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Early Events in Liver Allograft Rejection

Delineation of Sites of Simultaneous Intragraft and Recipient Lymphoid Tissue Sensitization

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The early events of liver allograft rejection in untreated rats were studied in the DA to BN rejection strain combination and compared with DA and BN liver isograft recipients. In the liver allografts, T-cell infiltration first occurred at 2 days after transplantation and localized to the portal triads and subjacent to the terminal hepatic venules (THV), regions rich in intensely Ia+ spindle and dendritic-shaped interstitial cells. Double staining showed distinct 'clustering' between donor Ia-positive dendritic-shaped cells and W3/25+ infiltrating lymphocytes, or to a lesser extent, 0X8+ cells. The infiltrating mononuclear cells underwent blastogenesis and proliferated in both the triads and THV regions at 3 and 4 days. Donor Ia-positive cells were also noted in the W3/25+ periarterial lymphatic sheath and marginal zone of the recipient spleen 1 day after transplantation. The number of these cells in the spleen peaked at 3 to 4 days, but were no longer detectable by 10 to 12 days. Mitotic activity became evident in these same regions by days 3 and 4. Paracortical blastogenesis (day 2) and proliferation (days 3 and 4) were also noted in the regional lymph nodes of liver allograft recipients, but no donor Ia+ cells were found in the mesenteric nodes or thymus of the allograft recipients. These results demonstrate that sensitization of the recipient lymphoid tissue to liver allografts can occur both peripherally (intragraft) and centrally (spleen and lymph nodes). Passenger leukocytes (donor dendritic cells) are likely the primary stimulators of the rejection reaction. Still, it is probable that other pathways of sensitization exist. *(Am J Pathol 1991, 138:609–618)*

Solid organ allografts are increasingly used as therapy for patients with end-stage isolated organ disease. However Medawar[1] recognized decades ago that an immunologic reaction (rejection) directed at the classical transplantation antigens was the principal barrier to ultimate success of this therapeutic approach. More recently, many studies have dissected the various phases of the rejection reaction, which can be broadly classified into three overlapping stages.[2,3]

The first phase of rejection involves recognition of the foreign alloantigen and initiation of an immune response. During the second or effector phase, mechanisms capable of destroying and eliminating the foreign antigens are generated. Finally, as in other immune responses, a regulatory phase ensues, which restores homeostasis to the organism. This study is concerned with the first of these three stages.

Bach and Hirschhorn[4] discovered that mixing hematopoietic cells from genetically different individuals *in vitro* (mixed leukocyte response [MLR]) results in proliferation of the lymphoid components of the mixture. Later, Hayry and Defendi[5] found that the MLR reaction also results in the generation of cytotoxic T lymphocytes (CTL). They concluded that the observed reaction was an *in vitro* analog of the rejection response.[5] Although many cells in an organ allograft may bear surface transplantation or major histocompatibility (MHC) antigens of the donor, a distinct population of 'passenger leukocytes,' which are intensely class II MHC+, are thought to initiate the reaction.[2,3] Alternatively release of MHC antigens by the organ, either as a soluble product or the result of cellular debris from the allograft, may be processed by recipient accessory cells and presented to the recipient lymphoid tissue.[2,3] This latter pathway, however, is thought to be of lesser significance.

With the discovery of dendritic cells, Steinman and Cohn[6] showed that this distinct family of hematopoietic cells were the most potent, and possibly the only, stimu-

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lators of virgin lymphocytes in a primary MLR.[7] They noted that dendritic cells avidly bound to distinct subpopulations of allogeneic lymphocytes during the early phases of an MLR. The binding resulted in cell clusters, formed by a central dendritic cell surrounded by responding lymphocytes, which undergo blastogenesis and ultimately proliferate. Applying this idea to solid organ allografts, Forbes et al[8] found *in vivo* evidence for clustering of recipient lymphocytes around donor interstitial dendritic cells in rodent cardiac allografts several days after transplantation. Thus the study of dendritic cells has assumed a prominent position in transplantation biology.[9]

Despite these advances, a controversy still exists about whether the recognition and inductive phase of the rejection reaction occurs primarily within the allograft (peripherally) or in the recipient lymphoid tissue (centrally). Recently, however, Larsen et al[10] have stressed the role of central sensitization by demonstrating cardiac-derived dendritic cells in the spleen of rodent heart allograft recipients. The following study is a sequential analysis of rejection in untreated rat liver allograft recipients. Daily histopathologic and immunohistochemical analyses of the graft and recipient lymphoid tissue, including mitotic indexes, were performed in an attempt to understand the early events associated with rejection as they apply to the liver.

Materials and Methods

Operative Procedures

Orthotopic hepatic transplantation was performed according to a method modified from that described by Kamada and Calne.[11] Briefly, after the donor animal was heparinized, the donor liver was perfused *in situ* through the abdominal aorta with 10 ml cold Ringer's lactate solution and removed. Cuffs were placed on the portal vein and the infrahepatic vena cava. The liver graft was transplanted orthotopically into the recipient rat. The suprahepatic vena cava was anastomosed with sutures. The portal vein and infrahepatic vena cava were connected with the cuff technique. The hepatic artery was not reconstructed. Bile duct continuity was achieved with a polyethylene tube, which was inserted into the lumens of both bile ducts and secured outside with sutures. All recipients were given a single intramuscular injection of cefamandole nafate (20 mg) immediately after the surgery. No further treatment was given. After operation food and water were allowed *ad libitum*.

