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# Sequential, morphological, and antidonor antibody analysis in a hamster-to-rat heart transplantation model

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**Abstract.** The pathogenesis and the mechanism of accelerated graft rejection in concordant xenotransplantation are unclear. The histopathological features and kinetics neither fulfill the criteria of classic hyperacute rejection nor resemble an accelerated type of first-set allograft reaction. The aim of this study was to investigate the mechanism of concordant xenograft rejection in relation to the early morphological changes in hamster hearts transplanted to unmodified rat recipients by sequential, immunohistological analysis of grafts, regional lymph nodes, and spleens and to correlate these results to the production of antidonor antibodies, as determined by a flow cytometric assay. Histopathological features were characterized by a gradually increasing myocytolysis with fragmentation and loss of myofilaments. The first slight signs were observed a few hours after transplantation. Later, vascular changes developed, evolving into a leukocytoclastic type of vasculitis, eventually with thrombosis. No significant interstitial lymphocyte infiltration was present, but neutrophilic granulocytes and macrophages appeared. In addition, a distinct increase in B cells in spleens and lymph nodes was noted. Low levels of preformed antidonor antibodies did not increase during the first 48 h; however, significant amounts of species-, but not donor-, specific antibodies were demonstrated at the time of rejection. These data, together with the morphological observations, indicate a primarily humoral xenograft rejection in this model. Minor damage to graft myocytes a few hours after transplantation, progressing to vascular changes within 24–48 h, further suggests that preformed antidonor antibodies directed against endothelial or myocyte determinants may play an initiating role in the pathogenesis of unmodified, concordant xenograft rejection.

**Key words:** Xenotransplantation, hamster-to-rat – Antidonor antibodies, in xenotransplantation – Xenograft rejection, hamster-to-rat

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Accelerated xenograft rejection is one of the main characteristics of concordant xenotransplantation between different combinations of species [1]. Corcordant – as opposed to discordant – was originally defined by Calne [3] to describe a species combination in which grafts are rejected at a tempo and with morphological characteristics similar to first-set allografts. Concordant further implies the absence of preformed antidonor antibodies.

Hamster-to-rat heterotopic cardiac transplantation has been studied by several groups as a model of concordant xenograft rejection [2, 5, 9, 10, 12, 13, 18, 19]. Graft survival data from these studies, including different treatment modalities, are not always consistent. Some groups achieved moderate to significantly prolonged graft survival with cyclosporin A (CyA) alone [10, 12], while in other reports CyA (up to toxic doses) could not improve graft survival [15, 19]. The combined treatment of CyA with total lymphoid irradiation (TLI) resulted in long-term graft survival for Knechtle et al. [12, 13]; however, these results could not be reproduced by others [5, 19]. Some groups found preformed lymphocytoxic antibodies [9, 10], while others were unable to detect antidonor antibodies [12, 15].

It is unclear whether the mechanism responsible for accelerated graft rejection in concordant xenogeneic combinations is the result of antibody-mediated rejection, the end effect of cell-mediated mechanisms of quantitatively stronger, but in principal identical, response to allogeneic rejection, or a qualitatively different mechanism.

Rosengard et al. showed, in a detailed, serial, histological analysis [17] using concordant cardiac transplantation in a hamster-to-rat combination, that neither the rejection tempo nor the morphological features of graft rejection resembled those observed in first-set allograft rejection. The histological observations indicated a significant role for induced humoral immune response. Recently, Thomas et al. demonstrated that T-cell-deficient nude rats rejected hamster hearts at the same tempo and with analogous histological features as recipients with normal T- and B-cell responses [21].

The aim of this study was to investigate the mechanism of concordant xenograft rejection in a sequential study of

the very early morphological changes of hamster hearts transplanted to unmodified rat recipients, including immunohistological analysis of grafts, regional lymph nodes, and spleens, and to correlate these results with the production of antidonor antibodies, as determined by a flow cytometric assay.

