Studies of Rejection in Experimental Xenotransplantation

BY

TOMAS LORANT
ABSTRACT

One main hurdle to xenotransplantation, i.e. transplantation between different species, is the immunological barrier that the organ meets in the recipient. The aim of this thesis was to characterise xenogeneic rejection mechanisms by using the concordant mouse-to-rat heart transplantation model.

Graft-infiltrating immune cells could be isolated from both rejecting and non-rejecting grafts using ex vivo propagation, a technique based on incubation of graft biopsies in culture medium for 48 hours. The numbers of recovered T lymphocytes were considerably higher in grafts undergoing cell-mediated rejection than in grafts undergoing acute vascular rejection (AVR) or in non-rejecting transplants. Thus, ex vivo propagation should be a valuable tool for further studies of cell-mediated rejection.

Cytokine patterns in the grafts, as measured by a quantitative real-time RT-PCR method, showed that AVR and cell-mediated rejection are associated with an increase of both pro-inflammatory cytokines (IL-1β and TNF-α) and more specific cytokines (IL-2, IL-10, IL-12p40 and IFN-γ). These data differed considerably from the patterns seen in the spleens of the recipients. Cell-mediated xenograft rejection was also found to be associated with a local accumulation of hyaluronan.

Oral administration of xenogeneic cells stimulated a production of antibodies that could induce hyperacute rejection of cardiac xenografts when passively transferred to graft recipients. This is in contrast to several models for autoimmune diseases and allogeneic transplantation where oral administration of antigens is an effective way to induce unresponsiveness. Hence, future attempts to induce oral tolerance in xenotransplantation should be done with caution.

Key words: Antibodies, cell culturing, cytokines, hyaluronan, immunosuppression, mouse, rat, rejection, T lymphocytes, xenotransplantation.
There is nothing more deceptive than an obvious fact

Sherlock Holmes
in “The Boscombe Valley Mystery”
by Sir Arthur Conan Doyle
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I  Isolation of mouse-to-rat cardiac xenograft-infiltrating cells by ex vivo propagation.
   Lorant T, Engstrand M, Tufveson G, Johnsson C.

II Intragraft cytokine mRNA-expression in rejecting and non-rejecting vascularised xenografts.
   Lorant T², Krook H², Wilton J, Olausson M, Tufveson G, Korsgren O, Johnsson C.
   *Xenotransplantation, in press*¹.

III Oral administration of xenogeneic erythrocytes induces production of antibodies that are capable of inducing hyperacute rejection of concordant vascularised xenografts.
   Lorant T, Wilton J, Olausson M, Tufveson G, Johnsson C.
   *Submitted*.

IV The graft content of hyaluronan is increased during xenograft rejection.
   Lorant T, Tufveson G, Johnsson C.
   *Submitted*.

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²The first two authors contributed equally to this work.
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<tr>
<td>AEC</td>
<td>3-Amino-9-ethyl-carbazole</td>
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<td>APC</td>
<td>Antigen-presenting cell</td>
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<td>AVR</td>
<td>Acute vascular rejection</td>
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<td>bw</td>
<td>Body weight</td>
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<tr>
<td>C'</td>
<td>Complement factor</td>
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<td>CD</td>
<td>Cluster designation (cluster of differentiation)</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>CyA</td>
<td>Cyclosporine A</td>
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<td>DSG</td>
<td>15-Deoxyspergualin</td>
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<td>dw</td>
<td>Dry weight</td>
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<td>FBS</td>
<td>Foetal bovine serum</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FSC</td>
<td>Forward scatter</td>
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<td>Gal-antigen</td>
<td>Galα1-3Galβ1-4Glc-NAc-R</td>
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<td>H-2</td>
<td>Mouse major histocompatibility complex</td>
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<td>HABP</td>
<td>Hyaluronic acid binding proteins</td>
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<td>HAR</td>
<td>Hyperacute rejection</td>
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<td>hDAF</td>
<td>Human decay accelerating factor</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>Interleukin</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>NK</td>
<td>Natural killer</td>
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<td>PAP</td>
<td>Peroxidase-antiperoxidase</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PE</td>
<td>Phycoerythrin</td>
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<td>RT</td>
<td>Reverse transcriptase</td>
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<td>RT1</td>
<td>Rat major histocompatibility complex</td>
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<td>SSC</td>
<td>Side scatter</td>
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<td>Th</td>
<td>T helper</td>
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<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>ww</td>
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INTRODUCTION

For several hundred years scientists have been fascinated about the possibility of moving whole organs, tissues or cells from one species to another, so-called xenotransplantation (Auchincloss and Sachs 1998). Back in those days it was used as a last alternative for people who had no other chance to recover from states of illness such as blood loss or kidney failure. In heroic attempts, foreign tissues such as blood from sheep, skin from frogs and kidneys from pigs were transplanted to humans. Later, during the end of the 19th century and beginning of the 20th century, several attempts of xenotransplantation were well documented. The organs transplanted involved sheep pancreas tissue as well as kidneys from several, widely disparate donor animals (White and Nicholson 1999). These transplants survived only for a very short time.

Nowadays, with a successful human-to-human transplantation programme the demand for xenogeneic organs has become more sophisticated. People with terminal failure of kidneys, liver, pancreas, heart or lungs are today successfully treated with human-to-human transplantation, i.e. allogeneic transplantation. However, many patients in need of a new organ have to wait for a long time before transplantation due to the lack of human donors (Grunnet et al. 2001). Patients waiting for a kidney transplant might be treated with dialysis, but those waiting for other organs have no equivalent alternative. With this background, other ways are needed to increase the organ supply. Several approaches have been seriously investigated and xenotransplantation appears to be one possible solution.

The animals that phylogenetically would suit well as donors are Old World monkeys (rhesus monkey, cynomolgous monkey and baboon), chimpanzee, gorilla, orang-utan and gibbon (Galili et al. 1987, Galili et al. 1988b). However, many ethical questions have been raised against the use of the monkey as donor. Additionally, their low reproduction rate with relatively long pregnancy and small litters is also a limiting factor. The general opinion, today, is that the best suited animal for organ donation is the pig. The pig is more phylogenetically distant from humans than the monkey, but its reproduction rate is high, and it is well accepted as a breeding animal by the general public.

To gain long-time survival of xenogeneic transplants one has to overcome several obstacles, some of which are discussed below.
HURDLES TO XENOTRANSPLANTATION

Several of the obstacles to xenotransplantation are related to the different biology of disparate species. As a result, organs differ in anatomical structure, size, orientation and morphology, factors that might affect the overall function of the organ in the recipient. The size of donor organs, such as heart and lung, has to be equivalent to the organs they are replacing since the size differences would otherwise alter the physiological characteristics in the recipient as well as complicate surgery. Additionally, e.g., even if a pig lung is of appropriate size for a human, it is naturally oriented in the horizontal level. When transplanted to a human it would be upright oriented, therefore affecting overall respiration (Hammer 1998).

Based on physiology, other factors of the donor organ also have to correspond to the recipient to ensure that vital functions are working properly. Blood pressure, temperature regulation and oxygen uptake do not differ much between mammals; however, important substances such as haemoglobin, interleukins and albumin are clearly divergent between species (Hammer 1998).

Another obstacle is the fear of transmission of pig endogenous retroviruses (PERV) to human cells (Weiss 1998), which theoretically could produce new viruses infecting other human beings. PERVs have the potential to infect human cells in vitro (Patience et al. 1997), yet no viruses have been detected in humans (Paradis et al. 1999) or primates (Loss et al. 2001) receiving pig transplants.

Further, several ethical aspects of using animal organs for donation have been discussed (Vanderpool 1998, Melo et al. 2001).

Another hurdle to xenotransplantation of considerable importance is the immunological barrier that the organ meets in the recipient after transplantation, as reviewed in (Auchincloss and Sachs 1998, White and Nicholson 1999, Cascalho and Platt 2001, Sachs DH et al. 2001). Summarised, it is based on the recognition of the organ as foreign by the recipient’s immune system. The humoral and cellular immune systems are involved at different stages in the course of events. These defence mechanisms result in the destruction of the organ and are termed hyperacute rejection (HAR) (Kissmeyer-Nielsen et al. 1966), acute vascular rejection (AVR) (Platt et al. 1998) (sometimes referred to as delayed xenograft rejection (Bach et al. 1996)), cell-mediated rejection (Platt 2002) and chronic rejection (Shen et al. 1998), and are further discussed below.
REJECTION OF VASCULARISED XENOGRAFTS

Hyperacute rejection (HAR)

After the transplantation of a vascularised organ, the first immunological obstacle is the HAR (Figure 1) (Kissmeyer-Nielsen et al. 1966, Perper and Najarian 1966a). The onset is immediate after the revascularisation, and it destroys the organ within minutes to hours. Transplantation models where HAR occurs are called discordant, whereas models where it does not occur are called concordant (Calne 1970). Complement activation is one important component in HAR. The activation is mediated either by naturally occurring xenoreactive antibodies (e.g. in pig-to-human or pig-to-baboon transplantation) or directly through the alternative pathway (e.g. in guinea pig-to-rat or pig-to-dog transplantation). Preformed antibodies bind mainly to endothelial cells (Platt et al. 1991) and initiate damage of the endothelium, interstitial haemorrhage, oedema and platelet aggregation in smaller vessels (Rose et al. 1991). Additionally, the polysaccharide heparan sulphate is released from the endothelial cells, which leads to increased coagulation and complement binding (Platt et al. 1990). HAR can be prevented in complement factor (C’)6-deficient mice (Brauer et al. 1993) or by treating recipients with cobra venom factor, a C3c analogue that depletes complement (Gewurz et al. 1967, Miyagawa et al. 1988, van den Bogaerde et al. 1991). Moreover, HAR may also be inhibited in primates (and probably also in humans) by transplanting organs from transgenic pigs expressing the human decay accelerating factor (hDAF) and CD59 on the cell surfaces (McCurry et al. 1995, Waterworth et al. 1997). These factors are complement regulatory proteins that protect the tissue from complement-mediated destruction.

The carbohydrate Galα1-3Galβ1-4Glc-NAc-R (Gal-antigen) is the most important antigen on pig cells against which humans have natural antibodies (Galili et al. 1984). These antibodies consist of immunoglobulin (Ig)M (Parker et al. 1994), IgG (Parker et al. 1994, Galili et al. 1995) and IgA (Hamadeh et al. 1995), where IgM is the primary antibody to induce complement activation (Sandrin et al. 1993).

Acute vascular rejection

In concordant models or when preventing HAR in discordant models, the grafts succumb due to an AVR (Figure 1) (Perper and Najarian 1966b, Magee et al. 1995) approximately one to five days post transplantation (Gannedahl et al. 1990, Valdivia et al. 1990, Lin SS et al. 1998). This is a process that is mainly mediated by the binding of antibodies to the graft endothelium. The endothelial cells are activated and increase their production of interleukin (IL)-1α, which further enhances the production of tissue factor. This leads to intravascular
thrombosis. Histologically, the grafts have thickened vessel walls, endothelial cell damage and thrombosis. Most parenchymal cells are well preserved. However, a small halo of tissue destruction and oedema surrounds most of the blood vessels, and a severe haemorrhage is seen in the interstitium. These processes are distinguished from the HAR since complement factors appear to be of low importance (van den Bogaerde et al. 1991, Brauer et al. 1993, Leventhal et al. 1993). There is, however, an activation of systemic complement at this time (Loss et al. 2000).

