XENOTRANSPLANTATION 1995; 2:46–51
Printed in the United States of America - all rights reserved.

XENOTRANSPLANTATION
ISSN 0908-665X

Functional cooperation of xenoproteins after hamster-to-rat liver transplantation: With particular reference to hamster C3 and secretory component for rat IgA

Celli S, Valdivia LA, Kelly RH, Demetris AJ, Fung JJ, Rao AS, Pan F, Tsugita M, Starzl TE. Functional cooperation of xenoproteins after hamster-to-rat liver transplantation, with particular reference to hamster C3 and secretory component for rat IgA. Xenotransplantation 1995; 2:46–51.

Abstract: Long-term survival after hamster-to-rat liver xenotransplantation has provided the opportunity to study the posttransplantation source of major serum proteins and the functional consequences of several different receptor-ligand interactions, where one or the other is a xenogeneic protein. We report here that serum albumin, α-1-antitrypsin, complement component 3, and other acute phase reactants switch from recipient to donor origin during the first week after transplantation while serum immunoglobulins remain largely that of recipient. Despite the disparate source of complement (hamster) and immunoglobulins (rat), these two proteins were able to cooperate effectively to produce lysis of sheep red blood cells. Moreover, rat IgA was successfully processed by hamster hepatocytes and biliary epithelial cells, being present in the bile of successful liver xenograft recipients within one day after transplantation. The ability of these liver xenograft recipients to survive long-term in conventional and viral-free animal facilities without grossly obvious morbidity or unusual susceptibility to stress, suggests that xenogeneic proteins are able to successfully interact with several different physiologic systems in the hamster-to-rat combination.

S. Celli,[1] L.A. Valdivia,[1] R.H. Kelly,[2] A.J. Demetris,[2] J.J. Fung,[1] A.S. Rao,[1,2] F. Pan,[1] M. Tsugita,[1] and T.E. Starzl[1]

Pittsburgh Transplantation Institute and the Department of [1]Surgery and [2]Pathology University of Pittsburgh Medical Center, Pittsburgh, PA, U.S.A.

Key words: xenoproteins – liver transplantation – complement – albumin – rat – hamster

Address reprint requests to Thomas E. Starzl, MD, PhD, Department of Surgery, University of Pittsburgh, 3601 Fifth Avenue, 5C Falk Clinic, Pittsburgh, PA 15213, USA

Accepted for publication 19 August 1994

Abbreviations: EDTA—ethylenediamine tetracetate; RID—radial immunodiffusion plate; PBS^{G}—PBS + 1% gelatin; PBS^{G2+}—PBS^{G} + 0.15 mM Ca^{++} + 0.5 mM Mg^{++}; PBS—phosphate buffered saline; SRBC—sheep red blood cell.

Introduction

Features such as resistance to humoral or antibody-mediated rejection and a "tolerogenic" influence on the recipient's immune system make the liver an attractive organ to use in breaking the immunologic barrier of animal-to-human xenotransplantation [1,2]. However, given the liver's key role in maintaining the biochemical homeostasis within the body, hepatic xenografts may also provide some of the most formidable challenges. Complex metabolic and immunologic functions require the liver, or its genetically encoded products [3], to integrate with a number of physiological systems in the body, where nonfunctionality, incompatibility, or even immunogenicity of xenogeneic proteins may pose major problems. For example, serum proteins are synthesized largely by hepatocytes and then released into the circulation where they interact with many other cells throughout the body. Other proteins are produced by and remain in the hepatocytes, where they serve as receptors for hormones, growth factors, and immunoglobulins, the ligands for which are recipient proteins.

The achievement of long-term survival in the hamster-to-rat hepatic xenograft model using combination immunosuppressive therapy has made feasible the study of the physiological effects of xenogeneic serum albumin and coagulation factors [4]. Both of these important physiologic proteins switch to donor origin within a few days after transplantation and remain so for the life of the graft/recipient [4]. The present study was undertaken to determine a) if as expected the liver was the ma-

jor source of complement component 3 (C3) after hepatic xenotransplantation; b) if the hamster C3 produced by the liver could interact with rat immunoglobulin G to produce cell lysis; and c) if hamster secretory component produced by hepatocytes and biliary epithelium, could successfully transport rat IgA from the serum into the bile.

