Studies of Acute Rejection Using Contrast Agents and Magnetic Resonance Imaging

EVA PENNO
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Abstract

Solid organ transplantation is today an established form of treatment for end-stage organ disease. Monitoring of graft function and pharmacological therapy constitutes a maze of clinical observations and histological evaluations of biopsy specimens; with the biopsy results playing a decisive role. The aims of this doctoral research were to investigate the feasibility of detecting acute rejection of transplanted organs and monitoring the effect of anti-rejection treatment, with the use of ultrasmall superparamagnetic iron oxide particles (USPIO) and magnetic resonance (MR) imaging with a clinical MR scanner.

Allogeneic and syngeneic heterotopic heart transplantations were performed in rats. Three different-sized USPIO were given to one allogeneic and one syngeneic group. The change in MR signal intensity (SI) over time was measured. An increase in SI was interpreted as damage to micro vessels due to the pronounced inflammatory reaction caused by acute rejection, which led to leakage of USPIO into the tissue. A decrease in SI was interpreted as normal vascular structure, since USPIO normally remains in the intravascular space. The same method, using one of the previously tested USPIO, was used in a treatment study in which acute rejection in transplanted rats was induced and subsequently treated. An attempt was also made to detect presence of macrophages in an acutely rejecting graft, since this cell type plays an important role in the acute rejection process; this was done by testing the ability of macrophages to phagocytose the USPIO compound.

In permeability studies with MR imaging all three USPIO tested discriminated between rejecting and non-rejecting grafts without any overlap of the groups. Factors that contributed to the ability to distinguish between grafts were the size and half-life of the particle. We were also able to monitor effects of anti-rejection treatment by studying the vascular permeability of USPIO and MR imaging. We did not succeed in detecting macrophages in the rejecting grafts with USPIO and MR imaging.

This thesis presents a novel approach to detection of acute rejection of transplanted organs and to monitoring the effects of anti-rejection treatment using different USPIO contrast agents and MR imaging in a clinical MR scanner.

Keywords: Magnetic resonance imaging, contrast agents, organ transplantation, acute rejection

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List of papers

This thesis is based on the following studies, which will be referred to in the text by their Roman numerals

I  Acute cardiac transplant rejection: detection and grading with MR imaging with a blood pool contrast agent - experimental study in the rat. 
Lars Johansson, Cecilia Johnsson, Eva Penno, Atle Björnerud and Håkan Ahlström. 
Radiology 2002;225:97-103.

II  Comparison of USPIO and low-molecular-weight contrast agents to detect rejecting transplanted hearts with MRI. 
Eva Penno, Cecilia Johnsson, Lars Johansson and Håkan Ahlström. 

III  Macrophage uptake of ultrasmall iron oxide particles (USPIO) for MR imaging in experimental acute cardiac transplant rejection. 
Eva Penno, Cecilia Johnsson, Lars Johansson and Håkan Ahlström. 

IV  Ultrasmall iron oxide particle contrast agent and magnetic resonance imaging can be used to detect the effect of anti-rejection treatment. 
Eva Penno, Lars Johansson, Håkan Ahlström and Cecilia Johnsson. 
Manuscript.

Reprints were made with permission of The Radiological Society of North America, (Study I), Lippincott Williams & Wilkins (LWW), (Study II), and Taylor & Francis, (Study III).
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ADC</td>
<td>Apparent diffusion coefficient</td>
</tr>
<tr>
<td>BOLD</td>
<td>Blood oxygen level-dependent</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>FOV</td>
<td>Field of view</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigens</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MPIO</td>
<td>Micrometer-sized paramagnetic iron oxide particles</td>
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<tr>
<td>MR</td>
<td>Magnetic resonance</td>
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<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>PAP</td>
<td>Peroxidase-antiperoxidase</td>
</tr>
<tr>
<td>POD</td>
<td>Post operative day</td>
</tr>
<tr>
<td>$r_1$</td>
<td>T1 relaxivity</td>
</tr>
<tr>
<td>$r_2$</td>
<td>T2 relaxivity</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>T</td>
<td>Tesla</td>
</tr>
<tr>
<td>T1 relaxation</td>
<td>Longitudinal relaxation time</td>
</tr>
<tr>
<td>T2 relaxation</td>
<td>Transverse relaxation time</td>
</tr>
<tr>
<td>T2*</td>
<td>Total transverse relaxation time</td>
</tr>
<tr>
<td>TE</td>
<td>Echo time</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>SI</td>
<td>Signal intensity</td>
</tr>
<tr>
<td>SPIO</td>
<td>Superparamagnetic iron oxide</td>
</tr>
<tr>
<td>USPIO</td>
<td>Ultrasmall superparamagnetic iron oxide</td>
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</table>
Introduction

Magnetic resonance imaging

Background

The physical phenomenon nuclear magnetic resonance (NMR) was first described independently in 1946 by Bloch (1) and Purcell (2), who shared the Nobel Prize in physics in 1952 for this discovery. In 1973 Lauterbur demonstrated for the first time how the NMR phenomenon could be converted into an image (3) and in 1976 made Mansfield produce the first images of living humans (4).

The physical principles are difficult to describe in detail and demand knowledge in quantum mechanics. For a short review, however, classical physics will suffice. When certain atoms, for example $^1$H, $^{31}$P, and $^{13}$C are exposed to an external magnetic field, a spin will arise. The spin can organize itself either in a parallel or an anti-parallel fashion and the sum of the magnetic moment will create a magnetic vector which is aligned with the external magnetic field. The level of energy in the spins can be changed by supplying energy in the form of a short radio-frequency (RF) pulse. If this is of the right frequency (Larmor frequency), resonance can be induced in the system, whereby the equilibrium will be altered and the net magnetization vector will change to a plane perpendicular to the external magnetic field. When the spins are returned to their initial state, a signal can be detected, the strength of which reflects the concentration of the spinning atoms and the relaxation times (T1 and T2), which in turn reflects, the molecular dynamics. By this means different signals will be induced in different tissues. In magnetic resonance tomography the protons in the water molecule and in fat the protons in the carbon-hydrogen chains in the lipids are utilized. A magnetic resonance image can thus be seen as a map of water and fat, depending on the parameters chosen (5).

To generate an image the signal has to be encoded, which is done by overlaying the main magnetic fields with gradient magnetic fields. Since the Larmor frequency is proportional to the field strength, the gradients give use to a distribution of Larmor frequencies. The RF pulses can then be repeated at different frequencies and with varying gradient strengths. This makes it possible to localize the spins spatially in the object one wants to depict.

Contrast agents

To further increase the contrast between tissues in the magnetic resonance (MR) images, a contrast agent can be administered. This increase can be achieved in two ways either by decreasing the time it takes for the net magnetization vector to revert to the direction of the main magnetic field (T1 relaxation) or by causing the spins that make up the vector to go out of phase with one another (T2 relaxation).
The efficacy of the contrast agent is measured by its relaxivity. The relaxivity is a constant which reflects the capability of altering the relaxation times per unit of concentration of the contrast agent. This assumes a linear relationship between the effect of the contrast agent and the concentration of the contrast agent, but this is only true in solutions and not for the *in vivo* situation. The two relaxivity constants are referred to as $r_1$ and $r_2$.

**Paramagnetic contrast agents**

The most commonly used contrast agents used are paramagnetic. These substances have a magnetic moment, but only when placed in an external magnetic field. They have unpaired electrons and the larger the number of electrons the stronger the paramagnetic effects. These compounds affect both T1 and T2 relaxation, but have their greatest effect on T1. For clinical practice today, low-molecular-weight gadolinium dominates the market. Gd$^{3+}$ has seven unpaired electrons, but is toxic and has to be linked to a carrier molecule when used *in vivo* (6). The result is a chelate with a molecular weight of 600-800 Daltons, which distributes intravascularly and into the extracellular space. The gadolinium chelates are eliminated inertly through renal filtration and the half-life in blood thus depends on the glomerular filtration rate, but is approximately 60-70 min. The time taken for the contrast agent to reach steady state between the intravascular and extravascular space is only a few minutes.

**Superparamagnetic contrast agents**

The other type of contrast agent in clinical use is the superparamagnetic type are based on iron oxide crystals. These can consist of magnetite (Fe$_3$O$_4$) or maghemite ($\gamma$ Fe$_2$O$_3$). Each crystal consists of thousands of Fe ions (Fe$^{2+}$ and Fe$^{3+}$) and the core usually has a diameter of 5-10 nm. The total diameter, which ranges between 15 and 200 nm, depends on the coating of the iron core. The reason why the iron oxide particles are called superparamagnetic is that the magnetic moment supersedes the sum of the individual iron ions. This group of contrast agents is also called intravascular, since, because of their size, they remain in the intravascular space and is eliminated through the reticuloendothelial cells in the liver, spleen, and bone marrow. The half-life in blood differs considerably between the compounds, depending on their size, chemical characteristics and type of coating. The superparamagnetic contrast agents can be divided into two groups, namely superparamagnetic iron oxide particles (SPIO) with a diameter of more than 50 nm, and ultrasmall superparamagnetic iron oxide particles (USPIO) with a diameter of less than 50 nm (7). The USPIO contrast agent are recognized within minutes by the reticuloendothelial cells and hence have a short intravascular half-life. They have been used mostly as a specific
contrast agent for the liver (22-24).

