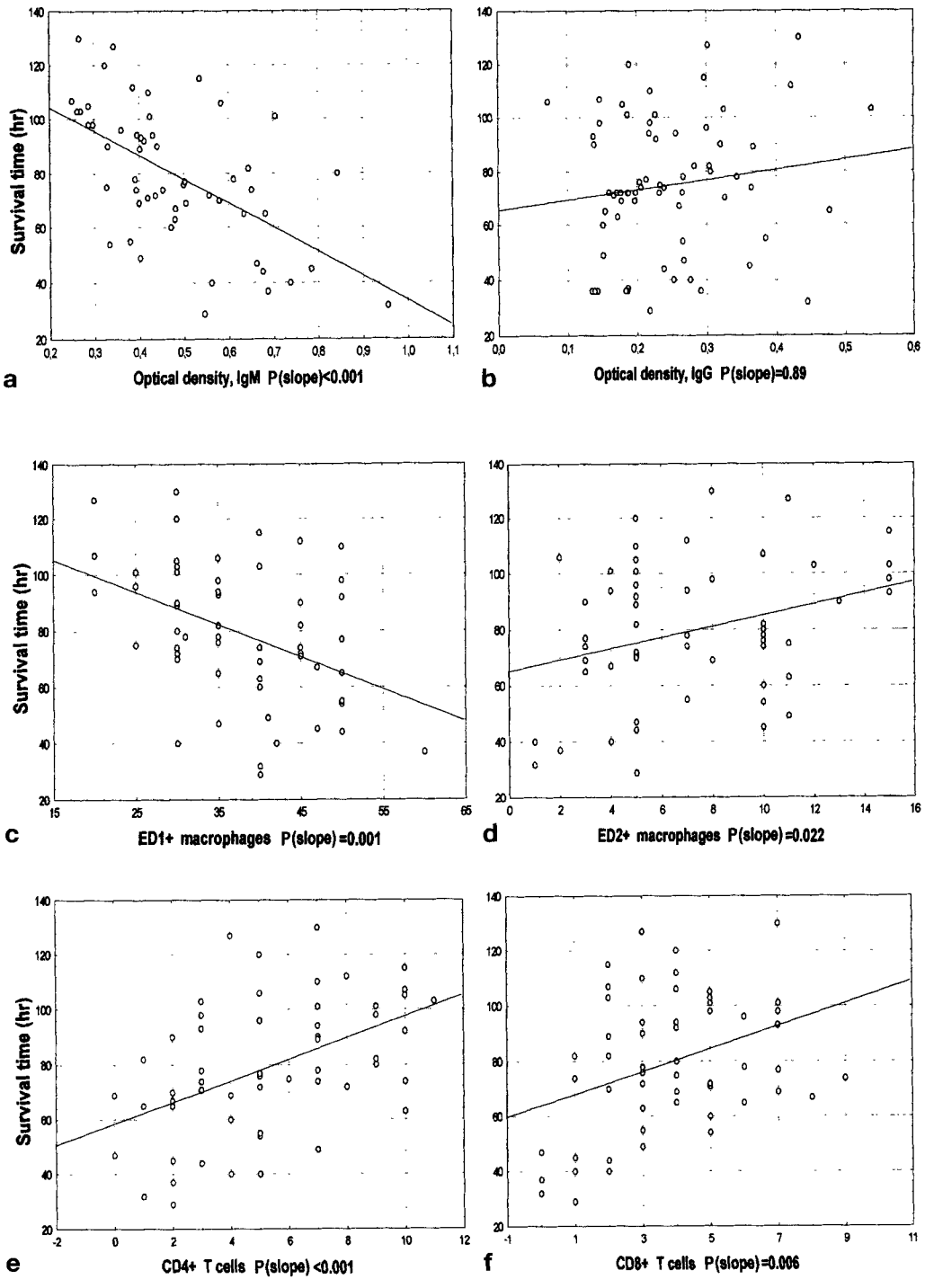

Fig. 3 Relationships between survival time and serum antibody levels (**a**, **b**) and survival time and the numbers of intragraft cells (**c–f**)

and both newly recruited and resident tissue macrophages. ED2 binds to a subpopulation of resident tissue macrophages, probably representing a more differentiated stage [6]. The intense infiltration of macrophages in the present study seems to indicate that macrophages play an active and specific role in the xenorejection process. Regression analysis showed a strong correlation between an increasing number of ED1 + macrophages and decreasing survival time. The opposite was true for the other cell types. An increasing number of ED2 + macrophages was related to increasing survival times. This finding may simply reflect the increased

time for cell differentiation in grafts with longer survival times.

The numbers of CD4 + and CD8 + cells were also inversely associated with survival. If grafts survive beyond DXR, a T-cell-mediated immune response will probably develop [22]. Some investigators believe this response will be suppressed by the immunosuppressive therapies used in allotransplantation, while others claim it will probably be more difficult to overcome. The increasing numbers of CD4 + and CD8 + cells may well reflect the initiation of such an induced response that, with longer graft survival, will contribute to the rejection process. It is interesting to note that this infiltration also occurred in groups treated with DSG combined with the T cell inhibitors CyA or FK 506.

The immunohistological picture in the rejected xenografts was characterized by vasculitis and cellular infiltration. Xenoreactive natural, and possibly elicited, antibodies are believed to play an important role in DXR by binding to the vascular endothelium leading to endothelial cell activation. The activated endothelium loses its ability to inhibit blood coagulation. Cytokine secretion and de novo expression of the adhesion molecules, such as E-selectin, promote the inflammatory process. Macrophages and NK cells can also cause endothelial cell activation, but the process is enhanced if antibodies are present [1]. In our model, macrophage infiltration might have been reduced if measures had been taken to remove rat anti-guinea-pig antibodies before transplantation.

In conclusion, our results indicate that both the humoral and cellular immune systems operate in xenograft rejection in the guinea pig-to-C6$^-$-rat model. DSG combined with splenectomy resulted in moderate prolongation of heart xenograft survival. The beneficial effects seem to be associated with suppression of xenoreactive antibody production. The complexity of xenograft rejection makes it unlikely that any one therapy will be effective, and ultimately combinations of therapies may be necessary.

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