Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Early biological and immune response to semi-identical liver or kidney allograft in miniature swine

Masayuki Otsuka,[1] Satoshi Ambiru,[1] Kenji Uryuhara,[1] Philippe Herman,[1] Stephanie Talpe,[2] Jean-Paul Dehoux,[1] François Jamar[3] and Pierre Gianello[1]

1 Laboratory of Experimental Surgery, Faculty of Medicine, Université Catholique de Louvain, Brussels, Belgium
2 Department of Pathology, Cliniques Universitaires Saint-Luc, Brussels, Belgium
3 Service of Nuclear Medicine, Cliniques Universitaires Saint-Luc, Brussels, Belgium

Keywords
immune and biological response, liver/kidney transplantation, miniature swine.

Correspondence
Pierre Gianello, Laboratory of Experimental Surgery, Univesité Catholique de LOVAIN CHEX 5570, 55, Avenue Hippocrate, 1200 Brussels, Belgium. Tel.: +32-2-7645586; fax: +32-2-7645589; e-mail: gianello@chex.ucl.ac.be

Received: 9 October 2003
Accepted: 24 May 2004

doi:10.1111/j.1432-2277.2004.00030.x

Summary

In inbred miniature swine, semi-identical liver allograft recipients survive up to 3 months without immunosuppression, whereas similarly mismatched kidney allografts are uniformly rejected within 2 weeks. The early biological and immunological events were assessed in this unique model. $SLA^{d/d}$ pigs (MGH, Harvard Medical School, Boston, MA, USA) received liver or kidney allograft from heterozygous $SLA^{c/d}$ miniature swine. Survival, graft function, histology, intragraft cytokines, peripheral lymphocyte and platelet count, plasma cortisol level and cellular/humoral anti-donor immune response were assessed. Kidney allografts were uniformly rejected within 2 weeks, whereas liver allografts survived for up to 87 days. After both liver and kidney transplantation, the peripheral lymphocyte count decreased during the first week concomitantly to a significant elevation of plasma cortisol level. Early decrease of peripheral platelet count was observed after liver but not renal transplantation. Up-regulation of transforming growth factor β1 (TGF-β1) and interferon-γ (IFN-γ) was observed during the first postoperative week in semi-identical liver allografts and IFN-γ as well as IL-10 in kidney allografts. In liver recipients, labelled autologous lymphocytes accumulated in the liver graft and native spleen, whereas after renal allograft, lymphocytes accumulated in the native spleen and liver but never in the kidney allograft. Specific cellular anti-donor unresponsiveness was observed from the first post-transplant day in both liver and kidney recipients, while the humoral anti-donor response remained intact. In semi-identical liver allograft, recipient rejection is milder and slower than in similarly matched kidney allograft. The intragraft up-regulation of TGF-β1 in semi-identical liver allograft might be one mediator to explain the modulation of rejection after liver transplant. The rapid, nonspecific accumulation of recipient lymphocytes in the liver allograft but not in kidney allograft might also play a role in the different survival time in this model.

Introduction

Liver transplant is less susceptible to acute cellular rejection than other primarily vascularized organs. In clinical practice, liver allograft resistance to rejection has been documented indirectly as randomized clinical trials were difficult to set up, but there is evidence that human liver and not kidney transplant resists poor human leucocyte antigen matching, ABO incompatibility and positive cross-matches [1,2]. The lower susceptibility of liver allografts to rejection has, however, been clearly evidenced in small animal models.

Although the underlying mechanisms of this unique characteristic of liver allograft is not fully understood,

several factors have been suggested to be involved, such as soluble immunoregulatory proteins, i.e. major histocompatibility complex, Fas, Fas-ligand [3,4], or immunoregulatory (suppressor) cells [5]. Activation-induced cell death (AICD) phenomena in the liver have been reported and this T-cell exhaustion is known as the 'killing field hypothesis' [6–8]. The incoming alloreactive T cells are activated by alloantigens in the liver allograft but encounter intrahepatic killing mechanisms and are eliminated, leaving the graft intact [8,9]. The liver is also a haematopoietic organ and provides a microenvironment able to grow donor or host cells [10]. The presence of dendritic cells (DC) in the liver that poorly expresses co-stimulatory molecules plays a major role to explain the absence of T-cell activation and proliferation in liver allograft. *Ex vivo*, the liver triggers a substantial trapping of activated T cells but not naive T cells [11]. The liver is therefore an organ which needs to address the delicate balance of tolerance to harmless antigens/pathogens and immunity against dangerous pathogens.

In large animal models, the comparison of spontaneous survival between similarly mismatched liver and kidney allografts has not been investigated. Long-term spontaneous liver-graft survival in swine has been previously reported between apparently unrelated breeds [12], but these results were controversial because of the lack of swine leucocyte antigen (SLA) genetics. In a well-defined SLA miniature swine model, only SLA-matched recipients of liver allograft could survive long-term without immunosuppression, but acute cellular rejection eventually occurred across class I, class II or full mismatches [13]. We similarly demonstrated in a semi-identical model that spontaneous liver or kidney graft survival was never obtained without immunosuppression [14,15].

In order to compare the early biological and immunological response after liver or kidney allograft, we achieved transplantation across a similar semi-identical SLA mismatch ($SLA^{c/d} \rightarrow SLA^{d/d}$) in a genetically well-defined swine model without immunosuppression. Semi-identical liver allografts were rejected more slowly and more mildly than kidney semi-identical grafts in terms of mean survival time. In both types of allografts, the specific anti-donor cellular immune response disappeared as soon as the first postoperative day (POD) after transplantation and during two postoperative weeks. The intragraft production of transforming growth factor β1 (TGF-β1) was evidenced in liver but not in renal allograft.

Materials and methods

Animals

Heterozygous $SLA^{c/d}$ (class $I^{c/d}$, class $II^{c/d}$) and homozygous $SLA^{d/d}$ (class $I^{d/d}$, class $II^{d/d}$) MGH miniature swine were bred in our facilities (by E. Collignon, Centre A. Marbaix, Louvain-La-Neuve, Belgium). $SLA^{d/d}$ were used as recipient and $SLA^{c/d}$ as donors. In this combination, one-haplotype class I plus class II (SLA^{c}) mismatch was achieved, while haplotype SLA^{d} is shared by donor and recipient. This SLA combination eliminated the possibility of graft-versus-host disease, which might interfere with the allograft rejection process [16,17].