This thesis will focus on USPIO contrast agents and their ability to detect acute rejection in experimental heart transplantation.

**Transplantation**

**History**

There has always been a fascination with the possibility of changing and replacing worn-out and non-functioning organs in the human body. Despite this it was only in the beginning of the 20th century that surgical advances led to transplantation, and it was in the 1950s, following the progress in immunology in the previous decade, that the first successful human renal transplantation was achieved. The development of successful solid organ transplantation has proceeded in parallel with the advances in pharmacological immunosuppression. Initially, the greatest obstacle to graft survival was acute rejection, and in attempts to overcome this problem a number of different treatments were tried. The great breakthrough came at the end of the 1970s with the introduction of cyclosporin A (25, 26). Nevertheless, even a single episode of acute rejection still remains a strong predictor of long-term graft failure (27). During the years since the 1970s the number of patients that remain rejection free in the first two years after a transplantation has increased tremendously (25).

Transplantation has today become a successful therapeutic option for patients with end-stage organ disease and the list of patients waiting for transplants far exceeds the availability of organs.

**Transplantation immunology**

The immune system of the body protects us from foreign agents. The defence is organized as a specific and a non-specific system. The non-specific defence protects us from previously unknown agents, while the specific defence consists of cellular and antibody immunity. Transplant immunity results after exposure of a recipient to different histocompatibility antigens. The major transplantation antigens are the human leukocyte antigens (HLAs). HLAs are controlled by a series of genes on chromosome 6, referred to as the human major histocompatibility complex (MHC). These genes have been classified into major categories. HLA-A and HLA-B are examples of class I genes and HLA-DR represents class II genes. These genes are highly polymorphic. It is therefore unlikely that two unrelated persons will have the same HLA type. Both HLA class I and class II alloantigens can induce transplant immunity at humoral (antibody) and cellular (T lymphocyte) immune levels. ABO incompatibility is important only in antibody-mediated injury of the graft.

Since transplant immunity leads to rejection, it seems best that the patient and donor be matched for histocompatibility antigens. This is possible for geneti-
cally identical twins (syngeneic grafts) and to a lesser extent between HLA- and ABO-identical siblings, but the vast majority of transplants come from incompatible, unrelated donors. Rejection can be mediated by antibodies, lymphocytes, or both and can occur in different forms: namely as hyperacute rejection, acute rejection, and chronic rejection.

Types of rejection

Hyperacute rejection

Hyperacute rejection may occur within minutes or a few hours after transplantation in pre-sensitized persons. This humoral presensitization may be caused by a previous transplant, blood transfusions, or pregnancy. The primary target of the donor-specific antibodies is the vascular endothelium of the transplanted organ, and the antigens mostly affected are HLA class I and ABO. Activation of the complement cascade leads to release of various inflammatory mediators and initiation of the coagulation and fibrinolytic systems. Hyperacute rejection is manifested by rapid vascular constriction, edema, and thrombotic occlusion. As a consequence of the vascular damage, the graft never becomes revascularized and it undergoes ischemic necrosis. This type of rejection is prevented with the current practice of cross-matching, and is no longer a significant clinical problem (28).

Acute rejection

An acute rejection may take place within days of transplantation, or appear suddenly months or even years later. In most cases it can be treated pharmacologically. Acute rejection is mediated by lymphocytes, which have become alloactivated against the donor antigens. The strongest stimulus is provided by donor dendritic cells (for example monocytes and macrophages) which directly activate the lymphocytes. The antigen-presenting function of the dendritic cells provides a strong signal to the HLA class II reactive CD4+ cells, which can stimulate the growth and differentiation of, for example, HLA class I reactive CD8+ lymphocytes. Graft-infiltrating lymphocytes mediate various effector mechanisms of allograft immunity, such as direct cytotoxic effects and recruitment of numerous immunocompetent cells, by releasing various cytokines. The activation of the immune system will lead to tissue damage and necrosis. The majority of the cells that accumulate in the graft are macrophages and T lymphocytes (28).

Chronic rejection

In this type of rejection, gradual impairment of the graft function and morphology occurs. Its pathogenesis probably involves both humoral and cellular immune mechanisms. The endothelial cells are damaged or destroyed. Histologically there is vascular inflammation, which is sometimes called transplantation arteriosclerosis, since it is similar to that observed for instance, in coronary arteriosclerosis, but there is also a progressive fibrosis in the parenchymal tis-
The condition cannot be treated and the prognosis has not been improved despite the recent years’ developments of immunosuppressive drugs. Many risk factors are known, for example previous acute rejection episodes, hyperlipidemia, and a high age of the donor, but there are probably many more (28).

**Immunosuppressive treatment**

Immunosuppressive drugs are given to control transplant immunity, so that acute rejection can be prevented or managed. This is a life long treatment in which a balance has to be achieved between suppression of the immune system to prevent upcoming rejections and tolerability of the drugs and their side effects (26). The immunosuppressive effect cannot be measured on an individual level. Neither can the individual need for immunosuppression be estimated. Hence the administered doses are based on an empirically average need. Tolerance of the graft will gradually occur, but not total acceptance; thus the therapy can never be totally discontinued (29).

**Diagnosis of acute rejection**

The diagnosis of acute rejection today is based partly on the sum of clinical observations and laboratory tests, but first and foremost on the result of a biopsy. However, biopsies are inconvenient for the patient and associated with certain risks (30-33). In renal transplantation some studies have shown computer tomographically verified hematomas after biopsy in 100% of the cases (34) and an incidence of arteriovenous fistula in 10-15% (35, 36). In heart transplantation biopsies are the gold standard and during the first year post transplantation a number of biopsy samples are taken for surveillance. Biopsies can also give some false negative results, where a rejection occurs but the biopsy is silent. This strengthens the view that lymphocytic graft infiltration may not be the only mechanism of cardiac rejection and that endomyocardial biopsies may fail to detect such episodes before hemodynamic compromise (37). A biopsy also provides only a limited view of the state of the entire graft, since only a small specimen of the tissue is obtained. Clinical signs of rejection include peripheral edema, bradycardia, atrial flutter, or more serious symptoms such as impaired systolic function. There may also be more subtle indications of rejection, such as gain in weight, shortness of breath, or fever (38). In 30-40% of heart transplanted patients, one or more acute rejections occur within the first post transplant year (38). In renal transplantation an elevated serum creatinine level can lead to a suspicion of rejection, but this is also observed in a number of other conditions, such as acute tubular necrosis, dehydration, accumulation of lymph fluid around the graft and problems with the arterial anastomosis. Clinical symptoms such as fever, decreased urine output, weight gain, increased blood pressure, or tenderness over the graft, which previously were thought to indicate possible rejection, have today almost disappeared, as a result of the new and better immunosuppressive drugs that have entered the market (29).
Detecting acute rejection of transplanted organs with magnetic resonance imaging

Different methods have been described for diagnosing acute rejection of transplanted organs with magnetic resonance imaging. In some studies native MR imaging, has been used, and in others different kinds of contrast agents have been tried. A short summary of some previous studies is given below.

Studies performed without contrast agents

Sadowski et al (39) using blood oxygen level-dependent (BOLD) MR imaging found significantly lower R2* values in the renal medullary region in patients with acute rejection compared to those in patients with acute tubular necrosis or normal renal function.

Cortical perfusion measured with arterial spin labeling has been described in experimental renal transplants at 4.7T (40). In that study the changes in tissue water magnetization were measured, using endogenous arterial water as a tracer, by magnetically labeling the in-flowing blood water protons in the suprarenal aorta. There was a difference between allogeneic and syngeneic grafts in that the perfusions in the allogeneic grafts were immeasurable as a result of damage to the microcirculation.

Studies using magnetic resonance spectroscopy (MRS) have been performed in experimental heart and renal transplants (41, 42) (43). There have been reports on clinical studies, on relatively small number of patients in which 31P-magnetic resonance spectroscopy has been used to demonstrate different relative concentrations of phosphate-containing metabolites in renal grafts due to acute rejection (44) (45) (46). MRS is a very cumbersome technique, which is highly sensitive to motion artefacts, but might be having a revival with the newer scanners and more refined methods.