Tissue Collection and Histopathologic Studies

Untreated DA to BN liver allograft recipients were chosen as the subjects of study because they represent a strong rejector strain combination with an average survival of 10 to 15 days.[12] Three of these recipient animals were killed on days 1, 2, 3, 4, and 5, and two each were killed on days 7, 10, and 12 for study. DA to DA liver isograft recipients were used as controls for findings within the liver and two animals each were killed on days 2, 3, 4, and 10. BN to BN liver isograft recipients were used as controls for observations within the spleen and mesenteric lymph nodes; three each of which were killed on days 1, 2, 3, 4, 5, and 10. A complete autopsy was done and tissue from all organs was processed for histologic examination.

For routine histopathologic studies, tissues were fixed in neutral buffered formalin, embedded in paraffin, sectioned at 4 μ, and routinely stained with hematoxylin and eosin (H&E). For immunohistochemical studies, tissue from the liver graft, spleen, thymus, and mesenteric lymph nodes were frozen in optimum cold temperature compound (Tissue-Tek, Ames Division Miles Laboratories, Inc., Elkhart, IN) and stored at −70°C until the staining was performed.

Mitotic Index Analysis

The number of mitotic figures in the liver allografts and spleen were enumerated using H&E-stained slides of the organs. In the livers, the total number of portal tracts (PT) and terminal hepatic venules (THV; ie, central veins) in the tissue sections were counted. A portal tract was defined as connective tissue containing a bile duct and branches of the hepatic artery and portal vein surrounded by hepatocytes. Terminal hepatic venules were recognized by their lumens and a small subjacent connective tissue matrix. The total number of mitotic figures present with in the confines of the triad or adjacent to the THV were counted using a light microscope (400×). The total number of PT mitoses was divided by the total number of PT within the specimen to control for the variable numbers of PT and THV in the samples from different animals. The same was done for the THV. The ratios were then multiplied by a factor of 10 for ease of graphical representation. Counts for PT, THV, and mitoses were repeated on five liver samples. The count for each was found to have an error rate of less than 10%.

For splenic mitoses, a small opaque cover with an adhesive backing containing a 2.5-mm diameter circular hole in the center was made (area = 4.9 mm^2). The hole permitted visualization of roughly 70% of the total splenic

area on the glass slide. The cover was then placed over the section of the spleen, which permitted visualization of only a standardized region. Mitotic figures visible through the hole were counted with a light microscope at 400×. In a typical field viewed through the hole, 6 to 8 periarterial lymphatic sheaths (PALS), accompanying marginal zones (MZ), and the intervening red pulp could be seen. The counts were repeated twice on five randomly chosen animals after moving the hole to a different portion of the spleen. The error rate was less than 10% in all instances. For statistical analysis the results obtained for the DA to BN allograft recipients were compared with the BN to BN isograft recipients using the paired *t*-test.

The same technique could not be used to count mitoses in recipient mesenteric lymph nodes because of the large variability in size between allograft and isograft recipient MLN and the relative restriction of mitotic figures to distinct anatomic regions.

Immunoperoxidase Studies

Immunoperoxidase studies of the liver allografts, recipient spleen, thymus, and mesenteric lymph nodes were performed using a panel of monoclonal antibodies. This panel included OX19 (pan T-cell marker, Accurate Chemical and Scientific Co., Westbury, NY), OX8(nonhelper subset, Accurate), W3/25 (T-helper/inducer and subsets of other accessory cells, Accurate) and L-21-6, a mouse anti-rat IgG monoclonal antibody that recognizes Ia antigens in DA, LEW, and a variety of other inbred rat strains, but not in BN tissue.[13] The primary antibodies were applied singly, and to determine the relationship between Ia-positive donor cells (L-21-6) and T-cell subsets, double staining for L-21-6 and W3/25 or OX8 was performed.

For single staining, a standard three-step avidin-biotin complex (ABC) method was used. Cryostat sections were cut at 5 μ, fixed in cold acetone for 5 minutes, air dried, and stored at −70°C until staining, at which time they were washed in phosphate-buffered saline (PBS) for 5 minutes. The sections were then placed in 0.6% H_2O_2 in methanol for 8 to 10 minutes to deplete endogenous peroxidase activity. This was followed by a 20-minute wash in PBS, incubation with nonfat dried milk for 45 minutes to block endogenous avidin-biotin activity,[14] washes in PBS (2×, 5 minutes), and incubation with protein blocking agent (Lipshaw Co., Detroit, MI) for 20 minutes. The sections were then incubated with the primary antibody for 45 minutes (L-21-6 supernatant; W3/25, 1:10; OX19, 1:10; OX8, 1:25), washed in PBS for 5 minutes (2×) and incubated with a biotinylated rat anti-mouse Fab_2 (Pel-Freez, Rogers, AK) fragment secondary antibody (1:2000) for 45 minutes. This was followed by a PBS wash for 5 minutes (2×) and incubation with ABC (Vector Lab., Inc., Burlingame, CA) for 30 minutes. Color development was achieved with aminoethylcarbazole. The tissues were counterstained with H&E and mounted with crystal mount (Biomedia, Corp; Foster City, CA).

