American Journal of Pathology, Vol. 143, No. 1, July 1993
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Humoral and Cellular Immunopathology of Hepatic and Cardiac Hamster-into-Rat Xenograft Rejection

Marked Stimulation of $IgM^{++bright}/IgD^{+dull}$ Splenic B Cells

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Normal Lewis rat serum contains antibodies (IgM > IgG) that bind to hamster leukocytes and endothelial cells. Transplantation of either the heart or liver from hamster rat results in release of hamster hematolymphoid cells from the graft, which lodge in the recipient spleen (cell migration), where recipient T- and B-cell populations initiate DNA synthesis within one day. There is marked stimulation of splenic $IgM^{++(bright)}/IgD^{+(dull)}$ B cells in the marginal zone and red pulp, which account for 48% of the total splenic blast cell population by 4 days after liver transplantation. $CD4^+$ predominant T-cell proliferation in the splenic periarterial lymphatic sheath and paracortex of peripheral lymph nodes occurs almost simultaneously. The effector phase of rejection in cardiac recipients is dominated by complement-fixing IgM antibodies, which increase daily and result in graft destruction in 3 to 4 days, even in animals treated with FK506. In liver recipients, combined antibody and cellular rejection, associated with graft infiltration by $OX8^+$ natural killer, and fewer $W3/25^+$ (CD4) lymphocytes, are responsible for graft failure in untreated recipients at 6 to 7 days. FK506 inhibits the T-cell response in liver recipients and significantly prolongs graft survival, but does not prevent the rise or deposition of IgM antibodies in the graft. However, a single injection of cyclophosphamide 10 days before transplantation effectively depletes the splenic $IgM^{++(bright)}/IgD^{+(dull)}$ cells and in combination with FK506, results in 100% survival of both cardiac and hepatic xenografts for more than 60 days. Although extrapolation of morphological findings to functional significance is fraught with potential problems, we propose the following mechanisms of xenograft rejection. The reaction initially appears to involve primitive host defense mechanisms, including an IgM-producing subpopulation of splenic B cells and natural killer cells. Based on the reaction and distribution of $OX8^+$ and $W3/25^+$ cells, antibody-dependent cell cytotoxicity and delayed-type hypersensitivity responses seem worthy of further investigation as possible effector mechanisms. Effective control of xenograft rejection is likely to require a dual pharmaceutical approach, one to contain T-cell immunity and another to blunt the primitive B-cell response. (Am J Pathol 1993, 143:85–98)

Restrictions imposed by the limited number of suitable human donor organs has recently curtailed the growth of transplantation as a readily available form of therapy. Attempts to use nonhuman primates as donors for human recipients in the 1960's were abandoned because of formidable immunological barriers, lack of effective immunosuppressive therapy, and the emergence of hemodialysis as an alternative treatment[1–2] for endstage renal disease.

With the recent introduction of FK506, transplantation of allografts once thought too difficult to at-

Accepted for publication January 18, 1993.

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tempt, such as the small intestine, has become possible.[3] Although in xenotransplantation the major immunological barrier is thought to be humoral,[4–7] recent success with FK506 as monotherapy in the hamster-to-rat hepatic xenograft model is quite promising.[8] In addition, the combination of FK506 or cyclosporine with "anti-proliferative" agents has resulted in excellent cardiac and hepatic xenograft survival in the hamster-to-rat model.[9,10]

Part of the success of FK506 in hepatic xenotransplantation can be attributed to the liver's natural resistance to antibody-mediated rejection.[11] On the other hand, survival of a graft with an arterial-only afferent vasculature, such as the heart, is not prolonged by FK506, except when combined with splenectomy[12] or drugs that inhibit the antibody response.[9,10]

The importance of antibodies as primary mediators of graft damage, and of the spleen[12–15] in the development of xenograft effector response notwithstanding, there are conflicting results regarding the immune mechanisms of xenograft rejection.[16] This is particularly true with studies based on *in vitro* analyses,[16] and *in vivo* studies are in need of further investigation.[17]

The goals of this study are two-fold: 1) to delineate the morphological correlates of the initiation and effector phases of xenograft rejection *in vivo*, with and without therapy; and 2) to define more accurately the role of the spleen. Our experiments were primarily with the liver, because this organ's natural resistance to antibody-mediated damage and natural tolerizing influences[18,19] make it an attractive target for clinical trials of xenotransplantation. However, the heterotopic heart transplant model also was studied, because it exemplified the susceptible end of the antibody-sensitive spectrum.

Materials and Methods

Operative Procedures

Male LVG hamsters (100–150 g) and male Lewis (LEW) rats (240–280 g) served as donors and recipients, respectively. Orthotopic liver transplantation was performed according to the cuff technique of Kamada and Calne.[20] Donor cholecystectomy was carried out at the time of grafting and the preparation lacked anastomosis of the hepatic artery. Heterotopic heart transplantation was performed by a microsurgical technique as described by Ono and Lindsey.[21]

Experimental Design

In the first set of experiments, a comparison was made of the histo- and immunopathology of rejection between FK506-treated and -untreated liver and cardiac xenograft recipients in the LVG hamster to LEW rat transplantation model. Untreated liver xenograft recipients were sacrificed on days 1 through 5 (n = 3 on each day), and untreated cardiac xenograft recipients were sacrificed on days 2, 3, and 4 (n = 1 on each day). The treated liver xenograft recipients were given FK506 (1.0 mg/kg/day), started on the day of transplantation and continued until the rats were sacrificed for study on days 3, 4, and 5 (n = 3 on each day).

In a second series of experiments, the effect of therapy on graft survival was studied. A single 500 mg/m^2 (~80 mg/kg) dose of cyclophosphamide (CYP) previously shown to deplete marginal zone B cells in the rat,[22] was given 10 days before the transplantation of heart or liver xenograft. At the time of transplantation, heart graft recipients received 2 mg/kg/day of FK506 for 15 days, 1 mg/kg/day for days 15 to 30, and 0.5 mg/kg every other day until day 100. Liver graft recipients received 1 mg/kg/day of FK506 for 30 days and 0.5 mg/kg/day every other day up to day 100. Animals treated only with that same dose of FK506 and untreated graft recipients were included as controls. The causes of graft failure and/or death were confirmed histologically.

Tissue Triage and Immunoperoxidase Studies

A complete autopsy was done, and tissue from all organs was frozen in optimum cold temperature compound (Tissue-Tek, Ames Division, Miles Laboratories, Inc., Elkhart, IN) for immunoperoxidase studies and formalin-fixed for routine histological examination. For immunostaining of the graft and recipient spleen, the following panel of monoclonal antibodies (MAbs) were used: OX8 (1:150; non-helper subset, Sera-lab), W3/25 (1:100; T helper/inducer and subsets of other accessory cells, Sera-lab), OX33 (1:100; B-cell marker, Serotec, Indianapolis, IN), MCA 190 (1:50; mouse anti-rat IgD heavy chain, Serotec), IgM^{bio} (1:75; anti-rat IgM-biotinylated, Accurate) and NKR-P1 (1:100; natural killer cell specific marker, courtesy of Dr. William Chambers, Pittsburgh Cancer Institute, Pittsburgh, PA).