In a resent attempt to look at diffusion imaging at 7T the ADC values were found to be significantly decreased in allogeneic renal transplants compared to syngeneic and native kidneys (47).

A retrospective clinical study on heart transplantation showed that patients with biopsy-confirmed moderate acute rejection had a T2 value higher than normal (≥ 56 ms), as a result of increased edema (48).

Studies performed with low-molecular-weight contrast agents

First pass perfusion with low-molecular-weight gadolinium contrast agent has been observed in pancreatic and renal human grafts (49-53). However, the studies were made on small numbers of patients and in some cases an overlap was seen between the rejecting and non-rejecting grafts; in other cases, no biopsies were correlated to the MR results.
Studies performed with iron oxide particle contrast agents

First pass perfusion with a few different USPIO contrast agents have been reported from experimental renal transplantation studies (54, 55). Some USPIO have been shown to be phagocytosed by rat macrophages and to accumulate in the vacuoles, which gives a strong susceptibility effect. In ex vivo experiments no signal intensity loss has been seen when the particles are dispersed. The accumulation of macrophages in transplanted organs has been monitored in experimental renal, heart, and lung grafts (56-60). All these studies were performed on a 4.7T scanner with gradient echo. The results are promising, but there is a time delay between injection of the contrast agent and observation of the result on the MR image. To my knowledge no clinical studies have been published with use of this method yet.

Background of the present studies

The idea of investigating rejecting transplanted organs with intravascular contrast agents and MR imaging was based on an experimental study by Daldrup et al (61) in which they used albumin-Gd-DTPA and a low-molecular-weight contrast agent in benign and malignant tumours. With the use of the macro-molecular contrast agents the permeability was higher in the malignant tumors than in the benign tumors, owing to the disrupted endothelial integrity of the microvessels in the malignant tumors. With the low-molecular–weight contrast agent no difference in permeability was seen between the two types of tumors. Since then a USPIO has been tested in tumor models in a similar way (62, 63).

Acute rejection of transplanted organs is a powerful inflammatory process with edema, cellular infiltration and finally cell death and necrosis. The idea was therefore to test the microvascular permeability of USPIO in an inflammatory model, to see whether discrimination between rejecting and non-rejecting grafts could be achieved.

Detection of the presence of macrophages in acutely rejecting transplanted organs has been achieved, as mentioned above, on a 4.7T scanner. This is made possible by the documented the fact that some USPIO are taken up by macrophages, and that a large proportion of the infiltrating cells in an acutely rejecting organ consists of macrophages (64). Thus it was of interest to test the applicability of this method on a clinical 1.5T scanner.
Aims of the study

General aim

The overall aim of this research was to investigate the feasibility of using USPIO contrast agents in MR imaging to detect acute rejection of transplanted organs.

Specific aims

To examine the possibility of detecting acute rejection of transplanted hearts by studying the vascular endothelial permeability of a USPIO contrast agent in MR imaging (study I).

Using the method applied in study I, to evaluate the ability of USPIO contrast agents, of different sizes, to detect acute rejection in transplanted hearts as compared with that of a conventional low-molecular-weight contrast agent (study II).

To investigate the feasibility of demonstrating the presence of macrophages in acutely rejecting transplanted hearts using a USPIO contrast agent and MR imaging (study III).

To attempt to demonstrate the effect of anti-rejection therapy, by using the method developed in study I and II, in a more clinically relevant acute rejection model (study IV).
Materials and Methods

Animals, transplantation and drug treatment

Animals and anesthesia

The following animals were used in all of the studies I-IV, male inbred Wistar/Kyoto rats from Taconic M&B (Ry, Denmark), Charles River (Sulzfeld, Germany), or M&B (Skensved, Denmark) and male inbred PVG rats from Taconic M&B (Ry, Denmark), M&B (Skensved, Denmark), Charles River (Sulzfeld, Germany), or B&K Universal (Sollentuna, Sweden). The animals were allowed to acclimatize for at least one week before the experiment started and had unlimited access to food and water throughout the experiment. At the beginning of the experiment the animals weighed between 180 and 340 g. For the heterotopic heart transplantation (see below) and MR imaging, anesthesia was induced by intraperitoneal injection of a mixture of chloral hydrate (180 mg/kg body weight [b.w.]), pentobarbital (40 mg/kg b.w.) and magnesium sulfate (90 mg/kg b.w.). All operations were performed under aseptic conditions. 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 regional ethical committee approved the experiments. Table 1 shows the experimental groups, contrast agents used, and doses given in the studies included in this thesis.

Heterotopic heart transplantation

In all studies (I-IV) the heart transplantations were performed heterotopically. In short: The caval and pulmonary veins of the donor are ligated. The aorta of the donor is connected to the carotid artery of the recipient and the pulmonary artery of the donor is connected to the jugular vein of the recipient. This is done with a non-suturing technique, where small plastic tubes are threaded over the carotid artery and the jugular vein. The vessels are then turned inside out over the tubes and fixed with ligatures. The donor aorta and pulmonary artery are then pulled over the tubes and secured with ligatures. The non-suturing technique is explained in Figure 1. The arterial supply for the organ is thus the aorta, and the venous outflow is the pulmonary artery. In detail, the path of the arterial blood supply will start in the aorta and pass through the coronary arteries to the right atrium and then to the right ventricle and finally through the pulmonary artery to the jugular vein. Very little blood will spill over to the left ventricle, which often will be trombotized. This is a general organ transplantation model and because of the altered blood flow it cannot be used as a specific model for heart transplantation. The model is more thoroughly described by Olausson et. al. (65). The technique allows a very short warm ischemic time of less than 10 min. Graft survival is checked by daily palpation.
<table>
<thead>
<tr>
<th>STUDY</th>
<th>TRANSPLANTATION</th>
<th>CONTRAST AGENT</th>
<th>DOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Allogeneic</td>
<td>NC100150 Injection</td>
<td>2 mg Fe/ kg bw</td>
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<tr>
<td></td>
<td>n=6</td>
<td></td>
<td></td>
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<td>I</td>
<td>Syngeneic</td>
<td>NC100150 Injection</td>
<td>2 mg Fe/ kg bw</td>
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<td></td>
<td>n=6</td>
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<td></td>
</tr>
<tr>
<td>II</td>
<td>Allogeneic</td>
<td>Unformulated NC100150</td>
<td>2 mg Fe/ kg bw</td>
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<td>II</td>
<td>Allogeneic</td>
<td>AMI-227</td>
<td>2 mg Fe/ kg bw</td>
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<tr>
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<td>AMI-227</td>
<td>2 mg Fe/ kg bw</td>
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<tr>
<td>II</td>
<td>Allogeneic</td>
<td>Gadodiamide</td>
<td>0.2 mmol/ kg bw</td>
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<tr>
<td>II</td>
<td>Syngeneic</td>
<td>Gadodiamide</td>
<td>0.2 mmol/ kg bw</td>
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<td></td>
<td>n=5</td>
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<tr>
<td>III</td>
<td>Allogeneic</td>
<td>AMI-227</td>
<td>2 mg Fe/ kg bw</td>
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<tr>
<td>III</td>
<td>Syngeneic</td>
<td>AMI-227</td>
<td>2 mg Fe/ kg bw</td>
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<tr>
<td></td>
<td>n=6</td>
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<td></td>
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<tr>
<td>III</td>
<td>Allogeneic</td>
<td>AMI-227</td>
<td>11 mg Fe/ kg bw</td>
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<td>III</td>
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<td>AMI-227</td>
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<td>IV</td>
<td>Allogeneic, treated group</td>
<td>NC100150 Injection</td>
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<tr>
<td>IV</td>
<td>Allogeneic, rejecting group</td>
<td>NC100150 Injection</td>
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Table 1.
The experimental groups and the contrast agents given in studies I-IV. The total number of heart-transplanted animals in this investigation was 83. The syngeneic and allogeneic groups receiving 2 mg Fe/kg bw of AMI-227 in studies II and III were the same animals.
Fig. 1.
The illustrations A to H explain the non-suturing cuffing technique schematically. A short plastic tube is pulled over the recipient vessel (A and B). The vessel is turned inside out and is folded over the plastic tube and secured with a ligature (C to F). The donor vessel is then threaded over the plastic tube that is connected to the recipient vessel and this is secured with another ligature (G and H).
Both allogeneic and syngeneic transplantations were performed. Wistar/Kyoto rats were used as recipients in all transplantations and as donors in the syngeneic transplantations. PVG rats were donors in the allogeneic transplantations. With this combination of rat strains the grafts will stop beating about 8 days post transplantation.

