A Controlled Serial Ultrastructural Tracer Study of First-Set Cardiac Allograft Rejection in the Rat

Evidence That the Microvascular Endothelium Is the Primary Target of Graft Destruction

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In order to define the serial morphologic correlates of unmodified first-set cardiac allograft rejection in an inbred rat strain combination, a series of Wistar–Furth cardiac allografts transplanted to normal nonsensitized Lewis recipients were studied as a function of time with the use of well-documented ultrastructural tracer techniques. Colloidial carbon was employed as a vascular label for detection of microvascular endothelial structural alterations, and horseradish peroxidase was used as a tracer probe for localization of cell-membrane permeability dysfunction of allograft endothelium and cardiocytes as well as of elements of the cellular infiltrate. Wistar–Furth to Wistar–Furth syngeneic heart grafts and Wistar–Furth recipients' own hearts provided appropriate control data. This study has demonstrated that severe diffuse loss of functional and structural integrity of the microvascular endothelium precedes the development of extensive damage to cardiac muscle cells and thus provides strong evidence that the allograft microcirculation is the primary target of immunologic injury. In addition, the sequential pattern of injurious changes present in the rejecting allografts was similar to that observed in certain models of delayed-type hypersensitivity and of skin graft rejection, raising the possibility that lymphokine-mediated mechanisms may be of major pathogenetic significance in this setting. (Am J Pathol 1983, 111:184–196)

HETEROTOPIC cardiac allografts exchanged across the major histocompatability complex of the rat provide an ideal model for controlled morphologic studies of rejection of primary vascularized organ allografts *in vivo*.[1,2] It has been shown that T lymphocytes are essential for functional rejection of first-set rat cardiac allografts.[3] However, the precise pathogenetic mechanisms involved are unknown. Although there is strong evidence that primary vascular injury is the critical determinant of first-set rejection of renal allografts[4] and of skin grafts,[5,6] some data have suggested that progressive primary immunologic injury to cardiac myofibers is the central mechanism of cardiac allograft destruction.[7,8] In the current investigation, rat cardiac allografts in unmodified combinations were studied at serial time periods with the use of well-documented ultrastructural tracer techniques for comparative assessments of microvascular and myocardial injury. Colloidal carbon was employed as a label for the localization and characterization of microcirculatory endothelial structural alterations.[1,9] Horseradish peroxidase, a protein tracer, was used as a probe for ultrastructural identification of altered permeability states of microcirculatory and myocardial components of the target organ as well as of elements of the cellular infiltrate.[10,11] Syngeneic heart grafts and recipients' own hearts provided the appropriate control obervations. These integrated tracer data clearly define the nature and progression of the injurious processes that culminate in functional rejection in this model, and serve as a detailed basis for further morphologic analysis of the pathogenetic effector mechanisms involved in this complex form of lethal organ injury.

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Materials and Methods

All rats used in this study were obtained from a commercial supplier (Microbiological Associates, Bethesda, Md) or bred in this laboratory and were of the Wistar-Furth (W/Fu) ($Rt1^u$) or Lewis (LEW) ($Rt1^l$) strains. W/Fu was selected as donor strain in this investigation because of its well-documented resistance to the mast-cell-damaging effects of horseradish peroxidase.[12,13] The microvascular surgical technique for heterotopic intraabdominal cardiac transplantation in the rat has been previously described.[1,2] All donor hearts removed prior to transplantation were maintained at 4 C for a period not exceeding 20 minutes. The rejection time of allografts in this inbred rat strain combination is 6–8 days.[14]

This study consisted of 20 W/Fu rat cardiac allografts transplanted to normal nonsensitized LEW recipients and 10 W/Fu to W/Fu syngeneic heart graft controls. All cardiac grafts were beating, and all recipients were alive at the time of the arbitrary conclusion of the studies. The investigation may be divided into two groups, based upon the separate experimental protocols employed for ultrastructural tracer analysis.

Colloidal Carbon Tracer Studies

Two W/Fu cardiac allografts and 1 W/Fu syngeneic heart graft were evaluated at each daily time period from Day 1 to Day 6 after transplantation. Thirty minutes prior to the arbitrary conclusion of each study, 2.5 ml of a 10% solution of colloidal carbon (Pelikan Shellack Free Biological Ink, Lot Number C11/14321a Gunther Wagner Werke, Hannover, Germany) filtered immediately before use,[1] was administered intravenously under ether anesthesia through the femoral vein. The methods employed for *in situ* aldehyde perfusion fixation and for subsequent processing and embedding of the inner two-thirds of the entire right ventricle of all heart grafts and all recipients' own hearts for ultrastructural evaluation of colloidal carbon vascular labeling patterns have been been given in a previous publication by this laboratory.[1]

Horseradish Peroxidase (HRP) Tracer Studies

Two W/Fu cardiac allografts and 1 W/Fu syngeneic heart graft were studied at each daily time period from Day 3 to Day 6 after transplantation. Horseradish peroxidase, Type II, RZ 1.0–1.5 (Sigma Chemical Co., St. Louis, Mo) was administered intravenously through the femoral vein under ether anesthesia in a dosage of 20 mg/100 g recipient body weight dissolved in 0.75 ml saline/100 g recipient body weight, 30 minutes prior to arbitrary conclusion of each study. One W/Fu allograft studied at each time period as well as all W/Fu syngeneic heart grafts and all their W/Fu recipients' own hearts were fixed *in situ* by aldehyde perfusion fixation methods previously described.[1] Additional single allografts removed at each time period were immediately fixed by immersion in a paraformaldehyde-glutaraldehyde fixative used for perfusion fixation. We carried out parallel immersion fixation of allografts for comparison in order to eliminate the possibility of *in situ* autolytic changes that could conceivably arise as a result of incomplete aldehyde perfusion during the course of perfusion fixation of late-surviving allografts. The methods employed for subsequent tissue fixation and sectioning for histochemical detection of HRP reaction product in the inner two-thirds of the entire right ventricle of all heart grafts and recipients' own hearts have been detailed by Rona et al.[11] The histochemical and embedding procedures used in this study were a modification of the Karnovsky method[10] as developed by Wissig and Williams,[15] which was altered only in that the Graham-Karnovsky medium was adjusted to pH 7.6. One-micron-thick Epon-embedded sections of all blocks were evaluated by light microscopy for an overall semiquantitative assessment of the number of cardiac muscle cells showing intracellular HRP reaction product and for selection of the appropriate areas for ultrastructural studies. Ultrastructural analysis was carried out in a Philips EM-300 electron microscope.