A standard avidin-biotin-peroxidase complex method was used as previously described.[23] A

Shandon Cadenza automated immunostainer was used for the actual staining (Shandon Scientific Limited, Cheshire, England).

Cell Trafficking Studies

Donor hamster cells were identified with polyclonal fluorescein isocyanate (FITC)-conjugated rabbit anti-hamster lymphocyte antibody (Inter-Cell Technologies, Inc., Hopewell, NJ). The neat antibody was diluted 1:10 and then absorbed with LEW (recipient) white blood cells from the spleen and lymph nodes to remove crossreactive antibodies. The LEW cells were prepared on a Ficoll gradient and washed (three times) with RPMI 1640 culture medium (GIBCO, Grand Island, NY) and pelleted. 5.0×10^6 cells were then resuspended in 500 µl of the diluted antibody and incubated for 15 minutes. The cells were then pelleted (1500 rpm for 10 minutes), the supernatant collected, and the entire procedure repeated twice.

Before staining, the nonspecific protein-binding activity in the tissue sections was inhibited by incubation with protein-blocking agent (Lipshaw Co., Detroit, MI) for 20 minutes. The absorbed antibody was then applied for 40 minutes, followed by two (5 minute) washes in phosphate-buffered saline (PBS) and mounting in anti-fade (Oncor, Gaithersburg, MD). Specificity of the absorbed antibody was confirmed by using normal LEW rat and LVG hamster spleens and livers as controls.

Identification of LEW rat cells was achieved by using the MAb L-21-6, which reacts with the invariant chain of the LEW rat, but not with that of the hamster.[24] Normal LEW rat and LVG hamster spleens and livers were used as controls.

Immunofluorescence Studies

An indirect immunofluorescent assay was used to evaluate the tissue-binding activity of preformed anti-hamster antibodies in the LEW rat. Cryostat sections of normal LVG hamster heart and liver were incubated with protein blocking agent for 20 minutes followed by incubation with undiluted normal LEW rat serum for 30 minutes. The sections were washed twice in PBS, incubated with goat anti-rat IgG or IgM, washed twice in PBS and mounted in anti-fade (Oncor). Normal hamster sera were substituted for the rat sera as a negative control.

A direct immunofluorescent assay was used to detect IgM, IgG, and C3 deposition in the xenografts and IgM^+ and IgG^+ cells in the recipient spleen. Cryostat sections of the grafts and recipient spleen were incubated with FITC-conjugated goat anti-rat IgG (heavy and light) (1:25, Jackson ImmunoResearch Labs., Inc., West Grove, PA), IgM (1:25, Jackson), or C3 (1:25, Cappel Research Products, Durham, NC), for 30 minutes, washed in PBS twice, and mounted in anti-fade (Oncor). The above antibodies were absorbed with 10% heat-inactivated normal hamster serum for 45 minutes before use. Normal rat spleen or lymph nodes served as the positive control. All of the above stains were reviewed on a Nikon fluorescent microscope.

Complement-Dependent Cytotoxicity

Complement-fixing lymphocytotoxic antibodies were measured in five normal LEW rats. Briefly, 30 minutes after incubation of the rat serum and complement (37 C, 95% O_2, 5% CO_2) with hamster lymph node cells (5×10^6/ml), the percentage of cells stained with trypan blue was calculated. The lymphocytotoxic antibody titer was defined as the highest serum dilution with more than 25% cell lysis. Baby rabbit serum (Accurate) diluted 1:10 served as the source of complement. Normal hamster serum served as the negative control. Hyperimmune serum was used as the positive control.

Proliferative Activity

To determine the sites of recipient lymphoid activation, as measured by proliferation, a mitotic index analysis was performed on each of the animals, as previously described.[23] Briefly, metaphase mitotic figures were counted in a standardized area of an H&É-stained section of the recipient spleen. In addition, tissues from one animal at each time point were stained with anti-proliferating cell nuclear antigen (PCNA) (Signet Laboratories, Inc., Dedham, MA) as previously described.[25]

Flow Cytometric Analysis

The spleens from FK506-treated (n = 3) and -untreated (n = 3) hepatic xenograft recipients sacrificed on days 4 and 5 were used to prepare a single-cell suspension of splenocytes for phenotypic analysis in cold RPMI 1640. Viability, determined by trypan blue exclusion, was always >90%. Single-color staining was carried out using the same unconjugated primary antibodies as used for

immunostaining above (OX8, 1:150; W3/25, 1:100; OX33, 1:100; NKR-P1, 1:500), with a rat anti-mouse IgG FITC (Accurate) secondary antibody. Two-color staining was done using goat anti-rat IgM $F(ab)_2'$ phycoerythrin 1:100 (Jackson) and IgD-FITC 1:50 (Serotec), incubated separately. The cell suspension was washed twice with Hanks' balanced salt solution (HBSS) (GIBCO) containing 1.0% sodium azide (St. Louis, MO) and 1.0% bovine serum albumin (GIBCO) before fixation in 1.0% paraformaldehyde. Samples were then stored at 4 C until analysis. Mouse IgG_1 FITC (Dako Corp., Carpinteria, CA), (Dako), and goat anti-rat $F(ab)_2'$ phycoerythrin mouse IgG_1 (Jackson) were used as negative controls.

To determine binding of rat IgG or IgM to hamster lymphoid cells, diluted (1:100 in HBSS with 1% bovine serum albumin and 0.1% sodium nitrate) normal LEW rat serum was incubated with hamster lymph node cells for 45 minutes at 4 C. Diluted (1:100) hamster serum or buffer alone served as controls. After incubation, the cells were washed and FITC-conjugated goat anti-rat IgG (Accurate) or IgM antibody (1:75 dilution) was added and the cells were incubated for another 30 minutes at 4 C. Cells were then washed twice and fixed in 1% paraformaldehyde.

Ten thousand cells from each sample were analyzed on a flow cytometer (FACS II, Becton Dickinson, Mountain View, CA). Splenocytes from a normal LEW rat served as a baseline control.

Results

Preformed Antibody Analysis

Lymphocytotoxic crossmatch studies were performed in five normal LEW rats before transplantation. The titer of anti-hamster cytotoxic antibodies present varied between 1:16 ($n = 2$) to 1:32 ($n = 3$). Flow cytometric studies using normal hamster lymph node cells as targets and normal LEW rat serum as the primary antibody revealed binding of IgM $\gg$ IgG. Lastly, serum from normal LEW rats bound to hamster endothelial cells (large vessels and microvasculature) in the heart and liver, with IgM much greater than IgG, using the indirect immunofluorescent assay. We could not, however, ascertain whether the binding in tissue sections was directed at surface or cytoplasmic constituents.

Routine and Immunopathology of Cardiac and Hepatic Xenograft Rejection

Cardiac Grafts

Untreated recipients showed classical features of humoral rejection, which gradually developed over the first 3 days. There was progressive platelet, fibrin, and neutrophil plugging of the microvasculature and margination in larger vessels, which showed endothelial cell hypertrophy and focal denudation. The process culminated on days 3 to 4 with widespread hemorrhagic necrosis (Figure 1). No mononuclear infiltrates were seen in any of the samples. Therefore, immunoperoxidase stains for infiltrating mononuclear cell phenotypes were negative, including L-21–6, used to identify class II MHC^+ recipient mononuclear cells. However, immunofluorescence studies showed daily increasing IgM $\gg$ IgG and C3 deposits in both larger vessels and diffusely throughout the microvasculature (Figure 1). FK506 did not significantly prolong graft survival or change the routine histologic or immunofluorescence findings.