Surgical procedures

Orthotopic liver transplantation

Orthotopic liver transplantation (OLT) was performed according to a previously described technique developed in our laboratory [18]. In the donor, *in situ* liver perfusion was achieved with Hartmann solution. Total cold ischaemic time was kept below 60 min. In the recipient, an indwelling central catheter was inserted in the external jugular vein for intravenous infusion and blood sampling. OLT was performed without veno-venous bypass and no recipient received blood transfusion. The portal-vein clamping time was uniformly below 20 min (15.0 ± 1.0 min).

In sham OLT, the liver was dissected out and portal vein, suprahepatic and infrahepatic vena cava were clamped for 30 min. During clamping, the liver was perfused with cold (4 °C) Hartmann solution *in situ* through a catheter inserted in the portal vein, and the outflow was recovered from infrahepatic vena cava. Animals were kept under anaesthesia for a total of 5 h, which was comparable with the situation of OLT. Similarly, control animals which did not undergo any surgery, were anaesthetized for a period of 5 h.

Heterotopic renal transplantation

The right kidney of the donor was used in each case. After harvesting, the kidney was perfused with cold (4 °C) Hartmann solution (1 l). Recipients underwent bilateral nephrectomy and received an heterotopic right renal transplantation. The renal artery was anastomosed end-to-side to the recipient aorta, and the renal vein end-to-side to the recipient vena cava. The ureter was implanted into the bladder via ureteroneocystotomy. The total cold ischaemic time was kept at <60 min. In the recipient, the insertion of an indwelling central venous catheter into an external jugular vein allowed frequent blood sampling for monitoring.

As sham-heterotopic renal transplantation (HRT), the left kidney was excised and the right kidney was isolated from the general circulation for 30 min. During this period, cold (4 °C) Hartmann solution was perfused *in situ* through a catheter inserted into the right renal artery and flow out from the right renal vein. Animals were kept

under anaesthesia for a total of 2 h, which was comparable with the situation of HRT.

Haematology analysis

Blood samples were taken daily and the total number of peripheral platelets and leucocytes analysed with a blood counter (MS9 vet; Melet Schloesing Laboratory, France).

Cortisol assessment

Plasma cortisol assessment was carried out by cortisol solid phase radioimmunoassay (Corti-Cote; Becton Dickinson, Erembodegem-Aalst, Belgium).

Graft tissue sampling

First, graft biopsies were taken after transplant unclamping (POD 0). Afterwards, percutaneous needle biopsies were collected sequentially at POD 1, 3, 5, 7, 10 and 14 under ultrasound (Siemens, Munich, Germany) control. Whether or not animals survived beyond 14 days, additional biopsies were performed once a month until being killed. Part of the sample was used for histological examination and the other was snap-frozen in liquid nitrogen for RNA preparation.

Histology

Graft specimens were fixed in Bouin and stained by haematoxylin and eosin. In the case of OLT, the grade of cellular rejection was assessed following the Hubscher scoring system [19] and for HRT by the Banff scoring system [20].

Semi-quantitative analysis of cytokine mRNA expression in the allograft

RNA preparation

Total RNA was extracted from serial graft biopsy samples using TRIZOL® reagent (Life Technologies, Paisley, UK). The purified RNA was dissolved in diethylpyrocarbonate-treated water and RNA concentration and purity were assayed by the measurement of the optical density at 260/280 nm wavelength.

cDNA synthesis

Total RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Life Technologies). One microgram of RNA was mixed with hexamer oligonucleotides, dNTP mixture, reverse transcriptase buffer and DTT in a total volume of 20 μl. This mixture was incubated at 37 °C for 1 h. The cDNA obtained was stored at −20 °C until use or directly subjected to polymerase chain reaction (PCR).

Real-time PCR

For real-time PCR, primers and internal fluorogenic probes (Table 1) were designed using the software primer express (Applied Biosystems, Foster City, CA, USA) to detect a housekeeping gene (i.e. hypoxanthine–guanine phosphoribosyltransferase) and cytokine genes [i.e. interleukin 2 (IL-2), IL-4, IL-10, IL-12 and interferon γ (IFN-γ)]. PCR reaction mixtures consisted of cDNA (corresponding to 25 ng of total RNA) in buffer A containing internal passive reference ROX, 250 μM dNTP mixture, 5 mM $MgCl_2$, 0.4 μM each of 5′- and 3′-specific primers, 0.2 μM probe and 0.625 U of TaqGold DNA polymerase in a final volume of 25 μl. For the evaluation of TGF-β1 mRNA, primers were developed and the SybrGreen PCR core reagents kit (Applied Biosystems) was used according the manufacturer's instructions. The amplification of cDNA was performed in the ABI prism 7700 sequencing detector system, which contains a gene-Amp PCR system 9600 (Applied Biosystems). The PCR conditions were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 10 s, and 60 °C for 1 min. Fluorescence emission was collected at the end of an annealing-extension step. The housekeeping gene expression was determined as an endogenous reference for each sample in parallel to the gene of interest in order to normalize the amount of cytokine cDNA. The sample on POD 0 served for calibration and the data were expressed as *x*-fold expression

Table 1. Primers and internal fluorogenic probes used for the PCR.

Cytokines	Primer A	Primer B	Internal probes
IL-2	5′GATTTACAGTTGCTTTTGAAGG3′	5′TGCTGAGTCAGAGTTTTTGCTTTG3′	5′TTTAGCACTCCCTCCAGAGCTTTGAGTTCTTCTACTAAA3′
IL-4	5′CACAGCGAGAAAGAACTCGT3′	5′GTCCGCTCAGGAGGCTCTTC3′	5′TCTGCCGGGCCTCGACTGTGC3′
IL-10	5′CTTGTTGCTGACCGGGTCTCT3′	5′TCGGCATTACGTCTTCCAGGT3′	5′TCTCTGACAAGGCTTGGCAACCCAGGT3′
IFN-γ	5′TTCAAAGATAACCAGGCCATTCA3′	5′CACTGATGGCTTTGCGCTG3′	5′CTTTAATCAGCTTTTCGAAGTCATTCAGTTTCCCAGA3′
IL-12	5′CTGGTGGCTGACAGCAATCA3′	5′GCCCTCCTGACACTCCACTCT3′	5′CAGTGTCAAAAGCAGCAGAGGCTCCAC3′
TGFβ1	5′AGCCCTGGATACCAACTA3′	5′AGCTGCACTTGCAGGAAC3′	
House keeping	5′TGACACTGGCAAAACAATGCA3′	5′TCCTTTTCACCAGCAAGCTTG3′	5′TGACCATCTTTGGATTATGCTGCTTGACCA3′

relative to the calibrator. Negative controls consisted of PCR reaction mixtures without cDNA subjected to the same amplification programme.