**Contrast agents**

NC100150 Injection (Clariscan™, Amersham Health, Oslo, Norway) was used as contrast agent in studies I and IV. This is a USPIO with an iron core of 6.43 nm, a starch coating (66), and a total diameter of 15 nm. The $r_1$ relaxivity at 60MHz was $13.4\text{ s}^{-1}\text{ mM}^{-1}$ and $r_2$ was $48\text{ s}^{-1}\text{ mM}^{-1}$. In study II, two different USPIO were used. One was unformulated NC100150, which has an iron core identical to that of NC100150 Injection but does not contain methoxy PEG, and was sterile filtered instead of autoclaved. This makes the total diameter slightly smaller than that of NC100150 Injection. The $r_1$ of unformulated NC100150 at 60 MHz was $10.7\text{ s}^{-1}\text{ mM}^{-1}$ and $r_2$ was $45\text{ s}^{-1}\text{ mM}^{-1}$, (information obtained from Amersham Health). The second contrast agent used in study II and also in study III was AMI-227 (Sinerem™, Laboratoire Guerbet, Aulnay, France), a blood pool agent with an iron core and a dextran coating, with a mean diameter of the whole particle of 35 nm and a mean core diameter of 6.3 nm. The $r_1$ of AMI-227 at 60MHz was $8.3\text{ s}^{-1}\text{ mM}^{-1}$ and $r_2$ was $73\text{ s}^{-1}\text{ mM}^{-1}$. All relaxivity values were determined in whole blood at 40°C.

In study II we also used a conventional low-molecular-weight gadolinium (Omniscan™, Amersham Health, Oslo, Norway).

The dose used for all USPIO in studies I-IV was 2 mg Fe/kg b.w. The dose was chosen in study I to maximize the T1-relaxation effect while maintaining the T2*-relaxation effect at a level at which it could be neglected for the chosen acquisition parameters (67). In study II-IV the same dose was used. In study III we also used a higher dose of AMI-227, 11 mg Fe/kg b.w., which is recommended by the manufacturer to compensate for the more rapid metabolism of the contrast agent in rats compared to man.

In study II the dose of the low-molecular-weight gadolinium contrast agent was 0.2 mmol/kg, which is a standard clinical dose.

All contrast agents were given intravenously through a catheter placed in the femoral vein.

**Magnetic resonance imaging**

The rat was placed in a supine position in study I and in a prone position in study II-IV, on top of a heating table with a 22-mm surface coil in a 1.5T clinical scanner (Gyroscan ACS-NT, Philips Medical Systems, Best, the Netherlands).
In study I, II, and IV, in those groups that received USPIO contrast agent, a 3D dynamic scan in 20 phases, with the first dynamic phase one minute after injection of the contrast agent as a reference point, was performed. Each dynamic scan lasted 130 s. The scan was a spoiled gradient echo with TR/TE 20/3.1 ms with a flip angle of 35°. FOV was 70 mm with a 256 x 256 matrix yielding an in-plane resolution of 0.27 mm, and slice thickness of 0.5 mm. The relative change in signal intensity (SI) over time, in percent, was calculated, with the first time-point after contrast agent injection as reference.

In study II in the group that received gadolinium a gradient echo sequence with a TR/TE 14/4.6 and a flip angle of 10° was performed. The FOV was 50 x 34.4 with an in-plane resolution of 0.78 mm, giving a dynamic scan of 720 ms. For viewing and analysis the images were interpolated to a 256 x 256 matrix. The slice thickness was 2 mm. The analysis was done as a calculation of the steepest slope produced by the first passage of the contrast agent. Because of the different kinetics between the low-molecular-weight contrast agent and the USPIO contrast agents, different scan protocols had to be used and different analyses performed.

In study III the sequence used was an rf-spoiled 3D gradient echo sequence with a TR of 26.8 and two echoes of 3.1 and 15 ms. The flip angle was 35° and FOV was 70 mm. The matrix was 256 x 256, yielding an in-plane resolution of 0.2734 mm. The slice thickness was 1 mm. Since half-scan of 62.5% and fold-over suppression in the slice direction were used, the total acquisition time was 2 min and 50 sec. T2* was calculated.

**Pharmacological treatment**

In study IV the animals received immunosuppressive treatment with cyclosporin A (Sandimmun Neoral®, Novartis, Basel, Switzerland), 10mg /kg b.w.. The drug was mixed with Intralipid® (Pharmacia & UpJohn, New Jersey, NJ, USA) and administered orally through a gastric tube at a total volume of approximately 1 ml. In the rat heart transplantation model used in the present studies this is optimal anti-rejection treatment.

**Histology**

In study I-IV hearts were fixed in 4% formalin, embedded in paraffin, and cut into 4-µm-thin sections. These were stained with hematoxylin-eosin and in study I-III they were graded blindly into five groups on an arbitrary scale based on the extent of morphological changes, taking into account cellular infiltration, edema, and areas of necrosis. The scale was rough, to be able to include both syngeneic and allogeneic transplants. Group 1 was defined as having almost normal morphology with no edema and few or no infiltrating cells. Group 2 had mild edema with some infiltrating cells. Group 3 grafts showed moderate edema and a moderate number of cells. Group 4 was defined as having moderate
to severe edema and areas of massive cellular infiltration. Lastly, group 5 had pronounced interstitial edema and massive infiltration of cells. The changes in group 4 and 5 corresponded to those seen at severe rejection. In study IV the hematoxylin-eosin sections were ranked on an ascending scale according to the extent of the morphological changes.

In study III part of the heart apex was snap frozen in cold isopentane and subsequently stored at -70°C until sectioned and stained immunohistochemically for detection of macrophages. The procedure was as follows: As primary antibody, ED1, which is directed against macrophages, monocytes and dendritic cells, was used. This antibody is of the IgG1 isotype and was acquired from Serotec, Oxford, UK. In short, the sections were stained by a peroxidase-antiperoxidase staining (PAP) method. The sections were incubated with 0.3% H₂O₂ in phosphate-buffered saline to inhibit endogenous peroxidase activity, and thereafter with normal goat sera to prevent non-specific background staining prior to incubation with the primary antibody. A secondary antibody, goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), was used and added in excess. In the next step, the sections were incubated with horseradish peroxidase-mouse antiperoxidase (Dakopatts, Glostrup, Denmark). Finally, H₂O₂ as substrate and 3-amino-9-ethyl-carbazol as electron donor were added to react with the horseradish peroxidase and produce a brown color. For counterstaining, Mayer's hematoxylin was used. In controls, the primary antibody was excluded.

In study III the formalin-embedded sections were also stained with Perl's Prussian Blue iron staining.

**Statistics**

In studies II, III and IV a region of interest (ROI) covering as much of the myocardium as possible was drawn by hand. In study I, a ROI covering a large part of the septum was drawn. For this commercially available software (ICE™Nordic Neurolab) was used. The mean signal intensity (SI) was used. In the grafts receiving USPIO in studies I, II and IV, the relative change in SI, in percent, was measured in each dynamic scan, using the first phase after injection of contrast agent as the reference point. This means that impermeability to the contrast agent would result in a decrease in SI over time, because of clearance from the blood pool, whereas a high permeability would lead to an increase in SI over time. The equation used was:

\[ \% \Delta SI = -100 \times \frac{(SI_t - SI_1)}{SI_1} \]

where SI₁ represents the first dynamic scan after injection of the contrast agent and SI₁ represents one of the subsequent 19 dynamic scans.
In study I and II, the allogeneic and the syngeneic grafts were compared over all times for each USPIO. The 95% confidence intervals for the differences in the means were calculated using bootstrap techniques (68) at every time point. In study IV the relative change in SI over time between each MR examination was calculated in each animal. The 95% confidence intervals for the differences in the means were calculated using bootstrap techniques at every time point.

In study II, in the groups that received gadolinium the early upslope of the contrast enhancement was used. The early upslope was defined as the four points forming the steepest part of the curve. A bootstrap sample was made out of the slope of the syngeneic grafts and the allogeneic grafts, and a 95% confidence interval was calculated for the differences between the slopes.

The bootstrap technique was used instead of non-parametric tests so as to avoid relying on asymptotic approximations, which is a potential problem with a limited number of samples.

