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A rat model of monitoring liver allograft rejection

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Abstract Rat models are often used to study liver allograft rejection. We have established a model for rat liver allograft rejection, monitored by fine needle aspiration biopsy (FNAB), in the strain combination PVG-to-BN with a mean survival time of 37 ± 20 days. In this model, we observed acute rejection with an intense peak of lymphoid blasts and lymphocyte-dominated inflammation in the FNAB [9.1 ± 3.0 corrected increment units (CIU)], and an eventual increase in macrophages (up to 4.2 ± 4.4 CIU), together with fibrosis and parenchymal necrosis in the graft. Markers of immune activation, such as an increase in IL-2-receptor (from 1 % ± 2 % to 21 % ± 13 %) and class II (from 20 % ± 9 % to 43 % ± 13 %) expressing lymphoid cells and induction of ICAM-1 in the graft, were consistent with the overall cellular response. The FNAB correlated well with parallel graft histology. In this rat model, the atraumatic monitoring makes a close follow-up possible without having to sacrifice the experimental animals. This saves work, animals, and costs in the study of liver rejection.

Key words Liver transplantation, rat model · Rat, liver transplantation, fine needle aspiration · Fine needle aspiration, liver, rat · Rejection, liver, rat

Introduction

Liver transplantation in the rat has been used as a model for studying rejection and the effects of various immunosuppressive therapies. The immunobiology of inbred rat strains is well characterized, and alloresponses in different strain combinations have been thoroughly studied [5, 6, 21]. In addition to choosing a suitable donor-recipient combination with a sufficient rejection response, it is necessary to obtain information about the graft. The course of acute rejection can only be defined by serial examination of liver specimens. Yet, obtaining serial histological samples without major complications is almost impossible, which is why acute rejection is usually established by examining specimens obtained at autopsy after sacrificing the transplant recipients. The effect of immunosuppressive drugs is generally correlated to the mean survival time. The time-related analysis of the rejection process makes it necessary to sacrifice a great number of animals.

In general, acute liver allograft rejection presents as infiltration of the portal tracts by mononuclear cells and damage to various structures of the liver. The inflammatory infiltrate consists mainly of lymphoid cells, but neutrophils, eosinophils, and monocytes are also present. The bile ducts and blood vessels are affected, and cholestasis is recorded. In the rat liver, mononuclear cell infiltration of portal areas is also the major histological finding in allograft rejection, together with parenchymal necrosis and endothelialitis, but the bile

ducts are not consistently affected [8, 15]. Fine needle aspiration biopsy (FNAB), originally developed at our clinic in Helsinki for the monitoring of kidney allografts [3, 13], is also used routinely in clinical liver transplantation and has been reported by several groups [2, 4, 9–11, 14, 17, 20]. The hallmark of acute liver allograft rejection, demonstrated by aspiration cytology, is the appearance of lymphocytosis and lymphoid blasts in the graft. In the more advanced stages, mononuclear phagocytes are abundant, and large numbers of macrophages are usually associated with irreversible rejection. A more detailed analysis of immunological activation in the FNAB may reveal so-called activation markers, such as an increase in class II and ICAM-1 expression, and the appearance of IL-2-receptors on lymphoid cells [2, 11, 17].

The aim of this study was to develop an experimental model of rat liver transplantation in which the rejection process could be followed up without having to sacrifice the recipients. To this end, we investigated whether repeated FNAB was atraumatic enough to be used to monitor acute rejection in the same animals. We also wanted to characterize the time-related cellular and immunological events of acute liver rejection in this rat strain combination, which are known to eventually lead to irreversible rejection after a mean graft survival of 37 ± 20 days. To use this strain combination for further studies, we wanted to know the baseline cytological profile of acute liver rejection in this model.

Materials and methods

Rats

A donor-recipient combination of PVG ($RT1^c$) to BN ($RT1^n$) was used. A mean survival time of 36 days without immunosuppression has previously been reported for this strain combination [21]. The rats were fed regular rat food and tap water ad libitum. The animals were treated according to the "Principles of laboratory animal care". The study was approved by the committee for experimental research of Helsinki University Central Hospital.