Materials and methods

Animals, operative procedures, and immunosuppression

Male Syrian Golden Hamsters (100–120 g) and male LEWIS rats (250–270 g) were purchased from Charles River Laboratories (Wilmington, MA) and used as liver donors and recipients, respectively. Orthotopic liver transplantation was according to the cuff technique [5] with modifications which included donor cholecystectomy [6]. After liver transplantation, the rats were maintained under standard clean conditions, having free access to rodent chow and water and given 1 mg/kg/day of intramuscular FK-506 (Fujisawa Pharmaceuticals, Japan) for one month, and 8 mg/kg/day of intraperitoneal Cyclophosphamide (Sigma Chemical Co., St. Louis, MO) for 6 days. All therapy was then stopped. This treatment results in 80% recipient survival for more than 100 days.

To ensure collection of plasma without complement breakdown for subsequent testing (see below), the abdominal portion of the aorta and inferior vena cava were mobilized, clamped at the level of the renal vessels and severed at their bifurcation. After inserting both free ends of the vessels into a test tube containing EDTA (Vacutainer 6384—Becton Dickinson, Rutherford, NJ) the clamp was removed and the sample collected.

Double and radial immunodiffusion assays

Double immunodiffusion was done using I.D. discs with indicators (Cappel-Organon Teknika, West Chester, PA). Briefly, 17.5 µl of serum from normal rat, hamster, and liver xenograft recipients obtained at 5, 36, 72, and 137 days after transplantation were tested against goat anti-rat IgG antibodies (Sigma). After 48 h of diffusion, the lines of precipitation between the anti-IgG sera in the central well and the test sera listed above were evaluated. Similar tests were carried out using mouse anti-hamster IgG and IgM monoclonal antibodies (Sigma).

The Mancini technique was used for radial immunodiffusion [7]. Twenty microliters of bile from a normal rat and hamster or from a liver xenograft recipient 1, 3, and 5 days after transplantation were placed in wells in an agarose gel radial immunodiffusion plate (RID) containing monospecific goat anti-rat IgA antibody (The Binding Site, Inc., San Diego, CA). The ring of precipitation formed after diffusion for 24 h at room temperature in different wells was compared to the ring of precipitation produced by a known amount of rat IgA.

Electrophoresis and immunofixation

Plasma proteins were electrophoresed for 30 min at 250 volts in 1% agarose gel (SEAKEM-ME, FMC, Rockland, ME) containing 75 mm Veronal buffer, and subsequently stained with a mixture of Amido black and Coomassie brilliant blue (Sigma) (80:20 vol/vol). In order to detect the location of the C3 migration band, electrophoresis was performed in two gels with the same three plasma samples (from normal rat, hamster, and liver xenograft recipient) running simultaneously under similar conditions. One gel was then immediately stained for standard detection of the migration bands, while the other gel was incubated with goat anti-rat C3 monoclonal antibody (mAb) (Cappel) for 1 hr at room temperature in a humidified chamber and then stained with Amido black/Coomassie blue.

Hemolytic assay

The ability of complement contained in sheep red blood cell (SRBC) absorbed serum obtained from 1) a liver xenograft recipient (hamster-to-rat) 36 days after transplantation; 2) a normal rat or 3) a normal hamster, to lyse SRBC in the presence of a known amount of rat antibodies was tested using a modified Mayer's method [8]. The test sera were prepared by absorption with fixed SRBC for 15 min at 4°C. The rat anti-serum was prepared by injection (×2) of SRBC into the peritoneal cavity of LEWIS rats at 2 week intervals. One week after the last injection, the animals were sacrificed and the anti-serum obtained was then diluted to 1:16 with PBS containing 0.01 M EDTA. The antibody solution was added with an equal volume of SRBC (5×10^6/ml) resuspended in PBS and 1% gelatin (PBS^G), incubated at 37°C for 30 min and washed (×3) with PBS^G containing 0.15 mM Ca^{++} and 0.5 mM Mg^{++} (PBS^{G2+}). One hundred microliters of the sensitized SRBC were then placed into each well of a 96-well U-bottomed microliter plate (Becton Dickinson, Lincoln Park, NJ) and as a source of complement, different volumes of absorbed sera (1:2 dilution), were added. Additional PBS^{G2+} was added to each well to bring the volume up to 200 µl/well, the plates were incubated for 9 h at 37°C, and then centrifuged for 2 min at 300*g*. The plates were read by measuring the absorbance of the supernatant at 405 nm using a VMAX Spectrophotometer (Molecular Devices, Menlo Park, CA). Wells that contained complement

plus antibodies but no SRBC's were included as background controls. Additional controls included wells containing only SRBC or SRBC and complement without antibodies to exclude the contribution, if any, of spontaneous hemolysis of the SRBC as well as activation of complement through the alternative pathway.