Tissues used for double staining for L-21-6 and W3/25 or OX8 were first stained with L-21-6 using the method described above except for the H&E counterstain. This was followed by incubation in 2% glacial acetic acid for 5 minutes to block endogenous alkaline phosphatase activity and to elute any residual immunoglobulins. After washes in PBS, the sections were incubated with the second primary antibody (OX8 or W3/25) for 45 minutes, PBS washes, incubation with a rabbit anti-mouse (DAKO, Carpenteria, CA) secondary antibody for 30 minutes, PBS washes, and incubation with alkaline phosphatase anti-alkaline phosphatase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 30 minutes. Enhancement of alkaline phosphatase staining was done by repeating steps 2 and 3 for 15 minutes each. The substrate was developed by dissolving 2 mg of naphthol AS-Mx phosphate (Sigma Chemical Co., St. Louis, MO), 0.2 ml of dimethylformamide (Sigma), and 9.8 ml of 0.1 mol/l (Molar) TRIS buffer (ph 8.2) with one drop of levamisole (Vector) per 5 ml Fast Blue BB (Sigma) was added immediately before use at 1 mg/ml and the mixture was incubated on the sections for 45 minutes at 37°C. The tissues were counterstained with methyl green and mounted with crystal mount (Biomedia).

Three sets of controls for the immunoperoxidase studies were used. In the first set, the primary antibody was omitted from the single procedure or both primary antibodies were omitted from the double staining procedure. The second group of genetic control studies evaluated the liver, spleen, and thymus from normal BN or BN liver isograft recipients for L-21-6 staining. None was found. Normal DA spleen and liver tissue served as the positive control for L-21-6 and both normal DA and BN spleen served as positive controls for the anti–T-cell antibodies.

Results

Routine Histologic Analysis

Liver Grafts

Allografts could not reliably be distinguished from the isografts 1 day after transplantation. By day 2, however, the allografts contained mononuclear cells in the portal triads (PT) and immediately subjacent to the terminal hepatic venules (THV) (Figure 1). No mononuclear cell accumulations were seen in the sinusoids between the PT

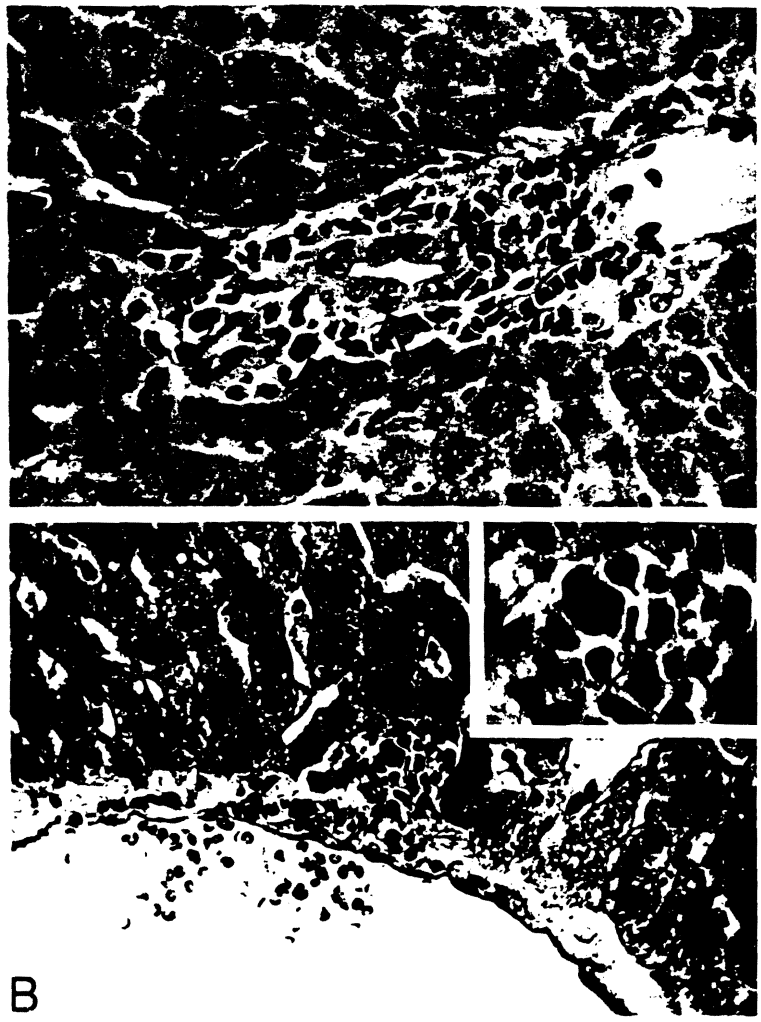


Figure 1. *Proliferating mononuclear inflammatory cells were evident in the portal tracts* (A; *H&E; 480×*) *and beneath the terminal hepatic venules* (B) *by 2 days after transplantation. As an example, in* A, *the 3 mitotic figures noted (arrows) were used to calculate a mitotic index for this animal. No mononuclear infiltrate or mononuclear mitoses were present in the isografts. In* B, *a distinct clustering of lymphocytes was noted around a larger central cell (H&E; ×480 inset, ×1200), located beneath the terminal hepatic venules.*