Transplantation

Liver transplantation was performed on 23 rats under ether anesthesia using the technique introduced by Kamada [6] but with reconstruction of the hepatic artery. An operating microscope was used. Briefly, the cuff technique was used for the portal and infrahepatic caval vein anastomoses. The bile duct was stented. The arterial anastomosis was performed end-to-side between the donor and recipient celiac trunks. The anhepatic phase lasted 15–20 min. Cold (4 °C) heparinized 0.9 % saline was used for perfusion and preservation of the graft. No immunosuppression was given after transplantation.

FNAB monitoring

Serial FNABs and corresponding peripheral blood samples were obtained from eight rats on postoperative days 1–2, 3–5, 7–9, 13–15, 20–21, and 28–30. For sampling, the animals were sedated with a fentanyl-fluanisone injection. The sample was aspirated with a 25-gauge needle under the right costal margin and transferred directly into cell culture medium. A few drops of blood, obtained from the tip of the tail, were placed into a similar culture medium to determine the blood background.

The processing of the aspirate and the blood sample was performed as described for clinical specimens [3]. The specimens were cytocentrifuged onto microscope slides and stained with May-Grünwald-Giemsa (MGG). The number of hepatocytes was determined by the FNAB. The intensity of inflammation associated with rejection was quantified from the aspirates with the increment method, as described previously [3]. Following this method, each inflammatory cell type is given a correction factor that reflects the diagnostic value of the cell type in acute rejection. Lymphoid blasts, plasma cells, monoblasts, and macrophages have the highest correction factor, 1.0; activated lymphocytes are 0.5, large granular lymphocytes and monocytes 0.2, and lymphocytes and polymorphonuclear cells 0.1. The result is expressed in corrected increment units (CIU). Total inflammation in the graft is expressed as total corrected increment (TCI), which is a corrected sum of inflammatory cells in the FNAB from which the blood background is subtracted. Altogether, 52 aspirates and corresponding blood specimens were obtained.

Activation marker analysis

The immune activation of rejection in the FNAB was further analyzed by studying the expression of cell surface molecules considered "activation markers". A three-layer, indirect, immunoperoxidase technique and monoclonal antibodies against rat MHC class II antigens (MAS 043, Seralab, Sussex, UK), IL-2-receptor (MAS 263, Seralab), and intercellular adhesion molecule-1 (ICAM-1, R&D Systems Europe, Abingdon, UK) were used. The cytocentrifuge preparations of the FNABs were first incubated with the monoclonal mouse antibody, then with peroxidase-conjugated rabbit anti-mouse antibody (Dako, Copenhagen, Denmark), and thereafter treated with a peroxidase-conjugated goat anti-rabbit antibody (Tago, Burlingame, Calif., USA). The reaction was revealed by AEC (3-amino-9-ethyl carbazole) solution containing hydrogen peroxide. Mayer's hemalum was used for counterstaining.The percentage of lymphoid cells expressing activation marker molecules was calculated, and the intensity of positive staining on hepatocytes was semiquantitatively scored from 1 (slightly positive) to 3 (intense staining).

Histology

In parallel to FNAB, graft histology was performed on another six recipients on days 1–2, and on an additional six on days 6–8 postoperatively by sacrificing the animals. The grafts of FNAB-monitored rats were later obtained for histology when the animals died or had to be sacrificed due to deterioration of their condition during ongoing acute rejection. All livers were fixed in 4 % buffered formalin solution and embedded in paraffin. Four-micron-thick sections were cut and stained with hematoxylin and eosin, and PAS. The characteristic histopathological changes in the graft were scored from + to + + +.

Statistical analysis

Data were expressed as mean ± SD, and for comparison of results, the *t*-test was used. Significances were calculated by comparing the results to the values recorded immediately (1–2 days) after transplantation.