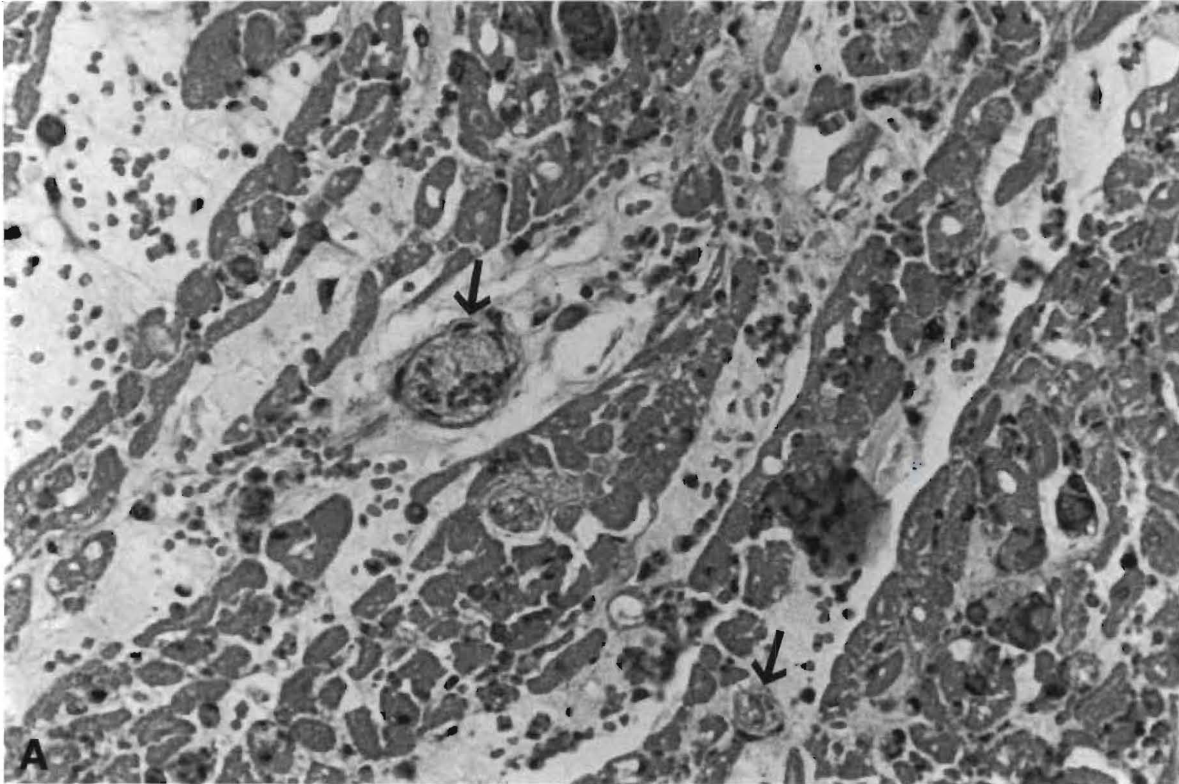
Hepatic Grafts

In the untreated recipients, the findings on day 1 were limited to focal platelet plugs, which were located in portal veins and scattered throughout the sinusoids. IgM $\gg$ IgG and C3 deposits were present on the endothelium of the microvasculature and larger vessels.

Day 2 was marked by the appearance of spotty hepatocyte necrosis with polymorphonuclear cell sludging in the peribiliary vascular plexus, occasional sinusoidal lymphocytes, a minimal mixed portal cellular infiltrate, and partially degranulated mast cells in the hilum. The phenotypic composition of the portal and scattered sinusoidal infiltrate consisted largely of $OX8^+$ cells with fewer $NKR\text{-}P1^+$ and $W3/25^+$ cells. IgM^+ cells were rare, and no $OX33^+$ or IgD^+ cells were present.

The findings on day 3 (Figure 2) resembled an escalated version of day 2 with a mild-to-moderately mixed portal infiltrate consisting of a significant increase of $OX8^+$ cells and a slight increase of $NKR\text{-}P1^+$ cells. Endothelial cell damage worsened and platelet/fibrin thrombi were scattered throughout the vasculature. Occasional IgD^+ cells also appeared in the triads.

By day 4, neutrophils and mast cells were less numerous, but a striking sinusoidal mononuclear infiltrate appeared, consisting predominantly of $OX8^+$