and THV. In the PT, the mononuclear cells were sludged in the small portal capillaries and clustered in the portal tract connective tissue. Similar clusters could be seen immediately subjacent to the THV, and these often assumed the appearance of small granulomas (Figure 1B). The infiltrative mononuclear cells in both regions were blastic, with enlarged nuclei and small nucleoli. Occasional mitotic figures could be seen, but were not plentiful.

By day 3, the number of PT and peri-THV mononuclear cells increased, as did the mitotic activity within this population. In addition, eosinophils sometimes could be recognized in both locations. The mononuclear cells continued to increase in number on day 4, and migration beneath the endothelium of the portal and THV was noted (Figure 2). An influx of nonlymphoid cells was also evident at this time, marked by the appearance of more eosinophils, neutrophils, and macrophages. Simultaneously, spotty hepatocellular necrosis was conspicuous in the lobules. We interpreted these latter findings as evidence of the beginnings of the effector phase of the rejection response, as tissue damage was indisputable. A description of the later stages of liver allograft rejection in this strain combination is given elsewhere.[15]

None of the isografts contained a significant mononuclear inflammatory infiltrate at any time after transplantation. The only abnormalities seen consisted of an occasional small infarct, mild portal edema accompanied by occasional neutrophils, and mild bile duct proliferation, which were noted on or after day 4.

Spleen

The spleen of liver allograft recipients could not be reliably distinguished from isograft recipients 1 day after transplantation by routine light microscopy. Both contained cellular debris, particularly in the PALs and MZ. By day 2 in the allograft recipients, however, the PALS was slightly expanded and appeared mottled because of blastic transformation and low-grade mitotic activity. The cellular debris had largely been cleared. Blastic transformation became more evident at the periphery of the

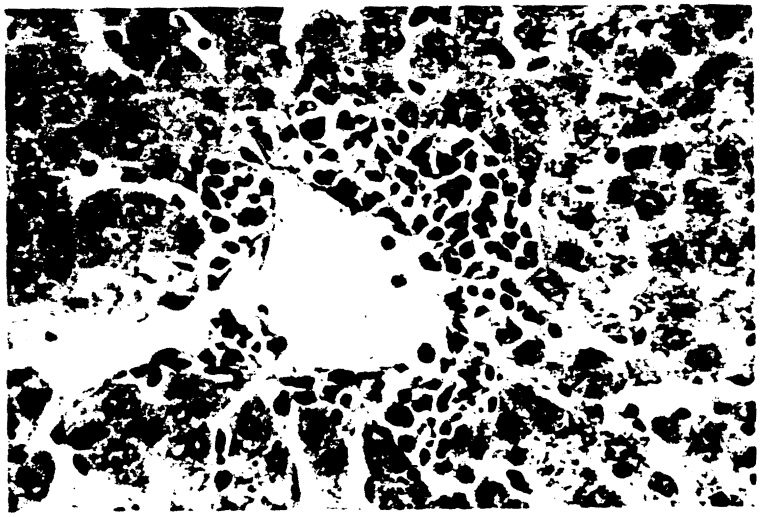


Figure 2. *Central "venulitis" was present by day 3 or 4 in the allografts (H&E, ×480). This lesion has been understressed in rejection, although it was originally described by Porter.[27] In addition to the mononuclear cells, red blood cell congestion and other leukocytes were also present.*

PALS on day 3, as did the mitotic activity in the same location. The prominent blastic transformation and mitotic activity in the PALS was not seen in isograft recipients. The histologic appearance of the spleen in allograft recipients was similar on days 4 and 5, when the activity at the periphery of the PALS continued. In addition, secondary follicles appeared in the PALS, as did small clusters of blasts and plasmacytoid lymphocytes in the cords of the red pulp and adjacent to the splenic trabeculae. Clusters of plasmacytoid cells also appeared in the red pulp cords and the paratrabecular region of isograft recipients, but to a lesser extent.

Mesenteric Lymph Nodes (MLN)

One day after transplantation, MLN from allograft recipients could not be reliably distinguished from isograft recipients. Both contained red blood cell congestion of the subcapsular and medullary sinuses. In addition, there was abundant cellular debris in the sinuses, germinal centers, and paracortex, often within tingible-body macrophages. By day 2, however, there was paracortical expansion in the allograft MLN but not in the isograft recipient MLN. The red cell congestion and cellular debris largely had been cleared. On closer examination, the paracortical expansion in the allograft recipients was because of blastic transformation of the lymphocytes, but few mitotic figures were noted. The findings on day 3 were similar to those on day 2 in the allograft recipients, although overall the nodes were much larger. The MLN from isograft recipients had not changed from day 2 and remained relatively constant thereafter. On day 4, large areas of the MLN of allograft recipients, which spanned the region of the paracortex to the medulla, were populated by mitotically active immunoblasts. These cells assumed a plasmacytoid appearance as they extended into the medulla.