^{111}Indium-oxine labelled lymphocyte scintillography

A 1×10^8 recipient pig peripheral blood mononuclear cells (PBMCs) were isolated, suspended in physiological water and incubated for 15 min with 780 μCi (28 860 MBq) of ^{111}indium-oxine at 37 °C. The complex is neutral, lipid soluble and penetrates the cell membrane of the lymphocytes. Within the cells, the indium attaches to cytoplasmic components. The labelled cells were washed with 50% autologous plasma in ACD (Acid Citrate Dextrase) buffer and the pellet was re-suspended in 4 ml of autologous plasma for injection. This labelled lymphocyte suspension was injected intravenously 5 h after allograft in one liver and one kidney transplant recipient, as well as in two swine which underwent either a laparotomy alone or only anaesthesia. Thoracic and abdominal scans were performed every 20 min for 1 h after injection, and radioactivity evidenced with a gamma-camera coupled to a computer with a medium-energy collimator. After 24 h, the four animals were killed and biopsies from liver, spleen, lymph nodes, intestine, kidney, lung, heart, thymus and peripheral blood were taken to assess *in vitro* residual radioactivity.

Mixed lymphocyte reaction

One-way mixed lymphocyte cultures were carried out using freshly isolated PBMCs as previously described [21]. The stimulation index (SI) was calculated as: SI = (experimental c.p.m. − antiself c.p.m.)/antiself c.p.m. The results were expressed as the ratio of SI based on the preoperative SI.

Anti-donor antibody production

In order to assess anti-donor immunoglobulin G (IgG) antibodies, sera from both OLT and HRT recipients were collected during the period of survival. SLA$^{c/d}$ cells were extracted from peripheral PBMC and mixed for 30 min with decomplemented sera from allograft recipients. After washing and incubation with polyclonal goat anti-swine anti-IgG (Kirkergaard and Perry, Gaithersburg, MD, USA) for 30 min, the presence of anti-donor IgG antibody was monitored by flow cytometry.

Results

Animal and graft survival

Kidney graft survival

The five one-haplotype mismatched renal allografts were uniformly rejected on days 14–17 and died from uraemia. Sham kidney recipients survived up to POD 14 (day of killing) (Table 2).

Liver graft survival

All liver allograft recipients survived longer (range 23–87 days: mean 49.5 ± 22.1 days) than kidney recipients. At the time of death or being killed, there were always signs of rejection plus additional complication such as pneumonia, gastric ulcer, sepsis and aerophagia. The sham-OLT recipients survived up to killing (Table 2).

Renal and liver function

All the kidney recipients maintained normal renal function for 1 week and then serum creatinine rose rapidly to 10–14 mg/dl in the second week. All animals died because of uraemia. Conversely, sham kidney transplant recipients maintained normal serum creatinine between 1 and 1.2 mg/dl during the two follow-up weeks.

The liver recipients demonstrated a significant increase in plasma total bilirubin 2 days after transplantation as well as an increase in hepatic enzymes (AST). AST (Aspartate Amino Transferase) levels varied during the follow-up of graft survival but were usually above normal values from a minimal to a moderate extent. Sham liver recipients maintained both bilirubin and hepatic enzyme levels at normal values.

Table 2. Animal survival and cause of death.

Recipient no.	Days survived	Cause of death	Histology at autopsy
Heterotopic renal transplantation			
dd194	14	Killed (uraemia)	Banff III
dd196	14	Killed (uraemia)	Banff III
dd197	14	Killed (uraemia)	Banff IIB
dd195	17	Killed (uraemia)	Banff III
dd188	14	Killed (uraemia)	Banff IIA
Sham-renal transplantation			
15443	>14	Killed	Normal findings
15510	>14	Killed	Normal findings
Orthotopic liver transplantation			
dd135	44	Killed (pneumonia)	Hubscher score: 4
dd142	61	Killed (aerophasia)	Hubscher score: 3
dd140	23	Killed (sepsis)	Hubscher score: 6
dd8455	37	Rejection (gastric ulcer)	Hubscher score: 6
dd8471	45	Rejection (sepsis)	Hubscher score: 5
dd8453	87	Rejection (pneumonia)	Hubscher score: 4
Sham-liver transplantation			
94	>14	Killed	Normal findings
101	>14	Killed	Normal findings
3206	>14	Killed	Normal findings

Peripheral lymphocyte count

The peripheral lymphocyte count rapidly decreased immediately after unclamping in both OLT and HRT (Fig. 1a and b). The leucocyte fall reached 50% of the preoperative value in both types of transplant, and OLT or HRT recipients recovered a preoperative peripheral lymphocyte count after 1 week. This phenomenon, however, was not transplantation-specific as similar events could be observed in animals which underwent sham OLT, anaesthesia only or sham HRT.

Peripheral platelet count

Peripheral platelet count fell rapidly (more than 50%) in both OLT and sham-OLT recipients, whereas there was no decrease of platelet count in renal and sham-renal recipients. OLT recipients needed at least one postoperative week in order to recover a preoperative value of platelet count. After 10 POD, the platelet count fell in both OLT and HRT concomitant to the acute cellular rejection process (Fig. 1c and d).

Plasma cortisol levels

After OLT, HRT, sham-OLT and HRT and anaesthesia only, plasma cortisol level quickly increased up to five times the preoperative value and then recovered a normal value after 48 h post-transplantation, postsurgery or post-anaesthesia. This result clearly emphasized the stress response to surgery or anaesthesia and was not related only to surgery or transplantation because a similar increase in cortisol was observed after anaesthesia only (Fig. 2a and b).

Histology

In HRT, morphological signs of rejection appeared from 5 to 7 days after transplantation and, according to the Banff classification, reached grade IA on POD 5–7, IB or