In order to demonstrate the specificity of the histochemical procedure for detection of HRP reaction product, selected sections of heart grafts and recipients' own hearts were incubated in Tris buffer alone and in Graham-Karnovsky medium in which H_2O_2 had been omitted. To document the presence of endogenous peroxidase within granules of cells infiltrating the allografts, one allograft transplanted to a recipient that received intravenous saline only was removed at Day 5 and was processed by the same procedures employed for the HRP study group. Incubation of selected samples in Tris buffer only and in Graham-Karnovsky medium in which H_2O_2 was omitted was also carried out. All control material was evaluated by light and electron microscopy.

For correlative histologic studies, serial transverse sections of the residual left ventricle of all heart grafts and selected recipients' own hearts were processed by routine methods and stained with hematoxylin and eosin and hematoxylin-phloxin-saffran.

Results

Correlative Histologic Studies

All cardiac allografts in this study were divided into three general groups on the basis of histologic assessment of the overall severity of the rejection process. Each allograft was assigned to one of these groups, and the cumulative ultrastructural tracer data derived from each group were compared. Group I consisted of all 3 allografts examined at Day 1 and at Day 2, and 4 of 5 allografts removed at Day 3. This group showed either no significant alterations or occasional minor perivenous and/or subendocardial mononuclear cell aggregates. Group II consisted of 1 of 5 Day 3 allografts, all 5 Day 4 allografts, and 4 of 5 allografts examined at Day 5. The predominent histologic features were the presence of mononuclear cells diffusely distributed throughout the microcirculation and perivascular interstitial spaces, variable extravasation of erythrocytes, and diffuse interstitial widening with cardiac muscle cell separation. Focal alterations of cardiocytes as indicated by localized segmental hypereosinophilia was an infrequent and inconsistent additional finding. Group III consisted of 1 allograft examined at Day 5 and all 5 allografts removed at Day 6. This group was characterized by diffuse infiltration by mononuclear cells, multifocal areas of necrosis and replacement of cardiocytes, prominent erythrocyte compaction and mononuclear-cell aggregation within the microcirculation, and widespread interstitial hemorrhage.

Syngeneic heart graft controls showed no significant microscopic alterations when compared with the recipients' own hearts, except for a variable degree of epicardial infiltration by mononuclear cells and granulocytes, which was attributed to the effect of local factors related to the nature of the transplantation procedure.

Ultrastructural Tracer Studies

Group I: Early Phase of Rejection

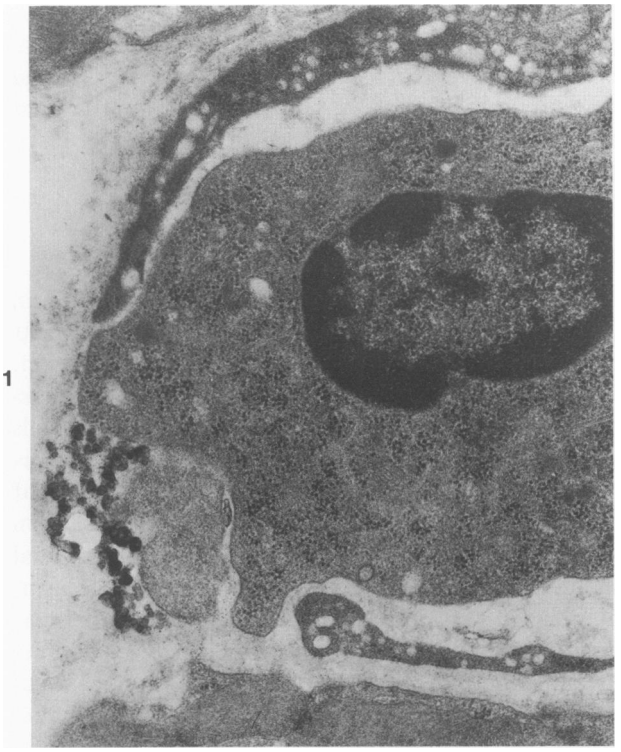
The ultrastructural characteristics of the microvascular endothelium of all allografts in this group were similar to those of syngeneic heart graft controls and of the recipients' own hearts. Colloidal carbon particles were exclusively confined to the lumens of incompletely perfused microvascular segments. Intravascular and extravascular fibrin was not observed. Horseradish peroxidase reaction product was localized to plasmalemmal vesicles and intercellular clefts of microcirculatory endothelial cells and also clearly delineated the extracellular interstitial space, including the extensive tubular network of the T system of cardiocytes. Tracer protein reaction product was not observed in the sarcoplasm of cardiac muscle cells by light or by electron microscopy in all of the material evaluated. Mononuclear cells were uncommonly encountered and were present largely within the lumens of microcirculatory segments or insinuated between the microvascular endothelium and the underlying basal lamina. Extensive mononuclear cell aggregates were rarely observed and were exclusively confined to the immediate perivenular interstitial spaces. In these foci, interdigitations between adjacent mononuclear cells as well as between mononuclear cells and ultrastructurally unaltered cardiocytes were clearly delineated by the extracellular HRP reaction product. Cells having ultrastructural features that have been attributed to lymphocytes[16] formed a conspicuous component of the cellular infiltrate. In addition, a significant number of mononuclear cells showed marked peroxidatic activity of discrete intracellular granules, which, on the basis of comparisons with the control histochemical data, indicated the presence of endogenous peroxidase. Horseradish peroxidase reaction product due to the presence of the exogenous tracer protein was also detected within membrane-bound vesicles and vacuoles of these cells but was otherwise excluded from other cellular constituents. Cells having the above ultrastructural characteristics were identified as exudate macrophages.[17,18] Peroxidatic staining patterns ascribed to resident macrophages and to resident-exudate macrophages[17]were not observed during the course of this study.

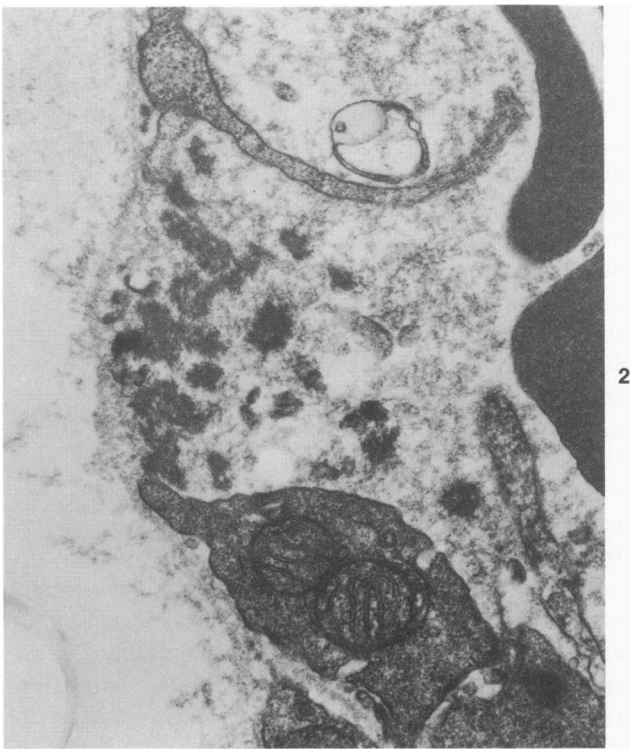
Group II: Intermediate Phase of Rejection