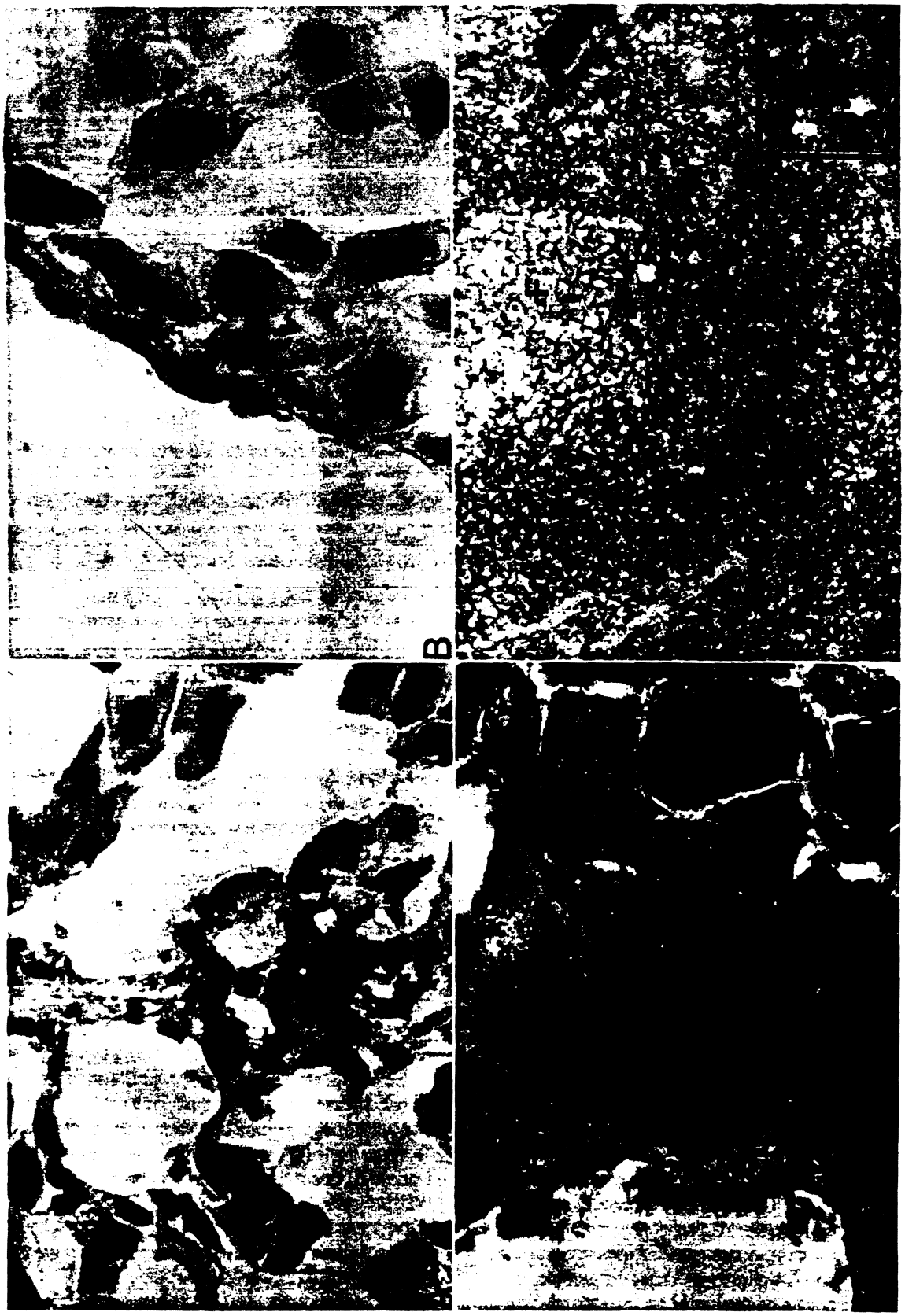
Immunoperoxidase Studies

Liver Tissue

The liver of normal DA and BN rats contained only rare OX19, OX8 or w3/25 positive mononuclear inflammatory cells, and no aggregates were noted. Similarly T cells were rare in both DA and BN isografts. Staining with L-21-6 in various DA tissues demonstrated a distribution of positivity typical for that of class II MHC antigens,[16] but no L-21-6 staining was seen in BN any tissues.[13] In the DA livers, intensely L-21-6+ spindle-shaped cells were present in the triads, often immediately subjacent to the bile ducts and in the periadventitia of portal arteries and veins (Figure 3). Similar cells were also seen in the connective tissue directly beneath the endothelium of the terminal hepatic venules (Figure 3) and in the liver capsule. Rarely, isolated L-21-6+ cells were seen in the sinusoids. No L-21-6+ cells were noted in any of the BN liver isograft recipients, nor was any staining observed in the negative controls (see Methods).