AVR is possible to overcome by using immunosuppressive drugs, such as 15-deoxyspergualin (DSG) (Gannedahl et al. 1990, Valdivia et al. 1990) or cyclophosphamide (Hasan et al. 1992, Murase et al. 1993), which prevent antibody formation. Furthermore, plasmapheresis has proved efficient to avoid AVR (Lin SS et al. 1998).

Circulating xenospecific antibodies do, however, not always have the capacity to reject the transplant. This phenomenon is termed accommodation (Soares et al. 1999) and was first observed in patients receiving ABO-mismatched kidney transplants (Alexandre et al. 1987). Although anti-blood group antibodies returned after plasmapheresis, the grafts continued to function. In small animal models such as the mouse-to-rat (Koyamada et al. 1998) and hamster-to-rat (Bach et al. 1997), accommodation may be induced by complement depletion in combination with T lymphocyte inhibition. These grafts survive in the presence of xenospecific IgM.

![Figure 1. Immunological obstacles to xenotransplantation.](image)

**Cell-mediated rejection**

When successfully inhibiting AVR, the next immunological barrier is the cell-mediated rejection (Figure 1). It has an early onset with infiltration of leukocytes 12 to 24 hours after the revascularisation of the graft (Steinbrüchel et al. 1992, Blakely et al. 1994). The infiltration gradually increases and culminates between one and two weeks after the transplantation. In many xenotransplantation models, the dominating cell-type is the ED1-positive (ED1+) macrophage (Blakely et al. 1994, Wallgren et al. 1995, Karlsson-Parra et al. 1996, Nagayasu

Of great importance is also the T lymphocyte, which is the second most abundant cell type during cell-mediated rejection. Both CD4-positive (CD4+) and CD8-positive (CD8+) T lymphocytes can activate rejection of vascularised xenografts (Fryer et al. 1995, Loudovaris et al. 1996, Lin Y et al. 1999, Obatake et al. 1999) and non-vascularised xenografts (Pierson et al. 1989, Click et al. 1994). Several experiments with transfer of these cell types, alone or in combination, induced rejection in T cell-deficient rats. Further, the transfer of CD4+ or CD8+ cells induced accelerated rejection of guinea pig heart grafts in splenectomised rats treated with cobra venom factor (Fryer et al. 1995).

Different T lymphocytes can also be distinguished based on their production of cytokines. The so-called T helper (Th)1 cells mainly produce IL-2 and interferon (IFN)-γ, whereas Th2 cells mainly produce IL-4, IL-5, IL-10 and IL-13 (Holgersson et al. 2002). In the hamster-to-rat heart transplantation model a Th1-associated cytokine response was found during a cell-mediated rejection, whereas a Th2-response was associated with accommodation and long-time survival (Bach et al. 1997, Soares et al. 1999). When cells producing Th2-associated cytokines, isolated on day 20 from accommodated hamster grafts, were transferred to newly xenotransplanted rats, the grafts became more resistant against rejection, increasing the percentage of grafts surviving for a long time (Wang N et al. 2001). These findings are somewhat contradicted by other works in the hamster-to-rat model where a Th2-associated cytokine response was absent during long-time survival (Brouard et al. 1998, Sebille et al. 2001).

Other immune cells are also highly involved in the cell-mediated rejection. Even if natural killer (NK) cells are found only in small numbers, they can reject hamster grafts transplanted to rats in the absence of T lymphocytes (Lin Y et al. 1997a). This rejection is as fast as rejection in the presence of T lymphocytes.

**Chronic rejection**

Chronic rejection in xenotransplantation (Figure 1) has only been briefly investigated. In long-time surviving mouse-to-rat heart grafts, several blood vessels had signs of intimal thickening, probably due to an ongoing vasculitis (Bersztel et al. 1998). Aortic hamster grafts transplanted to rats presented thickening of the intima, media and also the adventitia. The accelerated arteriosclerosis occurred extremely rapidly, faster than accelerated arteriosclerosis in allogeneic aortic grafts (Scheringa et al. 1996).

Recently, antibodies were found to contribute to the development of chronic rejection in allogeneic mouse cardiac grafts (Hancock et al. 1998). It can only be speculated whether this occurs in a xenogeneic situation. However,
since antibodies are highly involved in the induction of HAR and AVR, it is probable that they also will be significant in chronic xenograft rejection.

REJECTION OF CELLULAR XENOGRAFTS

The transplantation of xenogeneic pancreatic islets is most often done by the injection of the cells in the portal vein or under the kidney capsule of the recipient. These grafts are revascularised by angiogenesis of recipient endothelium, in contrast to vascularised xenografts where the donor blood vessels are directly connected to the recipient’s blood system (Simeonovic 1999). Injection of islets in the blood system triggers an instant blood-mediated inflammatory process, characterised by rapid consumption and activation of platelets, and activation of the coagulation and complement systems (Bennet et al. 2000).

The immediate revascularisation of vascularised xenografts leads to binding of preformed or newly produced antibodies to the graft endothelium, which initiates HAR or AVR. Since cellular implants are revascularised with recipient endothelium, they are not subjected to a similar antibody response. In fact, the anti-serum raised after acute rejection of cellular transplants cannot reject established islet transplants in recipients under anti-CD4 treatment (Simeonovic et al. 1998). Further, rejection of porcine islets in Ig µ-chain knockout mice is as fast as in the control mice (Benda et al. 1996). This indicates that antibodies do not have an important role in rejection of xenogeneic islet xenografts.

The B lymphocyte unresponsiveness that may be induced in vascularised heart xenotransplantation does not protect pancreatic islets from rejection (Bersztel et al. 2000). This lack of unresponsiveness may be due to differences in antigens expressed on the cell surfaces of the different tissues, or to the fact that an endothelial lining is necessary for the induction of unresponsiveness.

In rodents, the cell-mediated rejection of xenogeneic islet transplants is mainly mediated by T lymphocytes (Wilson et al. 1989, Benda et al. 1998) and macrophages (Wallgren et al. 1995). Also, eosinophils (Simeonovic et al. 1997) and NK cells (Karlsson-Parra et al. 1996) infiltrate the graft, but these cell types appear to have a less important role in rejecting the islets. Early in the rejection, the Th1-associated cytokines IL-2, IL-12p40 and IFN-γ heavily increase, and are followed by a Th2-response that coincides with the infiltration of eosinophils (Krook et al. 2002).
Further understanding of the problems causing xenograft loss, together with the introduction of the first immunosuppressive drugs, i.e. azathioprine and steroids, prepared the way for new attempts of xenotransplantations. In the 1960s, a new era started with clinical efforts to transplant foreign organs to humans. The transplantations were done mainly in a few individuals, and not in larger series. The first operations were transplantations of chimpanzee kidneys to humans, where one graft survived as long as nine months before the patient died, with the only pathological finding of electrolytic imbalance (Reemstma et al. 1964). Shortly after, six baboon kidney transplantations to humans were performed, with graft survival up to two months (Starzl et al. 1964).

In the late 1970s, the introduction of cyclosporine A (CyA) presented new hope for immunosuppression in xenotransplantation. A baboon heart transplanted to a child survived for 20 days (Bailey et al. 1985), while a baboon liver survived for 70 days in a patient treated with FK506 (Starzl et al. 1993). Later on, several transplantations of foetal porcine islets to humans were performed (Groth et al. 1994). In four of ten patients C-peptide production continued for several hundred days, and in one patient, intact islets were identified both morphologically and immunohistochemically; however, no patient became free from exogenous insulin. There was also an indication that foetal pig neural cells can survive in a human (Deacon et al. 1997). Recently, it was reported that one patient had become free from insulin treatment after transplantation of foetal porcine islets (Valdes-Gonzalaes et al. 2002).

To achieve long-time survival of vascularised organs, the genome of the pig most likely has to be modified in several ways to inhibit the expression of important xenogeneic antigens. Today, this can be achieved by so-called nuclear transfer (Polejaeva et al. 2000). Knock-out pigs lacking the enzyme α1,3-galactosyltransferase that is involved in the synthesis of the Gal-antigen are bred in ongoing research (Lai et al. 2002). However, it is probably not enough only to remove the most important xenoantigens. The removal of an important antigen could result in an increased antibody response against other antigens, which are not important in the non-transgenic animal. In such a scenario, HAR, but not AVR, would probably be overcome.

To induce long-time survival of xenografts, one also has to inhibit the cell-mediated and the chronic rejection. To study the underlying mechanisms of xenograft rejection, established small animal models such as the mouse-to-rat, the hamster-to-rat and the guinea pig-to-rat heart transplantation models are more practical than the large animal models.
MOUSE-TO-RAT HEART TRANSPLANTATION MODEL

The heterotopic transplantation of a mouse heart to a rat was originally described in a work by Gannedahl et al (Gannedahl et al. 1990). Surgically, the mouse heart is connected to the neck vessels of the rat by a non-suture technique (Olausson et al. 1984). Immunologically, this model is concordant, i.e. no HAR occurs; instead, the grafts succumb almost invariably on the third postoperative day (Gannedahl et al. 1990, Johnsson et al. 1997b, Koyamada et al. 1998). This rejection is characterised by perivascular necrosis, haemorrhage and thrombosis, a picture that corresponds to an AVR. At this time, both IgM and IgG have accumulated in the blood vessels (Gannedahl et al. 1994).

The rat has circulating natural antibodies, mainly IgM, against mouse antigens (Gannedahl et al. 1994). A small titre of preformed IgG also exists (Gustavsson et al. 1996). After the heart transplantation, an antiserum is raised against xenoantigens. These antibodies can induce HAR when transferred (Perper and Najarian 1967) to a new heart graft recipient (Gustavsson et al. 2001). Moreover, a second mouse graft is hyperacutely rejected (Gannedahl et al. 1994). One xenoantigen in this species combination has been identified as the Forssman antigen, which is a lipopolysaccharide (Springer et al. 1978, Gustavsson et al. 1996). In addition to the Forssman antigen, there appear to be at least three important antigens, of which two are peptides (Springer et al. 1978). Anti-Forssman antibodies do not induce HAR upon transfer (Wu et al. 1999, Gustavsson et al. 2001). The reason for this might be that the Forssman antigen is expressed on dendritic cells in the graft tissue and not on mouse vascular endothelium (Gustavsson et al. 2001).

DSG is an immunosuppressive drug well known for inhibiting antibody production (Valdivia et al. 1990), cytotoxic T lymphocytes (Fujii et al. 1992) and antigen presentation (Hoeger et al. 1994). In the mouse-to-rat model, DSG effectively inhibits the AVR and prolongs the graft survival to a mean of 8 to 9 days (Gannedahl et al. 1990, Johnsson et al. 1997b). These grafts are rejected in a picture of cell-mediated rejection. Furthermore, DSG prolongs the survival of a second graft from 2 min to 30 min (Gannedahl et al. 1994). On the other hand, no prolonged survival is seen with the T lymphocyte-inhibiting drug CyA or when transplanting mouse hearts to T cell-deficient rats (Gannedahl et al. 1990). The combination of CyA and DSG has proved to be successful in inducing long-time survival of mouse-to-rat heart grafts. When giving the recipients DSG for 4 weeks and CyA continuously, the graft survival is increased to over 100 days in nine out of ten animals (Johnsson et al. 1997a). A second graft, transplanted on day 56 after the first transplantation, survives more than 100 days in almost 50 % of the animals.
ORAL ADMINISTRATION OF ANTIGENS

In the field of experimental autoimmunity, the oral administration of autoantigens has effectively been used to induce antigen-specific tolerance (Thompson and Staines 1986, Bitar and Whitacre 1988, Higgins and Weiner 1988, Zhang ZJ et al. 1991, Wang ZY et al. 1993). In addition to experimental models, oral tolerance induction has been used clinically in patients suffering from rheumatoid arthritis, where chicken type II collagen given orally for three months markedly reduced the disease symptoms (Trentham et al. 1993). In allogeneic transplantation, oral tolerance has successfully been used in vascularised (Sayegh et al. 1992a) as well as non-vascularised experimental models (Buelow et al. 1995). However, only one study on oral tolerance in xenotransplantation has been published so far. Oral administration of rat splenocytes was shown to be effective in prolonging the survival of non-vascularised rat skin grafts in mice when combined with anti-CD4 treatment (Niimi et al. 2001). The graft survival with this treatment was increased from 12 days to approximately two months, whereas oral administration alone only prolonged the survival to 19 days.