Immunohistochemical studies

Liver tissues from a normal rat, hamster, and a rat liver xenograft recipient 30 days after transplantation were snap-frozen in OCT compound (Tissue-tek, Ames Division, Miles Laboratories, Inc., Elkhart, IN) and sectioned at 6 microns. Immunoglobulins within the liver tissue were localized with a standard avidin-biotin-complex technique using a monoclonal mouse anti-rat IgA (2360-MCA 191-Serotec-Accurate, Westbury, NY) as the primary immunoreactant.

Results

Origin of plasma proteins, including immunoglobulins and complement component 3

The electrophoresis profile of plasma proteins obtained from normal rat, hamster, and a liver xenograft recipient, 17 days after transplantation, are shown in Figure 1. Migration of albumin and α-1-antitrypsin from the liver xenograft recipient were almost identical to that from a normal hamster, indicating that major plasma proteins in the recipient have been replaced by those from the donor. Furthermore, there were two bands of identical migration patterns, which were localized to the β2-globulin region of the xenograft recipient and normal hamster (Fig. 1). Immunofixation studies using goat anti-rat C3 mAb (which crossreacts with both hamster and rat), highlighted only the upper band in the β2-globulin region both in the plasma from normal hamster and liver xenograft (hamster-to-rat) recipient, suggesting that the plasma C3 in the recipient is of donor origin. In contrast, this doublet was less distinct and C3 migration was somewhat retarded in the plasma of normal rat as compared to the same in normal hamster and liver xenograft recipient. These studies suggest that the plasma C3 detectable by electrophoresis and immunofixation in a liver xenograft recipient posttransplantation was of donor (hamster) origin.

In contrast to other major proteins, the bulk of the serum immunoglobulins in liver xenograft recipients remained that of the host (rat) (Fig. 2). This was confirmed by similar double immunodiffusion analysis using mouse anti-hamster IgG

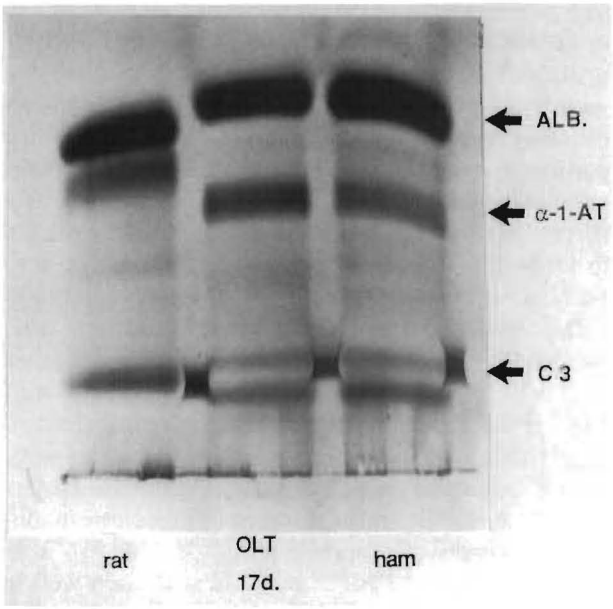

Fig. 1. Gel electrophoresis of plasma proteins shows that the profile of a liver xenograft recipient is more similar to the donor (hamster) than that to the recipient (rat). Immunofixation with goat anti-rat C3 mAb shows that the upper band in the β2-globulin region is C3 and is derived from the donor (hamster) and not the recipient (rat).