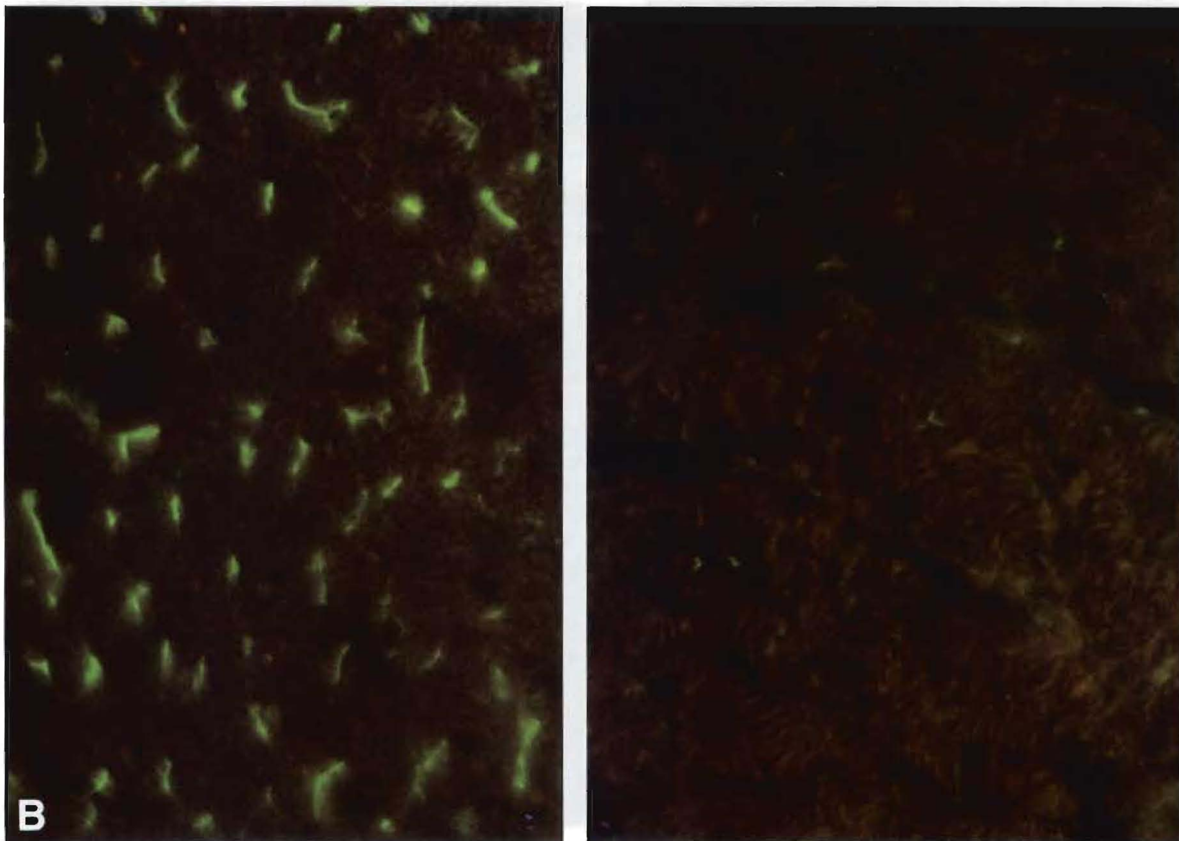
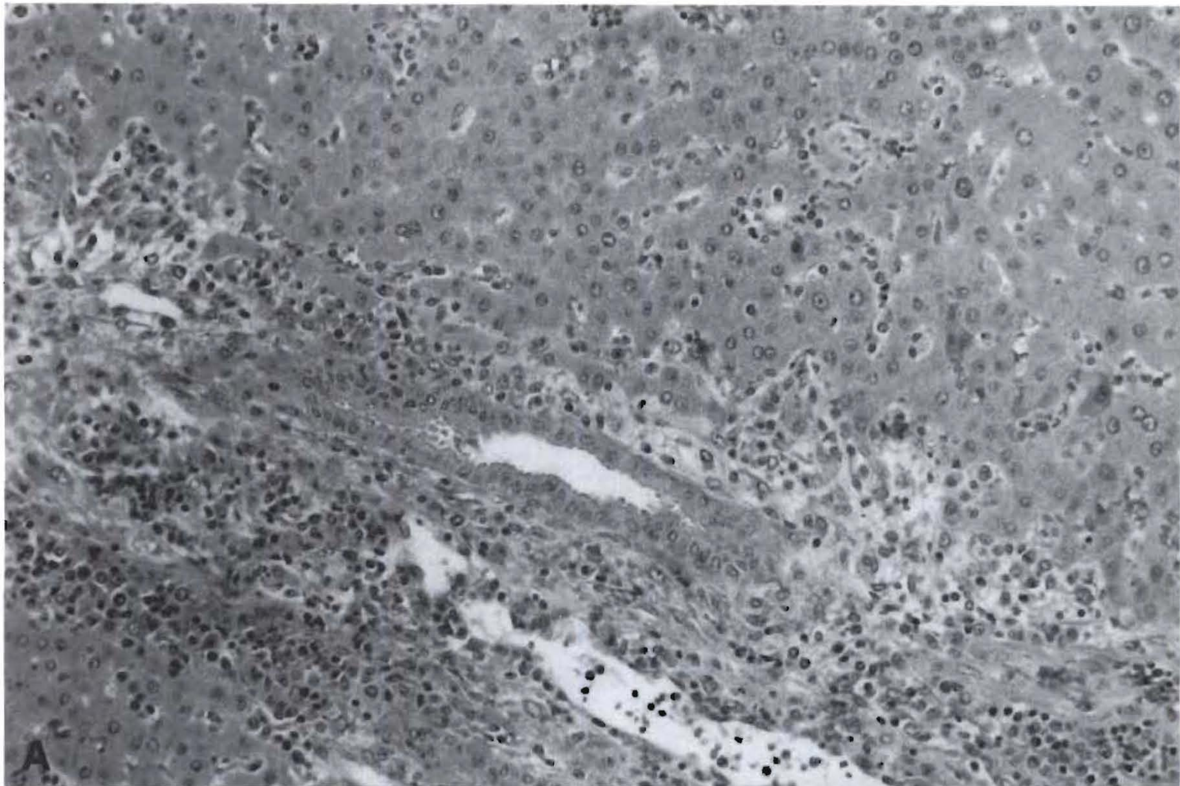


Figure 1. *Cardiac grafts uniformly failed in 3 to 4 days because of pure humoral rejection, with* (A), *platelet-fibrin thrombi in the microvasculature (arrow), widespread hemorrhagic necrosis* (a: *H&E, ×250) and* (B), *marked deposition of anti-hamster IgM antibodies* (left; *anti-rat IgM IF, ×100). IgG deposition was much less* (right *anti-rat IgG; ×100).*



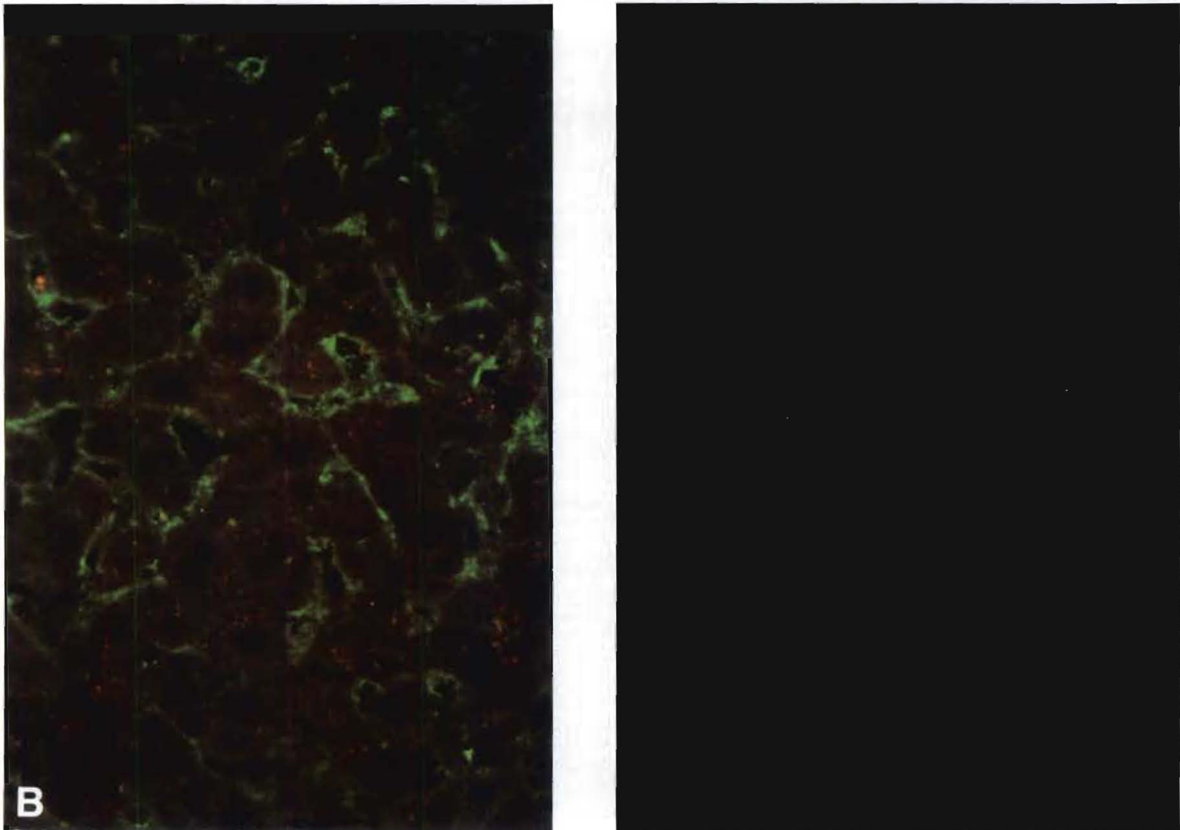


Figure 2. *Liver grafts in untreated recipients developed mixed humoral and cellular rejection* (A; *H&E, ×100*), *with* (B) *marked deposition of IgM* (left; *anti-rat IgM IF, ×100*) *and much less IgG* (right; *anti-rat IgG IF; ×100*). *Note the portal and sinusoidal mononuclear infiltrates.*