In study III the T2* value was calculated from the SIs according to the equation:

\[ T2* = \Delta T E / \ln(SI_1/SI_2) \]

where SI1 represents the signal intensity of the first echo and SI2 that of the second echo. The difference in T2* value, in percent, between pre-contrast images on postoperative day (POD) 6 and post-contrast images 24 h later, on POD 7, was calculated as:

\[ \% \Delta T2* = 100 \times (T2*_{POD7} - T2*_{POD6}) / T2*_{POD6} \]

Mean values and standard deviations were calculated for the allogeneic and the syngeneic groups. To test for significance of differences between these groups with contrast agent and with no contrast agent (controls), a Kruskal-Wallis test was used. This test was chosen because the assumptions of normal distribution and equal variances could not be met. Tests for significance were also performed between allogeneic groups that received and did not receive contrast agent, and similarly between corresponding syngeneic groups. A Sign Rank test was used to test whether the mean value differed from zero. In analyses of the intensity of the Perl's Prussian blue staining, Fisher's Exact Test was used, since this is suited for small sample sizes organized in a two by two table, and does not demand the assumption of a normally distributed sample.
Experiments and Results

Study I

Experiment

This study comprised one allogeneic group (n=9) and one syngeneic group (n=6), neither of which received any immunosuppressive treatment. The animals were examined with MR imaging on postoperative days (POD) 2 and 6. In our animal model we know by experience that at POD 2 no rejection process has started, but at POD 6 an immense acute rejection has developed. After the last MR imaging the hearts were recovered and the vascular permeability of the USPIO contrast agent, i.e., the development of SI change over time, was compared between the rejecting and the non-rejecting groups. The first post contrast image was used as reference. The mean SI was calculated on a group level at each time point and on both MR imaging occasions (Fig. 2). The differences in the means between the syngeneic and the allogeneic grafts at days 2 and 6 were tested for significance and the 95% confidence intervals were calculated. The histological sections were scored according to the extent of the morphological changes on an arbitrary five-graded scale. In addition, the MR results were correlated to the morphological findings. The mean SI change in the myocardium of the last ten time points in each dynamic scan was calculated and compared with the histological score to determine the correlation between SI change and morphological degree of rejection.

Results

On POD 2 the mean relative SI change did not differ between the allogeneic and syngeneic groups, but at POD 6 there was already a difference 4 min after the acquisition of the reference scan (Fig. 3). The distance of the difference increased over time. There was no difference in the blood signal either between the groups or between the days post surgery. A positive correlation was found between the mean SI change of the ten last time points and the histologically assessed degree of rejection at POD 6 (r = 0.89, p < 0.005, for the analysis of the allogeneic grafts alone and r = 0.94, p < 0.001, when also the syngeneic grafts were included) (Fig. 4). The morphological analysis showed severe rejection in the allogeneic grafts and only minor changes in the syngeneic grafts.
Fig. 2.
An example of an allogeneic graft receiving NC100150 Injection. The curve shows the SI change (a.u.) over time in myocardium, skeletal muscle and blood.
Fig. 3
The graph shows the difference in the mean relative SI change in the myocardium between the syngeneic and the allogeneic grafts on postoperative day (POD) 2 (◊) and POD 6 (□) as a function of time after injection of the contrast agent. Error bars indicate bootstrap 95% confidence intervals.

Fig. 4.
Correlation between histological rank and relative change in signal intensity (SI); \( r=0.94, \ p<0.0005 \)
Study II

Experiment

This study was a follow-up of study I and the aim was to test our newly developed method of detecting acute rejection of transplanted organs with two different-sized USPIO contrast agents, and if possible to find the one that would best discriminate between rejection and non-rejection. A further purpose was to compare the findings with the results obtained with a low-molecular-weight gadolinium contrast agent, since that is by far the most frequently used contrast agent in clinical practice. We used 36 rats which had undergone heart transplantation. They were divided into six groups, among which one allogeneic and one syngeneic group each received one of the three contrast agents. The rats did not receive any immunosuppressive treatment and were examined on POD 6. The MR examination on POD 2 performed in study I was excluded from study II, since it did not contribute to the discrimination between the rejecting and non-rejecting groups. After the radiological examination, the data were analysed and the change in SI over time was compared between the allogeneic and syngeneic groups receiving the same contrast agent. The calculation method was the same as that used as in study I for the groups that received the USPIO contrast agent. As mentioned in Materials and Methods a different scanning technique and method of data analysis was used when calculating the results from the low-molecular-weight gadolinium contrast agent experiments on account of the difference in kinetics between this compound and UPSIO contrast agents. The histological sections were analyzed in a way similar to that in study I, except that the correlation between the MR results and morphological data was not examined.

Results

In the groups that received unformulated NC100150, SI differed between allogeneic and syngeneic grafts at all times; (p < 0.001 Fig. 5). In the grafts receiving AMI-227 the SI change over time differed at all times except in three out of the first four measurements; (p < 0.05 Fig. 6). In the groups that received the low-molecular-weight gadolinium contrast agent, no difference was seen between the rejecting and the non-rejecting groups. The results of the morphological evaluation were similar to those in study I.
Fig. 5. The relative change in signal intensity (SI) in the myocardium in the groups that received unformulated NC100150, using the first point after contrast agent injection as reference point. Data shown are the mean SI change in each group, with error bars at 95% confidence.

Fig. 6. The relative change in signal intensity (SI) in the myocardium in the groups that received AMI-227, using the first point after contrast agent injection as reference point. Data shown are the mean SI change in each group with error bars at 95% confidence.
Study III

Experiment

In this study a different approach for detecting acute rejection was tested. Macrophages are important cells in the rejection process. They are among the first to enter the scene on account of the surgical trauma and reperfusion of the graft and they can play an important role in triggering the rest of the immune system (29). In the histological sections a large proportion of the infiltrating cells are macrophages. It has been confirmed that one of the blood pool contrast agents used in study II is phagocytosed by macrophages and it has been used in clinical trials for detecting lymph node metastases (11, 12, 14). To be able to detect the presence of macrophages in the tissue would therefore be an important indication of an acute rejection. Studies similar to ours have been carried out using a high field 4.7T MR scanner (56-59).

Thirty-six rats were used in study III. They were divided into six groups, among which one allogeneic and one syngeneic group each received either one of the two doses of the blood pool contrast agent, or no contrast agent at all. The two latter groups served as control groups. The lower dose of the contrast agent (2 mg Fe/kg b.w.) was chosen because the same dose was used in studies I, II, and IV. The higher dose (11 mg Fe/kg b.w.) was chosen to compensate for the shorter half-life of the contrast agent in rats compared to humans. The syngeneic and allogeneic groups that received the lower dose of the contrast agent were the same animals as were used in study II. The rats were examined with MR imaging on POD 6 before injection of the contrast agent. A new MR examination was performed 24 hours later. The reason for the time delay between the two examinations was to allow time for the contrast agent to reach the graft and for the macrophages to be able to phagocytose the compound. In studies concerning lymph node evaluation and identification of inflamed carotid plaques with USPIO, the optimal time delay was judged to be 24 hours (17, 18, 69). The percentage change in T2* between pre-contrast and post-contrast imaging was calculated in the analysis. The histological sections were evaluated regarding the extent of morphological changes as in studies I and II, but were also stained with iron staining and with respect to the number of macrophages.

Results

No difference in T2* was seen between the allogeneic and the syngeneic grafts in either of the dose groups, or between those groups and in the control groups. There was a difference between the allogeneic group receiving the higher dose and the allogeneic group that served as a control group; (p < 0.05 Fig. 7). The morphological changes in the allogeneic groups corresponded to severe acute rejection while the syngeneic grafts showed mild morphological changes, as in studies I and II. The amount of iron staining did not differ between any of the groups (allogeneic receiving USPIO, syngeneic receiving USPIO, allogeneic con-
controls not receiving USPIO, syngeneic controls not receiving USPIO). However, there was a difference in the intensity of the staining between the syngeneic and the allogeneic grafts in the low-dose groups (p = 0.002) and in the control groups (p = 0.02), the syngeneic grafts displaying the higher intensity coloring. The staining for macrophages revealed large numbers of macrophages in the allogeneic grafts and only a few in the syngeneic grafts.

**Fig. 7.**
The difference in T2* in percent between postoperative day (POD) 6 and 24 h later in all six experimental groups. No significant difference was seen between the allogeneic group and the syngeneic group with either dose or with no contrast agent at all. There was a significant difference between the allogeneic group that received 11 mg/kg of AMI-227 and the allogeneic group that did not receive any contrast agent at all (p=0.0374).
Study IV

Experiment

This study was undertaken to test our method developed in studies I and II, to see whether it was possible to follow the effects of the anti-rejection treatment and detect any reversal of the rejection process. In studies I, II and III the acute rejection was too pronounced to be able to reverse. In study IV, two groups of rats with allogeneic transplantation were included and were treated with optimal immunosuppressive therapy for the first 9 postoperative days. By experience, at POD 9 the reperfusion damage after transplantation would have healed and no acute rejection would have developed by virtue of the immunosuppressive treatment. At this time the immunosuppressive treatment was interrupted for five days, up to POD 15, to allow an acute rejection to develop.