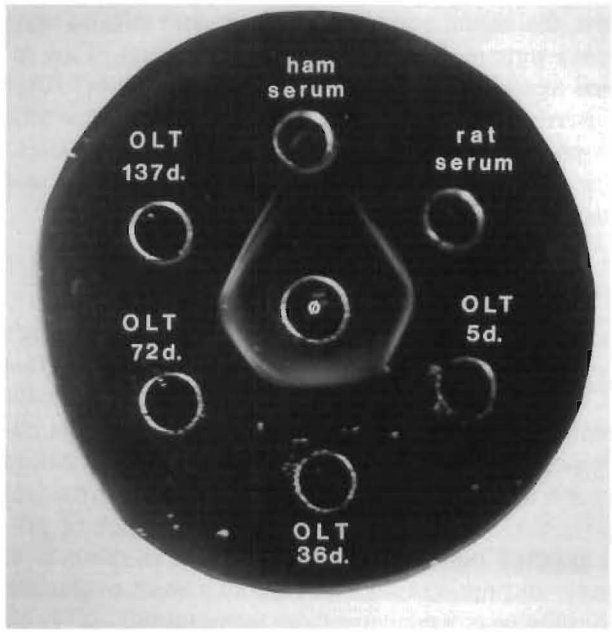

Fig. 2. Double immunodiffusion shows that the circulating immunoglobulins remain of recipient origin after hepatic xenotransplantation. The central well contains goat anti-rat IgG, which forms a line or precipitation with the serum from liver xenograft recipients at 5, 36, 72, and 137 days after transplantation. Note the absence of a precipitation line with the normal hamster serum.

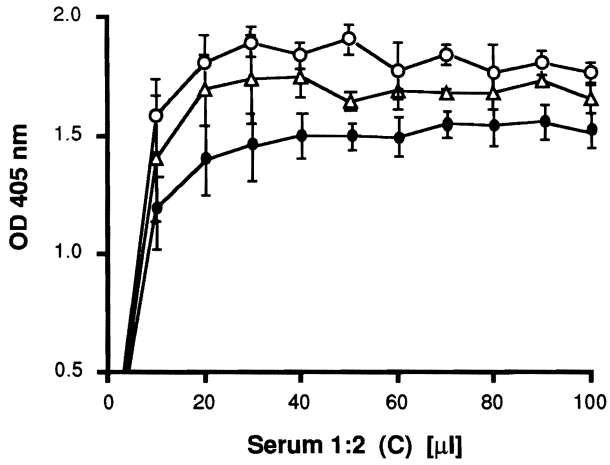

Fig. 3. Hemolytic assay. As a source of complement activity, serum from liver xenograft recipients 37 days after transplantation (△) was as efficient as normal rat serum (○) but more effective than normal hamster serum (●) in producing lysis of sensitized SRBC (see methods). There was no significant statistical difference in the activity of complement of normal and transplanted rats.

and IgM antibodies, which showed lack of precipitation line with the serum of liver xenograft recipients at several times after transplantation (data not shown).

Cooperation of rat immunoglobulins and hamster complement in a hemolytic assay

Since the circulating immunoglobulins in liver xenograft recipients are largely derived from the recipient (rat), whereas most of the complement components are of donor (hamster) origin, we tested whether serum, as a source of complement, from a liver xenograft recipient could lyse SRBC sensitized with rat immunoglobulins. The results shown in Figure 3 were compared to SRBC lysis produced by normal rat and hamster serum in the same assay. Serum from a liver xenograft recipient 36 days after transplantation mediated lysis of SRBC sensitized with rat immunoglobulin as efficiently as that produced by serum from a normal rat and more effectively than normal hamster serum.

Processing and biliary secretion of rat IgA by a successful hamster liver xenograft

The liver normally processes serum IgA by selective absorption, conjugation with the secretory component, transcellular processing, and finally, secretion into the bile. We determined the origin and if present, the concentration of rat IgA in the bile from liver xenografts 1, 3, and 5 days after transplantation. The results in Figure 4 show that within 1 day after transplantation, a small amount of rat IgA can be detected in the bile of a hamster-to-rat liver xenograft. The concentration of rat IgA reaches near normal levels by day 5 and can be immunohistochemically detected in the expected areas of deposition (canaliculi and bile ducts) by day 30 after transplantation (Fig. 5).