cells with fewer NKR-P1$^+$ and W3/25$^+$ cells, and only occasional OX33$^+$ and IgD$^+$ lymphocytes. OX8$^+$, W3/25$^+$, and IgD$^+$ cells were also increased in the triads. Congestion, bile duct proliferation, and small infarcts also appeared. All of these findings worsened on day 5, and were accompanied by large areas of hemorrhagic necrosis, presaging graft failure. By this time the daily increasing deposits of IgM ≫ IgG and C3 on the endothelium, bile ducts, and hepatocytes had become massive. Observations after day 5 were not systematically recorded because of the extensive necrosis.

In the FK506-treated animals sacrificed for histological examination during the first 5 days, the routine and immunopathological changes were qualitatively similar, but much less severe than described above. Scattered platelet/fibrin thrombi and a mild portal and sinusoidal inflammatory infiltrate appeared during the first 3 days, which was phenotypically similar to that seen in the untreated recipients. However, by day 5 the overall inflammatory infiltrate was noticeably less severe in the FK506-treated rats, and qualitatively different. The relative amount of OX33$^+$ and W3/25$^+$ cells was increased, whereas the OX8$^+$ cells were reduced. Despite the noticeable decrease in the cellular infiltrate, there was only a slight decrease in the IgM ≫ IgG and C3 deposits, which were easily detectable in the same distribution as in the untreated recipients.

Splenic Response

The splenic proliferative response was quantified and the phenotypic composition of the splenocytes was recorded. Proliferation evoked by the hepatic xenografts (day 4 peak) was significantly higher than that evoked by the cardiac graphs (day 3 peak) in untreated recipients (Figure 3). However, FK506 therapy alone significantly blunted the proliferation associated with liver transplantation. Nevertheless, increased mitotic activity remained, especially in the red pulp. The localization and the phenotypic profile of the responding splenocyte populations is presented in detail below.

Cardiac Graft Recipients

Blast transformation and mitotic activity of cells in the red pulp (IgM^{++}), marginal zone (MZ) (IgM^{++}/IgD$^-$), and at the MZ/periarterial lymphatic sheath (PALS) interface (W3/25$^+$ and NKR-P1$^+$) was evident within 2 days after transplantation. By days 3 and 4, clusters of mitotically active surface IgM$^+$ (sIgM) plasmablasts and cytoplasmic IgM$^+$ (cIgM)

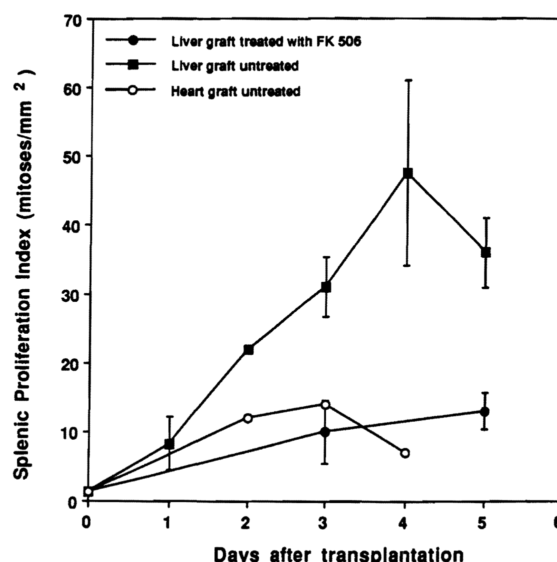


Figure 3. *Splenic proliferative index of untreated cardiac (○) and liver (■) and FK506 treated liver (●) xenograft recipients. The values shown represent the mean of three rats at each time point, except for the cardiac recipients (n = 1), and the error bars are the standard deviation.*

plasma cells with PCNA labeling of >60% appeared in the red pulp. IgG$^+$ cells constituted a distinct minority population.

Hepatic Graft Recipients

Marked blast transformation, mitotic activity, and PCNA labeling were seen in the IgM^{++}/IgD$^+$ cells in the MZ (Figure 4), and in small clusters of NKR-P1$^+$ and W3/25$^+$ cells at the MZ-PALS interface, one day after transplantation.

This pattern was accentuated on day 2 when, in addition, sIgM^{++} blasts with a PCNA labeling percentage >70%, and cIgM$^+$ plasma cells began to fill the red pulp. By days 2 to 3, the MZ had been depleted and blast transformation of the PALS was particularly brisk (Figure 4), resulting on day 4 in almost total replacement of the PALS by large, mitotically active cells. Meanwhile, the red pulp became engorged with the IgM$^+$ cells mentioned above (Figure 5). Surprisingly few IgG$^+$ cells were noted in the spleen.

FK506 muted the blast transformation of the PALS and the proliferative response to hepatic grafts described above. A well defined MZ and PALS were still easily discernable at 5 days. However, the population of mitotically active IgM$^+$ cells with a PCNA labeling of >60% still expanded the red pulp, but to a lesser degree than in the untreated animals.