Results

Eighty-seven percent of all the animals survived the operation and could be included in the study. Serial FNABs did not cause any complications, and 48 of 52 FNABs were representative, giving a success rate of 93%. The reasons for nonacceptance were intraperitoneal contamination or blood in the sample. The FNAB-monitored rats developed acute rejection and died or had to be sacrificed after a mean graft survival of 37 ± 20 days (range 16–59 days).

Cellular findings of rat liver rejection in the FNAB

All rats developed acute rejection, demonstrated by FNAB. The inflammatory profiles of acute rejection are summarized in Fig. 1. On days 1–2 after transplantation, no immune activation was seen in the aspirates. A high peak of inflammation associated with rejection was recorded in every recipient. The episode of acute rejection started on days 3–5 after transplantation. The intensity of the total inflammation was 0.7 ± 0.6 CIU on days 1–2; it increased significantly ($P < 0.0001$) to 6.7 ± 1.6 CIU on days 3–5 and reached a maximum of 9.1 ± 3.0 CIU on days 7–9 ($P < 0.0001$).

The inflammatory response was dominated by lymphoid blasts (2.0 ± 1.0 CIU on days 3–5 and 2.7 ± 1.5 CIU at the peak, $P < 0.0001$ and $P < 0.001$, respectively) and lymphocytes (0.6 ± 0.7 on days 1–2, 3.6 ± 1.1 on days 3–5, $P < 0.0001$, and 3.7 ± 1.3 on days 7–9, $P < 0.0001$) in the early phase of rejection. Thereafter, lymphoid activation decreased and mononuclear phagocytes began to dominate the graft infiltrate. The number of macrophages increased slowly, from neglible on days 3–5 (0.6 ± 0.5 CIU) to remarkably high on days 7–9 (2.0 ± 1.1 CIU, $P < 0.001$); they reached a peak (4.2 ± 4.4 CIU, $P < 0.05$) on days 20–21.

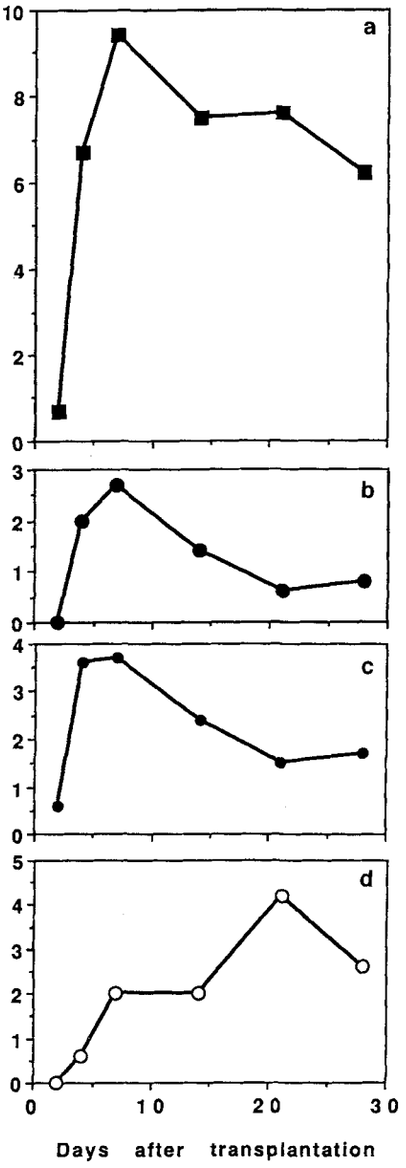

Fig. 1 a Summary of the inflammation profiles (mean values) of eight rat liver allografts monitored by FNAB and expressed corrected increment in units (TCI = —■—) units. **b–d** The major inflammatory cell components: lymphoid blast cells (*Blasts* = —●—), lymphocytes (*Ly* = —●—), and macrophages (Mp = —○—)