The microcirculation of all allografts in this group showed more conspicuous and diffuse margination and emigration of mononuclear cells. The microvascular endothelium of capillaries and venules[19] showed three distinct forms of ultrastructural alteration:

1) Short gaps separated otherwise ultrastructurally unaltered endothelial cells of capillaries and venules which were particularly well delineated by colloidal carbon tracer particles. These carbon-labeled interendothelial gaps were commonly but not exclusively encountered in relation to the advancing pseudopods of emigrating mononuclear cells, or in close proximity to mononuclear cells sequestered within the immediate perivascular interstitial space (Figure 1). Fibrin, a natural tracer, was encountered in relation to the exposed basal lamina at sites of interendothelial gaps (Figure 2) as well as diffusely dispersed throughout the interstitial spaces, particularly in allografts examined at Day 5 (Figures 2–5 and 8).

2) Markedly hypertrophic endothelial cells were frequently observed lining microvascular segments.





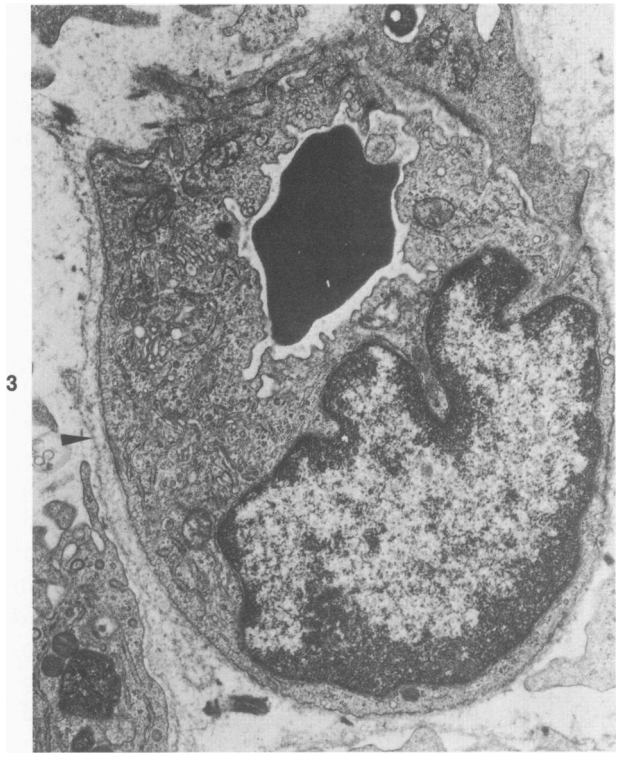


Figure 1 — Allograft at Day 4 showing the advancing pseudopod of an emigrating lymphoid cell at site of a carbon-labeled interendothelial gap. (Uranyl acetate en bloc–lead citrate, × 26,000) **Figure 2** — Allograft at Day 5 showing an interendothelial gap within a venular segment. Fibrin and carbon particles label the exposed segment of basal lamina. (Uranyl acetate en bloc–lead citrate, × 38,250) **Figure 3** — Allograft at Day 4 showing a venular segment lined by hypertrophic "activated" endothelium. There is marked reduction in luminal calibre and reduplication of the basal lamina (*arrow*). Extravascular fibrin is present. (Uranyl acetate en bloc–lead citrate, × 15,000)

These cells were characterized by numerous ribosomes, a well-developed rough endoplasmic reticulum, conspicuous lamellas of the Golgi apparatus, a variable number of dense bodies and multivesicular bodies, and prominent complex intercellular junctions that were commonly labeled by colloidal carbon. The underlying basal lamina often showed evidence of reduplication (Figure 3). Uncommonly, ultrastructurally unaltered endothelial cells appeared to be separated by short interendothelial gaps from

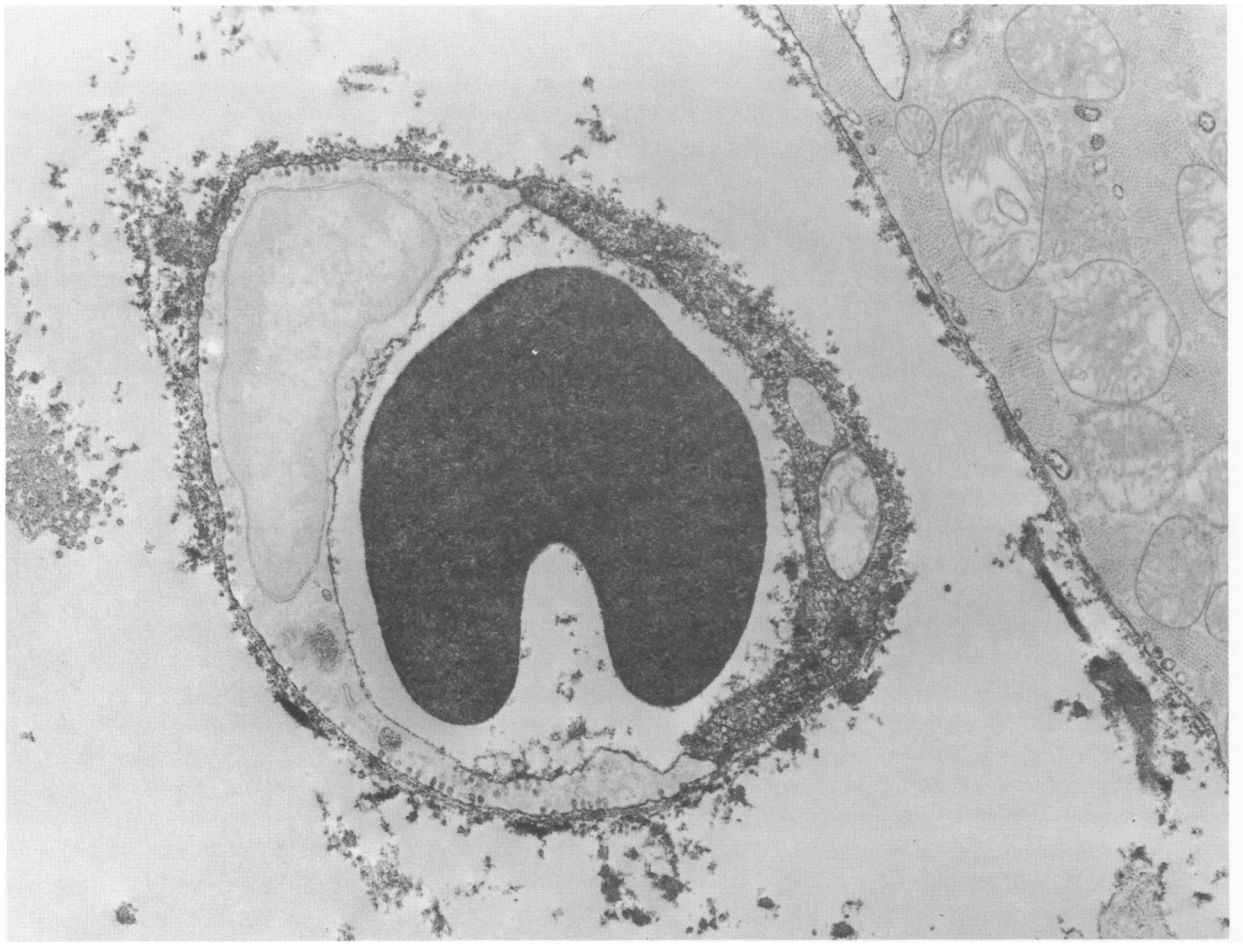
the apparent advancing folds or processes of hypertrophic endothelium.