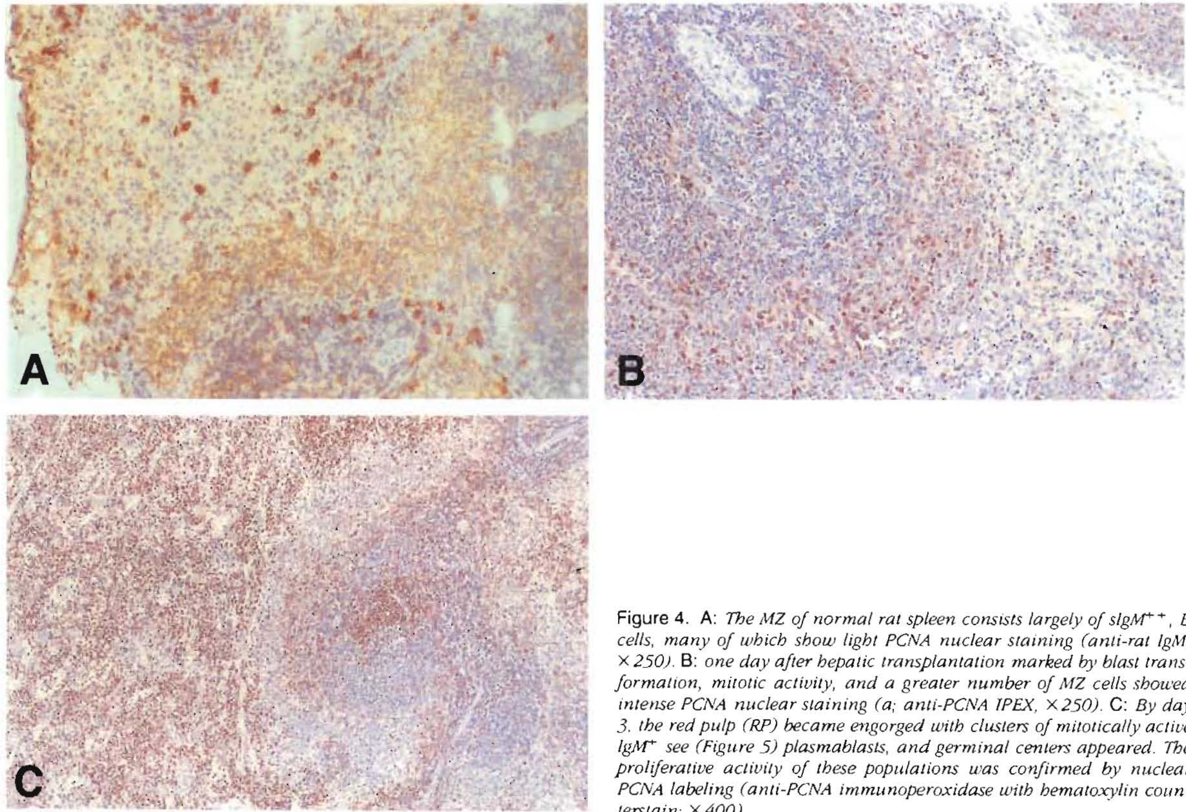


Figure 4. A: *The MZ of normal rat spleen consists largely of sIgM++, B cells, many of which show light PCNA nuclear staining (anti-rat IgM; ×250).* B: *one day after hepatic transplantation marked by blast transformation, mitotic activity, and a greater number of MZ cells showed intense PCNA nuclear staining (a; anti-PCNA IPEX, ×250).* C: *By day 3, the red pulp (RP) became engorged with clusters of mitotically active IgM+ see (Figure 5) plasmablasts, and germinal centers appeared. The proliferative activity of these populations was confirmed by nuclear PCNA labeling (anti-PCNA immunoperoxidase with hematoxylin counterstain; ×400).*

Flow Cytometry

Analyses were performed on days 4 and 5 after hepatic transplantation, which corresponded to the peak proliferative response. Compared with normal controls, recipients of hepatic xenografts developed a population of cells with a larger forward and wide-angle scatter, approximating the blast cell population observed histologically (Figure 6). The blast population accounted for 34% of the total in untreated recipients and 18% in treated recipients. The phenotypic composition of the blasts in both groups are shown in Table 1.

The overall proportion of blasts as well as the percent of IgM^{++}/IgD^{+} cells decreased in the FK506-treated recipients. However, up to 25% of the blasts in the treated population still consisted of IgM^{++}/IgD^{+} cells.

Peripheral Lymph Node Response

Blast transformation and mitotic activity was evident in the paracortex and interfollicular regions of untreated cardiac and hepatic xenograft recipients within one day after transplantation. This response continued to expand, and by days 3 to 4, germinal centers appeared and the paracortex was markedly enlarged by a mixture of $W3/25^{+}$ and $sIgM^{+}$ cells, whereas the medulla was engorged with $sIgM^{+}$ plasmablasts and $cIgM^{+}$ plasma cells.

The lymph node response in the FK506-treated recipients was markedly diminished. Little if any blast transformation in the paracortex was noted, and engorgement of the medulla with IgM^{+} cells and germinal center formation was almost completely inhibited.

Cell Migration Studies

Recipient spleens were examined 1, 3, and 5 days after hepatic xenografting, and 2 days after cardiac transplantation. Hamster cells were present in the splenic PALS and most numerous (approximately five to six cells/PALS cross-section) on day 1 after hepatic transplantation (Figure 7). In the untreated cardiac and liver graft recipients on days 3 and 5, hamster cells were difficult to find, or absent.

Effects of Therapy on Survival

Two animals treated with a single dose of CYP were sacrificed for splenic histology and flow cytometric analysis 10 days after injection. There was striking

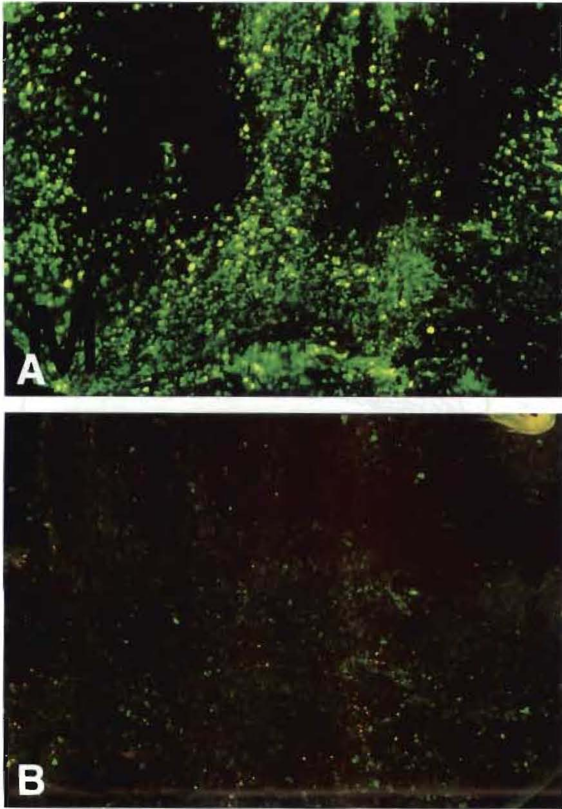


Figure 5. *The cells filling the splenic red pulp of liver xenograft recipients 3 days after transplantation consisted mostly of IgM⁺ cells* (top: *anti-rat IgM; ×40) with many fewer IgG⁺ cells* (bottom: *anti-rat IgG IF; ×40). The unstained areas in the top photomicrograph are the periarterial lymphatic sheaths.*

depletion of the IgM^{++}/IgD^{+} cells in the marginal zone, which represented only 3% of the splenocyte population, in comparison to 12% in normal controls. The percentage of other splenic lymphoid cells ($Ox8^{+}$, $W3/25^{+}$. $NKR\text{-}P1^{+}$, IgD^{++}/IgM^{+}) in CYP-treated rats were all within normal limits, consistent with the observations of Kumararatne et al[22] (Figure 8).

Heart and liver graft survival after the various forms of therapy is shown in Table 2. FK506 therapy alone did not significantly prolong survival of the cardiac xenografts, which failed, as in the untreated controls, because of humoral rejection. However, the CYP therapy alone increased survival from 3 to 6 days, at which time the failed grafts showed a dense cellular infiltrate, typical of cellular rejection. The combination of FK506 and CYP resulted in excellent graft survival.

In contrast, FK506 therapy alone resulted in a significant prolongation of liver xenograft survival (Table 2). Histologic examination of the failed grafts revealed changes of biliary obstruction and grossly, "bilomas" were noted. CYP therapy alone did not enhance liver graft survival, but combination of the two drugs yielded excellent results.

Discussion

Humoral effector mechanisms appear to be responsible for cardiac xenograft failure and cause significant hepatic xenograft injury in this model. The response appears immediately after transplantation and is dominated by complement-fixing IgM antibodies, consistent with the studies of Hasan et al[10] and Platt et al.[26] Although we were unable to determine with certainty whether the predominant IgM or IgG fraction was the more destructive, Platt and others have shown that the IgM antibodies[4–7,10,26] are largely responsible for graft damage.