In allotransplantation, different allopeptides, i.e. major histocompatibility complex (MHC) class I (Nisco et al. 1994, Zavazava et al. 2000) and MHC class II (Sayegh et al. 1992a) antigens, or intact cells, i.e. splenocytes (Sayegh et al. 1992b, Niimi et al. 2000), dendritic cells (Niederkorn and Mayhew 2002), corneal cells and keratinocytes (He et al. 1996) have been used as antigen source.

Usually, a low antigen dose results in suppression, whereas a high dose leads to anergy or deletion of CD4+ T lymphocytes (Friedman and Weiner 1994, Chen et al. 1995). Oral administration of antigens also leads to inhibition of CD8+ effector T lymphocytes and antibody responses, while stimulating CD8+ suppressor cells (Ke and Kapp 1996). The coupling of the antigen to a mucosal adjuvant, such as cholera toxin, enhanced the tolerance-inducing effects in some models (Sun et al. 1994).

Antibody responses are reduced in allogeneic situations by oral administration of antigens. In rats sensitized with skin grafts 5 days before cardiac transplantation, oral administration of donor splenocytes saved vascularised heart grafts from accelerated rejection and significantly reduced the depositions of IgM, IgG and C3c in the grafts (Sayegh et al. 1992b).
HYALURONAN

Hyaluronan is a large, negatively charged glycosaminoglycan that is built up of the repeating disaccharide N-acetyl-D-glucosamine-D-glucuronic acid (Laurent 1970). It is mainly produced by fibroblasts, which are found in the extracellular matrix. In loose connective tissue, hyaluronan functions as a stabiliser of the organ tissue. A characteristic of hyaluronan is that it binds large amounts of water (Comper and Laurent 1978). Nowadays, hyaluronan is regarded as a marker for inflammatory processes since the local content of hyaluronan increases strongly during various inflammatory responses such as alveolitis (Nettelbladt et al. 1989), myocarditis (Waldenström et al. 1991), pancreatitis (Johnsson et al. 2000a), Crohn’s disease (Colombel et al. 1989), ischemia (Johnsson et al. 1996) and allograft rejection (Hällgren et al. 1990a, Hällgren et al. 1990b). During rejection of allogeneic transplants, hyaluronan is correlated not only to the water content, but also to the interstitial pressure (Johnsson et al. 2000b).

Besides the strong water-binding capacity, hyaluronan also exerts effects on the immune system through its interaction with the cell surface receptor CD44. For example, activated T lymphocytes express the hyaluronan-binding isoform of CD44 (DeGrendele et al. 1997b). The T lymphocytes can thus target both hyaluronan bound to CD44 on endothelial cells (DeGrendele et al. 1997a) and hyaluronan present in the extracellular matrix. Blockade of the hyaluronan-CD44 interaction increases the survival of allogeneic grafts (Zhang W et al. 2000).

Hyaluronan is also found to bind different cytokines to the tissue causing a local accumulation at the inflammatory site (Fernandez-Botran et al. 1999). Inhibition of this interaction prolongs the survival of allogeneic, vascularised rat skin grafts (Fernandez-Botran et al. 2002).
Aims of the Investigation

The general purpose of this study was to further characterise xenogeneic rejection mechanisms.

The specific purposes were to:

– investigate whether ex vivo propagation can be used to isolate xenograft-infiltrating cells.

– characterise the graft-infiltrating cells by means of different cell-surface markers.

– characterise the cytokine patterns in the graft and spleen during rejection and non-rejection.

– explore the antibody responses during rejection and non-rejection.

– examine any potential antibody response after oral administration of xenogeneic cells.

– investigate hyaluronan content and distribution during rejection.

All studies were performed in the concordant mouse-to-rat heart transplantation model.
MATERIALS AND METHODS

ANIMALS, TRANSPLANTATION AND DRUG TREATMENT

Animals and anaesthesia (Papers I - IV)

For all experiments, inbred male Lewis rats (RT1\textsuperscript{l}) and outbred male NMRI mice (H-2\textsuperscript{k}) were obtained from M&B, Ry, Denmark. They were allowed to settle for one week before operation and had unlimited access to standard diet and water throughout the experiment. Rats weighing 150-200 g were used as recipients and mice weighing 20-35 g were used as donors. Both recipients and donors were anaesthetised with an intraperitoneal injection of a mixture of chloral hydrate (180 mg/kg body weight (bw)) and pentobarbital (40 mg/kg bw), except for the transplantation studies in paper III. In those animals, anaesthesia was induced with an intraperitoneal injection of a mixture of Hypnorm\textsuperscript{®} (fluanisone 10 mg/ml and fentanyl 0.315 mg/ml; Janssen Pharmaceutica, Beerse, Belgium) 1 ml/kg and Stesolid Novum\textsuperscript{®} (diazepam; Dumex-Alpharma, Stockholm, Sweden) 5 mg/kg and maintained with Hypnorm\textsuperscript{®} 0.5 ml/kg and Stesolid Novum\textsuperscript{®} 2.5 ml/kg after 1 hour. All animals were handled in accordance with the Guide for the care and use of laboratory animals published by the National Research Council in 1996, and the experiments were approved by the local ethical committees.

Heart transplantation (Papers I - IV)

The donor heart was heterotopically transplanted to the recipient using a non-suture cuff technique (Olausson et al. 1984) previously described (Gannedahl et al. 1990). Briefly, the right common carotic artery and the right internal jugular vein of the recipient were dissected, cross-clamped caudally, ligated cranially and then divided. Short plastic tubes were threaded on the vessels, which were then turned inside and out over the tubes and fixed with ligatures. The ascending aorta of the donor heart was pulled over the carotic tube of the recipient, and the pulmonary artery over the jugular tube, respectively, and both were fixed with a ligature. After revascularisation of the heart, a single dose of cefuroxim (Zinacef\textsuperscript{®}, GlaxoWellcome, Greenford, UK), 20 mg/rat, was administered intramuscularly. Graft function was monitored by palpation of the graft. Rejection was defined as the time when the xenograft stopped beating.

Pharmacological treatment (Papers I, II and IV)

Cyclosporine A (CyA; Sandimmun Neoral\textsuperscript{®}, Novartis, Basel, Switzerland) was mixed with Intralipid\textsuperscript{®} (Pharmacia & UpJohn, New Jersey, USA) and administered orally through a gastric feeding tube (CH 5) at a total volume of
approximately 1 ml/administration. A daily dose of 20 mg/kg bw was given from day -1 until day 4, followed by 10 mg/kg bw daily from day 5 until day 7. The grafts were removed on day 2 or day 8.

15-Deoxyspergualin (DSG; Behringwerke, Marburg, Germany) was diluted in sterile saline and injected intraperitoneally. A dose of 10 mg/kg bw was given on day -1 to day 4, followed by a dose of 5 mg/kg bw from day 5 until day 28. In papers I and II, the grafts were removed on day 2 or day 8, whereas in paper IV, the grafts were removed on day 5 or at the time of terminal rejection.

Hyaluronidase type V from sheep testes (Sigma Chemical Co., St. Louis, MO, USA) was mixed with phosphate-buffered saline (PBS) with 2% albumin (Centeon Pharma GmbH, Marburg, Germany), and given in the left femoral vein of the rat at a dose of 20,000 U/kg bw on day 0 immediately after transplantation. Animals used for hyaluronan and water measurements were then given 20,000 U/kg bw daily from day 1 to day 5 in a tail vein. The last dose was given two hours before graft removal. For the survival studies, 20,000 U/kg bw was given daily from day 0 to day 9.

INFLTRATING CELLS AND GRAFT MORPHOLOGY

Culturing and counting of ex vivo propagated cells (Papers I and II)

Ex vivo culturing of graft biopsies was performed with some modifications to previous protocols (Mayer et al. 1985, Miceli et al. 1985, Engstrand et al. 1999). At harvest, one part of the graft was cut with a razor into 12 sections and placed on a 12-well tissue culturing testplate (TPP, Trasadingen, Switzerland). Three ml of complete culturing media (RPMI-1640 with 10% foetal bovine serum (FBS; Sigma-Aldrich), 1% HEPES (Statens Veterinärmedicinska Anstalt (SVA), Uppsala, Sweden), 1% penicillin/streptomycin (SVA) and 1% glutamine (SVA)) was added to each well. The plates were incubated in 5% CO₂ at 37°C for 48 hours.

Thereafter, cell propagation was inspected in a phase contrast light microscope (Olympus CK30 Culturing Microscope, Olympus, Tokyo, Japan) after which the cells were aspirated from all wells and pooled. The pooled cells were centrifuged at 300 x g for 5 min, and erythrocytes were then lysed with 1 ml of lysing solution (0.15 M NH₄Cl, 0.01 mM KHCO₃ and 0.1 mM Na₂EDTA in dH₂O). After washing in PBS, live cells were counted in a Bürker chamber using Trypan Blue exclusion. The quotient of T lymphocytes among the live cells was deduced from the flow cytometric analyses (see below), and the results presented as the amount of T lymphocytes per mg tissue.
Control biopsies were taken from hearts from normal NMRI-mouse, normal Lewis-rat and naive heart from Lewis-rat transplanted with NMRI-heart under DSG treatment.

**Immunohistochemistry** (Papers I and II)

Frozen sections of the grafts, 6 μm thick, were cut on a cryostat, fixed in cold acetone for 10 min, and then stained with monoclonal antibodies (Table 1) according to the peroxidase-antiperoxidase (PAP) method (Sternberger 1979). Briefly, endogenous peroxidase and non-specific background were blocked by incubation with 0.3% H₂O₂ and normal goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), respectively. Incubation with the primary antibody was followed by a secondary antibody, goat anti-mouse-IgG (Jackson ImmunoResearch Laboratories Inc.), and mouse PAP. The reaction was completed by addition of H₂O₂ as substrate and 3-amino-9-ethyl-carbazole (AEC) as electron donor. Counterstaining was performed with Mayer's haematoxylin. Primary antibodies were omitted from negative controls.

Sections were analysed blindly in a light microscope (Nikon Microphot-FX®, Nikon, Ohta-ku, Tokyo) and graded semiquantitatively according to a four-step scale (1 to 4): 1 = occasional infiltration; 2 = mild infiltration; 3 = moderate infiltration; 4 = massive infiltration. The various antibody stainings were ranked separately, i.e. the results of one antibody staining cannot be directly compared with that of the others.