Sequential analysis of the DA to BN allografts disclosed a gradual increase in the number of OX19-, OX8-, and W3/25-positive cells in the triads and subjacent to the THV over the first 3 days. The T cells were localized in the triads and beneath the THV. Subset analysis showed that W3/25+ cells were slightly more prevalent at 2 days, but both OX8+ and W3/25+ cells were present on days 2 and 3. By days 5 through 7, however, OX8+ cells outnumbered the W3/25+ cells.

Staining for L-21-6 in the DA to BN allografts on day 1 was similar to that seen in normal DA rats. By days 2 and


B

3, the spindle-shaped L-21-6+ cells in the PT and THV regions were larger and dendritic shaped. Mononuclear cells were seen adjacent to these cells, but were L-21-6−. No intensely L-21-6+ cells were noted in the allografts by day 7, but by days 10 through 12, weakly L-21-6+ sinusoidal, and occasional portal vein endothelial cells were observed. No definite L-21-6+ bile ducts were detected in the allografts. Examination of DA to DA liver isografts on day 4 was similar to the DA controls; no decrease in the number of L-21-6+ cells was noted. Double staining for L-21-6 and OX8 or W3/25 demonstrated distinct clustering between central L-21-6+ dendritic cells and surrounding W3/25+ mononuclear cells (Figure 4). L-21-6-W3/25 clusters were predominant, but L-21-6-OX8 clusters also were seen.

Recipient Lymphoid Tissue

The spleen of normal DA and BN rats showed a distribution of staining for OX19, OX8, and W3/25 similar to that previously reported.[17] L-21-6+ cells were noted in the DA to BN liver allograft recipient spleens 1 day after transplantation (Figure 5). They localized preferentially near the periphery of the PALS and in the MZ. L-21-6+ cells were rare in the red pulp. Double staining showed that the L-21-6+ cells localized almost exclusively in the W3/25+ PALS and MZ of the spleen (Figure 5). The number of L-21-6 in the recipient BN spleens peaked at days 3 to 4, and such cells were no longer detectable by 10 to 12 days. No staining for L-21-6 was seen in the spleens of any of the BN liver isograft recipients. In addition, we could find no evidence of L-21-6+ cells in the mesenteric lymph nodes or thymus of the DA to BN liver allograft recipients.

Mitotic Indexes

Liver Allografts and Isografts

No mononuclear cell mitotic activity was noted in the liver allografts or isografts on day 1. Thereafter inflammatory cell mitoses occurred only in the allografts. By day 2, despite the presence of a mildly blastic mononuclear PT and peri-THV infiltrate in the allografts, only occasional mitotic figures were seen. By day 3, however, although only a slight increase in the total number of inflammatory cells was noted, mitotic figures were abundant. The mononuclear cell mitoses were noted only in the PT and peri-THV regions, not in the intervening sinusoids or parenchyma. The brisk mitotic activity continued on day 4, accompanied by an influx of nonlymphoid inflammatory cells. Liver mitotic indexes were not calculated after day 4. The results for the liver mitotic indexes are shown graphically in Figure 6.

Within the liver allografts, there was no statistically significant difference between the mitotic indexes calculated for the triads and the peri-THV regions ($P > 0.05$) on any of the first 4 days. The only mitotic activity seen in the isografts was an occasional mitosis in parenchymal cells, which were easily distinguishable from the mononuclear cell mitoses noted in the allografts. Even if the rare parenchymal cell mitotic figures were counted, they totaled less than two per liver slide in all the DA liver isograft recipients.

Splenic Mitotic Activity

A baseline of 1 mitosis/mm^2 was seen in control BN and BN to BN isograft spleens. No statistically significant increase in mitoses was seen in the allografts or isografts on day 2, although the actual number was slightly higher. By day 3, however, there was a statistically significant increase in the number of mitotic figures in the allograft compared with isograft recipients, which peaked at 14 mitoses/mm^2 (Figure 7) on day 4. Thereafter, the rate of mitotic activity decreased slightly but remained elevated in the allografts until the terminal stages of rejection. The mitotic figures were most conspicuous at the interface between the PALS and the MZ. These cellular populations eventually expanded into the red pulp, where they began to display plasmacytoid characteristics.

Mitotic activity in the recipient MLN was clearly higher in the allograft compared with the isograft recipient by days 3 and especially on day 4, although these were not quantitated.

Discussion

The results of this study show that sensitization to liver allografts, as measured by the proliferative activity in re-

Figure 3. **A:** *In normal DA rats intensely Ia+ (L-21-6) spindle-shaped cells were concentrated in the portal triads as shown here (L-21-6 immunoperoxidase with hematoxylin counterstain, ×1200) A small bile ductule is present in the center of the photomicrograph.* **B:** *Similar cells were seen immediately subjacent to the terminal hepatic venules as shown here and in the liver capsule, but were rare in the sinusoids (L-21-6 immunoperoxidase, hematoxylin counterstain; ×1200)*

Figure 4. *Bottom left Double staining for donor dendritic cells (L-21-6+, red) and W3/25 (blue) in the portal triads of DA to BN liver allografts revealed distinct clustering in the portal triads on day 2 as shown here (L-21-6, red, W3/25, blue; double immunoperoxidase staining, methyl green counterstain, 1200×) Similar clusters were seen subjacent to the terminal hepatic venules.*