In the animals in the treated group (n=5), an MR examination was performed at POD 15 and subsequently anti-rejection treatment was re-introduced for five days to try to reverse the acute rejection that we presumed had developed. At POD 19 a new MR imaging was performed, after this the rats were sacrificed and the grafts prepared for histology. The rats in the rejecting group (n=7) were examined with MR at POD 9 to get a baseline value after the initial immunosuppressive treatment. At POD 15 a further MR examination was performed in this group and the hearts were subsequently recovered and prepared for histological analysis (Fig. 8). As in studies I and II, the mean relative SI change over time was calculated. The difference in the SI change between the two MR examinations performed was then calculated. Each rat was its own control and hence

![Diagram](image)

Fig. 8.
Time course describing the design of the experiment. Treated group (n=5) and rejecting group (n=5). POD = postoperative day.
the vascular permeability pattern in each individual rat was compared between the two MR examinations. The histological evaluation of the morphology was performed as a ranking of the morphological changes in the sections.

**Results**

In the treated group which went from an expected acute rejection, through treatment, to a reversed condition, the difference in the mean SI change over time was significant at 17 of the 19 measurements (Fig. 9). In the rejecting group which went from the expected normal function at POD 9 to an expected acute rejection at POD 15, the difference in the mean SI change over time was significant at 16 of the 19 measurements (Fig. 10).

In the ranking of the histological sections the grafts from the two groups were very randomly dispersed along the ranking scale.

**Fig. 9.**
The difference in mean SI change over time between the MR imaging at postoperative day (POD) 19 and 15, in the treated group (n=5). The error bars represent the bootstrapped 95% confidence interval.
Fig. 10.
The difference in mean signal intensity (SI) change over time between the MR imaging at postoperative day (POD) 15 and POD 9, in the rejecting group (n=7). The error bars represent the bootstrapped 95% confidence interval.
Discussion

Permeability to USPIO as a measure of vascular damage

In studies I and II a new technique is presented for detection of acute rejection of transplanted organs. MR imaging and injection of USPIO contrast agents were used and the percentage SI change over time after injection was measured. In normal vessels the USPIO contrast agents remained in the intravascular space, but if the normal vessel endothelium is altered for some reason, the contrast agent leaks into the tissue. It is believed that the SI change observed in the rejecting grafts after injection of contrast agent represents the discontinuity of the microvessel endothelium, which allows the contrast agent to leak into the tissue. The reason for the damage to microvessels is the pronounced inflammatory reaction caused by the acute rejection. This slow leakage of the contrast agent into the extracellular space gives it a greater distribution volume and hence the SI over time increases. If no leakage of the compound occurred, a decrease in SI would be expected owing to the half-life of the compound. The measurement starts when the contrast agent has reached steady state so as to try to minimize the perfusion component and only measure the permeability factor of the different contrast agents. This also allows a longer time for imaging to improve the resolution and get smaller motion artefacts in comparison with low-molecular-weight gadolinium contrast agent, where there is less time for image acquisition. In studies I and II three different sizes of USPIO were used, to see which one could discriminate best between the rejecting and non-rejecting grafts. The result was that all three were able to discriminate between rejecting and non-rejecting grafts without an overlap of the groups. Combined data for all three contrast agents are presented in Figure 11 and 12.

With NC100150 Injection the SI curve increases over time in the rejecting grafts and the rejecting and non-rejecting groups are best discriminated at the end of the scan after 45 min, when the difference in mean SI change between the rejecting and non-rejecting grafts is approximately 25%.

A different SI pattern is seen for unformulated NC100150. Here the maximum mean SI change in rejecting grafts is reached after roughly half the scan and, like NC100150 Injection, is about 18%. On the other hand the curve has a steeper slope initially possibly because unformulated NC10015 is slightly smaller in diameter than NC100150 Injection, which means that its passage through the vessel wall will be faster than with a larger particle. This permits discrimination between rejecting and non-rejecting grafts at an earlier stage than with NC100150 Injection. The latter part of the curve declines and according to our calculations the half-life, was found in study II to be 79 min, this influences the curve. The maximum difference in mean SI change between the rejecting and non-rejecting groups is, as for NC100150 Injection, 25%, but this is reached after half the scan, i.e., after 20 min.
Fig. 11.
Combined data for three USPIO contrast agents. The curves show the mean difference in SI over time in the myocardium of rejecting grafts.

AMI-227 is a larger particle than the other two tested. Its maximum SI change in rejecting grafts is reached at the end of the scan and is 7%. The slope of the curve is more level than the curves of the other contrast agents and the maximum difference in mean SI change between rejecting and non-rejecting groups reaches 11% at the end of the scan. The lower slope is interpreted as being due...
to the larger size of the particle, which causes slower leakage through the vessel wall than with the other contrast agents. The half-life of this compound is longer than for the other two agents tested, namely 386 min calculated in study II. If the scan had been longer, better discrimination between the groups might have been possibly.

It is concluded that the smaller the particle used, the sooner the ability to discriminate between rejecting and non-rejecting grafts. An important factor to consider is also the half-life of the compound, since this can influence the SI change considerably, to an extent depending on the length of the scan. If, when analyzing the results, a two compartment model had been used, which is a common and well established method in low-molecular gadolinium studies (70), but also have been used in USPIO applications (62, 63), a function of all the data could have been obtained, and not a function for each individual time point, as was done here. This might have increased the discrimination between rejecting and non-rejecting grafts with all three contrast agents. With that way of analyzing the results the half-life could have been taken into account, which might have been most favourable for unformulated NC100150, since this has the shortest half-life and therefore influences the result the most.

Similar applications of USPIO contrast agents have been described in tumor models and experimental ischemic heart studies. In reperfused ischemically injured rat myocardium there was an increase in SI after USPIO administration compared to normal myocardium (71), which was considered to be due to vascular damage. In that study, the same contrast agent as in study I was used. In tumor models albumin-Gd-DTPA and a low-molecular-weight contrast agent were evaluated with respect to permeability. When the macro-molecular contrast agent was used the permeability was increased in the malignant tumors compared to the benign tumors. With the low-molecular –weight contrast agent there was no difference between the two types of tumors. The results were interpreted as being due to an increase in microvascular permeability, caused by the disruption of the endothelial integrity of the microvessels, associated with a higher histopathological tumor grade (61). Since then, the same contrast agent as was used in study I has been evaluated similarly in experimental and clinical breast tumors where the results have pointed in the same direction (62, 63).

In study II low-molecular-weight-gadolinium contrast agent was used, since this is the far most commonly used contrast agent for MR imaging today. As it has completely different kinetics than USPIO contrast agents, a different MR sequence and a different way of analysing the results were used. First pass perfusion measurements were performed and the steep upslope of the curve was the measurement tool. The results showed no difference between the rejecting and non-rejecting grafts. Technical problems during MR scanning prevented us from using a more complete SI curve for analysis, with more parameters to compare, as has been done in other similar studies (49, 51). If the experimental conditions had been optimal the method would have been given more justice.
and the results might have been different. On the other hand, with USPIO contrast agents the method seems to be easier to interpret and more robust. The most suitable compound tested for the purpose of studies I and II was NC100150 Injection. It had the same ability to discriminate between rejecting and non-rejecting grafts as unformulated NC100150, but with unformulated NC100150 the half-life interfered with the interpretation of the curve. AMI-227 was also able to discriminate significantly between the groups, but to a lower extent. If the scan time had been shortened, the design would have been different and perhaps unformulated NC100150 might proved the most suitable, but in this setting NC100150 Injection seemed the most reliable. On the other hand, if the scan time had been longer it is possible that AMI-227 might have come out the best.

The method for detecting acute rejection has some obvious limitations. In this study there was a pure rejection model with no complication factors. The rejection had also progressed far beyond the point of possible reversal with pharmacological treatment. In solid organ transplants a number of complications can occur, which have to be differentiated from acute rejection. Acute rejection is an inflammatory reaction, and the occurrence of other inflammatory reactions might cause an increase in permeability similar to that seen in acute rejection, in which case our method would not be able to determine the reason for graft failure. The most common inflammatory reactions after organ transplantation are ischemia and infections. Ischemia at the time of transplantation results in ischemic-reperfusion damage and can affect the microvessels. The diagnosis is often evident from of the clinical and surgical observations. The histological examination reveals inflammatory changes.

Infections can also give rise to inflammatory reactions and the diagnosis is established by immunohistochemistry of the biopsy specimen; further, infection might be suspected from alterations of the epithelial cells (29). Viral infection, in particular, may cause problems and there is an association between viral infections, graft rejection and immunosuppression (72). It is therefore common to give prophylactic antiviral treatment at the time of transplantation, especially to children. In conclusion, viral infections might be difficult to discriminate from acute rejection with our method.