**Table 1. Antibodies**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Specificity/Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cell receptor α/β</td>
<td>R73</td>
<td>α/β chain of T cell receptor (97% of peripheral T lymphocytes)</td>
</tr>
<tr>
<td>CD4</td>
<td>W3/25</td>
<td>Helper T cells and macrophages</td>
</tr>
<tr>
<td>CD8</td>
<td>OX-8</td>
<td>Cytotoxic/suppressor T cells and most NK cells</td>
</tr>
<tr>
<td>ED1</td>
<td>ED1</td>
<td>Monocytes and most macrophages</td>
</tr>
<tr>
<td>ED2</td>
<td>ED2</td>
<td>Resident macrophages</td>
</tr>
<tr>
<td>MHC class II</td>
<td>OX-6</td>
<td>MHC class II antigens</td>
</tr>
<tr>
<td>IL-2 receptor (CD25)</td>
<td>OX-39</td>
<td>Stimulated rat T cells</td>
</tr>
<tr>
<td>-</td>
<td>OX-33</td>
<td>B cells and bone marrow (weakly)</td>
</tr>
<tr>
<td>-</td>
<td>3.2.3</td>
<td>Rat NK cells, lymphokine activated killer (LAK) cells and polymorphonuclear cells</td>
</tr>
</tbody>
</table>

Antibodies were acquired from Serotec (Oxford, UK)

**Flow cytometry analysis of ex vivo propagated cells** (Papers I and II)

Ex vivo propagated cells and splenocytes from graft recipients were incubated with antibodies (Table 1) and analysed by flow cytometry (Holmes and Fowlkes 1995). Briefly, all propagated cells from one graft were diluted in 700 μl PBS (containing 2% FBS and 0.1% NaN₃) and split into 7 tubes (5-ml polystyrene tubes; Beckton Dickinson Labware, Franklin Lakes, NJ, USA). Splenocytes
were diluted in PBS and 2x10^6 cells were distributed to each of 7 tubes to a volume of 100 µl/tube. The CD4/CD8-ratio and the percentage of T lymphocytes expressing MHC class II antigens (MHC II) or CD25 (α chain of the IL-2 receptor (IL-2R)) were determined by double stainings using monoclonal antibodies conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (Table 1). The antibodies were added and the tubes were incubated at 4°C for 10 min. The cells were washed once with PBS and were then fixed in a 1% paraformaldehyde-PBS-solution. To determine possible autofluorescence of the analysed cells, antibodies were omitted from one of the tubes. Two tubes were used for single stainings with each of the two fluorophores for compensation procedures (Otten et al. 1995).

Analysis was performed on a FACSCalibur® flow cytometer (Becton Dickinson, San José, CA, USA) using the Cellquest® software. Lymphocytes were identified and gated according to a forward scatter (FSC)/side scatter (SSC) dot plot with linear scales. A quadrant gate was used with FL1 on the x-axis and FL2 on the y-axis to be able to separate unstained, single stained and double stained cells from each other according to fluorescence intensity.

Morphology (Papers I - IV)
Formaldehyde fixed graft tissue was embedded in paraffin and 4-µm sections were cut on a microtome. Thereafter, the sections were stained with Mayer's haematoxylin and eosin, and examined under a light microscope (Nikon Microphot-FXA®, Nikon, Ohta-ku, Tokyo).

CYTOKINES

Real-time quantitative RT-PCR (Paper II)
One section of the rat spleen, 2 mm thick, and four biopsies from the graft (taken with a stainless steel biopsy punch (1 mm Ø; Tiemann, New York, USA)), were snap-frozen in liquid nitrogen, and then stored at -70°C. cDNA synthesis from these heart and spleen tissue samples was performed on oligo(dT)-coated manifold supports (Hagberg et al. 2000). Primers and probes for IL-1β, IL-2, IL-4, IL-10, IL-12p40, IFN-γ, tumour necrosis factor (TNF)-α and β-actin (Table 2) were used to run 5' nuclease assays for quantitative analysis of the generated complementary DNA (cDNA) by means of the ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The primers and probes for IL-1β and TNF-α react with both rat and mouse sequences, whereas the other primers and probes are purely rat specific.

The results were presented as threshold cycle values (Ct values), which are estimates of the amplification cycle number when the fluorescence exceeds a
specified threshold value (Gibson et al. 1996, Heid et al. 1996). Known amounts of amplicons, generated by the different primer pairs, were diluted and run in all PCR amplifications. Standard curves were then created by plotting $C_t$ values versus the log of the amount of cDNA template in the respective dilution. These curves were then used to calculate the initial quantity of cDNA template in the tissue samples.

### Table 2. Oligonucleotides

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward primer sequence (5'-3')</th>
<th>5' nuclease probe (5'-3')</th>
<th>Reverse primer sequence (5'-3')</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1$\beta$</td>
<td>M98820</td>
<td>TGTCTGTGTGTGATG</td>
<td>ACACCCACCCCTGCAGCTG</td>
<td>TGGGTATTGTGTG</td>
<td>69</td>
</tr>
<tr>
<td>IL-2</td>
<td>M22899</td>
<td>GAAAGACGG</td>
<td>GAGAGT</td>
<td>GAATTT</td>
<td>75</td>
</tr>
<tr>
<td>IL-4</td>
<td>X53087-88</td>
<td>CCAGCCTCTTACG</td>
<td>AAGACCGGAAGACAGAG</td>
<td>AGCACCCTGGAAGC</td>
<td>77</td>
</tr>
<tr>
<td>IL-10</td>
<td>L02926</td>
<td>GTAAGACTAAGAAGA</td>
<td>AGCGTTGAAGAATGATT</td>
<td>TAAAATAGCTTCAAGAGA</td>
<td>85</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>NM022611</td>
<td>GGAGGCCAGCAGA</td>
<td>CATCATCAACCAGACCC</td>
<td>AACCTTGGAGGGAGAA</td>
<td>180</td>
</tr>
<tr>
<td>IFN-$\gamma$</td>
<td>AF010466</td>
<td>AACAAGGACAGGAGA</td>
<td>TCTGAAGCATCGCAAGG</td>
<td>TAGCTTGAGTCTGTG</td>
<td>71</td>
</tr>
<tr>
<td>TNF-$\alpha$</td>
<td>L00981</td>
<td>AGAGCTACTCAGT</td>
<td>TGCCACAGGTGCTAGAA</td>
<td>GCTGTGCTAGCC</td>
<td>105</td>
</tr>
<tr>
<td>$\beta$-actin</td>
<td>V01217</td>
<td>GCCCTGCTCTCTA</td>
<td>TGAAGACTAAGTCAGGG</td>
<td>CCACCAATCCACAC</td>
<td>73</td>
</tr>
</tbody>
</table>

### ANTIBODIES AND COMPLEMENT

**Immunofluorescence** (Papers II and III)

*Detection of antibody deposits in the xenografts*

Acetone-fixed sections, 6 $\mu$m thick, of frozen transplants were incubated with FITC-conjugated antibodies reactive with rat IgM or IgG (sheep anti-rat IgM and sheep anti-rat IgG; Serotec, Oxford, UK) for 30 min at room temperature. The slides were analysed blindly in a fluorescence microscope (Nikon Microphot-FXA®).

*Detection of antibodies in serum from rats receiving mouse cardiac grafts*

To detect circulating xenoantibodies capable of binding to mouse heart tissue, acetone-fixed frozen sections, 6 $\mu$m thick, of normal mouse heart were incubated with serum from each recipient for 30 min at room temperature. Next, rat antibodies were detected by 30 min incubation with FITC-conjugated sheep anti-rat IgM and IgG (Serotec), respectively.
Detection of complement deposits in the xenografts

Complement deposits in the grafts were analysed by incubating acetone-fixed sections, 6 μm thick, of frozen grafts with a sheep anti-mouse/rat complement factor 3 (C3c) antibody (The Binding Site, Birmingham, UK) for 30 min at room temperature. The slides were then incubated with a FITC-conjugated rabbit anti-sheep antibody (Jackson ImmunoResearch Laboratories Inc.) for 30 min at room temperature. Sections from normal mouse heart, as well as sections where the primary antibody was omitted, were used as controls.

Haemagglutinating xenoantibodies (Papers II and III)

Blood from an anaesthetised NMRI mouse was extracted from the heart with a heparinised syringe, diluted in PBS and centrifuged at 300 x g for 10 min. Subsequently, the erythrocytes were centrifuged at 400 x g at +18ºC for 35 min over a density gradient using 4 ml Ficoll Paque Plus® (Amersham Pharmacia Biotech AB). The purified erythrocytes were diluted in PBS to a 2%-erythrocyte suspension.

Heat-inactivated serum (+56°C for 30 min) (Triglia and Linscott 1980) from each of the transplant recipients were placed in round-bottomed 96-well microtitre plates (TPP) and a two-fold dilution series was performed in PBS in 12 wells. A 50 μl 2%-erythrocyte suspension was added to each of the wells, after which the plates were incubated for 2 hours at +4°C, and then visually inspected for haemagglutination (Adler and Adler 1980).

Binding of xenoantibodies to mouse erythrocytes (Paper III)

Mouse erythrocytes were prepared as described above and fixed by incubating 500 μl of the erythrocyte pellet with 2.5 ml of buffered 0.5% formalin solution (pH 7.3) at +4°C for 20 min. Thereafter, the cells were washed twice and diluted in 10 ml PBS and stored at +4°C overnight. The next day, the cells were washed twice and diluted in PBS to a final concentration of 50 x 10⁶ cells/ml. A total of 0.5 x 10⁶ cells were incubated with 50 μl of each of the heat-inactivated sera in 5-ml round-bottomed polystyrene tubes (Beckton Dickinson Labware) for 10 min at +4°C, and subsequently washed once in PBS. Monoclonal antibodies against IgM (mouse anti-rat IgM, FITC-conjugated; BD Pharmingen, San Diego, CA, USA), IgG (F(ab)₂ goat anti-rat IgG, PE-conjugated; Serotec) and IgA (mouse anti-rat IgA, FITC-conjugated; Serotec) respectively, were added to the tubes and incubated for 10 min at +4°C. The cells were then washed once in PBS and immediately analysed using a FACSCalibur® flow cytometer (Becton Dickinson) with the Cellquest® software. Erythrocytes were identified and gated according to a FSC/SSC dot plot with linear scales. Fluorescence intensity was then analysed using histogram plots for each of the antibodies.
Binding of xenoantibodies to mouse mononuclear cells (Papers II and III)
The spleen of an anaesthetised NMRI mouse was removed and gently pressed through a metal net with small perforations. The splenocytes were then washed once in PBS. Mononuclear cells were obtained after centrifugation over a density gradient using 4 ml Ficoll Paque Plus® (Amersham Pharmacia Biotech AB). After washing 3 times in PBS, the cells were counted in a Bürker chamber and diluted in PBS to a final concentration of 2 x 10^6 cells/ml.

A total of 2 x 10^5 mononuclear cells were incubated with 50 µl heat-inactivated serum from each of the Lewis transplant recipients in 5-ml round-bottomed polystyrene tubes (Beckton Dickinson Labware) for 10 min at +4°C and thereafter washed once in PBS. Monoclonal antibodies against IgM (mouse anti-rat IgM, FITC-conjugated; BD Pharmingen), IgG (F(ab)₂ goat anti-rat IgG, PE-conjugated; Serotec) and IgA (mouse anti-rat IgA, FITC-conjugated; Serotec) respectively, were added to the tubes and incubated for 10 min at +4°C. The cells were washed in PBS, and then fixed in a 1% paraformaldehyde solution (diluted in PBS).