Expression of activation markers

The lymphoid activation associated with acute rejection was further demonstrated by the surface molecular changes (Fig. 2 a). The expression of IL-2-R molecules on lymphoid cells in the FNAB followed the overall pattern of the cellular rejection response. The number of IL-2-R-positive cells was low before the onset of inflammation, only 1.0% ± 1.7% on days 1–2, and then increased rapidly to 21.3% ± 12.9% ($P < 0.005$) on days 3–5 and to 21.0% ± 10.5% ($P < 0.001$) on days 7–9, together with the peak of the cellular response. Thereafter, some decrease in the number of IL-2-R-expressing lymphoid cells was recorded in the more advanced stage of the rejection response. The increase in

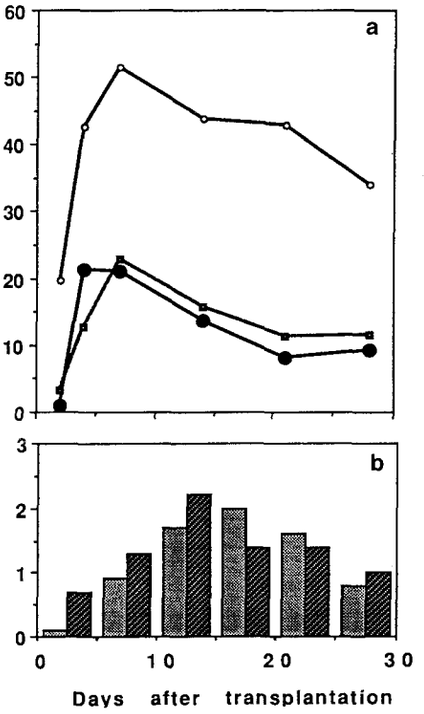

Fig. 2 **a** Summary of activation marker expression (IL-2-R = —●—, class II = —○—, ICAM-1 = —■—) on lymphoid cells in the FNAB of the eight monitored rat liver allografts, expressed as a percentage of positive lymphocytes. **b** The intensity of class II and ICAM-1 expression (scored from 1 to 3) on parenchymal cells in the aspirates. ▦ Class II, ▨ ICAM-1

Table 1 Histological alterations, scored from + to +++

	Postoperative days		Late phase of acute rejection (> 16 days
	1–2	6–8	
Portal inflammation	+	++/+++	++
Fibrosis	–	–/+	++/+++
Endotheliitis	–/±	++	–/+
Bile duct proliferation	–	–/+	+/++
Cholangitis	–	–/±	–/+
Parenchymal inflammation	–	+/++	+/+++
Parenchymal necrosis	+	+/++	+/+++

class II-positive lymphoid cells in the FNAB correlated with the peak of inflammation: it went from a low of 19.6 % ± 8.9 % on days 1–2, to a significantly elevated 42.7 % ± 13.0 % ($P < 0.01$) on days 3–5, and reached its maximum of 51.5 % ± 16.3 % ($P < 0.005$) on days 7–9 at the peak. ICAM-1 expression on the lymphoid cells in the FNAB was also linked to the immune activation of acute rejection. The number of ICAM-1-expressing cells increased significantly from a level of 3.2 % ± 1.3 % on days 1–2 to 12.7 % ± 1.6 % ($P < 0.0001$) on days 3–5, and up to 23.0 % ± 9.8 % ($P < 0.005$) on days 7–9.

Induction of class II antigens and ICAM-1 on the surface of liver parenchymal cells was also recorded in association with lymphoid activation in FNAB (Fig. 2b). Class II expression was not detectable before rejection on days 1–2 but usually appeared on hepatocytes at the beginning of immune activation on days 3–5. The intensity of class II expression was faint (semiquantitatively scored 0.9 ± 0.7, $P < 0.05$), but it increased significantly (1.7 + 1.0, $P < 0.01$) during the course of rejection. ICAM-1 induction on hepatocytes was also recorded. The intensity of ICAM-1 expression increased in association with the lymphoid response (0.7 ± 0.5 on days 1–2, 1.3 ± 0.8 on days 3–5, and 2.2 ± 0.8 on days 7–9, $P < 0.005$), corresponding to the overall pattern of immune activation.