## Materials and methods

### *Animals*

Outbred Spraque-Dawley rats (SPF, from Møllegard Breeding Center, Copenhagen, Denmark) served as recipients and inbred Syrian hamsters (albino variant, from the Department of Odontological Research, Göteborg University, Göteborg, Sweden) as donors. Lymphocytes from inbred Chinese hamsters and inbred LEW rats were used as control target cells for flow cytometry. All animals were maintained under conventional standard laboratory conditions and received humane treatment.

### *Surgical procedure*

Concordant heart xenotransplantations were performed using a modified heterotopic model. Briefly, donor hearts were harvested after being flushed with 5 ml aqueous heparin solution (50 IU/ml). End-to-side anastomosis with 9-0 running sutures was performed between the donor aorta and the recipient aorta abdominalis and between the donor pulmonary artery and the recipient inferior vena cava. In contrast to the original description by Ono and Linsey, the heart was turned 180 degrees – with the apex pointing to the right – resulting in our experience in an improved venous outward flow [19].

### *Experimental design*

Graft recipients were sacrificed 4–6 h (group 1, $n = 9$), 24 h (group 2, $n = 10$), or 48 h (group 3, $n = 7$) after transplantation. In an additional group (group 4, $n = 5$), the graft recipients were observed until spontaneous cessation of graft function (day 3 or 4).

### *Histology*

Heart grafts, spleens, and regional para-aortic lymph nodes were harvested for morphological examination. Histological procedures included routine hematoxylin and eosin staining of formalin-fixed tissue. Tissue for immunohistological examination was snap-frozen in isopentane, cooled by dry-ice, and blocked in Tissue Tek before 4-μ sections were cut at −24°C. Immunohistology was performed using an ABC complex/HRP staining technique. Sections were fixed in acetone for 10 min at room temperature, air-dried, and incubated with normal horse serum, diluted 1:5 in TRIS-buffered saline (TBS, pH = 7.4). After TBS removal, the sections were incubated for 30 min at room temperature with optimal dilutions of murine anti-rat monoclonal antibodies, consisting of MRC OX-8 (anti-CD8), MRC OX-19 (anti-CD5), MRC OX-38 (anti-CD4), MRC OX-39 (anti-IL-2R), and anti-kappa (Sera Lab) [4, 11, 16]. The MRC OX antibody hybridoma cell lines were a kind gift from Dr. A. F. Williams, Oxford, UK.

As secondary antibody, biotinylated horse anti-mouse IgG antibodies (Vector BA-2001), diluted 1:200 in 0.2% rat serum (TBS), were used. They were incubated for 30 min at room temperature, followed by ABC complex/HRP (Dakopatt no. K355) and peroxidase substrate solution (carbazole) incubation. The sections were then rinsed for 10 min with distilled water and counterstained with Mayer's hematoxylin.

In order to exclude possible crossreactivity of the different antibodies used, control incubation procedures – with or without primary or secondary antibodies – were routinely performed.

### *Flow cytometry*

Target cells were donor lymphocytes (DL) from spleens or lymph nodes. Lymph node and spleen tissue was chopped into RPMI 1640 (Gibco), squeezed in a syringe, and incubated for 30 min at 37°C with 0.5 mg/ml collagenase (Sigma C-1030) and 0.5 mg/ml hyaluronidase (Sigma H-3506) [20]. DL were separated by centrifugation of the supernatant over lymphopaque, density 1.086 g/ml (Nyegaard, Oslo, Norway). Mononuclear cells were recovered from the interphase layer and washed three times. Erythrocytes were shock-lysed with sterile water during the second wash.

*Antibody assay.* Donor mononuclear cells ($2 \times 10^5$) in 100 μl RPMI 1640 were incubated for 30 min at room temperature with 15 μl of recipient serum (dilutions 1:1, 1:4, and 1:16). After two washes, 50 μl FITC-conjugated rabbit-anti-rat Ig + antibody (Dakopatts), diluted 1:25, was added. The cells were incubated for 20 min at 4°C, washed twice, and resuspended in 300 μl of Hanks solution, ready for analysis.

Control sera consisted of the donors' own sera, and lymphocytes from Chinese hamsters and Lewis rats, prepared in the way described above, served as control target cells.