3) In studies utilizing HRP as tracer, the cytoplasm of endothelial cells of occasional microvascular segments showed diffuse labeling by HRP reaction product, although tracer permeation of the mitochondrial and nuclear membranes was not present. A dense precipitate of HRP reaction product was commonly encountered on the surfaces of these cells as well as on the external aspects of plasmalemmal vesicles. Frequently a single endothelial cell showing diffuse cytoplasmic dispersion of HRP reaction product was sharply demarcated at the intercellular clefts from adjacent endothelial cells in which the HRP reaction product was confined exclusively to luminal and abluminal plasmalemmal vesicles (Figures 4 and 5). These microcirculatory changes were principally confined to allografts examined at Day 5.

Horseradish peroxidase reaction product clearly delineated the extravascular interstitial spaces and was bound to myofilaments of approximately 2% of cardiocytes in 3 of 4 allografts studied with this tracer. A single allograft examined at Day 5 showed no evidence of cardiac muscle cell penetration by HRP reaction product when assessed by both light and electron microscopy, although all forms of microvascular injury detailed above were clearly present. Individual cardiocytes which were permeable to HRP appeared focally and randomly scattered, and their ultrastructural integrity was generally otherwise well maintained. Mononuclear cells were diffusely dispersed throughout the interstitial spaces and commonly lay in close complex apposition to cardiocytes which were impermeable to the tracer, the interface of which was clearly demarcated by extracellular HRP reaction product (Figures 6 and 7). Infiltrated cells were also occasionally observed in close relation to cardiocytes showing intracellular HRP (Figure 9), although this was a highly inconsistent finding. Cells having ultrastructural features that have been ascribed to lymphocytes[22] formed a major component of the infiltrate. Exudate macrophages[23,24] were also commonly encountered (Figures 6–8). A striking feature was the presence of diffuse sequestration of HRP reaction product within the cytoplasm of occasional cells virtually all of which were ultrastructurally identified as exudate macrophages (Figure 9). The intracytoplasmic HRP appeared to be bound primarily to ribosomes and to external membranes of organelles (Figure 9, inset). Tracer permeation was excluded by the nuclear membrane, external mitochondrial membranes, lamellas of the Golgi apparatus, and other internal membranous structures. These cells were randomly dispersed, showed no apparent direct anatomic relationship to sites of microvascular or myocardial permeability alterations, and commonly lay in close proximity to mononuclear cells and to cardiocytes in which cytoplasmic HRP sequestration was clearly absent (Figure 9).

Group III: Late Preterminal Phase of Rejection

All of the microvascular alterations observed in Group II were conspicuous features of the allografts examined during the immediate prerejection period. Dense deposition of diffusely dispersed intracytoplasmic HRP reaction product within endothelial cells was commonly encountered throughout the microcirculation and was the cardinal morphologic feature of this group. Floccular precipitation of the reaction product was present on the luminal and abluminal surfaces of some cells as well as within the cytoplasm, obscuring overall structural continuity. In some areas the endothelium showed evidence of focal ultrastructural disintegration (Figure 10). All of these changes were equally conspicuous in allografts fixed by *in situ* aldehyde perfusion and by immersion. Endothelial cell denudation of extensive segments of the microcirculation was an additional feature. Compacted erythrocytes and aggregated mononuclear cells commonly lay enclosed within a circumferential scaffolding formed by the residual basal lamina (Figure 11). Intravascular fibrin was frequently encountered. Aggregated platelets were rarely observed. Epon-embedded 1-μ-thick sections of allografts showed intrasarcoplasmic HRP reaction product within approximately 10% of cardiocytes in a randomly scattered multifocal pattern. Some cardiac muscle cells permeated by the tracer showed HRP binding to myofilaments but were otherwise ultrastructurally unremarkable. Others showed tracer binding to markedly hypercontracted myofilaments, while a minority of cells showed advanced disintegration with coarse floccular intrasarcoplasmic deposition of the tracer, loss of ultrastructural continuity of sarcolemmal membranes, and the presence of fibrin within the sarcoplasm. Fibrin, erythrocytes, and dense clusters of lymphocytes and exudate macrophages were diffusely distributed throughout the interstitium. Granulocytes were infrequently observed. Deposition of HRP product was commonly encountered within the free cytoplasm of scattered mononuclear cells, many of which also showed variable degrees of ultrastructural disintegration as well as intranuclear penetration by the tracer. The majority of altered cells could be readily identified as