Figure 5. *Bottom right Double staining for L-21-6 (red) to detect donor-derived cells, and W3/25 (blue, with methyl green counterstain, ×480) showed the presence of donor DC in the periphery of the W3/25+ PALS and in the MZ of the recipient spleen (F = follicle, P = periarterial lymphatic sheath, MZ = marginal zone, arrows = donor-derived cells) The inset (1200×) shows a closer view of the donor-derived cells.*

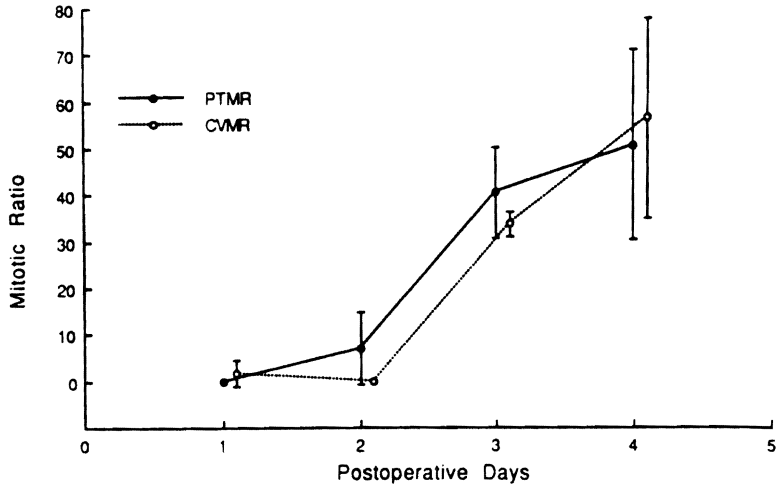


Figure 6. *Mononuclear mitotic indexes in liver allografts were calculated for the first 4 days. The results are expressed as the mean for 3 animals at each point ± the standard deviation. There was no difference noted for the PT or THV regions. Mitotic indexes were not calculated in isografts because no mononuclear infiltrates were noted. PTMR = portal tract mitoses ratio; CVMR = central vein mitoses ratio.*

cipient lymphoid tissues, occurs simultaneously in the liver allograft (peripherally) and recipient lymphoid tissue (centrally). Common to both the liver and recipient spleen was the presence of intensely L-21-6+ donor spindle- and dendritic-shaped cells, respectively, during the early stages of the rejection. No such cells were identified in the recipient mesenteric lymph nodes or thymuses, although blastic transformation and proliferative activity within the recipient regional MLN were noted within the same time frame after transplantation.

The intense L-21-6+ staining combined with their shape and localization in normal livers and allografts strongly suggest that the cells mentioned above belong to the dendritic cell family.[7,16] In fact, it has been stated that all Ia-positive spindle-shaped cells in the rat liver are dendritic cells (DC).[16] Although sinusoidal (Kupffer) cells may be induced to express Ia antigens, such induction did not occur until days 10 through 12. At this time, the L-21-6+ cells were no longer detectable in the spleen.

This study showed that a proportion of the hepatic DCs transported with the donor liver are released in the recipient circulation shortly after transplantation. Others remain in the liver. It is unlikely that peripheral blood DCs contamination from the donor liver significantly contributed to those found in the recipient spleen. Perfusion of the donor liver before transplantation, as was done in these animals, would remove the donor blood from the vasculature.

It is also unlikely that the L-21-6 staining in the spleen represented nonspecific uptake of noncellular donor Ia

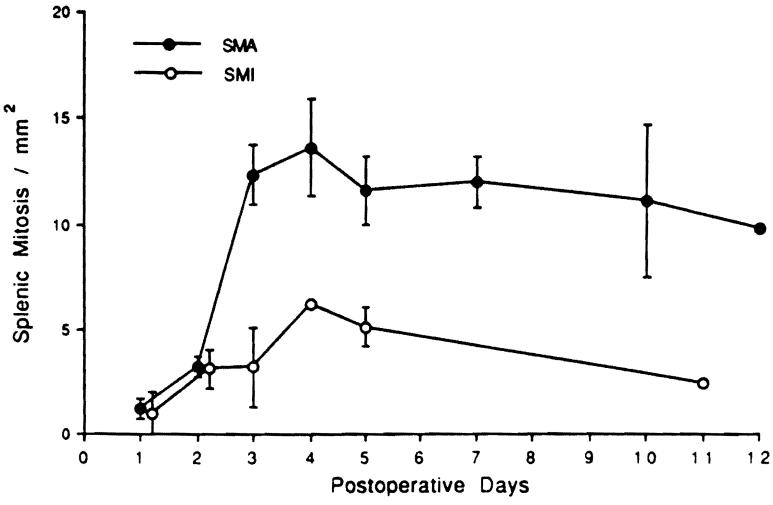


Figure 7. *Splenic mitotic indexes were calculated for both allograft and isograft recipients using the described methods. The results are expressed as the mean number of mitotic figures per mm² for 3 animals for each point ± the standard deviation. The difference between the allografts and isografts was statistically significant ($P < 0.05$) for all times after day 3.*

antigens from the graft. First, the staining appeared largely on the surface of the cells and secondly, such staining was no longer detectable by 10 to 12 days.