Flow cytometric analysis was performed using an Epics Profile Counter. Lymphocytes were gated according to forwad and 90° side scatter parameters. Then, $10^4$ cells/sample were counted and the percentage of FITC-positive cells and their mean channel number (MCN) was determined on a logarithmic scale from 1 to 256.

*Data analysis.* Ninety-five percent confidence limits were calculated on the basis of test runs with donors' own sera. The results were calculated as the ratio between percentage of positive cells from test sera and percentage of positive cells from controls. Values greater than 1.4 (defined by the 95% confidence limits) were considered as significantly increased antibody levels.

## Results

### *Histology*

*Group 1.* Four to six hours after transplantation, hamster heart grafts were characterized by slight interstitial edema and focal areas with myocytolysis with fragmentation and loss of myofibrillar elements, but with preserved sarcoplasmatic membranes (Fig. 1). No necrosis or hemorrhage was observed. Capillaries were normal, apart from slight endothelial swelling. Small and larger arteries showed no morphological changes and no cellular infiltration was seen.

*Group 2.* The type of morphological changes found 24 h after transplantation were essentially the same as those in group 1, but with increased interstitial edema, more widespread areas with myocytolysis, and eventually with small foci of necrotic cells. Vascular changes were characterized by perivascular edema, endothelial swelling, and moderate granulocyte infiltration in the vessel walls (Fig. 2).

*Group 3.* Forty-eight hours after transplantation, vascular changes were predominant with edema and focal necrosis of vessel walls with leukocyte infiltration, resembling a leukoclastic lesion (classified as leukoclast-like vasculitis; Fig. 3). In addition, scattered interstitial infiltration with granulocytes was observed, and in both capillaries and minor arteries, exfoliation of endothelial cells as well as thrombosis appeared.