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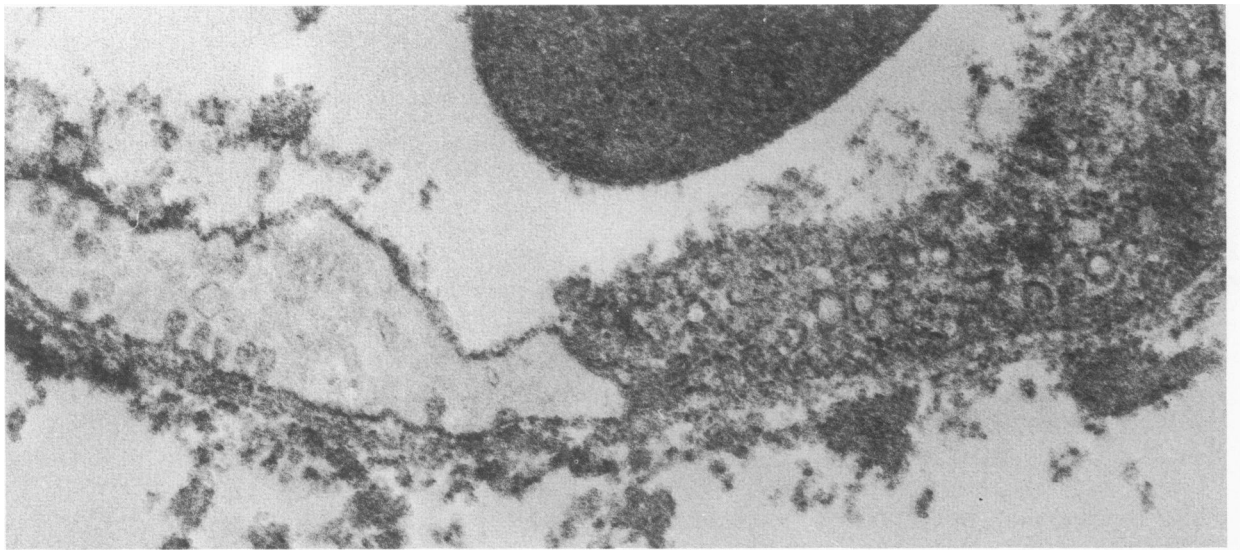

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Figure 4—Capillary segment of an allograft at Day 5 showing diffuse cytoplasmic labeling of an endothelial cell by HRP reaction product, which is sharply demarcated at the intercellular clefts from an adjacent endothelial cell showing normal tracer distribution. Interstitial fibrin is present. (Ferrocyanide-reduced osmium tetroxide, × 19,000) **Figure 5**—Interendothelial junction in Figure 4 at higher magnification. Precipitation of HRP reaction product is present in relation to luminal and abluminal cell surfaces as well as on the external surfaces of plasmalemmal vesicles of the tracer-permeated endothelial cell. The overall structural continuity is obscured. An adjacent endothelial cell shows reaction product localization confined to the interior of plasmalemmal vesicles. Extravascular fibrin is present. (×60,000)

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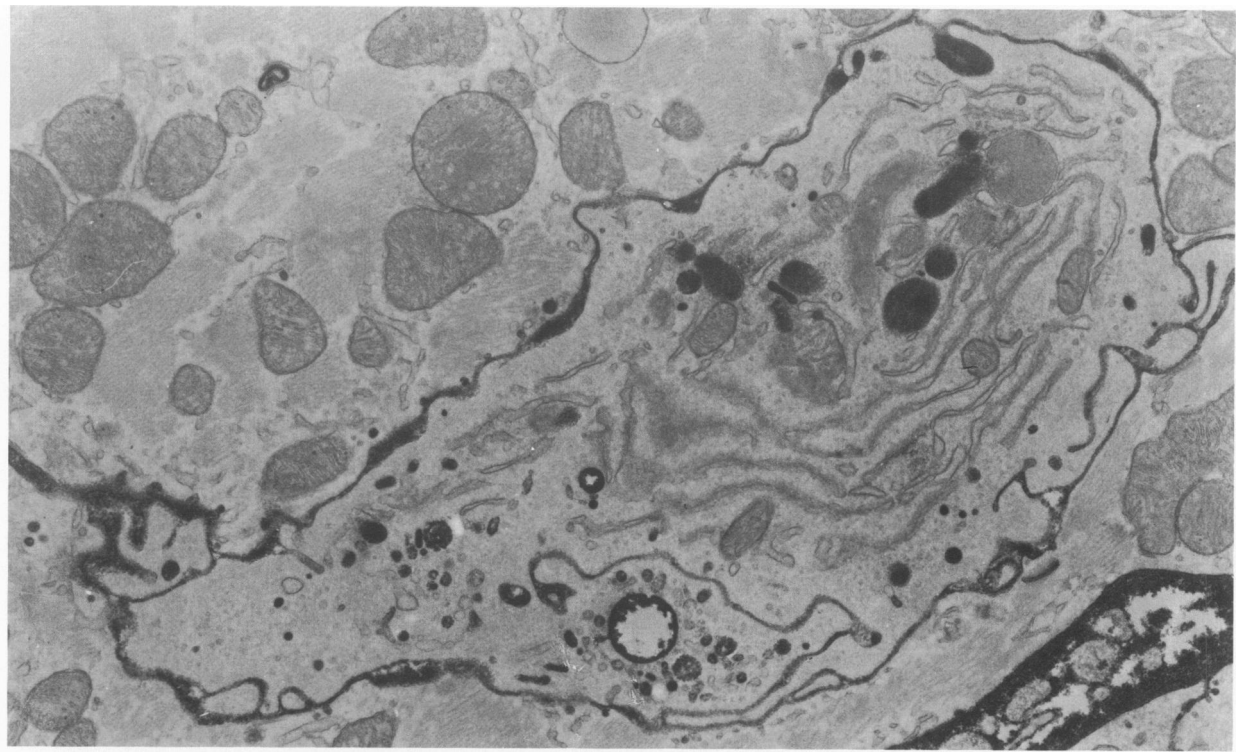