Discussion

These results confirm and extend our previous observations that liver xenografts retain the metabolic specificity of the donor after placement into a new host [4] in the same way as was originally shown for allografts almost 30 years ago [9,10]. This fact

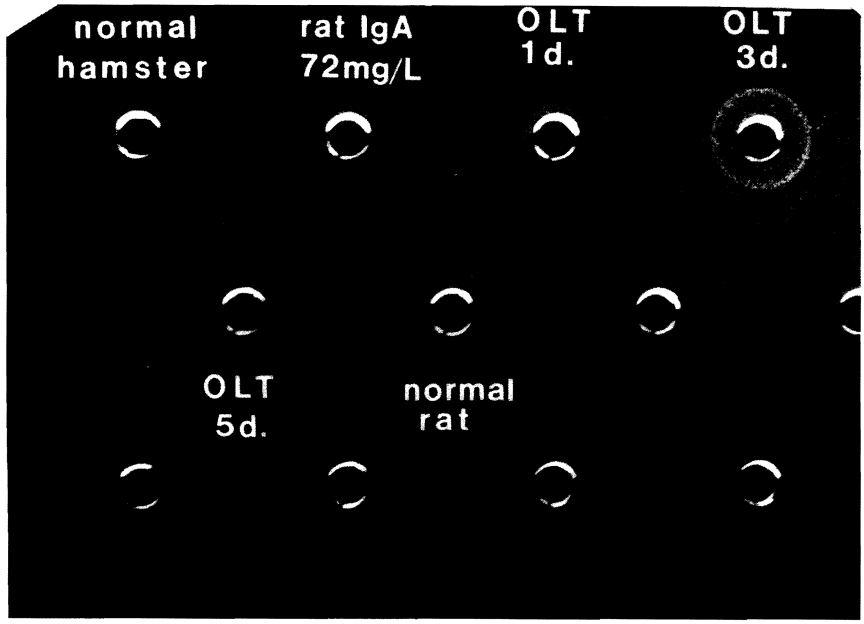

Fig 4. Radial immunodiffusion shows the presence of rat IgA in the bile of liver xenograft recipients. In this test, the agar plate contained goat anti-rat IgA. The wells (from left → right) were filled with normal hamster bile, 72 mg/L of rat IgA, (positive control), bile from hepatic liver xenograft recipients day 1, 3 and 5 after transplantation and normal rat bile.