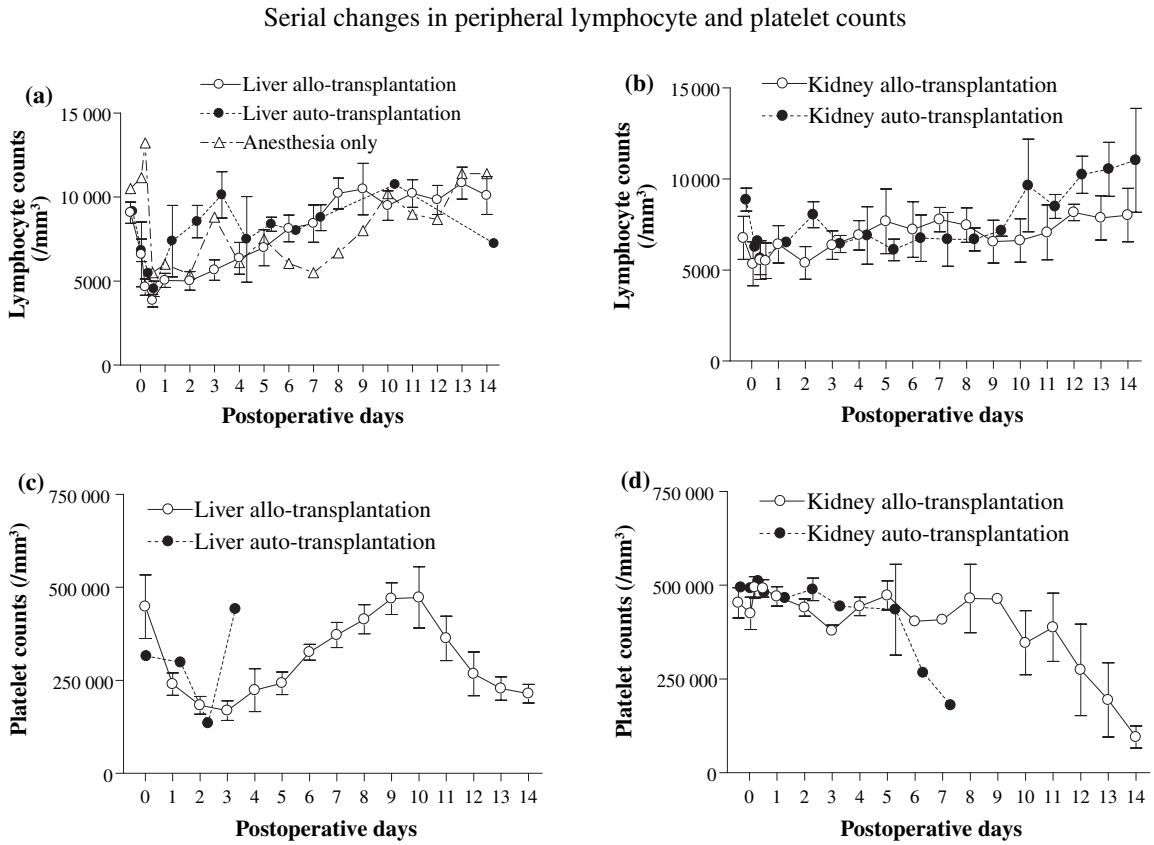

Figure 1 Changes of peripheral lymphocyte count after liver transplantation (a) and renal transplantation (b). Lymphocyte counts were rapidly decreased in pigs undergoing surgery, as well as anaesthesia only. Changes of peripheral platelet count after liver transplantation (c) and renal transplantation (d). In animals undergoing liver and sham-liver transplantation, platelet counts fell early after surgery, whereas, at this time point, no decrease of platelet count was found in recipients of renal and sham-renal transplantation.

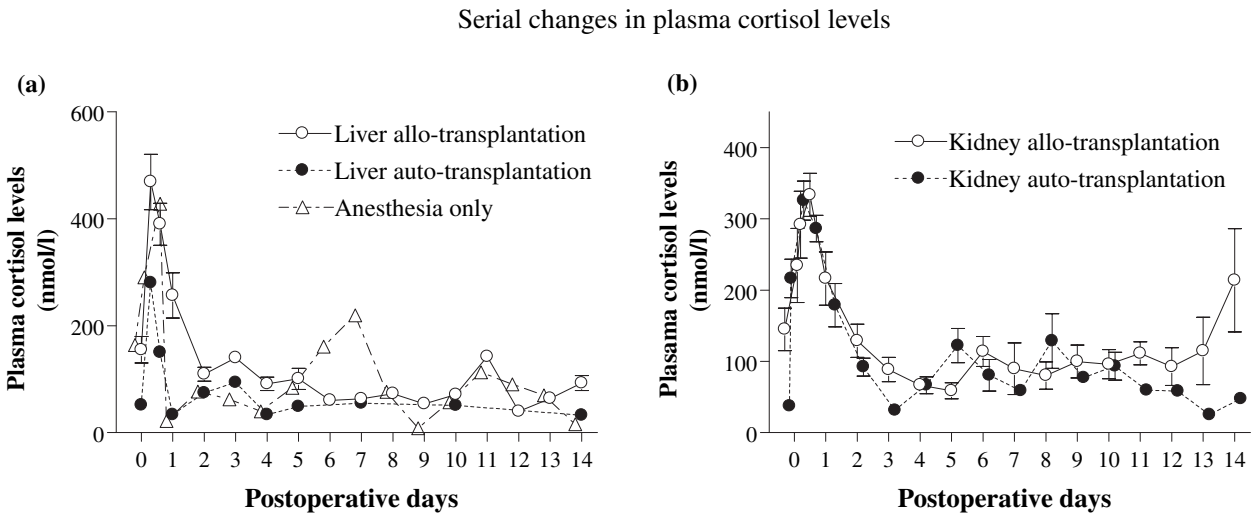

Figure 2 Postoperative kinetics of plasma cortisol levels. (a) Animals received heterotopic renal or sham-renal transplantation. (b) Animals received orthotopic liver or sham-liver transplantation, or anaesthesia only. Elevation of plasma cortisol levels was observed immediately after operation independently of the type of surgery, as well as anaesthesia only.

IIA on POD 10, and IIA, IIB, or III on POD 14 (Table 2). These histological findings were concomitant to the progressive increase in serum creatinine levels and renal-function deterioration. In sham HRT, there were no signs of rejection at the time of killing.

In OLT, serial biopsy examination demonstrated signs of rejection from POD3, especially cell infiltration in the portal tract and the bile duct. As assessed by Hubscher scoring, signs of rejection were maximal 2 weeks after transplantation and persisted for 4 weeks (Table 2). In some animals, these morphological signs of rejection occurred spontaneously and subsided thereafter. In sham-OLT, there were no signs of rejection at the time of killing.

Mixed lymphocyte reaction

All recipients demonstrated a significant proliferative response to both donor and TP (third party) antigens prior to liver or renal allografts. After OLT, recipients quickly lost the anti-donor proliferative response as early as the next day after transplantation (Fig. 3a). This early specific unresponsiveness usually persisted for up to 10–14 days after transplantation. This anti-donor cellular response partially recovered slowly thereafter in survivors, but never reached preoperative values prior to death or killing. The response to TP antigens transiently decreased from POD 5 to POD 10, but never reached the level of unresponsiveness and then regained the preoperative level. Sham OLT maintained a positive anti-TP antigen Mixed Lymphocyte Reaction (MLR) throughout the two follow-up weeks.

Similarly, kidney recipients demonstrated an early anti-donor specific unresponsiveness up to POD 10 and then the anti-donor response increased at the time of rejection, but the level was still below preoperative values. The anti-TP MLR did not decrease after HRT. In sham-HRT animals, however, the ratio of SI transiently decreased for 2–3 days after transplantation and then regained the preoperative values (Fig. 3b).