As noted above, DCs are the most potent stimulators of the MLR, whereas other class II MHC-positive cells such as B lymphocytes and other accessory cells are poor stimulators, or are inactive, in a primary MLR.[7] In the rat, capillary endothelia are normally class II MHC antigen-negative[16] and T cells infiltrating the allograft livers in the first 2 to 3 days localized exclusively in the regions containing the L-21-6+ cells. Therefore it is likely that donor DCs (passenger leukocytes) are the primary stimulators of rejection in the liver.

It is not particularly surprising that L-21-6+ DCs transported with the liver allografts and released into the recipient circulation localized to the recipient spleen. Studies of the traffic patterns of dendritic cells from the peripheral circulation of normal rodents using tracer dyes have noted a similar splenic homing.[18,19] As mentioned, donor DCs from cardiac allografts have also been seen in the recipient spleen[10] within days after transplantation.

In studies of primary rodent MLRs *in vitro*, lymphocyte DC 'clustering' occurred 20 to 40 hours after coculture, and proliferative activity began after an additional 48 hours.[7,20] These kinetics are identical to the morphologic observations and mitotic indexes calculated in the liver allografts and spleens in this study. By contrast, primed blastic alloreactive T lymphocytes reinitiate DNA synthesis 14 to 18 hours after restimulation with the appropriate alloantigen.[7,20] Therefore the origin of the proliferating cells in the allografts after 4 days is less certain than those seen during the first 3 days, because they may have been stimulated elsewhere.

Classical studies suggest that W3/25+(CD4) cells are the principal responders in an MLR and are crucial for the development of a rejection reaction.[2,3] Although we noted a predominance of W3/25+ cells in the clusters in the liver, OX8+ clusters were seen as well, in the first 3 days. Inaba et al[21] showed that DCs are capable of directly inducing mitogenesis and eliciting CTL activity in CD8+ cells without the help of CD4+ lymphocytes. Although the reaction was not dependent on the CD4+ cells for its initiation, the response was short-lived if soluble products from activated CD4+ cells were not added.[21]

Because the normal life span of DCs in the periphery is relatively short (approximately 5 days),[22] it was also not surprising that the donor DCs had disappeared 7 to 12 days after grafting. Active destruction and normal senescence are likely responsible for their attrition. Dendritic cells are likely the primary stimulators in rejection, but are probably not required for perpetuation of the response, as stimulatory requirements for unprimed lymphocytes differ from those of primed responder cells.[20] Induction of alloantigens on nearby parenchymal cells by cytokines released from lymphocytes stimulated within the clusters would be sufficient to perpetuate the response in primed cells. We have confirmed this suspicion *in vitro* by showing that human bile duct cells, which are targets in liver rejection, were capable of eliciting a proliferative response in alloprimed T cells but were ineffective in a primary MLR.[23]

This study also shows that DCs are probably not the only pathway for recipient lymphoid tissue sensitization after solid organ transplantation. Lymphocyte transformation and proliferation were also noted in the regional lymph nodes of allograft but not isograft recipients, yet donor dendritic cells could not be found in this location. Although speculative, it may be that soluble or particulate donor MHC antigens released by the graft may be processed by recipient accessory cells in the regional lymph nodes and, in turn, presented in an MHC-restricted fashion.

The observations made in this study also have direct relevance to humans. The morphologic events associated with early phases of liver allograft rejection appear identical in the rat and human.[15] The inflammatory infiltrate associated with rejection in both species is exclusively localized to the PT and THV regions. Dendritic cells have also been identified in human livers, in the same distribution as is seen in the rat.[24] Therefore it is likely that DCs are also primary stimulators of liver rejection in both. In many strict morphologic studies of human liver allograft rejection, the alterations around the terminal hepatic venules have been attributed to ischemia and a variety of other causes. This study has shown, however, that THV region is a site of primary sensitization in rejection.

One should also be aware of the fact that, in humans, capillary endothelia are constitutively class II MHC antigen positive. They appear to be capable (albeit less so) of eliciting a primary MLR if induced by gamma interferon beforehand.[25] Dendritic cell contamination of such endothelial cell populations, however, cannot be readily excluded.[25] Modulation of cells nearby the clusters by cytokines is likely important in recruitment and perpetuation of the response.

Finally it has been a dream of those in transplantation to reduce or eliminate the antigenicity of the graft and thereby eliminate the need for immunosuppression. A number of studies have pursued this line of reasoning, by pretreating donor organs in an attempt to destroy the dendritic cells. Recently Brewer et al[26] reported encouraging results in human renal allograft recipients, where the donor kidneys were treated with several anti-CD45 monoclonal antibodies. It seems of value to apply similar techniques to donor livers in humans, because liver grafts appear to be somewhat less 'immunogenic' than other solid organ allografts. Such treatment appears to

have the potential to reduce, but not eliminate, the immunogenecity of the graft.

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