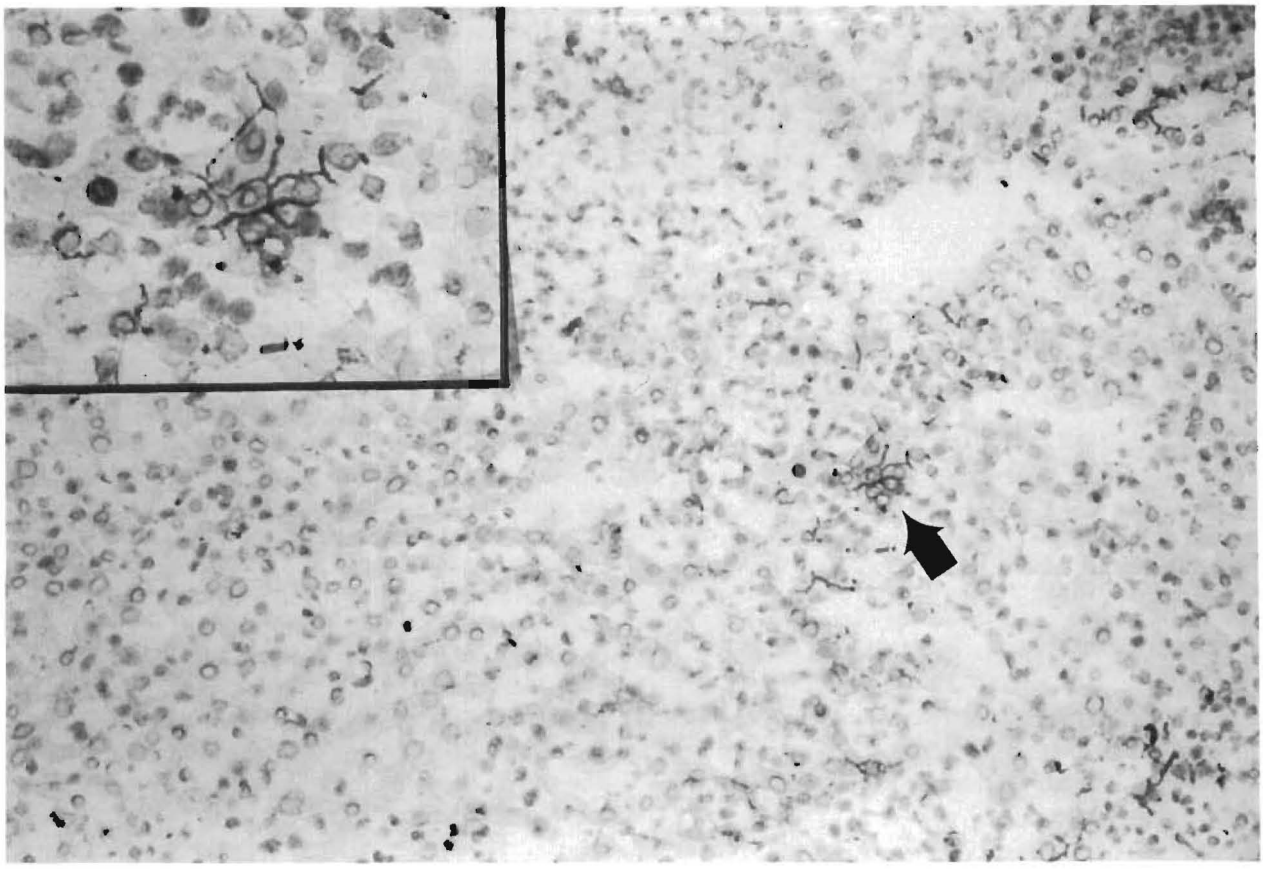

Fig. 5. Rat IgA (arrows) could be seen in the normal areas of deposition (biliary canaliculi) in this normally functioning hamster-to-rat liver xenograft, 30 days after transplantation. The inset (original magnification 400×) shows the staining detail of rat IgA (large arrow). *No* staining was detected in a control animal hamster liver (not shown).

has been used as a rationale for the treatment of many hepatic-based metabolic diseases with a liver transplant, which conversely, can also transfer a metabolic disorder from the donor to the recipient. Because receptor-ligand pairs derived from two separate species could result in molecular deficits or incompatibilities, the potential metabolic risks of hepatic xenotransplantation must be carefully studied. These observations prompted us to undertake more detailed examination of ostensibly healthy rats bearing hamster livers, beyond that previously reported [4].

The structures of albumin and α-1-antitrypsin secreted in the xenogeneic liver recipient (rat) were genetically encoded in the donor (hamster) hepatocytes genome. Their expression, however, was influenced by recipient's extrahepatic factors such as thyroid and growth hormones, glucocorticoids and insulin [11]. Similarly, donor C3 and other xenogeneic acute phase reactants also appeared to respond to recipient cytokines, such as IL-6 in that there was no obvious evidence of a breakdown of these interactions.

Conversely, the bulk of the serum immunoglobulins in these animals remained of rat origin. However, despite a complete switch-over of important serum oncotic proteins and acute phase reactants to that of a donor with retention of recipient's immunoglobulins, no edema, wasting syndrome, or unusual susceptibility to stress or infection was observed during postoperative follow-up of the liver xenograft recipients [12]. These gross observations suggested that the hamster xenoproteins were properly functioning in the rat recipients maintained in either open or viral-free animal facilities.

A more specific example of functional cooperation between hamster and rat xenoproteins was provided herein by the in vitro lysis of sensitized SRBCs using rat immunoglobulin and SRBC absorbed liver xenograft recipient's serum as a source of complement. Alper et al., [13] have previously demonstrated that the liver is the major site of C3 biosynthesis with an electrophoretic shift of the same from the recipient FS type to the donor SS type after hepatic allotransplantation in man [14]. However, there is contrasting evidence that suggests

the existence of extrahepatic sites of complement synthesis [15]. Although, by definition, a mixed milieu of complement is present, the sensitivity of electrophoresis and immunofixation techniques used in our studies was probably not high enough to detect any C3 in the circulation except that of hamster (donor) origin. The trace quantities of rat complement from extrahepatic sources could therefore account for the slightly more efficient lysis of SRBC by the hamsterized recipient serum than by normal hamster serum. It should also be noted that nonhepatic complement may be more important in local inflammatory responses, such as those involved in autoimmune processes [16].