Analysis was performed on a FACSCalibur® flow cytometer (Becton Dickinson) using the Cellquest® software. Mononuclear cells were identified and gated according to a FSC/SSC dot plot with linear scales. Fluorescence intensity was then analysed using quadrant gates or histogram plots for all the antibodies.

Haemolysis by complement-binding antibodies (Paper III)
Mouse erythrocytes were prepared for the haemolytic assay in the same way as described above. Heat-inactivated sera from the recipients were two-fold diluted in PBS in 96-well round-bottomed microtitre plates to a volume of 66 µl per well. An equal volume of the 2%-erythrocyte solution was added, followed by the addition of 7 µl rabbit complement (Biotest AG, Dreieich, Germany). Deionised water was added to some wells to induce total haemolysis for positive control. Erythrocyte suspension was used as negative control. The dilutions were made in duplicates to have one of the two dilutions (no addition of complement or cells) as a reference for background haemolysis. The plates were incubated at +37°C for 1 h, and thereafter centrifuged at 700 x g for 10 min. The supernatants were moved to another 96-well microtitre plate to measure free haemoglobin at λ=414 nm using a microplate photometer (Thermo Labsystems iEMS Reader MF, Helsinki, Finland) with the DeltaSOFT® 3 software (BioMetallics, Princeton, NJ, USA).

To obtain the results of the pure antibody-mediated complement lysis, the background haemolysis in the sera were subtracted from the results of the wells that contained serum, erythrocytes and rabbit complement.
Viability of mononuclear cells (Paper III)
Mouse mononuclear cells were isolated as described above. Rat sera were heat-
inactivated and two-fold diluted in PBS in 96-well round-bottomed microtitre
plates to a volume of 50 µl per well. To each well, approximately 2 x 10^5
mononuclear cells were added and incubated with 5 µl rabbit complement
(Biotest AG) at 37°C for 1 h. Subsequently, the cells were washed in PBS and
centrifuged at 700 x g for 10 min.

Cell analyses were performed on a FACSCalibur® flow cytometer (Beckton
Dickinson) with the Cellquest® software. Lymphocytes were identified and
gated according to a FSC/SSC dot plot with linear scales. Prior to the analysis,
the content of each well was moved to a 5-ml round-bottomed polystyrene tube
(Beckton Dickinson Labware), and 10 µl of propidium iodide (0.1 mg/ml) was
added. Viability of the cells was measured by gating of the propidium iodide-
negative mononuclear cells, thereafter the number of these gated cells was
compared with the total number of mononuclear cells.

CELL AND SERUM TRANSFER

Oral and intravenous administration of xenogeneic erythrocytes (Paper III)
Mouse erythrocytes were administered orally or intravenously to rats as follows:
Blood was collected by heart puncture of anaesthetised normal NMRI mice with
a heparinised syringe. The blood was diluted in PBS and centrifuged at 300 x g
for 10 min. To ensure erythrocyte purity, the cells were diluted in PBS to a total
volume of 5 ml and centrifuged (400 g at +18°C for 35 min) over a density
gradient with 4 ml of Ficoll Paque Plus® (Amersham Pharmacia Biotech AB).
Thereafter, the erythrocytes were washed 3 times, counted in a Bürker chamber
and diluted in PBS to a final concentration of 5 x 10^9 to 6 x 10^9 erythrocytes/ml.
Rats received mouse erythrocytes orally either during one or two episodes of 6
days. The erythrocyte solution was administered through a gastric feeding tube
(CH5) at a total volume of 0.8 ml per day (approximately 4 x 10^9 to 5 x 10^9
erythrocytes/day). Erythrocytes were given on days 0-5, with serum collected on
day 10, or on days 0-5 and 20-25, with serum collected on day 29.

Other rats received intravenous administration of erythrocytes on day 0,
with serum collected on day 9, or on days 0 and 20, with serum collected on day
29. All intravenous immunizations were performed by injecting 0.8 ml of
erythrocyte solution (approximately 4 x 10^9 to 5 x 10^9 erythrocytes/injection) in
either the left or the right femoral vein under anaesthesia.
Passive transfer of immunity (Paper III)
Mouse grafts newly transplanted to rats were allowed to stabilise for 15 min while the rats were still anaesthetised. The left femoral vein of the recipient was exposed to allow passive transfer of serum. All rats were given two injections, with 20 min in-between, of 0.8 ml heat-inactivated serum from rats that had received mouse erythrocytes orally or intravenously. The grafts were visually monitored, and rats with grafts still beating after 90 min were sutured and allowed to wake up. Thereafter, the xenografts were monitored by palpation by regular intervals. Rejection was defined as the time when the xenograft stopped beating.

HYALURONAN AND OEDEMA

Determination of water content (Paper IV)
Graft tissue was put on filter paper immediately after harvest and weighed 3 min later (wet weight, ww). The graft sections were then lyophilised overnight and weighed again (dry weight, dw). The residual tissue was used for subsequent extraction of hyaluronan, and the relative water content was calculated as 100 x (ww-dw)/ww and thus expressed as percent water of the total weight of the tissue. Normal NMRI hearts were used as controls.

Quantification of hyaluronan (Paper IV)
The lyophilised graft tissue from DSG- and hyaluronidase+DSG-treated rats and tissue from normal NMRI-hearts (control) were ground, and hyaluronan was subsequently extracted by 16 h cold incubation in 0.5 M NaCl under continuous rotation. After centrifugation for 15 min at 2000 x g, the supernatants were analysed for hyaluronan content by a radiometric assay (Pharmacia Diagnostics, Uppsala, Sweden). In parallel, sera from the same animals were analysed. The technique is based on the binding of hyaluronan to specific hyaluronic acid binding proteins (HABP) (Tengblad 1980). Briefly, 100 µl samples were incubated with 200 µl 125I-labelled HABP for 60 min at 4-7°C, followed by the addition of 100 µl hyaluronan-sepharose and cold incubation for another 45 min. Next, 2 ml of washing solution was added before centrifugation at 2000 x g for 10 min. After decantation, the radioactivity in the pellet was measured in a gamma counter. A standard curve was constructed from samples with known amounts of hyaluronan. All samples were analysed in duplicates.

Histochemical analysis of hyaluronan distribution (Paper IV)
The detection of hyaluronan is based on an avidin-enzyme biotin-protein system, as previously described (Hällgren et al. 1990a). In brief, 4-µm paraffin
sections were incubated with 0.1% trypsin in 0.05M Tris-HCl for 90 min. Subsequently, the sections were incubated with 3% H₂O₂ in PBS to block endogenous peroxidase, and with bovine serum albumin (10 mg/ml, Fraction V; Sigma Chemical) to block non-specific binding sites. Incubation for 1 h with biotin-labelled HABP (Seikagaku Corporation, Tokyo, Japan) was followed by 1 h incubation with ABC Vectastain Reagent (Vector Laboratories, Burlingame, CA, USA). Finally, H₂O₂ (substrate) and AEC (electron donor) were added, and the sections were counterstained with Mayer’s haematoxylin. Control sections were incubated for 2 h with Streptomyces hyaluronidase, and thereafter with HABP.

STATISTICAL ANALYSES

Statistics (Papers I - IV)
All data are shown as mean values with standard errors of the mean, except for the immunohistochemical analyses in paper I which are presented as medians and ranges. Statistical difference for unpaired data was evaluated using the non-parametric Mann-Whitney $U$-test, except for hyaluronan and water contents, which were evaluated using the unpaired Student’s $t$-test. Paired data were compared using the paired Student’s $t$-test. In paper IV, the correlation between hyaluronan and water content was evaluated by linear regression. A $p$-value of less than 0.05 was considered statistically significant. The following levels of significance were used: $p<0.05$ (*); $p<0.01$ (**); $p<0.001$ (***).
EXPERIMENTS AND RESULTS

Paper I
In this study, we explored whether the technique of ex vivo propagation can be used to study immune cells involved in xenograft rejection. Isolated graft infiltrating T lymphocytes were characterised by flow cytometry using various antibodies against cell surface markers. Biopsies were obtained from beating cardiac xenografts with ongoing AVR or cell-mediated rejection, and from grafts protected by immunosuppressive treatment previously shown to result in long-time survival. Grafts from untreated recipients (AVR) were obtained on day 2, whereas grafts from DSG- (cell-mediated rejection) and CyA+DSG-treated (long-time survival) recipients were obtained on day 8. In addition, three control groups (CyA-, DSG- and CyA+DSG-treated rats with grafts obtained on day 2) were analysed and presented in paper II.

In the visual analysis, propagated cells were seen around all biopsies in all groups, irrespective of treatment protocol. The counting of the propagated cells revealed many more cells around the biopsies of cellularly rejecting grafts than around biopsies from grafts with an ongoing AVR (untreated) or under effective immunosuppression (CyA+DSG) (Figure 2). Still the number of cells isolated from biopsies in the CyA+DSG group was between two and three times larger than in the untreated group.

The percentage of T lymphocytes expressing the activations markers MHC II and IL-2R in grafts and spleens was analysed in a flow cytometer. In comparison to T lymphocytes in spleen isolated from the recipients, the ex vivo propagated cells expressed more of both activation markers on their cell surfaces. This was seen irrespective of treatment and time.

Immunohistochemical stainings of graft biopsies for different cell phenotypes showed a similar pattern as found in the flow cytometry analysis of ex vivo propagated cells, i.e. increased numbers of T lymphocytes, CD4+, CD8+, MHC II+, and IL-2R-positive (IL-2R+) cells during cell-mediated rejection as compared with the other experimental groups. Additionally, the immunohistochemical studies revealed that ED1+ macrophages were the most abundant cells found in the grafts irrespective of treatment, followed in numbers by the T lymphocytes. CD4+ T lymphocytes were more numerous than CD8+ T lymphocytes in all groups.

Morphologically, the grafts undergoing AVR showed typical changes with perivascular necrosis, haemorrhage and occasional necrosis. During cell-mediated rejection the necrosis and cellular infiltration was pronounced, even though the grafts were still beating. CyA+DSG preserved a considerable amount
of heart tissue from destruction and reduced the amount of infiltrating immune cells.

**Figure 2.** Number of ex vivo propagated graft infiltrating T lymphocytes isolated from mouse-to-rat cardiac grafts. p<0.05 (*) and p<0.01 (**) vs DSG day 8.

**Paper II**

This paper was a follow-up study of paper I, with the main purpose was to investigate the occurrence of cytokines, both pro-inflammatory and more immune cell specific, in grafts during AVR, cell-mediated rejection and under effective immunosuppression. To also obtain a picture of the cytokines in the central immune system, the spleens of the recipients were examined. In parallel, antibody responses were characterised by several methods (immunofluorescence, haemagglutination and flow cytometry).

Grafts subjected to AVR were removed on day 2, whereas grafts subjected to cell-mediated rejection or under effective immunosuppression were removed on day 8. Additionally, three control groups were included: CyA-treated, DSG-treated and CyA+DSG-treated recipients, all with graft removal on day 2. All cytokine analyses were performed by using a reverse transcriptase RT-PCR method.