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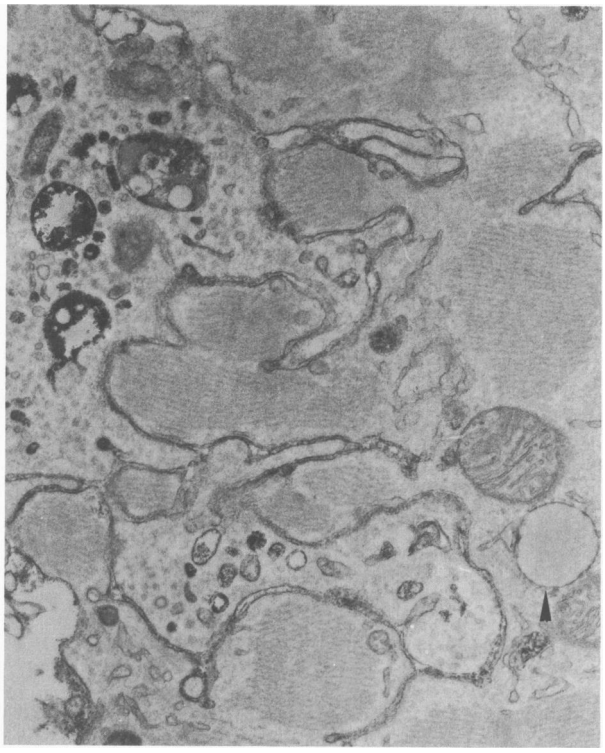
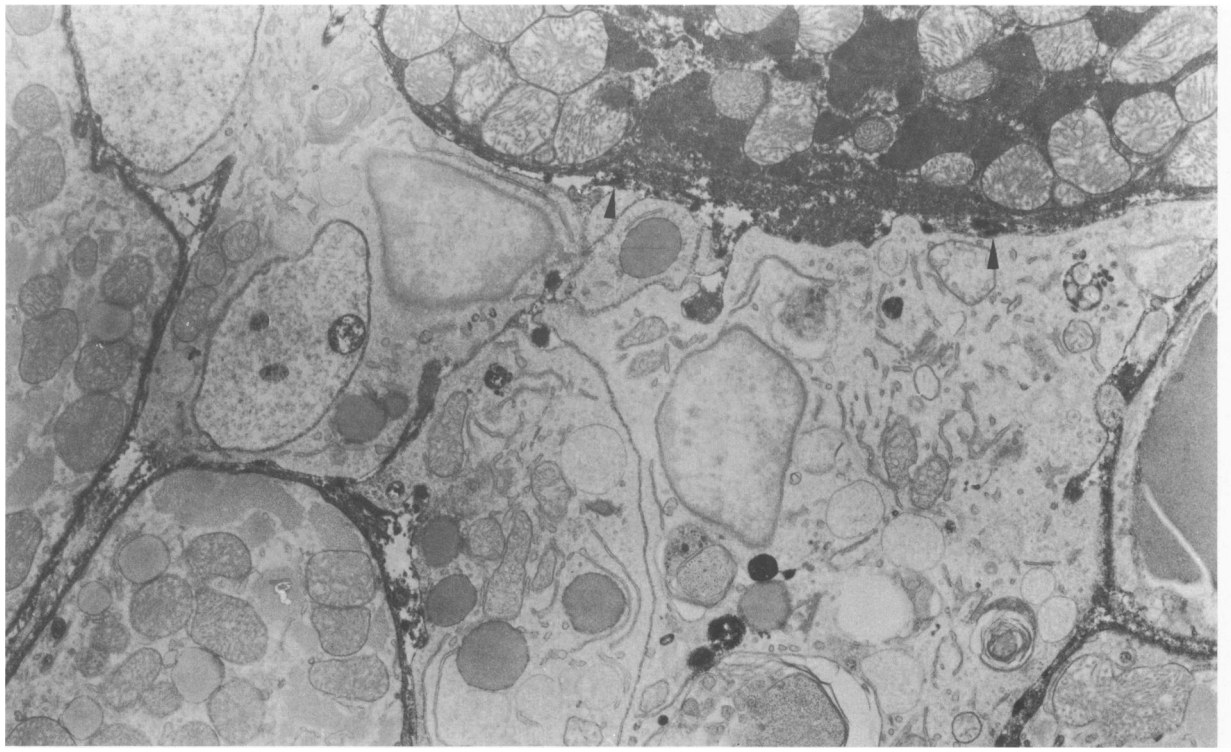


Figure 6—Allograft at Day 5 showing an exudate macrophage with prominent peroxidatic granules and vesicles in close apposition to an adjacent cardiocyte. Cellular interdigitations are sharply defined by extracellular HRP reaction product. There is no evidence of sarcoplasmic permeation by the tracer. (Ferrocyanide-reduced osmium tetroxide ×15,300) **Figure 7**—Allograft at Day 5 showing complex cellular interdigitations between the processes of an exudate macrophage with discrete peroxidatic granules and vacuoles and an adjacent cardiocyte, the extracellular interface of which is labeled by HRP reaction product. The intensity of staining of peroxidatic granules can be compared to that of an osmiophilic liposome of the cardiocyte (*arrow*). There is no evidence of sarcoplasmic permeation by the tracer. (Ferrocyanide-reduced osmium tetroxide, ×20,000)



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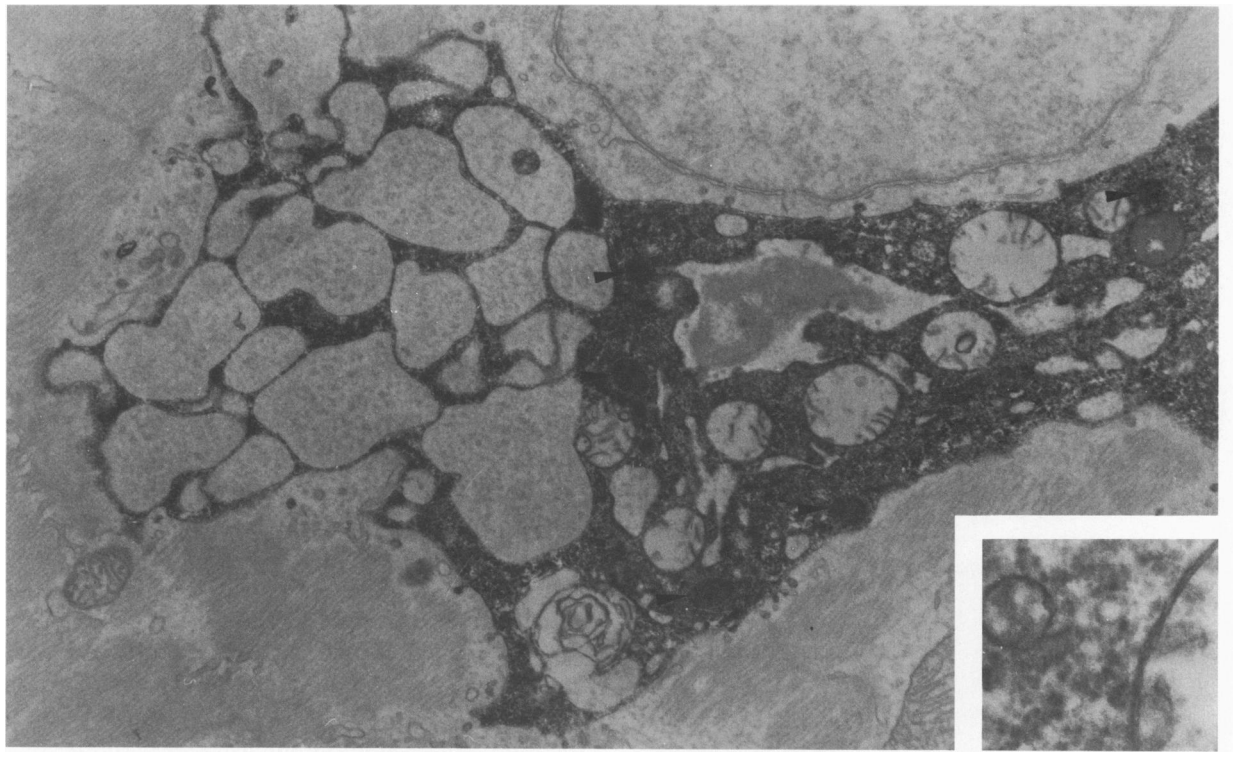