Histopathological findings

The histological findings of rat liver allografts are summarized in Table 1. In the livers of the animals sacrificed on days 1–2, no signs of acute rejection were present. Only minor postsurgical histological changes, such as some hepatocellular vacuolization and occasional focal parenchymal necrosis, were recorded.

All livers obtained on days 6–8 showed a histological picture consistent with moderate to severe acute rejection, with an intense portal mononuclear inflammatory reaction. The portal tracts were greatly enlarged, their outlines were constantly blurred, and inflammatory streaks with mononuclear cells were seen radiating from the portal areas. Endotheliitis was observed in all animals, both in the central and portal veins, but was more pronounced in the portal tracts. Proliferation, swelling and vacuolization of the central and portal venous endothelial cells were observed in five out of six of the cases. The portal tract arteries showed no significant alterations. Marginal cholangitis was observed in four of six animals, but only minimal ductal proliferation and practically no bile duct destruction or cholestasis were recorded. The parenchyma showed some focal inflammatory reaction, mainly pericentrally or paraseptally. Slight to moderate patchy necrosis and hepatocellular vacuolization were seen. Kuppfer cells showed some proliferative activity.

The end-stage livers of the eight FNAB-monitored animals were obtained when the animals died or had to be sacrificed on postoperative days 16–59. In these grafts, the portal areas were still heavily infiltrated by mononuclear cells, including macrophages, in six of eight animals, and in two cases the portal inflammation was of moderate intensity. Fibrosis was a prominent finding in these grafts. The portal tracts were enlarged and fibrotic in seven out of eight livers, and in some of

them there was bridging fibrosis connecting adjacent portal fields. Proliferation of bile ducts was common; some ductal epithelial degeneration and marginal cholangitis were also seen. Endothelial lesions were mild or absent, except for some single focal proliferation. The parenchyma showed inflammatory infiltrates and hepatocellular vacuolization in all animals and necrosis of varying degrees in most cases.

Discussion

The strain combination used in this study demonstrated an intense alloresponse and provided an ideal model for studying acute rejection in the rat. The cellular findings in the FNAB were comparable with those described for human liver allografts, as well as those in another model of liver rejection in the pig [12, 20]. The time-related course of the irreversible rejection process was relatively slow, and the appearance of each cellular component of the cascade was easily recognized. The immunological activation markers – the appearance of IL-2-R, class II increase, and ICAM-1 induction – supported the cellular findings. The appearance of IL-2-receptor expression in the graft has also been shown to correlate with acute rejection of the rat liver [18]. The upregulation of class II and ICAM-1, recognized in human and rat allografts [1, 14, 16, 17, 19], followed the overall course of immune activation in the FNAB.

Graft histology, obtained immediately after transplantation, demonstrated surgery-related changes only. Grafts harvested at the peak of immune activation showed remarkable portal inflammation, together with parenchymal changes. Thus, the time-related cellular findings in the FNAB correlated well with histology, but damage to the various structures of the organ and fibrosis were observed by histology only. The histological findings of acute rat liver allograft rejection are comparable with those of humans, although the biliary alterations and vascular changes may vary. Thus, this animal model can be considered representative enough to be used to study liver allograft rejection.

The FNAB did not cause any complications, such as bleeding or infection. It was possible to follow up the same recipients by frequent sampling. A five- to sixfold increase in the number of experimental animals would have been required to perform as close a follow-up with histological examination of the graft as with the FNAB method. Because acute allograft rejection is a time-related, multiphase process, there is an obvious need for a monitoring method in the rat model. It would be quite helpful to be able to follow the immunological response in detail, together with the cell surface molecular changes during acute cellular rejection and the early acute response leading to chronic changes, in the same recipient.

Acute rejection has been widely studied using the rat liver transplantation model. Yet, recording only the end point after acute rejection may be inaccurate and misleading. Even a relatively short prolongation of the survival of a test animal has been regarded as an indication of the influence of different treatments. In studying various immunosuppressive regimens, it is useful to monitor the effect at different stages of the rejection cascade, not only the final outcome of the grafts. In our rat liver rejection model, this can be done with considerably fewer animals, less manual work, and lower costs.

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