The pro-inflammatory cytokines IL-1β and TNF-α were found in largest amounts during AVR and cell-mediated rejection, whereas grafts under effective immunosuppression in the long-time surviving group had low levels of these cytokines. Likewise, the Th1-associated cytokines IL-2, IL-12p40 and IFN-γ
showed a similar pattern, as did IL-4 and IL-10. Notably, IL-2 could only be identified in three of six grafts in the untreated group with ongoing AVR, even though the average number of cytokine mRNA transcripts was large. Additionally, IL-4 was only detected in two of six grafts in the untreated group and three of ten grafts in the DSG-treated group (day 8). IL-1β and TNF-α were the only cytokines expressed in grafts from the three control groups, and these expressions were at a low level.

Analyses of cytokines in spleens from graft recipients under DSG or CyA+DSG treatment on day 8 showed that the levels of IL-2, IFN-γ and IL-4 were reduced compared to control spleens from non-transplanted rats. Further, the level of IL-2 was even lower under effective immunosuppression (CyA+DSG) than during cell-mediated rejection (DSG). AVR was associated with a reduction in splenic IL-4 expression.

Examination of grafts from the three control groups (grafts obtained on day 2) revealed that the number of ex vivo propagated cells (Figure 2) was at the same low level as found in paper I during AVR on day 2. The low numbers of ex vivo propagated cells were confirmed by immunohistochemical stainings.

Much IgM and IgG were found during immunofluorescent staining of grafts subjected to AVR or cell-mediated rejection. Sera from some of the recipients in these groups contained low titres of xenospecific antibodies, but in most of the recipient sera we were unable to detect any antibodies directed against mouse antigens. With CyA+DSG treatment, no or only small antibody deposits were seen, and no xenospecific antibodies were detected in the recipient sera.

**Paper III**

This paper focused on characterising antibody responses after oral administration of xenogeneic antigens. The aim of the study was originally to certify that no antibodies were produced after oral administration of xenogeneic cells. However, due to an early identification of a xenospecific antibody response we changed our focus and instead aimed to characterise these antibodies and to evaluate what kind of impact they could have on vascularised xenografts.

Two groups of animals received mouse erythrocytes orally, either during one episode (days 0-5) with serum obtained on day 10, or during two episodes (days 0-5 and 20-25) with serum obtained on day 29, as shown in Table 3. As positive control, other rats received mouse erythrocytes intravenously on day 0, with serum obtained on day 9, or on day 0 and day 20, with serum obtained on day 29 (Table 3).
Haemagglutinating and haemolytic assays were used for antibody analyses, where a titre of 1:2 or higher is considered positive. A positive titre was found in all animals (1:16-1:256 in either assay) irrespective of administration pathway (n=20), except for two animals receiving mouse erythrocytes orally for one period (PO1). The sera from all animals with a positive titre in each group were pooled and further analysed. All four pooled sera contained IgM and IgG with specificity for mouse erythrocytes and mononuclear cells, when analysed by flow cytometry. Two periods of oral administrations also raised an IgA-production against erythrocytes, whereas the rest of the sera were negative for IgA.

All heat-inactivated sera had the capacity to severely reduce the viability of mononuclear cells. This complement-mediated destruction was analysed by adding rabbit complement and, close to analysis, also propidium iodide to each sample. The flow cytometric analyses indicated that IV1 and IV2 could damage the cell surfaces of the mononuclear cells slightly more than PO1 or PO2.

Mouse heart-specific IgM and IgG were labelled by using FITC-conjugated anti-rat antibodies after application of serum to cryosectioned, normal, mouse heart tissue. IgM was identified in all sera by mild staining of the blood vessels and the sarcolemma. Staining for rat IgG showed large deposits with PO1 and IV1; the deposits were even stronger with PO2 and IV2. IgG was located in the blood vessels and in the sarcolemma. Staining for complement showed that most C3c was located in the vessels, whereas only mild staining was seen in or between the muscle cells. C3c-binding in the grafts was similar irrespective of experimental group.

All the sera were found to induce HAR when transferred to rats that shortly before had been transplanted with a mouse heart. The graft survival time ranged from 24 to 196 min (Figure 3). IV1 rejected the grafts the fastest, whereas PO1 rejected the grafts the slowest. Transfer of normal rat serum results in a graft survival time of approximately three days (Gustavsson et al. 2001).

### Table 3 Administration of mouse erythrocytes to rats

<table>
<thead>
<tr>
<th>Serum</th>
<th>Route of administration*</th>
<th>Time of administration</th>
<th>Serum collection</th>
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<tr>
<td>PO1</td>
<td>oral</td>
<td>days 0-5</td>
<td>day 9</td>
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<tr>
<td>IV1</td>
<td>intravenous</td>
<td>day 0</td>
<td>day 9</td>
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<tr>
<td>PO2</td>
<td>oral</td>
<td>days 0-5, 20-25</td>
<td>day 29</td>
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<tr>
<td>IV2</td>
<td>intravenous</td>
<td>day 0, 20</td>
<td>day 29</td>
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<td>IV1-1/4†</td>
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* Approximately 4 x 10^9 to 5 x 10^9 purified mouse erythrocytes were given daily.
† IV1 diluted 1:4 in serum from untreated, non-transplanted Lewis rats.
Figure 3. Graft survival after intravenous injection of serum from rats receiving mouse erythrocytes orally or intravenously.

Paper IV
In this paper, the role of hyaluronan was investigated in the early course of cell-mediated xenograft rejection. Additionally, the effects of hyaluronidase on hyaluronan content and graft survival were evaluated.

Rats receiving mouse hearts were treated with DSG or with both hyaluronidase and DSG. In some rats, grafts were removed on day 5 to analyse hyaluronan, water and morphology, whereas other rats were used to study graft survival.

On day 5, the hyaluronan content, as measured by a radiometric assay, was markedly higher in grafts undergoing rejection than in control mouse heart tissue (Figure 4). Also the relative water content was strongly increased during ongoing rejection (Figure 4). In five of six grafts histochemical analysis revealed large deposits of hyaluronan mainly in or surrounding large foci with myocytolysis. The remaining graft in the group presented a diffuse distribution of hyaluronan with mild staining throughout the whole section.

Adding hyaluronidase to DSG treatment effectively reduced the hyaluronan content in comparison to DSG treatment alone; however, it did not affect the relative water content (Figure 4). In four of five grafts in this group hyaluronan was diffusely distributed along both well-preserved myocytes and in areas with myocytolysis. This staining was mild throughout the sections. One graft had large deposits of hyaluronan in several foci, whereas the remaining tissue showed the same diffuse pattern as the other grafts in the group. The hyaluronan quantity in sera from the recipients was strongly increased in the group receiving hyaluronidase as compared with that receiving DSG alone.
Morphological examination showed a similar appearance of the graft tissue in both treatment groups.

Some animals were observed until the grafts stopped beating. The mean survival of the grafts in DSG-treated rats was 11 ± 1 days; in the hyaluronidase+DSG group, 16 ± 3 days. Three of the individuals in the hyaluronidase+DSG-treated group had grafts that survived for one or up to almost three weeks longer than the longest surviving graft after DSG treatment. However, there was no statistical difference in mean graft survival between the groups.

**Figure 4.** Hyaluronan (white bars) and water (grey bars) contents in mouse xenografts obtained on day 5.
One of the major findings in this thesis is that xenograft-infiltrating cells, in small as well as large numbers, can be isolated by the use of ex vivo propagation. This technique has the advantage of providing easy, fast access to infiltrating cells without the use of chemical agents, which may otherwise alter antigen expression on immune cell surfaces (Van Damme et al. 2000).

In our study, recipient immune cells were located in the grafts as early as two days after transplantation, both during AVR and non-rejection. This finding of an early onset of cellular infiltration is in agreement with previous immunohistochemical studies showing infiltration of mononuclear cells two to three days post transplantation in the hamster-to-rat heart (Steinbrüchel et al. 1992) and lung (Nagayasu et al. 1996a, Nagayasu et al. 1996b) models. The number of cells isolated by ex vivo propagation during cell-mediated rejection was approximately six times higher than during AVR, and almost three times higher than under effective immunosuppression, thus reflecting a cell-mediated rejection response against the graft. Immunohistochemical stainings and morphological analyses confirmed these observations.

The technique of ex vivo propagation was first introduced in 1985 in attempts to monitor rejection of renal transplants in humans (Mayer et al. 1985, Miceli et al. 1985, Carlquist et al. 1988, Kirk et al. 1992, Webber et al. 1998). Clinical signs of rejection were found to correlate to the number and cytotoxic activity of ex vivo propagated cells (Frisman et al. 1991). In the field of xenotransplantation, the method was previously applied by culturing heart biopsies for two weeks in the presence of both recombinant IL-2 and irradiated autologous blood mononuclear cells (Michler et al. 1996). The technique has recently been modified, resulting in a protocol where the addition of cytokines or stimulatory cells is unnecessary (Engstrand et al. 1999). Even if the method has great advantages, it can be questioned whether it presents a true image of the actual rejection process or whether it is biased by external factors. Even though many cells migrate out from the biopsy, the graft probably still contains cells that escape the cell analysis. Perhaps the most activated cells are those remaining in the graft, but this remains to be elucidated. What happens with the cells while they are in the culture media is another relevant question. Ex vivo propagated cells removed after 24, 48 and 72 hours of incubation of the biopsies were shown to contain a similar percentage of activated cells (unpublished results). The actual amount of cells after 48 hours of incubation was markedly higher than after 24 hours. No difference was, however, detected between 48
and 72 hours of incubation. Whether the difference in cell number between 24 and 48 hours incubation is due to larger propagation or to proliferation of the cells is unclear.

Recently, we managed to isolate a few resident T lymphocytes and ED1+ macrophages from large biopsies (approximately 175 mg) taken from native rat hearts (unpublished results). This confirms that the method should be sensitive enough to isolate infiltrating cells present in the tissue in low amounts. In the small biopsies (approximately 80 mg) used in paper I, no immune cells were detected in control hearts. Hence, to study a few cells and thus increase the sensitivity of the method, the biopsies have to be rather large.

The cell infiltration in untreated grafts obtained on day 2 primarily consisted of ED1+ macrophages and T lymphocytes, but other cell types such as B lymphocytes and NK cells were also found in small amounts. The same cell types were also located in grafts subjected to cell-mediated rejection on day 8, except that the total number of ex vivo propagated cells was increased six times. Several other studies show unanimous results that the macrophage is the dominating cell type during the first two post transplantation weeks in mouse-to-rat cardiac grafts (Bersztel et al. 1998), in hamster-to-rat cardiac grafts (Lin Y et al. 1997a, Lin Y et al. 1997b), in guinea pig-to-rat cardiac grafts (Blakely et al. 1994), in hamster-to-rat aortic grafts (Scheringa et al. 1996) and in porcine islets in mice (Karlsson-Parra et al. 1996) and rats (Wallgren et al. 1995). CD4+ or CD8+ T lymphocytes can activate rejection of both vascularised (Fryer et al. 1995, Lin Y et al. 1999, Obatake et al. 1999) and non-vascularised (Pierson et al. 1989, Click et al. 1994) concordant xenografts in the presence or absence of other immune cells, thus indicating the capacity of the T lymphocyte to cause a rejection process. Our result that the number of T lymphocytes increases considerably on day 8 after DSG treatment hence strengthens the notion that this cell type is involved in cell-mediated rejection in a concordant model. Rejection of xenografts might, however, also be induced in the absence of T lymphocytes. In T cell-deficient rats it has been shown that NK cells and macrophages can in fact induce rejection on their own (Candinas et al. 1996, Lin Y et al. 1997a).