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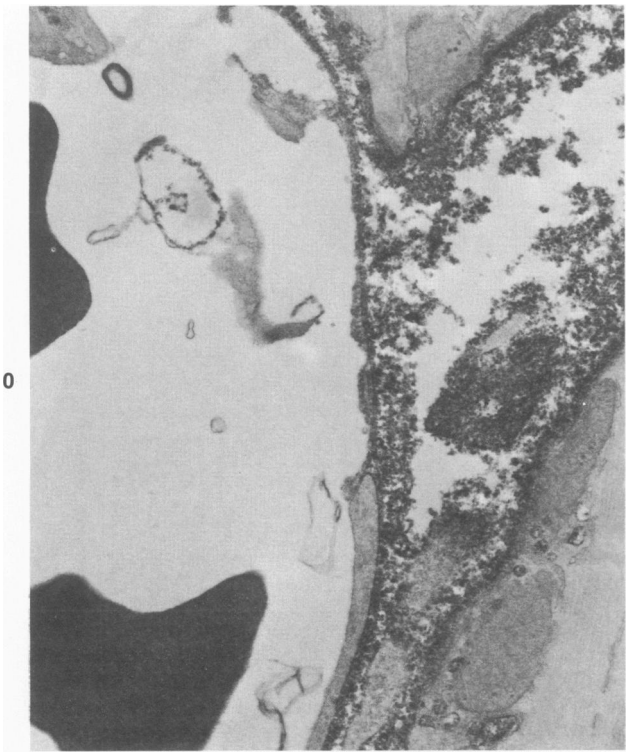
Figure 8—Allograft at Day 5. Aggregated interstitial mononuclear cells, some of which show peroxidatic activity of discrete granules and vacuoles, in close apposition to a cardiocyte in which there is intense myofilamentous binding of HRP reaction product. Fibrin is present in relation to the mononuclear-cell–cardiocyte interface (*arrows*). (Ferrocyanide-reduced osmium tetroxide, ×10,640) **Figure 9**—Allograft at Day 5. An exudate macrophage with peroxidatic activity of discrete granules (*arrows*) as well as diffuse cytoplasmic permeation of HRP reaction product lies immediately adjacent to a mononuclear cell and to a cardiocyte, which show no tracer permeation. The intracytoplasmic HRP reaction product appears to be primarily bound to ribosomes and to external membranes of mitochondria and other membrane-bound structures (**inset**). (Ferrocyanide-reduced osmium tetroxide, ×8740; **inset** ×54,000)



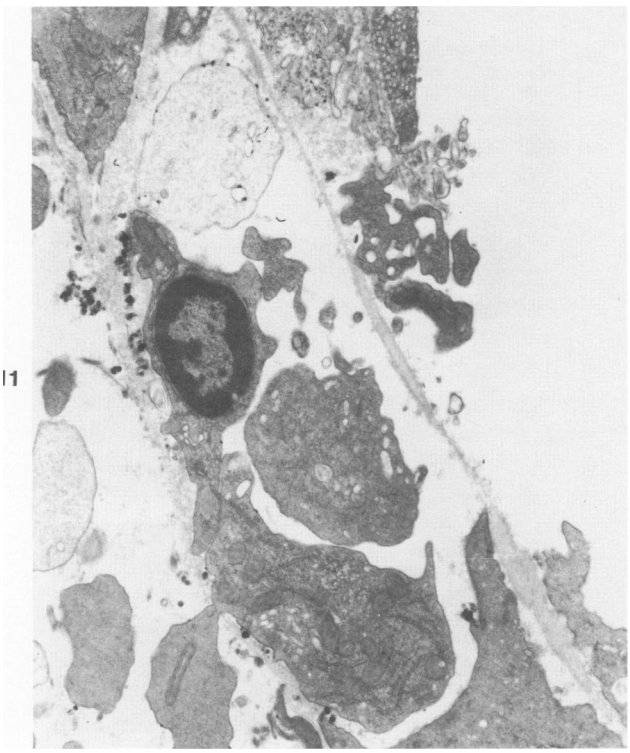


Figure 10—Allograft at Day 6 showing a microvascular segment in which an endothelial cell has undergone ultrastructural disintegration. Poorly defined discontinuous cellular remnants are sharply demarcated from an adjacent ultrastructurally unaltered endothelial cell. (Ferrocyanide-reduced osmium tetroxide, ×18,900) **Figure 11**—Allograft at Day 6 showing extensive segmental endothelial denudation. The lumen is occluded by aggregated mononuclear cells, one of which is emigrating to the interstitial space. Colloidal carbon labels the widely exposed basal lamina. Extravascular fibrin is present. (Uranyl acetate en bloc–lead citrate, ×13,000)

exudate macrophages.[17] These cells were widely and randomly dispersed throughout the infiltrate, were present in close approximation to ultrastructurally unaltered cardiocytes (Figure 12), as well as to cardiocytes showing myofilamentous HRP reaction product deposition, and commonly formed complex interdigitations with adjacent mononuclear cells, which often contained peroxidatic granules but remained impermeable to the tracer.

Syngeneic Heart Grafts and Recipients' Own Hearts

Syngeneic heart grafts showed no microvascular alterations. Colloidal carbon particles were confined to lumens of incompletely perfused microvascular segments. Horseradish peroxidase reaction product was detected exclusively within endothelial plasmalemmal vesicles, along interendothelial clefts, and throughout the extravascular interstitial spaces. The T-tubular system of cardiocytes was outlined by the HRP reaction product, but the tracer was uniformly excluded from cardiac muscle cells. Mononuclear cells, sometimes containing peroxidatic cytoplasmic granules, were only occasionally observed within the microcirculation and were rarely encountered in the immediate perivascular spaces. Intracytoplasmic dispersion of HRP was not present within intravascular or perivascular cells. The ultrastructural features of the recipients' own hearts were similar to those of the syngeneic heart graft controls.