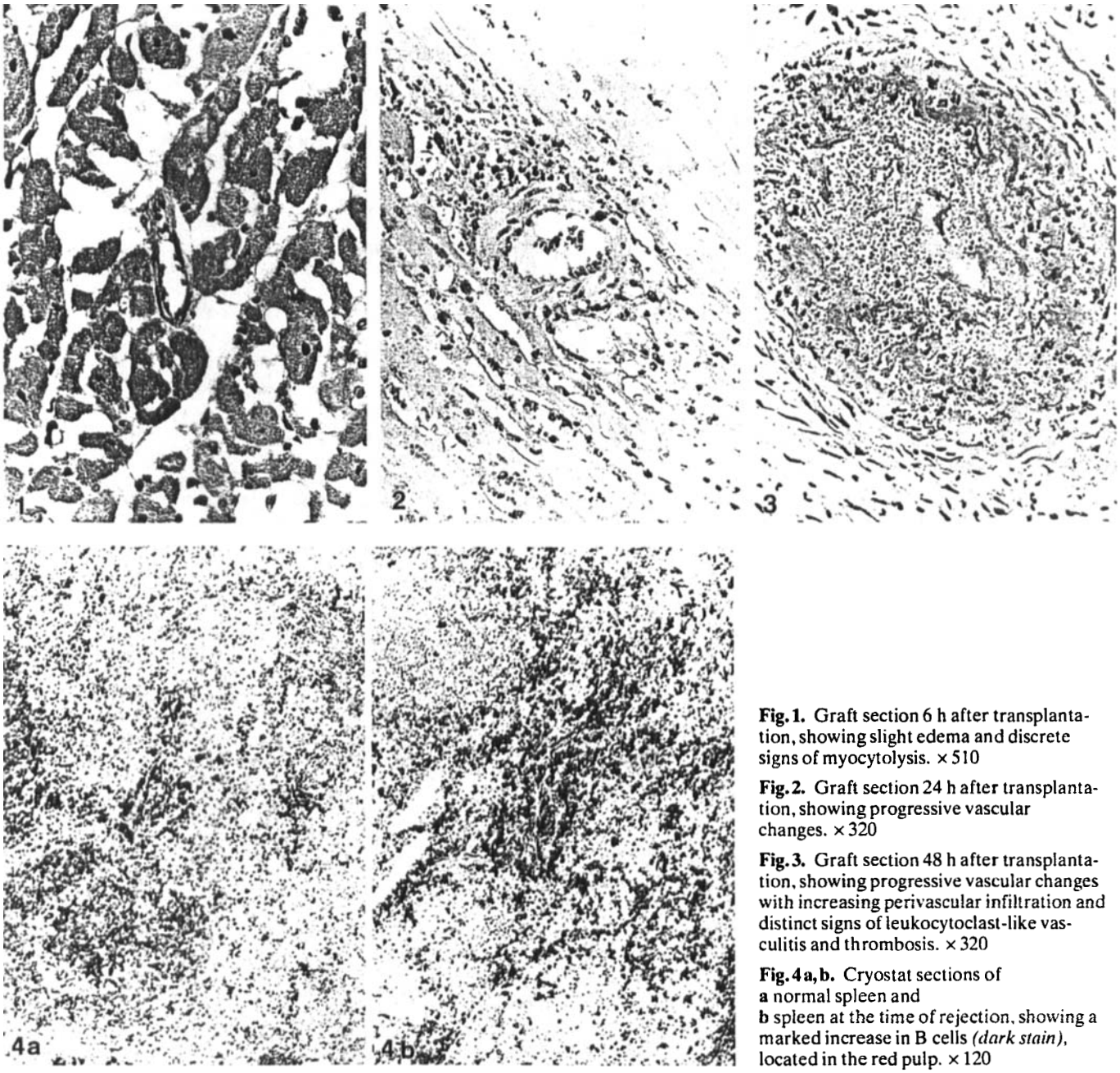


**Fig. 1.** Graft section 6 h after transplantation, showing slight edema and discrete signs of myocytolysis. × 510

**Fig. 2.** Graft section 24 h after transplantation, showing progressive vascular changes. × 320

**Fig. 3.** Graft section 48 h after transplantation, showing progressive vascular changes with increasing perivascular infiltration and distinct signs of leukocytoclast-like vasculitis and thrombosis. × 320

**Fig. 4 a, b.** Cryostat sections of
**a** normal spleen and
**b** spleen at the time of rejection, showing a marked increase in B cells *(dark stain)*, located in the red pulp. × 120

*Group 4.* Rejected grafts were characterized by total acute infarction, pronounced subendocardial and epicardial inflammation, and vascular rejection with endothelial cell proliferation and granulocyte infiltration in the vessel walls.

Infiltration with mononuclear lymphocytes was almost absent, and the few lymphocyte-like cells present could not be positively stained by immunohistological analysis of heart grafts when using the antibodies described above. *Spleens and lymph nodes.* In general, the morphological changes were slight, and no essential differences were found between groups 1, 2, and 3. However, lymph nodes and mainly spleens from graft recipients at the time of rejection (group 4) showed a distinct shifting of T-cell subsets and the number of B cells. Apart from slight acute congestion, spleen architecture was normal. T-cell areas showed a moderate decrease in CD5 + cells. CD4 + cells, which in normal spleens constitute 75 %–90 % of T cells, were reduced to 25 %–50 %. The number of CD8 + cells increased from 25 %–50 % to 50 %–75 %, the percentage of IL-2R + cells from 1 %–5 % in normal spleens to 5 %–25 %. The number of B cells was markedly increased (Fig. 4).

*Flow cytometric antidonor antibody analysis*

While sera from all graft recipients before transplantation showed a moderate increase in the percentage of Ig + target cells (average ratio 1.33, range 1.18–1.51) versus do-

nors' own sera, the mean channel number (MCN) was unchanged, indicating a low level of preformed, unspecific, antidonor antibodies. On days 1 and 2 after transplantation, antidonor antibody levels, like MCN, remained unchanged. However, at the time of rejection, the ratio between positive cells after incubation with test sera and donor controls increased to an average of 1.98 (range 1.88–2.12), and a shift of MCNs of 38 on a logarithmic scale was noticed (usually a channel shift of more than 10 is considered to be significant [8]), demonstrating a significant production of antidonor antibodies. Expressed in terms of Ig + cells, significant numbers of antidonor antibodies were demonstrated on 78%–88% of donor lymphocyte surfaces at the time of rejection. Weak, unspecific staining due to background crossreactivity was found on 35%–41% of donor lymphocytes when incubated with autologous test sera.