The presence of rat IgA in the bile after hepatic xenotransplantation mirrored another example of successful collaboration between xenogeneic proteins, in this case involving a recipient immunoglobulin whose uptake, processing, and conjugation is dependent on a xenogeneic secretory component [17]. Although no evidence of metabolic incompatibilities between xenogeneic proteins has been uncovered to date, there may be subtle changes, particularly with disparate species that could ruin the long-term results of human liver xenotransplantation. The extent of this problem, if it at all exists, and its implications can be more comprehensively analyzed by evolving better strategies that will mitigate the consequences of complement activation syndrome, one that has frustrated the practical application of xenotransplantation for decades [18].

Acknowledgments

This work was supported by NIH Grant DK-29961.

References

1. Starzl TE. Orthotopic heterotransplantation. In: Starzl TE, ed. Experience in Liver Transplantation. Philadelphia: Saunders. 1969, p 408.
2. White DJG. Transplantation of organs between species. Intern Arch Aller Immun 1992; 98:1.
3. Crick FHC, Barnett L, Brenner S, Watts Tobin RJ. General nature of the genetic code for proteins. Nature 1961; 192:1227.
4. Valdivia LA, Lewis J, Celli S, et al. Hamster coagulation protein activities in hamster liver transplanted rats. Transplantation 1993; 56:489.
5. Kamada N, Calne RY. Orthotopic liver transplantation in the rat: Technique using cuff for portal vein anastomosis and biliary drainage. Transplantation 1979; 28:47.
6. Valdivia LA, Monden M, Gotoh M, et al. Prolonged survival of liver xenografts from hamster to rat by splenectomy and cyclosporin administration. Transplantation 1987; 44:759.
7. Mancini G, Carbonara A, Heremans J. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 1965; 2:235.
8. Mayer M. Hemolytic assay. In: Kabat MM, ed. Experimental Immunochemistry. Springfield: CC Thomas, 1965, p 133.
9. Kashiwagi N, Groth CG, Starzl TE. Changes in serum haptoglobin and group specific component after orthotopic liver homotransplantation in humans. Proc Soc Exp Biol Med 1968; 128:247.
10. Starzl TE, Marchioro TL, Rowlands DT, et al. Immunosuppression after experimental and clinical homotransplantation of the liver. Ann Surg 1964; 160:411.
11. Shafritz DA, Yap SH, Stair RH. Regulation of albumin synthesis in the rat liver. Mol Biol Rep 1979; 5:71.
12. Valdivia LA, Fung JJ, Demetris AJ, Starzl TE. Differential survival of hamster-to-rat liver and cardiac xenografts under FK 506 immunosuppression. Transplant Proc 1991; 23:3269.
13. Alper CA, Johonson AM, Birtch AG, Moore FD. Human C3: Evidence for the liver as primary site of synthesis. Science 1969; 163:286.
14. Alper CA, Raum D, Awdeh ZL, et al., Studies of hepatic synthesis in vivo of plasma proteins including orosomucoid, transferrin, α-1-antitrypsin, C8 and factor B. Clin Immunol Immunopathol 1980; 16:84.
15. Colten H, Strunk R. Synthesis of complement components in liver and at extra hepatic sites. In: Whaley K, Loos M, Weiler JM, eds. Complement in Health and Disease (sec. edit.). London: Kluwer Academic Publishers, 1993, p 127.
16. Perlmutter D, Colten H. Complement: Molecular genetics. In: Gallin J, Goldestein I, Snyderman R, eds. Inflammation: Basic principles and clinical correlates. New York: Raven Press, 1988, p 85.
17. Abbas K, Lichtman AH, Pober JS. Antibodies and antigens. In: Abbas AK, Lichtman AH, Pober JS, eds. Cellular and molecular immunology. London: Saunders, 1991, p 37.
18. Starzl TE, Valdivia LA, Murase N, Demetris AJ, Fontes P, Rao AS, Manez R, Marino IR, Todo S, Thomson AW, Fung JJ. The biologic basis of and strategies for clinical xenotransplantation. Immunol Rev 1994; 141:213–244.