As seen in paper I, there were more propagated cells expressing the activation marker MHC II around biopsies with an ongoing cell-mediated rejection than around graft biopsies from rats receiving effective immunosuppression. In contrast, no difference was seen in the number of the IL-2R expressing T lymphocytes. Even though the percentage of activated T lymphocytes might not be altered, the actual number of these activated cells is markedly increased during cell-mediated rejection. Therefore, counting of the cells more correctly relates to the state of rejection than the percentage of activated cells, as previously stated (McKay et al. 1994, Engstrand et al. 1999).
Several methods, e.g. mixed lymphocyte culturing (Kruisbeek and Shevach 1997) and chromium-release assay (Wunderlich et al. 1997), are used to study immune responses during xenograft rejection, and are based on the use of organs in the central immune system, i.e. spleen, thymus and lymph nodes. Our findings of a difference in the expression of the activation markers MHC II and IL-2R between graft-infiltrating T lymphocytes and T lymphocytes from the spleen point to the importance of analysing the local processes in the graft. In fact, the examination of cytotoxic T lymphocytes in blood from patients that received heart transplants has been shown to be somewhat misleading in reflecting the actual rejection process (Vaessen et al. 1992).

During cell-mediated rejection, there were large interindividual differences in graft morphology as well as in the numbers of cells possible to isolate by ex vivo propagation. The number of cells ranged from 600 to 7,000 T lymphocytes per mg heart tissue. The reason for this might be found in the immunosuppressive protocol, where DSG is used to avoid AVR on day 3. Fulminate rejection after DSG treatment occurs in most of the grafts between day 9 and day 14, whereas some grafts survive as long as three weeks (Gannedahl et al. 1990, Valdivia et al. 1990, Johnsson et al. 1997b, Haga et al. 2000). The large variation in cellular infiltration could, therefore, be a result of differences in the overall effectiveness of DSG. However, DSG treatment always resulted in a lower CD4/CD8 ratio in the grafts as compared with all the non-DSG-treated grafts.

CYTOKINE PATTERNS

Both AVR and cell-mediated rejection were in our model associated with an increase of the pro-inflammatory cytokines IL-1β and TNF-α as well as of the more immune cell-specific cytokines IL-2, IL-10, IL-12p40 and IFN-γ in the grafts, shown in paper II. Under effective immunosuppression with CyA+DSG, the mRNA expression of all immune cell-specific cytokines were reduced to a near zero level, whereas IL-1β and TNF-α were present in low numbers. IL-4 was undetectable in most grafts.

Since the genetic sequences for IL-1β and TNF-α are highly conserved between rats and mice, the primers and probes used in this study quantify transcripts in both species. The early increase (day 2) in the pro-inflammatory cytokines could, hence, be a result of the local inflammatory response, and thus reflect cytokine production in mouse cells, as well as production by infiltrating recipient cells.
The differentiation of T lymphocytes after activation leads to the development of so-called Th1 or Th2 cells. These cells are discriminated by their different cytokine production, where Th1 cells mainly produce IL-2 and IFN-γ, whereas Th2 cells mainly produce IL-4, IL-5, IL-10 and IL-13 (Holgersson et al. 2002). IL-12p40, which is mainly produced by presenting cells (APC) (Gately et al. 1998), stimulates the differentiation of Th0 to Th1 lymphocytes. The expression of IL-12p40 seen in the grafts from untreated and DSG-treated rats should, thus, be a product of the large number of infiltrating macrophages (Hsieh et al. 1993, Macatonia et al. 1995, Desmedt et al. 1998). Further, the large numbers of IL-2 and IFN-γ expressed during both AVR and cell-mediated rejection indicate that xenograft rejection occurs in the presence of a Th1-associated immune response.

The mRNA expression of the Th2-associated cytokine IL-10 was found to be upregulated during rejection of the xenotransplanted mouse hearts. However, it has been postulated that IL-10 during cell-mediated xenograft rejection is not a part of a traditional Th2 response, but instead a product of the infiltrating macrophages (Feng et al. 1993, Brouard et al. 1998, Sebille et al. 2001, Krook et al. 2002). In this study, a high IL-10 expression, almost the same as during cell-mediated rejection (rats treated with DSG), was found during AVR (untreated rats) although the cell-mediated rejection was associated with a six-fold higher infiltration of immune cells, as seen in paper I.

In paper I, the presence of macrophages and T lymphocytes in rejecting and non-rejecting grafts was analysed by ex vivo propagation and immunohistochemistry. Together with the cytokine patterns described in study II, these data present important features of the rejection process. During both AVR and cell-mediated rejection, the expression of cytokines was strong even though AVR was associated with considerably fewer infiltrating cells than cell-mediated rejection. With CyA+DSG treatment, the cytokine transcription was effectively reduced whereas macrophages and T lymphocytes were still present. CyA is well known for its ability to suppress IL-2 (Andrus and Lafferty 1981, Elliott et al. 1984) and DSG is an inhibitor of antigen presentation (Hoeger et al. 1994). Since IL-2 and antigen presentation are two important factors in the activation of the immune cells, their inhibition could result in a reduction of other cytokines as well. This could explain the pattern with almost no cytokines detected during CyA+DSG therapy.

In long-time surviving concordant xenografts in recipients treated with CyA and cobra venom factor, IL-4 has been shown to be upregulated (Bach et al. 1997, Lin Y et al. 1999, Lin Y et al. 2000). Based on these data, it was proposed that Th2 cells were important in achieving long-term survival. However, our study indicates that a Th2-associated cytokine response is not a requirement for long-
time xenograft survival. This is in agreement with other studies where CyA in combination with the DSG analogue LF 15-0195 (Sebille et al. 2001), or in combination with rapamycin (Miyatake et al. 1998a), also induced long-term survival without any Th2-associated responses. Thus, instead, the Th2-associated cytokine response appears to be a parameter that follows from the immunosuppressive regimen with cobra venom factor.

The data from the analyses of spleen cytokines differed markedly from the patterns seen in the grafts, which, once again, suggests that rejection is best monitored locally in the grafts, rather than in the central immune system. The spleen may still present important information, and in the present study we showed that the spleen levels of IL-2 and IFN-\(\gamma\) were reduced in both DSG- and CyA+DSG-treated animals. Further, the reduction of IL-2 during CyA+DSG treatment was even stronger than during DSG treatment. This strong IL-2 reduction might correspond to the importance of a systemical inhibition of T lymphocyte activation to achieve long-term survival of the grafts.

One consideration with the technique used in this study is whether the quantification of cytokine mRNA transcripts really is correlated to the cytokine effects, and whether it corresponds to the actual rejection process. Arguably, measuring the cytokine proteins, rather than its coding mRNA, would be a more correct way to study cytokine patterns. However, a previous study on mouse heart allograft rejection showed that the rejection process was better monitored by RT-PCR analysis of cytokines than by intracellular cytokine staining analysed in a flow cytometer (Spriewald et al. 2000). RT-PCR was proposed to portrait the overall cytokine milieu instead of just the intracellular contents. The technique also eliminates the risk of measuring stored cytokines in intracellular vesicles not involved in the overall cytokine effects. RT-PCR is also highly applicable when using small tissue volumes.

Cytokines are known to be produced with onset and cease within hours after APC contact with the T lymphocyte (Slifka et al. 1999). Since T lymphocyte-specific cytokines are only produced as long as the T lymphocytes are in contact with APCs (Slifka and Whitton 2000), the production is obviously dependent on cell-to-cell interaction. Rapid ceasing in cytokine production is achievable because cytokine mRNA is highly unstable and quickly degraded (Lindstein et al. 1989, Sachs AB 1993). As a result, mRNA detection might be correlated to the amount of activated cells and their activation state, rather than to infiltrating cells in general. This is in agreement with the findings that grafts in the CyA+DSG group contained many infiltrating cells even though no immune cell-specific cytokines were detected. The long-time survival could, therefore, better be explained by the absence of cytokines in these grafts rather than by the low number of infiltrating cells.
Antibody responses after transplantation

All grafts with an ongoing rejection, i.e. untreated and CyA-treated grafts obtained on day 2 (AVR) and DSG-treated grafts obtained on day 8 (cell-mediated rejection), had deposits of IgM and IgG mainly according to a sarcolemmal distribution, as presented in paper II. CyA has been shown to reduce IgG production in the hamster-to-rat model (Miyatake et al. 1998b). In our study, this was seen by somewhat smaller deposits of IgG in grafts from CyA-treated recipients than in grafts from untreated recipients on day 2. However, treatment with CyA alone is clearly not sufficient to overcome AVR (Gannedahl et al. 1990, Miyatake et al. 1998a). DSG inhibits antibody production and macrophages (Tufveson and Gannedahl 1994) and effectively increases graft survival (Gannedahl et al. 1990, Valdivia et al. 1990, Johnsson et al. 1997b, Haga et al. 2000). With DSG treatment, almost no antibody deposits were seen in the grafts on day 2. However, during cell-mediated rejection both IgM and IgG antibodies were present. This indicates that DSG delayed, rather than inhibited, AVR, i.e. the cell-mediated rejection also has an AVR component. Combining CyA and DSG effectively reduced antibody deposition throughout the experiment.

Rat serum contains preformed IgM and a low titre of IgG antibodies against mouse antigens (Gannedahl et al. 1994, Gustavsson et al. 1996), and despite this no HAR occurs. Some explanations for this could be that 1) the preformed antibody titre is too low to induce terminal rejection; 2) the preformed antibodies have too low avidity towards mouse antigens to induce complement effectively; and 3) induction of IgG is required for HAR to occur. The avidity of the antibodies towards the antigen regulates their ability to activate the classical complement cascade (Cooper 1985). Thus, even if the antibodies produced are somewhat ineffective in their binding to the antigen, and hence also their complement activation, a large number of antibodies could probably exert effects on the graft. After the transplantation of an organ, B lymphocytes are relatively slowly activated to increase their antibody production. This process depends on the activation of memory B lymphocytes to differentiate into antibody-secreting plasma cells (Berek et al. 1987), which takes approximately two to three days. In our model, the antibody-mediated AVR thus coincides with the peak of plasma cell formation. In paper III, we show that antibody quantity is one important factor when determining the capacity of a serum to induce HAR. Passive transfer of anti-serum, diluted 1:4 in normal rat serum, to a rat transplanted with a mouse heart resulted in a delay of HAR from 29 min to 291 min.
In the pig-to-human situation, the Gal-antigen is the most important epitope towards which humans have preformed circulating antibodies (Galili et al. 1984). These antibodies consist of IgM (Parker et al. 1994), IgG (Parker et al. 1994, Galili et al. 1995) as well as IgA (Hamadeh et al. 1995). In humans, IgM is the primary natural antibody that induces complement activation when bound to pig antigens (Sandrin et al. 1993). In the concordant situation, the differences between IgM and IgG in inducing rejection are not yet completely understood. In previous studies, IgM induced HAR of hamster-to-rat heart grafts (Miyatake et al. 1998b), but not of mouse-to-rat heart grafts (Gustavsson et al. 2001), upon passive transfer to graft recipients. Both studies indicated, however, that IgG has a strong ability to induce HAR. According to paper III, the strength of the xenoantibodies to induce rejection was better measured by the ability of antibodies to activate complement-mediated haemolysis than by a haemagglutination assay. It thus indicated that complement activation by the xenospecific antibodies is important in predicting antibody-mediated rejection.