Analogous incubations with target cells from Chinese hamsters showed no preformed antibodies (ratio 0.99, range 0.91–1.15). Test sera from rejecting graft recipients increased the number of positive target cells to a ratio of 1.27 (range 1.11–1.39) and an average MCN increase of 21, indicating a significant interspecies crossreactivity of these antibodies.

Control incubations with Lewis rat lymphocytes as target cells showed no evidence of anti-rat reactivity.

## Discussion

The pathogenesis and the mechanism of accelerated graft rejection in concordant xenotransplantation are unclear. The histopathological features and the kinetics of rejection neither fulfill the criteria of classic hyperacute rejection [6] nor resemble an accelerated type of first-set allograft reaction [7]. In a morphological, sequential analysis, Rosengard et al. [17] suggested that humoral immunity is largely responsible for acute xenograft rejection. This suggestion was recently confirmed by Thomas et al. [21], who demonstrated that nude, T-cell-deficient rats rejected hamster hearts at the same tempo as immunologically unmodified graft recipients.

Our results confirm these data, in which the histopathological features of concordant xenograft rejection in this hamster-to-rat model were primarily characterized by a gradually increasing myocytolysis with fragmentation and loss of myofilamentary elements, with the first signs of damage appearing 6 h after transplantation. Subsequent vascular changes progressed to a leukocytoclastic type of vasculitis, eventually with thrombosis. The very early morphological changes related to cardiomyocytes seemed to be rejection-specific, since similar features were not found in control, syngeneic cardiac transplantation after simple, cold cardioplegic ischemia. This is in accordance with Lindal et al. [14], who found that rat hearts, after 2 h of cold cardioplegic ischemia, followed by reperfusion for 45–60 min, showed no ultrastructural myocyte damage apart from slight intracellular edema.

Spleens and regional lymph nodes from rejecting animals showed a slight decrease in CD4- and CD5-positive T cells, a moderate increase in CD8- and IL-2R-positive cells, and a marked increase in B cells. The significance of these shifts in lymphocyte populations is unclear but suggests a possible antidonor antibody production in recipient spleens and lymph nodes. This assumption is indirectly confirmed by data on improved graft survival and reduced antidonor antibody formation after splenectomy in a cardiac hamster-to-rat model [15]. However, we have recently shown that anti-CD4 monoclonal antibody therapy, in combination with cyclosporin A, can improve graft survival significantly in this model if graft recipients are preoperatively conditioned with total lymphoid irradiation [20]. This indicates that cellular immunity may influence the long-term outcome of graft survival in recipients with strongly impaired immune function.

The question of the existence and role of preformed antibodies in the hamster-to-rat model is controversial, possibly due to differences in donor and recipient combinations, as our results demonstrate. Some groups have found preformed lymphocytotoxic antibodies [9, 10] while others have not [12, 15]. The flow cytometric assay has proven to be a very sensitive method of detecting antidonor antibodies [8], and we were able to demonstrate moderate levels of preformed antibodies using this model. The antibody production did not increase during the first 48 h after transplantation; however, at the time of rejection (day 3 or 4), significant levels of specific antilymphocyte antibodies were found. These antibodies reacted with target cells from a different hamster strain as well, indicating that antibody reactivity was species-, but not donor strain-, specific, a fact which is usually not focused upon.

Moreover, target cells in antibody assays are almost exclusively lymphocytes, focusing on MHC incompatibility expressed on cell surfaces. This seems appropriate in allogeneic models, but in xenogeneic combinations, MHC incompatibility may play a less dominant role.

Even if our results do not directly demonstrate that species-specific antibodies, directed against donor lymphocytes, are responsible for graft rejection, the histopathological features indicate a primarily humoral xenograft rejection in this model. Damage to graft myocytes and vascular changes a few hours after transplantation further suggest that preformed antidonor antibodies against endothelial or myocyte determinants as direct target may play an initiating role in the pathogenesis of unmodified concordant xenograft rejection.

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