In renal transplantations the possibilities of acute tubular necrosis and cyclosporin A nephrotoxicity are matters of concern. In this diagnostic method one would expect to have a normal permeability pattern in conditions such as acute tubular necrosis, since this is a dysfunction of the tubuli and does not affect the microvessels. In a study of first pass perfusion of low-molecular-weight gadolinium, no significant difference in renal perfusion was seen between normal renal function and acute tubular necrosis, whereas the perfusion was decreased in acute rejection (51). BOLD MR imaging has also shown that acute tubular necrosis has a different etiology and can be differentiated from acute rejection but not from normal function (39).

Cyclosporin A nephrotoxicity might also exhibit endothelial changes and it
is possible that it might give results similar to those of rejection with use of our method with USPIO contrast agents and MR imaging. However, preliminary studies on small numbers of patients have shown a difference in the perfusion pattern with low-molecular-weight gadolinium between cyclosporin A toxicity and acute rejection (52, 53). In MR spectroscopy studies different spectra have been seen in grafts subjected to rejection, acute tubular necrosis and ischemia, but cyclosporin toxicity could not be distinguished from normal renal function (42, 45, 46). However, MR spectroscopy is cumbersome to perform and difficult to interpret.

**Monitoring of anti-rejection treatment based on permeability to USPIO**

In study IV the method developed in studies I and II was tested for monitoring anti-rejection treatment. The results illustrate that the method can provide an indication of whether the anti-rejection treatment has started to reverse an ongoing rejection. The level of immunosuppressive treatment is a matter of balancing so as to give as much drug as possible to avoid rejections without getting too many side effects, as it is impossible to measure the individual need of such treatment (29). Some immunosuppressive agents are monitored by their side effects, for example corticosteroids, while others are monitored by 12- or 24-hour serum troughs, such as calcineurin inhibitors, and some are still monitored empirically, such as antiproliferative agents (73). In the individual patient measurements of serum levels of immunosuppression are not sufficient when monitoring the treatment, and side effects such as diabetes, osteoporosis, hyperlipidemia and gingival hyperplasia also need to be taken into account (73).

The effect of an anti-rejection treatment is also very difficult to measure at an early stage. As mentioned before the surveillance of heart grafts is carried out by taking protocol biopsies, at least during the first post transplant years (37). The spectrum of immunosuppressive drugs available in the last few years has increased rapidly (26, 74); hence, if an indication of poor a clinical response can be obtained at an early stage there are several possibilities of switching to a different treatment regime. Studies have shown that there is a delay of infiltrating cell withdrawal even after successful rejection treatment (75) and thus morphological evaluation of graft biopsies may be insufficient. For that reason there is a need for a method for monitoring the anti-rejection treatment that would save patient suffering and money. Engstrand et al have described a method for propagating lymphocytes ex vivo from graft biopsies in an attempt to get an indication of the effectiveness of anti-rejection treatment, but it takes a few days for the results to be obtained and the method is invasive, since a graft biopsy is needed (76).

In the present study IV an acute rejection was allowed to develop and then treated pharmacologically. Two MR examinations in each animal were performed, one before and one after anti-rejection treatment. The change in SI
after contrast agent injection was compared between these two occasions, with each animal as its own control. This enabled us to get a baseline value in each animal to see the extent to which the microvessels were damaged by the time of the initial examination. A limitation of the study was that no biopsy was taken at the time of the first MR examination to have as a reference. Instead another experimental group, which was examined at the time of optimal immunosuppressive treatment and then again after termination of the treatment, served as a reference on group level. The results indicate that monitoring of anti-rejection treatment is possible and that the results can be measured even before the biopsy specimens have shown withdrawal of infiltrating cells. This analysis is based on the fact that no correlation between the histological ranking and the SI change in the MR examinations was seen, but there were clear differences between the MR examinations before and after treatment. The result is strengthened by the fact that only minor morphological changes were observed in three grafts where there was a decrease in SI change at the MR examination by the time when an acute rejection was expected to have developed and hence, when an increase in SI change was expected. This showed that in these three grafts no acute rejection had developed even though no immunosuppressive treatment had been given for a number of days. By experience, there is a greater individual variability in the time for rejection to occur among transplanted animals with immunosuppression than in transplanted animals without immunosuppression, like in study I and II.

In study IV we also achieved a milder acute rejection than in studies I and II. This was accomplished by transplanting allogeneic grafts and initially treating the animals with optimal immunosuppressive treatment and then discontinuing the treatment and letting an acute rejection develop. In this model the animal was kept untreated for five days in order to get a reversible acute rejection. This approach is easier to monitor than giving the animals suboptimal immunosuppressive treatment immediately after the transplantation and our laboratory has previous experience of this kind of experimental design in this animal model. The results confirmed that the MR method did not only work on an end-stage acute rejection but also in a setting closer to the clinical reality. As mentioned concerning the other experimental group, a limitation was that no histological reference was available by the time of the first MR examination, since no biopsy was taken. Instead, previous knowledge of how this animal model works, and the results from the MR examination, where all animals showed a decrease in SI change, as expected from the hypothesis, served as enough confirmation to rely on the results. The fact that the method is successful in a milder acute rejection is also a prerequisite for further development in the future to design a clinical study.

If the method were to be proven successful in clinical studies, it would probably be as a complement to histological analysis. Taking biopsies is a well established method which will always be able to give more information than MR examinations, as different kinds of staining and focused preparation of histo-
logical sections can give answers to many different questions. Even if the tested method proves to indicate the result of treatment more quickly than biopsy the overall final goal is to reduce the number of biopsies, and as a consequence the patient morbidity, and not to replace it. Especially in heart transplantation the method might have a great advantage, since the surveillance of the graft is based on protocol biopsies and clinical symptoms are often difficult to interpret, and because of the fatal consequences of severe clinical symptoms the aim is to fore-stall them.

Detection of the presence of macrophages in acutely rejecting grafts using USPIO

In study III a different approach to detection of acute rejection was tried. Identification of macrophages in an acutely rejecting organ is an appealing idea for getting closer to the diagnosis. Macrophages are important activators of the immune system and will enter the graft at an early stage (77). In acute rejection a substantial proportion of the infiltrating cells consists of macrophages, in human biopsies 38 – 60% (64). Several animal studies have been performed with use of USPIO on different transplanted organs, and all have pointed in a promising direction (56-59). These latter studies have all been performed on high field MR scanners (4.7T). The results are available 24 hours after the injection of the contrast agent and many particles have to be present in the voxel to allow detection of the susceptibility effect with MRI. An even newer technique has recently been introduced, where cells have been labeled with micrometer-sized paramagnetic iron oxide particles (MPIO). These particles are much bigger and studies have been carried out to detect single particles in vitro (78, 79). Comparisons between USPIO and MPIO have been made in a transplantation setting where the time to diagnosis has been reduced to only a few hours with use of MPIO (60), in these studies, however, a very high field strength, 11.7T, has been used, which is far from that of clinical scanners available today.

This attempt to apply the technique for detection of macrophages using a clinical 1.5T scanner encountered many problems. The results were highly variable and pointed in different directions. There was only one statistically significant relationship and that was between the allogeneic group that received the higher dose of AMI-227 and the allogeneic group that did not receive any contrast agent at all. This correlation indicates, however, that the method does have potential after all, since the only factor that differed between these groups was the injection of contrast agent in one of the groups. In that group the change in T2* decreased 24 hours after contrast agent injection, which is what we expect if there is contrast agent present in the infiltrating macrophages. In the control group that did not receive contrast agent the T2* increased between the MR examinations, a finding which could have been due to the progression of edema, which is a part of the rejection process. In these two groups the presence and degree of hemorrhage, which causes problems in the MR interpretation, should
have been the same and therefore ought to have cancelled out each other. In the other studies performed with similar methods, a higher field MR scanner was used, where the susceptibility effect is greater than in our clinical 1.5T scanner and the iron oxide particles would induce a larger T2* effect and consequently a greater difference between rejecting and non-rejecting grafts.

Not reported for in study III, unformulated NC100150 was also investigated with the same method as with AMI-227, and a significant difference between the rejecting and non-rejecting group; (p < 0.05) was observed (Fig. 12). A dose of 2 mg Fe/kg b.w. was used, which was the same as the lower dose used for AMI-227 (Fig. 13). AMI-227 has been shown to be phagocytosed by macrophages in experimental studies (80) and is used in clinical studies for detection of lymph node metastases (11, 13, 14) and visualization of vulnerable carotid plaques (81). Unformulated NC100150, however, has not been tested to see if it is phagocytosed by macrophages. Studies with NC100150 Injection, which is similar to unformulated NC100150, have failed to verify macrophage uptake which can be a result of the type of coating and/or the short half-life. Given this result, unformulated NC100150 might be phagocytosed by macrophages, although this has not been tested experimentally. With the same dose of AMI-227 as of unformulated NC100150, no significant result was seen. The reason for this may be that AMI-227 has a larger size than unformulated NC100150, and therefore the slower permeability might give the macrophages too short exposure to the compound to be able to phagocytose it, compared with unformulated NC100150.