**Oral administration of xenoantigens**

When rats received fresh mouse erythrocytes orally, large numbers of xenospecific antibodies were produced. The strong antibody response consisted of IgM and IgG, and had similar effects as the powerful antibodies raised after intravenous administrations. In addition, two oral administration episodes also stimulated a xenospecific IgA production. Anti-serum raised by oral administration induced HAR of mouse cardiac grafts after intravenous injection to rat recipients in the same manner as intravenously raised anti-serum. These data, presented in paper III, indicate that the oral route of administration can be an effective immunization pathway in a xenogeneic situation rather than a tolerance induction pathway.

Oral administration of antigens mainly is an effective way of inducing unresponsiveness to specific antigens in experimental models for autoimmune diseases, allogeneic transplantation and non-vascularised xenotransplantation. However, despite all positive immunosuppressive effects, oral administration is apparently not favourable in all situations. One paper recorded that oral administration of the autoantigen actually induced autoimmune diabetes in mice (Blanas et al. 1996). The underlying mechanism was the induction of antigen-specific cytotoxic T lymphocytes. In paper III, we showed that potent antibodies are produced after the oral administration of mouse erythrocytes to rats. With two episodes of feeding, a higher erythrocyte-binding antibody titre was induced than with two intravenous administrations. However, the anti-serum raised after intravenous injection of mouse erythrocytes had a somewhat stronger capacity to induce complement-mediated destruction of mononuclear cell membranes than the anti-serum raised after oral administration. Thus, it appears that both B
lymphocytes, as presented in paper III, and cytotoxic T lymphocytes (Blanas et al. 1996) may be induced against orally given antigens.

One possible explanation for the observed phenomenon of oral immunization, rather than tolerance, could be the molecular differences and origin of the antigens involved in the stimulation of the gut immune system. In autoimmune diseases and allogeneic transplantation, the antigens are proteins or peptides of different sizes, whereas in xenotransplantation, the foreign antigens also involve carbohydrate structures, such as glycoproteins and glycolipids (Oriol et al. 1993, Gustavsson et al. 1996). Some of these carbohydrate structures resemble bacterial antigens, e.g. the Gal-antigen in the pig-to-human situation (Galili et al. 1988a). The oral administration of xenogeneic cells could, therefore, be similar to an immunization against bacteria-like antigens rather than a possible way of inducing tolerance.

Mouse erythrocytes were chosen as antigen source based on previous works, where feeding of sheep erythrocytes to mice induced antigen-specific unresponsiveness. The gut immune system reacted by inducing suppressor T lymphocytes (Mattingly et al. 1980), resulting in inhibition of antibody responses (Kagnoff 1977, Kagnoff 1978b) and delayed-type hypersensitivity reactions (Kagnoff 1978a). We have also tested the use of mouse mononuclear cells for oral administrations to rats. These cells express antigens on their surfaces that are similar to antigens found on mouse erythrocytes (Gustavsson et al. 2001). Mononuclear cells could also induce an antibody response in most animals. These antibodies did not agglutinate mouse erythrocytes well, indicating that either fewer antibodies or antibodies with less avidity were produced, or that antibodies were raised mainly against antigens that are not expressed on erythrocytes.

The interpretation of our results could be complicated by the fact that we used intact cells, rather than isolated antigens, as antigen source. The antigens causing an antibody-mediated rejection are still not identified in the mouse-to-rat model (Springer et al. 1978, Wu et al. 1999, Gustavsson et al. 2001). This will hopefully be done in the near future, and after isolation the more specific antigens should be used in this model to investigate their capacity to induce antibody responses. Still, our findings clearly indicate the potentials of the oral route for immunizations, and that oral administration of antigens might indeed be a dangerous pathway when trying to prevent xenogeneic rejection.

HYALURONAN IN XENOGRAFT REJECTION

In xenografts with an ongoing cell-mediated rejection (under DSG treatment) the hyaluronan content is considerably increased, as introduced in paper IV. At
the same time (on day 5 post transplantation) a severe increase of the relative water content was also seen. Further, on both day 2 and day 8 an increase in the pro-inflammatory cytokines IL-1β and TNF-α was observed in paper II. These phenomena together give further strength to the opinion that hyaluronan functions as a marker for inflammatory processes, as previously observed in both clinical and experimental situations, including allograft rejection (Colombel et al. 1989, Nettelbladt et al. 1989, Häggren et al. 1990a, Waldenström et al. 1991, Johnsson et al. 1996, Johnsson et al. 2000a).

Hyaluronan is synthesised mainly by fibroblasts. The fibroblasts are affected by different cytokines produced by immune cells, such as T lymphocytes and macrophages. TNF-α and IFN-γ can increase the proliferation rate of fibroblasts isolated from rejecting allografts, and IFN-γ also raises the hyaluronan production of these cells (Hellkvist et al. 2002). These cytokines are clearly involved in the rejection process of xenografts, as seen in paper II. Both TNF-α and IFN-γ are augmented during cell-mediated rejection, and the increase of IFN-γ might therefore be involved in the stimulation of fibroblasts to produce hyaluronan during xenograft rejection. In contrast, hyaluronan and other glycosaminoglycans are found to bind different cytokines to the tissue causing a local accumulation at the inflammatory site (Fernandez-Botran et al. 1999). Inhibition of this interaction prolongs the survival of allogeneic vascularised rat skin grafts (Fernandez-Botran et al. 2002).

When studying the distribution of hyaluronan, different patterns are seen when comparing the xenografts and the allografts. In the xenogeneic situation, hyaluronan is found inside and around large areas of destroyed musculature that are surrounded by a halo of interstitial oedema. A rejecting allogeneic heart transplant, on the other hand, has a more pronounced interstitial oedema between the heart muscle cells with severe accumulation of hyaluronan along the muscle cells (Häggren et al. 1990a, Johnsson et al. 1999).

During allogeneic rejection, the hyaluronan and relative water contents are directly correlated with each other. Likewise, the increase in relative water content reflects an interstitial oedema. Thus, treatment with the hyaluronan-degrading enzyme hyaluronidase decreases the relative water content as well as the interstitial oedema and the interstitial pressure of the allograft (Johnsson et al. 1999, Johnsson et al. 2000b). This clearly differs from the xenogeneic situation where hyaluronidase effectively decreased the hyaluronan content, but did not lower the average water content.

Since the hyaluronidase-mediated reduction of hyaluronan did not affect graft oedema when studying graft morphology and relative water content, it could be questioned whether hyaluronan actually has an important function in xenograft rejection. However, when linear regression analysis was used, a correlation between hyaluronan and relative water content was actually found in
the hyaluronidase+DSG group. Hence, we conclude that hyaluronidase therapy really does affect the water content by reducing it slightly. This indicates that a hyaluronan-induced oedema occurs during xenograft rejection, but that this is less important than other factors that might cause interstitial or cellular oedema. A hyaluronan-independent cause of interstitial oedema could be the antibody-mediated destruction of myocytes and the following leakage of intracellular fluid into the interstitium. Perhaps xenograft-specific antibodies affect ion channels in the cell membranes, causing an increase in intracellular sodium ion content. This could lead to a passive inflow of water into the cells, hence resulting in a cellular oedema.

Another factor that distinguishes the xenogeneic from the allogeneic situation is that no differences in cellular infiltration were observed in the xenografts when comparing the two therapies. In rejecting allografts, hyaluronidase does not only ameliorate interstitial oedema but also reduces the cellular infiltration (Johnsson et al. 1999). This is probably mediated through the interaction between hyaluronan and CD44, an interaction that, when blocked, can increase the survival of allogeneic grafts (Zhang W et al. 2000). Activated T lymphocytes express the hyaluronan-binding isoform of CD44 (DeGrendele et al. 1997b) and can thus target hyaluronan on endothelial cells (DeGrendele et al. 1997a) and in the extracellular matrix. In xenotransplantation, the failure of hyaluronidase to decrease cellular infiltration could be due to higher concentrations of cytokines and chemotactic factors in xenografts than in allografts. This would increase the expression of other types of adherent cell-surface molecules on the endothelial cells and also increase the chemotactic signals to the immune cells. As a result, the relative importance of the binding of cell surface CD44 on activated T lymphocytes to hyaluronan would decrease.

No difference in mean graft survival time was seen between the two treatment groups. Still, there were three grafts in the hyaluronidase+DSG group beating one to three weeks longer than the longest surviving graft in the DSG group. As presented in papers I and II, the interindividual variation in cell infiltration, morphology and antibody deposition during DSG treatment is significant. Hence, when optimal effects with DSG are achieved, hyaluronidase appears to contribute to an increase in graft survival.

Paper IV is the first study to investigate the involvement of hyaluronan during xenograft rejection and our data suggest that hyaluronan might contribute to the rejection process of xenografts. However, to get a more complete picture and to explain whether hyaluronan has similar functions in the xenogeneic situation as in the allogeneic, different xenotransplantation models and pharmaceuticals have to be used.
Future perspectives
The introduction of the knockout pig lacking both genes that encode the \(\alpha 1,3\)-galactosyltransferase is considered to be an important step towards inhibiting HAR and AVR in the pig-to-human situation. However, one has to be cautious about drawing the conclusion that this will be the solution to antibody-mediated xenograft rejection. Other epitopes than the Gal-antigen might become more relevant, thus taking the Gal-antigen’s place as the most important antigen. Even if humans only have a few preformed antibodies against other antigens and HAR thus is avoided, the antibodies could hypothetically cause an AVR. This further strengthens the use of concordant models, such as the mouse-to-rat, to study rejection processes that are relevant in a discordant situation where HAR is inhibited. Since the cell-mediated and chronic rejections are indicated to be stronger in the xenogeneic than in the allogeneic situation, these rejections have to be specifically studied to understand the underlying mechanisms and to find out how rejection is best avoided.

In this thesis, some of the potentials of ex vivo propagation of graft-infiltrating lymphocytes are highlighted. This technique should be highly useful in future studies of cell-mediated xenograft rejection mechanisms. Further, the content of the tissue stabilising molecule hyaluronan was found to be heavily increased during early xenograft rejection. The involvement of hyaluronan in later rejection processes is still unknown, but has to be explained during later cell-mediated processes as well as during chronic rejection.

Oral tolerance induction has effectively been used as a complement to immunosuppressive treatment in experimental allogeneic transplantation. In xenotransplantation, tolerance induction should be favourable since the strong immunosuppression otherwise needed might have fatal side-effects. However, as shown in this thesis, one has to be cautious when trying to induce oral tolerance in xenotransplantation since oral administration of xenoantigens could actually be harmful by inducing powerful antibody responses.
CONCLUSIONS

– Xenograft infiltrating immune cells can be isolated by ex vivo propagation.

– The numbers of recovered T lymphocytes are considerably higher in grafts undergoing cell-mediated rejection than in grafts undergoing acute vascular rejection or in non-rejecting transplants.

– Acute vascular rejection as well as cell-mediated rejection of concordant vascularised xenografts are associated with an increased cytokine mRNA expression of both pro-inflammatory cytokines (IL-1β and TNF-α) and more specific cytokines (IL-2, IL-10, IL-12p40 and IFN-γ).

– Cytokine patterns and expression of activation markers on T lymphocytes differ between the xenograft and the spleen of the recipient.

– Oral administration of xenogeneic cells induces production of mainly IgM and IgG antibodies that are capable of inducing hyperacute rejection of vascularised xenografts when passively transferred to graft recipients.

– The hyaluronan and water contents of the graft increases during an ongoing cell-mediated rejection. Hyaluronidase effectively reduces hyaluronan, but does not affect the water content.
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