Anti-donor antibody production

As assessed by flow cytometry, OLT recipients produced anti-donor IgG antibodies in the month after transplantation. The kidney recipients similarly developed anti-donor IgG antibodies in the two postoperative weeks and until rejection (data not shown).

Cytokine mRNA expression

In the allograft, IFN-γ mRNA expression started to increase on POD 1 in HRT and POD 3 in OLT. In HRT, the IFN-γ expression was progressively up-regulated until graft rejection, while in OLT IFN-γ expression peaked on POD 5 in OLT and decreased thereafter. IL-10 gene expression increased in all animals (both HRT and OLT recipients) throughout the experimental period, and expression was higher in HRT than in OLT. TGF-β1 expression also significantly increased on POD 1 and peaked on POD 7 in OLT. This expression eventually decreased thereafter and returned to control levels on POD 14. In HRT, however, up-regulation of TGF-β1 mRNA was never seen during the same period.

Interleukin 2 (IL-2) and IL-12 mRNA were not modified compared preoperative values in liver or renal

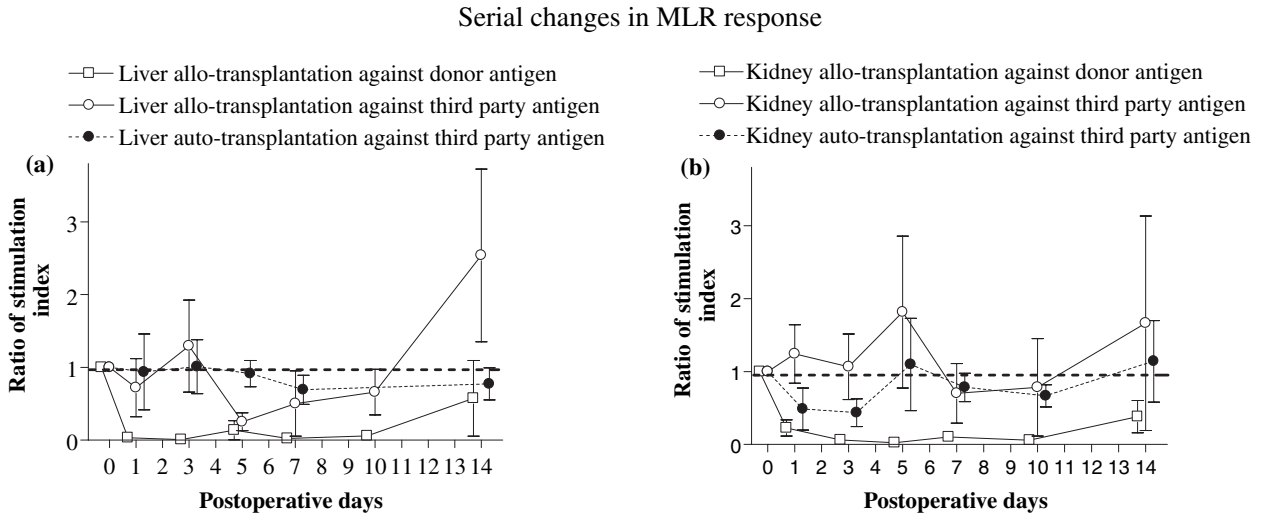

Figure 3 Post-transplant mixed lymphocyte reaction to donor and third party antigens. The anti-donor cellular response disappeared as early as the next day after both liver (a) and kidney (b) transplantation, and this specific unresponsiveness usually persisted up to 10–14 days. The anti-third party response, although decreased during the first postoperative week, persisted.

allografts in this model. IL-4 expression was not increased in animals which underwent OLT or HRT, although was sometimes detected at low levels (data not shown) (Fig. 4).

Indium-oxine labelled lymphocyte scintillography

Five hours after the procedure, four animals underwent injection of autologous PBMC labelled with ^{111}indium-oxine, and radioactivity was assessed by scintillography as excess counts. As shown in Fig. 5, radioactivity did not increase in the lungs, whereas in the spleen, the excess count significantly rose in the four animals. The increase of radioactivity was also significant in the liver during 10 min after injection in pigs which underwent anaesthesia only, laparotomy only and kidney transplant. After this early increase, there was a plateau during 60 min in these three animals, thereby suggesting that the accumulation of PBMC was significant in the liver, but to a lower level than in the spleen. However, the accumulation of labelled PBMC was significantly higher in the liver in the OLT recipient than in the three other animals in which the liver was native. In this OLT recipient, radioactivity followed exactly the same increase as in the spleen, which was different in comparison with the three other animals. Radioactivity was never observed in the native kidney or kidney allograft in these animals.

Twenty-four hours after injection, most radioactivity was localized in the spleen for all animals. However, tissue 24 h uptake/100 g was significantly higher in kidney or liver allograft recipients than in anaesthetized and laparotomized animal, suggesting an increased activity in spleen after transplantation of an allograft. The 24-h uptake in liver was much lower and comparable with uptake in lymph nodes and lungs (Fig. 5).

Discussion

In the current study, we confirmed that recipients of semi-identical liver allograft could survive for prolonged periods (23–87 days) without immunosuppression, although rejection eventually occurred [14]. Conversely, semi-identical kidney allografts were uniformly rejected within two postoperative weeks. In a well-defined miniature swine model, these results confirm that the rejection process is attenuated in liver allograft across semi-identical SLA mismatch, whereas rejection occurred rapidly and uniformly after semi-identical kidney allograft.

The initial histological picture exhibits the same tempo in OLT as in HRT recipients. However, the morphological changes in OLT were not always associated with graft dysfunction as was the case after HRT. Similar discrepancy is often observed in rodent models in the study of spontaneously tolerated liver allografts [7,22]. Even in humans, Schlitt *et al.* [23] reported that typical morphological signs of rejection were evidenced without clinical correlate, mostly within the first 2 weeks after transplantation. This may be attributable to difference in early immune response after OLT and HRT. In SLA-mismatched kidney recipients, we previously demonstrated that intragraft infiltrates by mononuclear cells was very