![Unformulated NC 100150](image)

**Fig. 13.**
The difference in T2* in percent between postoperative day (POD) 6 and 24 h later in the syngeneic and allogeneic group receiving unformulated NC10015 (p = 0.0285).

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In an acutely rejecting graft there is a high pressure caused by the edema. This will increase as the acute rejection progresses. The edema can also affect the function and the viability of the graft. Studies have demonstrated very low renal cortical perfusion in severe rejection (40). Results from study II, where perfusion of low-molecular-weight gadolinium studies were performed, showed that approximately 30% of the grafts had visible areas with no perfusion, which can be explained by the necrotic areas seen in the histological sections. In the MR image edema counteracts the reduction of T2* caused by the macrophage uptake of the USPIO. Hyaluronan is a glycosaminoglycan which plays a critical role in the development of inflammatory edema. Studies have shown that reduction of hyaluronan by hyaluronidase treatment can decrease the edema (82). In the present setting treatment with hyaluronidase might give a better idea of the amount of T2* change that is due to the injected contrast agent and the degree of opposite change that is due to increasing edema.

An additional concern in the analysis is the result of the iron staining. This staining was experienced in all the grafts that did not receive any contrast agent at all. One explanation could be the occurrence of hemorrhages. In acutely rejecting grafts hemorrhages occur if the rejection has proceeded far enough and the surgical trauma can produce bleeding. This will release iron into the tissue which the macrophages can phagocytos and we will not be able to determine the site of origin of the iron (83). Other groups may have been successful because they used a milder rejection model. To confirm the validity of the iron staining one rat, without a transplant, was injected with a much higher dose of a USPIO. After 24 hours the heart, liver, and spleen were harvested. The sections were then stained with Perl's Prussian blue and were found to display the expected results with iron staining in the areas where the macrophages were located. Below is an image of the spleen of the above-mentioned animal (Fig. 14).

In summary, no differentiation was achieved between acutely rejecting and non-rejecting transplanted hearts by measurements of T2* after myocardial macrophage uptake of AMI-227 in a clinical 1.5T scanner. Many factors will have contributed to the result, however. The technique might have potential in a more clinically relevant acute rejection model where the rejection process is less advanced.

Available contrast agents

A few superparamagnetic iron oxide particles (SPIO) are on the market at the moment (22, 24). They have a larger diameter than USPIO, namely > 50 nm. These compounds are similar to USPIO but are recognized within minutes by the reticuloendothelial system and are used as liver specific contrast agents and hence are not suitable for our purpose. Unfortunately, at the present time there is no USPIO on the market that is suitable for measuring the effect of anti-rejection treatment or identifying acute rejection. However, there are compounds in clinical phase III trials, in which angiographic studies have been made (8), that
might fit this method. There is also a gadolinium-based contrast agent which is bound to human albumin registered in Sweden (Vasovist, Schering AG) for MR angiography (84, 85) which may suit the purpose of permeability studies of acute rejection.

**Future perspectives**

The next step in this project would be to make an enlarged study on monitoring anti-rejection treatment to verify the results of study IV. It is hoped that a suitable contrast agent will soon be registered for clinical use to allow a clinical study both on diagnosis of acute rejection and monitoring of anti-rejection treatment.

Today the technical skills of performing human transplantation are well established and the limitation lies, rather, in access to donated organs. The indications for getting a new organ are increasing, which means that the demand of organs will further increase in the future. The availability of MR scanners is
also expected to increase and in contrast to most other radiological modalities MR imaging does not give any ionizing radiation and has no known side effects. Thus MR imaging would seem a potential valuable screening method for transplanted organs. To perform an MR examination shortly after transplantation and have the result as a baseline value for future examinations to evaluate the condition of the graft when clinical signs give indications of failure, would be an advantage. A prerequisite, of course, would be the availability of a contrast agent of which repeated injection would be allowed.

In a more general perspective, acute rejection is just one part that decides the long-term survival of a solid organ graft. Despite the recent years of improvement in the allograft lifespan in the short perspective, with the 1-year survival in renal transplantation > 90% (86), the long term lifespan of the grafts has not been so favorable. From the previous situation where chronic rejection was the major threat to graft dysfunction, the hypothesis of cumulative damage to the graft due to alloimmune, ischemic and inflammatory stimuli has now become a matter of great concern which will be the next challenge to be met in transplantation medicine (87).
Conclusions

This thesis has demonstrated a novel approach to detection of acute rejection in experimentally transplanted organs using USPIO contrast agents and a clinical MR scanner. The method measures the change in signal intensity over time with a dynamic MR scan as an indication of the microvascular damage to the graft due to the acute rejection. Three different USPIO contrast agents have been investigated and all three have been able to discriminate between rejecting and non-rejecting grafts.

Detection of the presence of macrophage in a rejecting graft using the uptake of USPIO and a clinical MR scanner was not possible in this setting.

Anti-rejection treatment of transplanted hearts can be monitored by analyzing the permeability to a USPIO contrast agent by MR imaging. The method is non-invasive, performed on a clinical 1.5T MR scanner, and enables the clinician to get a result within an hour of the examination.
Sammanfattning på svenska

Detta avhandlingsarbete handlar om hur man kan diagnostisera en akut avstötning-ningsreaktion av ett transplanterat organ med hjälp av en typ av kontrastvätska och undersökning i magnetkamera (MRT). Idag måste man ta ett vävnadsprov från det transplanterade organet och undersöka det i mikroskop för att få rätt diagnos vad som orsakar funktionsnedsättningen. Denna procedur kräver ett invasivt ingrepp i patienten och ger inte hela bilden av det transplanterade organet. Att följa behandlingen av ett organ som har fått en akut avstötningreaktion är också mycket svårt p.g.a. att de verktyg som finns tillgängliga är trubbiga och även här ofta innefattar ett vävnadsprov.

I alla delarbeten använder vi oss av en hjärttransplantationsmodell på rätta där vissa transplantationer utförs mellan genetiskt identiska individer där ingen avstötningreaktion uppträder, och andra transplantationer utförs mellan genetiskt olika individer och en avstötningreaktion förväntas ske. De kontrastvätskor för MRT undersökning som används är små järnpartiklar som, när de injicerats i blodbanan, cirkulerar i kroppens blodkärl och normalt sett inte läcker ut i vävnaden. Detta är i motsats till de mest använda kontrastmedlen för MRT som är så små att de lätt läcker ut i vävnaden.

Vår hypotes är att i ett organ där det pågår en akut avstötningreaktion är blodkärlen skadade så att de kontrastvätskor som normalt sett stannar i blodbanan nu läcker ut i vävnaden. Vi försöker då mäta läckaget som en ökad signal på MRT undersökning. I delarbete I och II undersökte vi tre partikulära kontrastvätskor av olika storlek för att se om de kunde skilja mellan ett transplanterat organ där det pågick en avstötningreaktion och ett organ som fungerande normalt. Resultatet blev att alla kontrastvätskor som vi testade kunde skilja de två tillstånden åt.
I delarbete IV använde vi oss av samma metod som vi utarbetat i delarbetena I och II, men denna gång utvecklade vi en lite mildare avstötningsreaktion av det transplanterade organen än i tidigare försök. Därefter behandlade vi avstötningsreaktionen farmakologiskt. Två MRT undersökningar utfördes, en innan behandlingen sattes in och en efteråt. Samma kontrastvätska som användes i delarbete I provades nu igen. Resultatet blev att läckaget av kontrastvätskan minskade efter att behandlingen hade satts in. Vi tolkade detta som att skadan på blodkärlen i de transplanterade organen minskade på grund av den farmakologiska behandlingen och därför blev läckaget av kontrastvätskan ut i vävnaden mindre efter behandling.

I delarbete III använde vi oss av ett annat angreppssätt för att diagnostisera en avstötningsreaktion. Makrofager är en viktig celltyp som ansamlas i det transplanterade organet vid en avstötningsreaktion. De kontrastvätskor som vi tidigare använt oss av tas upp av just makrofager. Vi försökte då visa förekomst av makrofager i det transplanterade organet som en skillnad i MRT signal mellan två MRT undersökningar; en innan kontrastvätskan hade givits, och en 24 timmar efteråt. Resultatet blev att vi inte kunde påvisa denna celltyp med hjälp av kontrastvätskan och MRT undersökning. Det var många faktorer som spelade in i bedömningen.

Slutsatsen av detta avhandlingsarbete är att vi visat en ny metod att diagnostisera en akut avstötningsreaktion av ett transplanterat organ med hjälp av partikulära kontrastvätskor och MRT undersökning. Vi kunde även med samma metod monitorera farmakologisk behandling av en akut avstötningsreaktion.
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