The deposition of antibodies is quickly followed in the liver grafts by a cellular infiltrate enriched in $OX19^{+}/OX8^{+}$(CD8) cells, with fewer $NKR\text{-}P1^{+}$ (natural killer) cells, in the sinusoids and portal tracts, the sites of antibody deposition. This is different from liver allograft rejection, where the initial infiltrate is portal or perivenular.[23] Lastly, portal and perivenular $W3/25^{+}$ cells appear. Although we can only speculate as to the functional significance of the observations above, the role of antibody-dependent cellular cytotoxicity in xenograft rejection is certainly worthy of further investigation.

At least one explanation for the importance of the spleen in xenograft rejection is based on the marked stimulation of IgM^{++}/IgD^{+} B cells, which in the rat are preferentially localized in, and thought to be confined to, the MZ and red pulp of the spleen.[27–29] This response could be elicited by release of soluble antigens, emigration of donor (hamster) hematolymphoid cells, or both. The "sensitizing potential" of passenger leukocytes from hepatic or cardiac allografts has recently been suggested.[23,30] It should be noted, however, that the migratory donor cells lodged first in the periphery of the PALS, but the earliest evidence of proliferation was detected in the MZ.

The relative ability of soluble or particulate noncellular xenoantigens to elicit MZ splenocyte proliferation, the need for accessory cell processing, and/or T-cell help for xenoantigens cannot be directly determined from this study. However, the splenic proliferative activity appears to be at least partially T-cell dependent, since FK506 markedly diminishes it, but does not abolish it. Nevertheless, the location and phenotypic profile of the responding recipient splenocytes suggest that a subpopulation of B cells that mediate T-independent B-cell immunity[31] are probably involved.

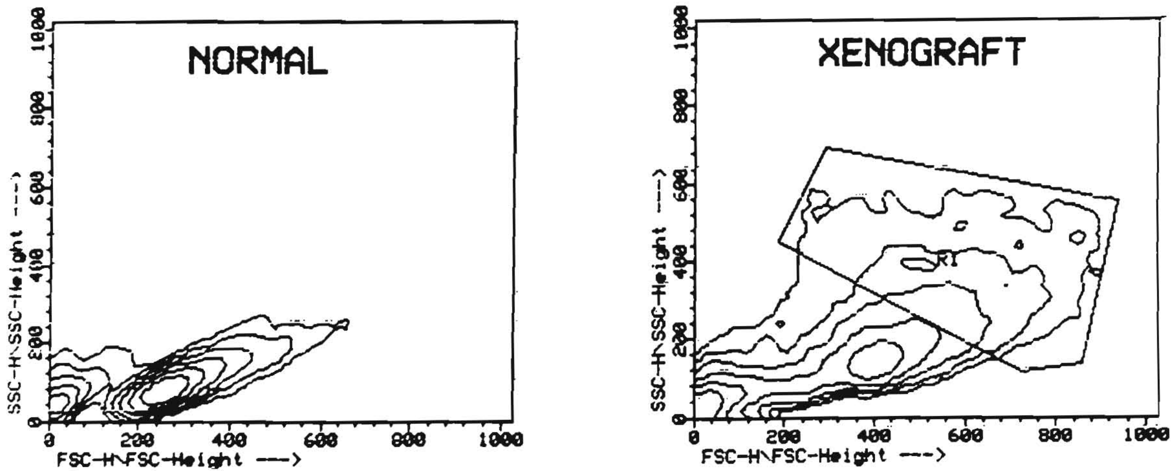


Figure 6. *Liver grafting (4 days after, no treatment) resulted in the appearance of a population of splenocytes with a larger forward and wide-angle scatter profile* (right) *on flow cytometry compared to normal controls* (left). *This population (R1) was gated for phenotypic analysis shown in Table 1.*

Table 1. *Flow Cytometric Analysis of Splenocytes 4 Days after Hepatic Xenografting from Recipients without and with FK506 Therapy, Compared with Normal Controls*

	Untreated recipients (n = 3)	FK506-treated recipients (n = 2)	Normal LEW rat (n = 3)
Blasts/total splenocytes (%)	34	18	<2
	Blast phenotype (% of blasts)		Normal size lymphocyte phenotype (% of total)
IgM^{bright}/IgD^{dull}	48	25	12
IgM^{dull}/IgD^{bright}	20	27	30
$W3/25^+$	18	24	41
$OX8^+$	10	16	17
NKR-$P1^+$	6	2	25

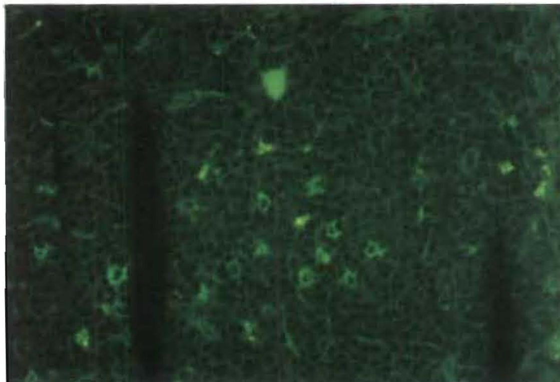


Figure 7. *Hamster cells (green fluorescent) were detectable in the PALS and MZ of the rat spleen one day after transplantation (anti-hamster IF ×250). Similar cells were difficult to detect in cardiac xenograft recipients and were absent by day 5.*

This same subpopulation of B cells forms the host's primitive defense against blood-borne polysaccharide antigens, such as those encountered on bacteria and bacterial products like lipopolysaccharide.[32–38] We are unsure whether the red pulp B cells represent a later maturational phase of those in the MZ, since the relationship between the two, if any, is currently unknown.[33–39] However, it is tempting to speculate that splenic IgM^{++}/IgD^+ B cells are the rat analog of human $CD5^+$ B cells and mouse Ly 1+ B cells.[33–39] These B-cell populations, along with natural killer cells, are thought to represent an earlier evolutionary stage of the immune system designed to provide broad-spectrum recognition of harmful foreign materials.[32] It is not unreasonable to suggest that rat IgM^{++}/IgD^+ splenic B cells are the source of the destructive xenoreactive antibodies in this model. Bach et al[40] have also suggested that B1 ($CD5^+$) cells may be the source of xenoreactive antibodies, based on their polyreactive nature.[41]

We tested the importance of $IgM^{++(bright)}/IgD^{+(dull)}$ B cells in xenograft rejection using a single 500 mg/m^2 intraperitoneal dose of CYP, which selectively ablates this splenic B-cell popula-