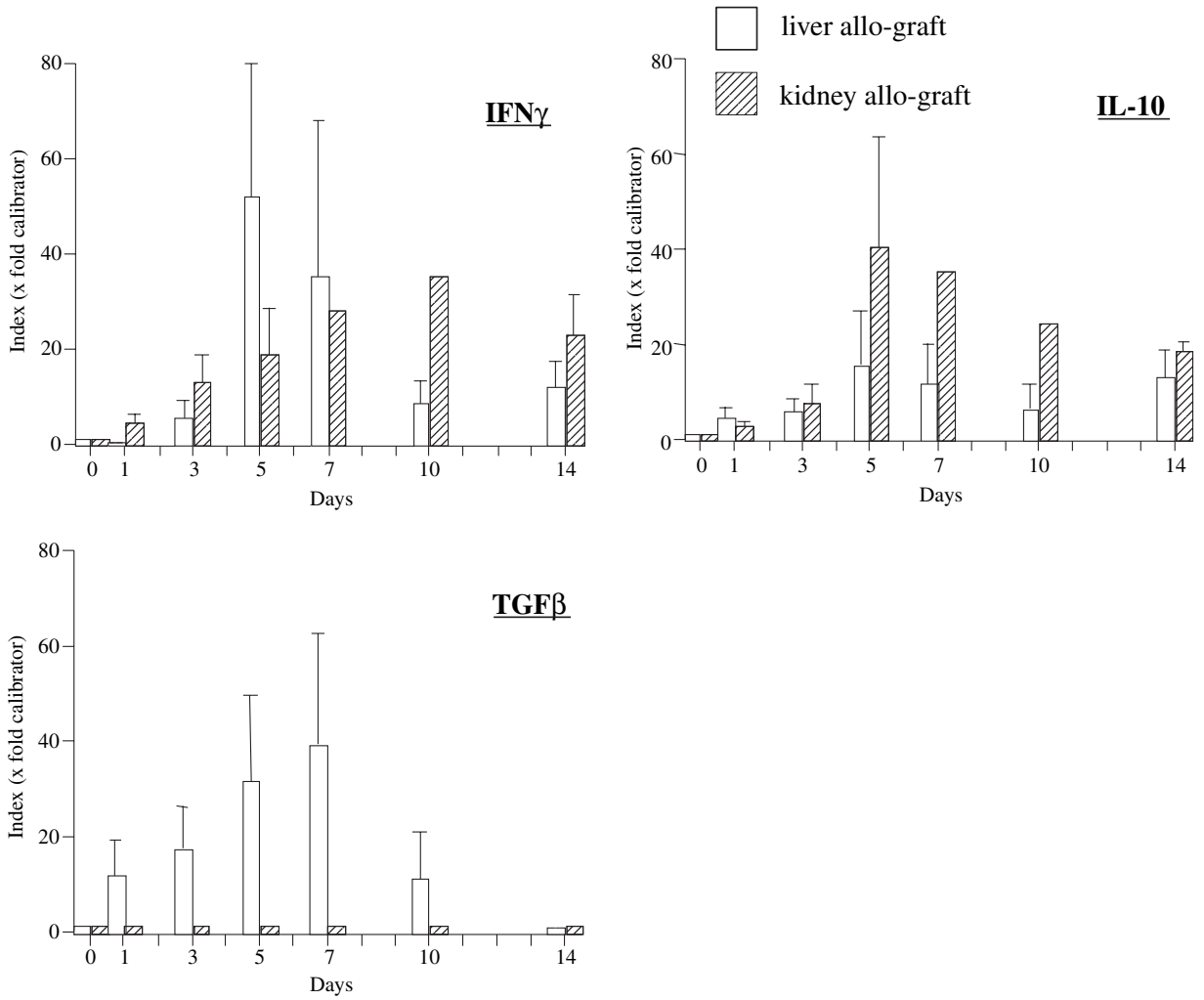

Figure 4 The time course of cytokine mRNA expression in liver and renal allografts early after transplantation. Interferon-γ mRNA expression was progressively up-regulated in both kidney and liver allografts during the first postoperative week. Expression of transforming growth factor-β1 gene increased with a peak of seven postoperative days in liver allografts and IL-10 in renal allografts.

significant (up to 75%) in animals which progressively went into tolerance and rejection. This demonstrates that infiltrating cells have a different activity in these two situations, such as producing immunomodulatory Th2 cytokines and having an anergized profile in tolerant animals [24,25].

Early after transplantation, we observed that the peripheral count of platelets fell significantly after OLT, but this phenomenon was probably related to the surgical procedure and the liver ischaemia, because a similar result was found after sham-transplantation. In contrast, kidney allograft recipients did not evidence a decrease in peripheral platelets immediately after transplantation. This difference is important because, in the follow-up of human liver transplant recipients this early platelet decrease could be misinterpreted as a sign of rejection, while at this early change it seems more related to ischaemia–reperfusion of the liver transplant.

Interestingly, in the 48 h after the procedure, the serum cortisol level increased threefold to fivefold after OLT, HRT, sham-transplantation and anaesthesia only. Therefore, this significant increase in cortisol, even after anaesthesia alone, seemed aspecific and unrelated to surgery. This result, however, questions the rationale for injecting intraoperative corticoids during transplantation in human transplant recipients. Cortisol is known to reduce T-cell numbers and function [26]. In the current study, we observed a significant early fall in peripheral lymphocytes after OLT and concomitantly to the elevation of plasma cortisol level. One of the mechanisms for the decrease in PBMCs could therefore be the elevation of the cortisol level in the blood [21].