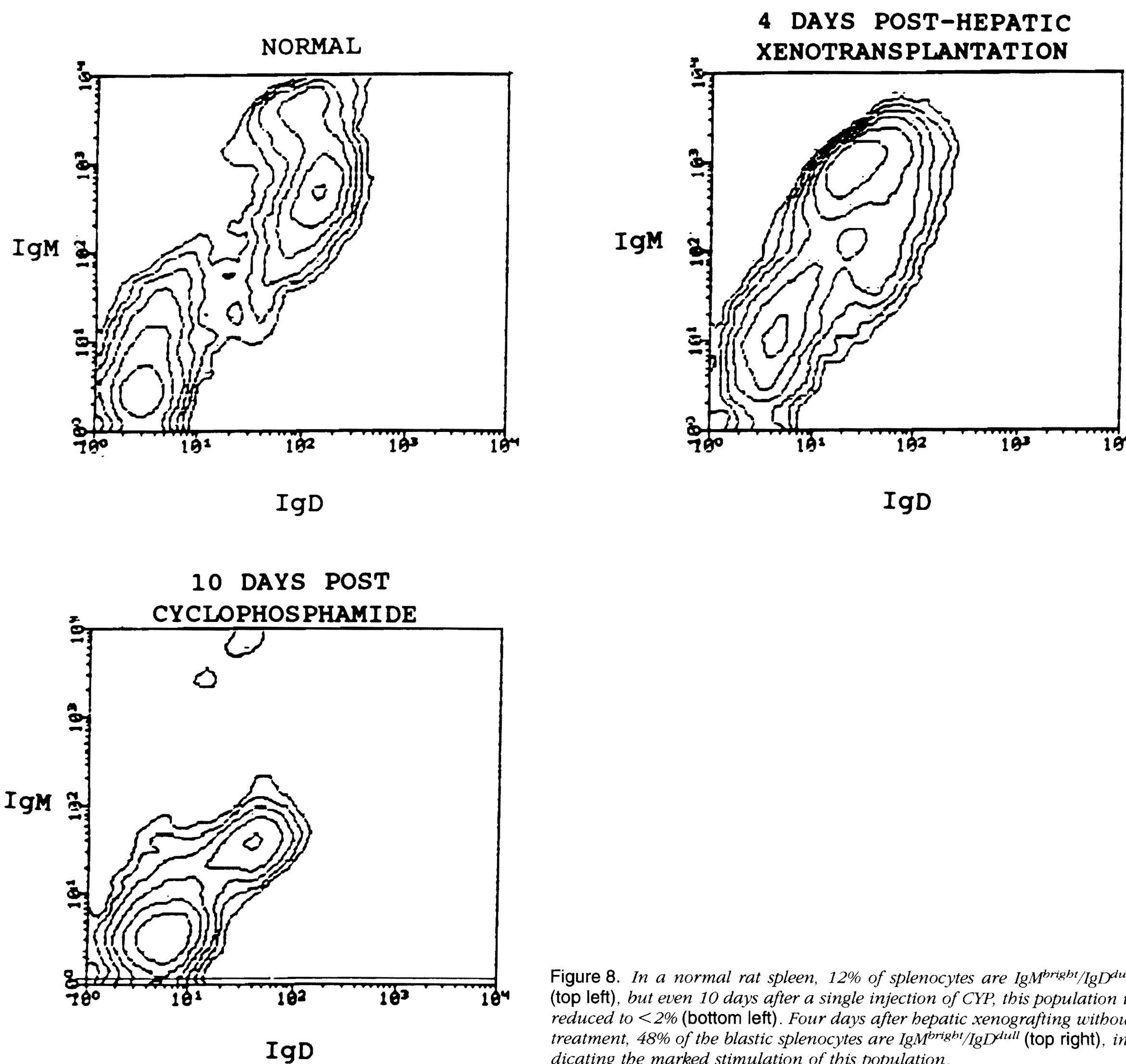


Figure 8. *In a normal rat spleen, 12% of splenocytes are IgM^bright/IgD^dull* (top left), *but even 10 days after a single injection of CYP, this population is reduced to <2%* (bottom left). *Four days after hepatic xenografting without treatment, 48% of the blastic splenocytes are IgM^bright/IgD^dull* (top right), *indicating the marked stimulation of this population.*

tion.[22,38] Although CYP kills all dividing cells, including other lymphocytes, 10 days after a single injection the T-cell zones and IgD^+ B cells are replenished, whereas the MZ IgM^{++}/IgD^+ cells are still markedly depleted.[22]

Lastly, preliminary studies in nude rats, which are T cell deficient but retain this fully functioning B-cell population,[39] find that they are able to reject cardiac xenografts in the normal fashion.[42] Liver xenograft rejection is only slightly delayed in these animals, except after splenectomy, in which case 100% of cardiac and 40% of hepatic grafts are accepted long term without therapy (L.A. Valdivia, personal communication). Examination of the nude rats[3] spleens after hepatic or cardiac xenografts confirmed the persistence of these B cells and their participation in xenograft rejection (unpublished observation). Overall, the evidence provided herein, combined with other published studies,[9,10,12,15,42,43] suggests that splenic IgM^{++}/IgD^+ B cells play an important effector function in hamster-to-rat xenograft rejection.

Nevertheless, the CYP protocol used in this study, like splenectomy, when used alone was unable to sufficiently control the T cell-mediated graft damage, so that examination of the rejected cardiac grafts in these recipients showed a heavy lymphocytic infiltrate. The addition of FK506 to CYP, however, resulted in excellent xenograft survival. Presumably this therapeutic regimen controls both arms of the immune system, as has been shown by Murase et al[9] and Hasan et al,[10] who suggested a direct role for the "natural" antibodies in graft rejection.[10]

Table 2. *Survival Results of Heart and Liver Xenograft Recipients Treated with Cyclophosphamide Given as a Single Agent 10 Days before Surgery or in Combination with FK506*

Xenograft type*	CYP 80 mg/kg, day −10	FK506	Survival (days)†
HTX	−	−	**3,3,3,3**
HTX	+	−	**6,6,6,6,7**
HTX	−	+	**3,3,3,3,4**
HTX	+	+	>74,[‡] >74, >68, >68, >66
OLT	−	−	**6,7,7,7,8**
OLT	+	−	**6,7,8,8,9**
OLT	−	+	**27,33,48,66,102,164**
OLT	+	+	>65, >66, >66, >66

* HTX, heart xenografts; OLT, liver xenografts.
† The survival days in bold type represent cessation of graft heart beat in the cardiac heart recipients and death in the liver recipients.
‡ > means that the animal is still surviving after the indicated number of days.

The tremendous advantage of a dual pharmaceutical approach to optimal control of allograft rejection, as well as the ability of CYP to inhibit arterial fibrinoid necrosis in renal allografts (a humoral insult), was first shown by Starzl et al.[44–47] This lesson has also been the conclusion of several experimental xenograft models.[5,9,10,12,14,42,43,48] In our experience, FK506 has been the most important drug for xenotransplantation,[8,9] presumably because of its effect on T cells, which indirectly also mutes the B-cell response. However, FK506 alone was ineffective in the cardiac model. In the liver model, FK506 alone increased survival, but the majority of recipients suffered late graft failure from biliary obstruction. We consider this late complication to be a delayed manifestation of earlier humoral injury to the biliary tree.[49]

If many of the "natural" antibodies, which are now a barrier to xenotransplantation, are shown to arise from this primitive B-cell population, more selective and less toxic treatment strategies can be designed. Once the primitive host defense mechanisms have been overcome, antibody accommodation[7] may permit recovery of this B-cell system and protection against common bacterial pathogens, without graft damage.[9] In addition, Murase et al[50] have recently shown the establishment of microchimerism after hepatic xenotransplantation, which may offer some degree of long term protection against xenograft rejection.

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