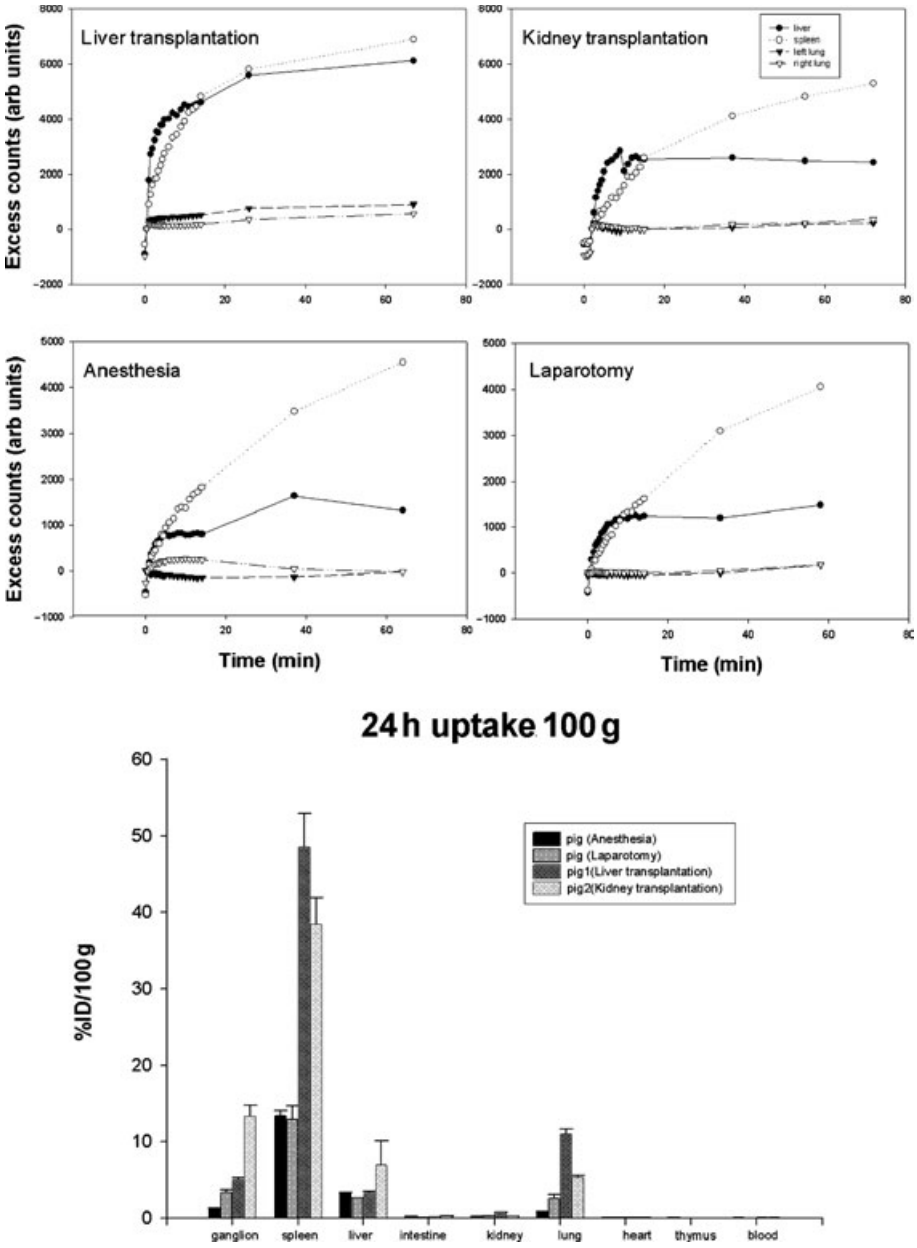

Figure 5 Indium scanning. The excess counts represent the labelled lymphocytes that were detected in liver, spleen and lungs. In the case of liver transplantation, the excess count increased similarly in both liver allograft and spleen. In the case of kidney transplantation, as well as either laparotomy alone or anaesthesia only, the excess count increased in spleen but less significantly in naïve liver. No accumulation of radioactivity was seen in the kidney, even in case of kidney allograft. There was thus an accumulation of lymphocytes into the liver allograft after hepatic transplantation, which is different from that of kidney allograft. After 24 h, the indium uptake was significantly higher in the spleen after both OLT and HRT when compared with nontransplanted animals, thereby suggesting that recipient lymphocytes reside first in the recipient liver and then in the spleen after OLT, and mainly in the spleen only after HRT.

Another possible mechanism for the reduction of peripheral lymphocytes might be the capture of peripheral lymphocytes in some organs. Current indium scanning data revealed that the spleen and the liver are two major sites where lymphocytes are trapped early after surgery or anaesthesia. Trapping of lymphocytes in these organs might be a nonspecific phenomenon because trapping in the spleen has been shown in all experimental animals. There was, however, a significantly more important trapping in the liver in the hepatic transplant recipient than in the kidney recipient. It has been demonstrated *ex vivo* that activated T cells but not resting T cells were trapped

in a liver allograft, whereas neither activated nor resting T cells are trapped through a kidney allograft [11]. In this *in vivo* experiment, we clearly demonstrated that mononuclear cells reside in the liver allograft and not in the kidney allograft for at least 1 h after injection. The trapped lymphocytes in the liver allograft might then undergo apoptosis or immunoregulation, which could explain why a liver allograft is more resistant to T-cell infiltration than a renal allograft. Recently, several studies suggested that AICD or apoptotic death of alloreactive T cells occur predominantly within the liver graft and that this phenomenon, triggered by donor alloantigens, is associated with a mechanism underlying the acceptance of liver allografts [8]. Graft-derived DC may at least in part induce this apoptotic cell death in case of OLT. Although mature DC are widely regarded as the most potent antigen-presenting cells, most DC resident within the liver are immature and therefore deficient in expression of cell-surface co-stimulatory molecules [27]. It is therefore likely that accumulated alloreactive lymphocytes early after transplantation are activated by alloantigens in the liver and subsequently eliminated through apoptosis, resulting in the mild and slow rejection process in liver-allograft recipients. In this particular model, the intragraft course of recipient lymphocytes should therefore be studied in order to assess whether these cells undergo apoptosis in liver transplant and not in kidney allograft.

In this model, mRNA expression of TGF-β1 gene was significantly increased in OLT recipients, but not in HRT recipients. In addition to permitting DC growing, TGF-β1 is known to be an important mediator of immunoregulation and usually down-regulates the activity of immune-system cells [28–30]. *In vitro* studies have demonstrated that TGF-β1 can prevent T-cell proliferation [28], inhibit cytotoxic T-cell activity [29] and inhibit differentiation of IFN-γ producing T cells [30]. These results suggest that the intrahepatic production of TGF-β1 after liver allograft could immunomodulate the early immune response in OLT, but the early production of IFN-γ could also be important as it has recently been shown that expression of IFN is required in liver allograft to avoid early necrosis [31]. In HRT allograft the over-expression of IL-10 did not seem to immunomodulate the acute cellular rejection of such allografts.

In a previous report, PBMCs from FK506-treated recipients lost the capacity to mount a cellular response against donor antigens as early as the next day after OLT, whereas anti-TP response remained detectable [14]. Similar results were shown in untreated recipients of liver allograft in this study, as well as in animals receiving a kidney allograft. These results suggest that the assessment of peripheral cellular immune response using MLR in an early phase after transplantation does not represent a marker for discriminating animals which will tolerate or reject an allograft. However, the exact mechanisms of this early donor-specific unresponsiveness remain unclear. Although there was aspecific cellular unresponsiveness after both OLT and HRT, the anti-donor humoral response was intact, as both OLT or HRT recipients produced anti-donor IgG antibodies.

In summary, although an initial immunological response seems to occur in liver allografts at the same tempo as kidney allografts, recipients of semi-identical OLT could survive for prolonged periods when compared with those receiving HRT. The longer survival of liver allografts might be attributable to intragraft production of concomitant IFN-γ and TGF-β1, which avoids liver necrosis and modulates the early immune response. The specific trapping of peripheral lymphocytes in the liver but not in the renal allograft might represent another factor which explains why the rejection process is milder and slower in liver allograft recipients. Mechanisms of AICD in the liver allograft should be assessed in this model. There are some biological events which need to be observed in this experimental model without immunosuppression. This would avoid misinterpretation of signs after human transplantation such as platelet fall in OLT recipients but not in HRT, and a significant decrease in peripheral lymphocytes, which as shown in this study may be related to high cortisol production and intragraft trapping but not to immunosuppressive drugs. These results must be taken into consideration to tailor immunosuppression in human recipients.

References

1. Rydberg L. ABO-incompatibility in solid organ transplantation. *Transfus Med* 2001; **11**: 325.
2. Chen M, Wade J, Levy GA, Greig PD. Effect of HLA matching a. *Transplant Proc* 1994; **26**: 2695.
3. Buelow R, Burlingham WJ, Clayberger C. Immunomodulation by soluble HLA class I. *Transplantation* 1995; **59**: 649.
4. Krams SM, Fox CK, Beatty PR, *et al.* Human hepatocytes produce an isoform of FAS that inhibits apoptosis. *Transplantation* 1998; **65**: 713.
5. Gassel HJ, Hutchinson IV, Engemann R, Morris PJ. The role of T suppressor cells in the maintenance of spontaneously accepted orthotopic rat liver allografts. *Transplantation* 1992; **54**: 1048.
6. Sharland A, Yan Y, Wang C, *et al.* Evidence that apoptosis of activated T cells occurs in spontaneous tolerance of liver allografts and is blocked by manipulations which break tolerance. *Transplantation* 1999; **68**: 1736.
7. Qian S, Lu L, Fu F, *et al.* Apoptosis within spontaneously accepted mouse liver allografts. *J Immunol* 1997; **158**: 4654.

8. Crispe IN, Dao T, Klugewitz K, Mehal WZ, Metz DP. The liver as a site of T-cell apoptosis: graveyard, or killing field? *Immunol Rev* 2000; **174**: 47.
9. Mathew JM, Marsh JW, Susskind B, Mohanakumar T. Analysis of T cell responses in liver allograft recipients. Evidence for deletion of donor-specific cytotoxic T cells in the peripheral circulation. *J Clin Invest* 1993; **91**: 900.
10. Taniguchi H, Toyoshima T, Fukao K, Nakauchi H. Presence of hematopoietic stem cells in the adult liver. *Nat Med* 1996; **2**: 198.
11. Mehal WZ, Juedes AE, Crispe IN. Selective retention of activated CD8+ T cells by the normal liver. *J Immunol* 1999; **163**: 3202.
12. Calne RY, Sells RA, Pena JR, *et al.* Induction of immunological tolerance by porcine liver allografts. *Nature* 1969; **223**: 472.
13. Flye MW, Pennington L, Kirkman R, Weber B, Sindelar W, Sachs DH. Spontaneous acceptance or rejection of orthotopic liver transplants in outbred and partially inbred miniature swine. *Transplantation* 1999; **68**: 599.
14. Oike F, Talpe S, Otsuka M, *et al.* A 12-day course of FK506 allows long-term acceptance of semi-identical liver allograft in inbred miniature swine. *Transplantation* 2000; **69**: 2304.
15. Gianello PR, Sachs DH. Effect of major histocompatibility complex matching on the development of tolerance to primarily vascularized renal allografts: a study in miniature swine. *Hum Immunol* 1996; **50**: 1.
16. Pageaux GP, Perrigault PF, Fabre JM, *et al.* Lethal acute graft-versus-host disease in a liver transplant recipient: relations with cell migration and chimerism. *Clin Transplant* 1995; **9**: 65.
17. Starzl TE, Demetris AJ, Rao AS, Thomson AW, Trucco M, Murase N. Migratory nonparenchymal cells after organ allotransplantation: with particular reference to chimerism and the liver. *Prog Liver Dis* 1994; **12**: 191.
18. Oike F, Uryuhara K, Otsuka M, *et al.* Simplified technique of orthotopic liver transplantation in pigs. *Transplantation* 2001; **71**: 328.
19. Hubscher S. Diagnosis and grading of liver allograft rejection: a European perspective. *Transplant Proc* 1996; **28**: 504.
20. Racusen LC, Solez K, Colvin RB, *et al.* The Banff 97 working classification of renal allograft pathology. *Kidney Int* 1999; **55**: 713.
21. Gianello PR, Lorf T, Yamada K, *et al.* Induction of tolerance to renal allografts across single-haplotype MHC disparities in miniature swine. *Transplantation* 1995; **59**: 884.
22. Farges O, Morris PJ, Dallman MJ. Spontaneous acceptance of liver allografts in the rat. Analysis of the immune response. *Transplantation* 1994; **57**: 171.
23. Schlitt HJ, Nashan B, Krick P, *et al.* Intragraft immune events after human liver transplantation. Correlation with clinical signs of acute rejection and influence of immunosuppression. *Transplantation* 1992; **54**: 273.
24. Gianello P, Fishbein JM, Sachs DH. Tolerance to primarily vascularized allografts in miniature swine. *Immunol Rev* 1993; **133**: 19.
25. Blancho G, Gianello PR, Lorf T, *et al.* Molecular and cellular events implicated in local tolerance to kidney allografts in miniature swine. *Transplantation* 1997; **63**: 26.
26. Parrillo JE, Fauci AS. Mechanisms of corticosteroid action on lymphocyte subpopulations. III. Differential effects of dexamethasone administration on subpopulations of effector cells mediating cellular cytotoxicity in man. *Clin Exp Immunol* 1978; **31**: 116.
27. Thomson AW, Lu L. Are dendritic cells the key to liver transplant tolerance? *Immunol Today* 1999; **20**: 27.
28. Mule JJ, Schwarz SL, Roberts AB, Sporn MB, Rosenberg SA. Transforming growth factor-beta inhibits the in vitro generation of lymphokine-activated killer cells and cytotoxic T cells. *Cancer Immunol Immunother* 1988; **26**: 95.
29. Smyth MJ, Strobl SL, Young HA, Ortaldo JR, Ochoa AC. Regulation of lymphokine-activated killer activity and pore-forming protein gene expression in human peripheral blood CD8+ T lymphocytes. Inhibition by transforming growth factor-beta. *J Immunol* 1991; **146**: 3289.
30. Schmitt E, Hoehn P, Huels C, *et al.* T helper type 1 development of naive CD4+ T cells requires the coordinate action of interleukin-12 and interferon-gamma and is inhibited by transforming growth factor-beta. *Eur J Immunol* 1994; **24**: 793.
31. Mele TS, Kneteman NM, Zhu LF, *et al.* IFN-gamma is an absolute requirement for spontaneous acceptance of liver allografts. *Am J Transplant* 2003; **3**: 942.