Histochemical Controls for HRP and Endogenous Peroxidase

Selected sections of heart grafts and recipients' own hearts from the HRP study group which were incubated in Tris buffer alone or in Graham–Karnovsky medium in which H_2O_2 was omitted showed no peroxidatic staining. A single W/Fu cardiac allograft examined at Day 5 in which intravenous saline only was administered to the recipient prior to graft removal was processed in the same manner as the HRP study group. Endogenous peroxidatic activity of discrete cytoplasmic granules was observed in a proportion of mononuclear cells constituting the infiltrate. Dense staining of mononuclear cell granules was abolished by the omission of H_2O_2 from the reaction medium, where staining intensity was comparable to that of material incubated in Tris buffer alone. Staining of mononuclear cell granules under these latter

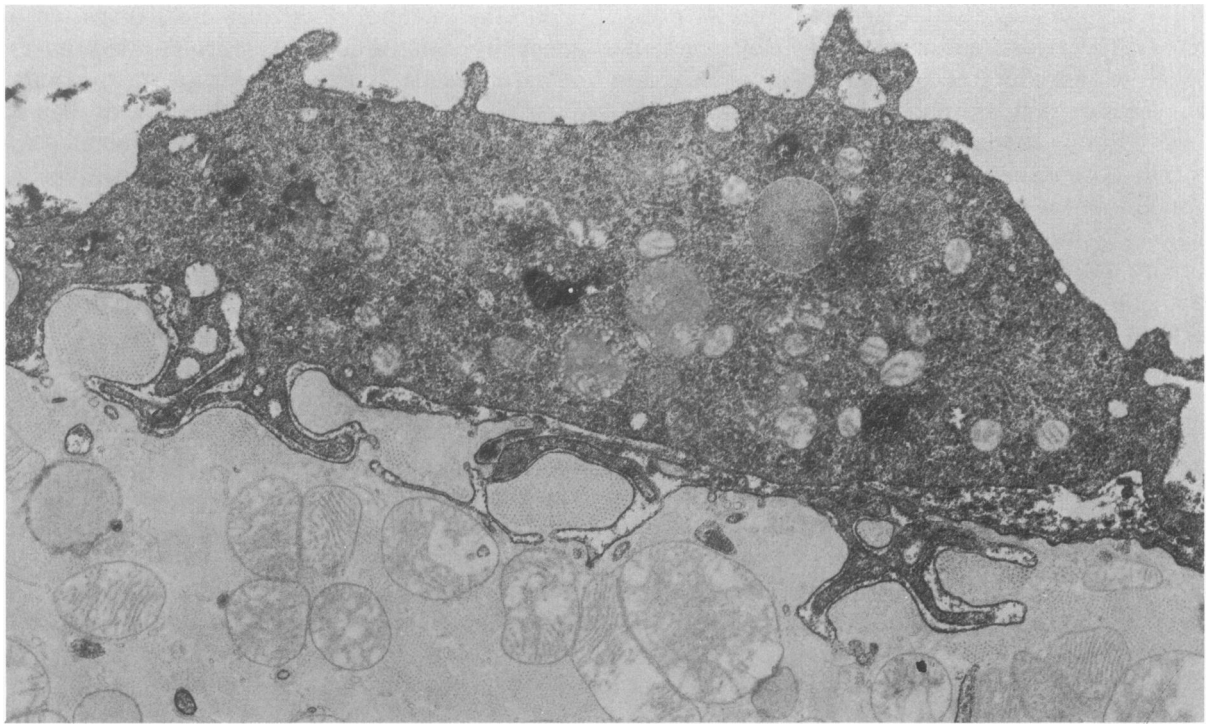


Figure 12—Allograft at Day 6 showing an exudate macrophage with discrete peroxidatic granules as well as diffuse cytoplasmic permeation of HRP reaction product forming complex cellular interdigitations with a closely apposed cardiocyte. The heart-muscle cell shows extracellular tracer labeling of elements of the T-tubular system, but sarcoplasmic permeation is not present. (Ferrocyanide-reduced osmium tetroxide, × 17,500)

conditions appeared less intense than that of osmiophilic liposomes of adjacent cardiac muscle cells.

Discussion

This controlled serial ultrastructural tracer study has clearly demonstrated that diffuse microvascular-endothelial injury is a characteristic feature of first-set rat cardiac allograft rejection and that profound microcirculatory changes precede the development of widespread permeability alterations and ultrastructural disorganization of cardiocytes. These data thus strongly suggest that the loss of integrity of the allograft microcirculation is the critical determinant of functional rejection in this model and that cardiac muscle cells are not the principal primary target of graft destruction.

The development of short gaps between adjacent ultratructurally unaltered endothelial cells of allograft capillaries and venules and the presence of high activated microvascular endothelium appeared to coincide with extensive mononuclear cell emigration during the intermediate and late phase of rejection. Similar microcirculatory alterations have been reported in ultrastructural tracer studies of a model of delayed-type hypersensitivity, where it was concluded that interendothelial gap formation was induced by an undefined permeability factor, which acted independently for leukocyte emigration.[20] No such distinction could be made in this investigation. Although the topographic distribution of the endothelial gaps observed in this study did not appear to coincide with the specific sites of action of histamine, serotonin, or bradykinin,[21] it is possible that these ultrastructural changes could directly result from the release of lymphokines or other mediator systems[21,22] by the infiltrating cells, since the morphologic effects of such agents upon the microcirculation remain uncharacterized. High activated endothelium, similar to that documented in the current study, has been shown to represent an ultrastructural manifestation of endothelial proliferation[23] and has been observed lining the microvasculature of rejecting renal allografts[24] and skin grafts[5] as well as in several forms of delayed-type hypersensitivity,[25,26] where its presence has been closely correlated with the degree of mononuclear cell infiltration.[27] The possible relationships that may exist between these microvascular changes and specific elements of cellular infiltrate or their products have not been clarified by the current investigation.

The most striking reflection of microvasculator

endothelial injury observed in this study was the presence of homogeneous dispersion of HRP reaction product within the free cytoplasm of endothelial cells, a feature that was particularly widespread and extensive during the late preterminal phase of rejection. The HRP tracer probe has clearly demonstrated the presence of a severe form of endothelial cell dysfunction resulting in altered plasma membrane permeability to blood-borne macromolecules, which appeared to progress directly to a state of overt ultrastructural disintegration. The abrupt onset and rapidly progressive nature of these changes, as well as the overall absence of close apposition of involved microvascular segments to elements of the mononuclear cell infiltrate, suggests mediation by membrane-active agents or other cell products rather than by cytotoxic mechanisms involving direct and persistent anatomic contacts. A similar pattern of cytoplasmic HRP sequestration has been reported in the endothelium of the rat cerebral microcirculation following administration of the polycation protamine sulfate, which was attributed to an induced reduction of anionic sites on the endothelial cell surface.[28] A similar pathogenetic mechanism could be involved in the current study. The possibility that circulating alloantibodies[29] could also play a mediator role cannot be entirely excluded. The relationship of interendothelial gap formation with this progression of lethal endothelial cellular injury remains unclarified.

Despite the marked alterations present within the allograft microcirculation during the late preterminal phase of rejection, occlusive platelet aggregates were only rarely encountered, although compacted erythrocytes and aggregated mononuclear cells commonly occluded the vascular lumens. Similar observations have been made by others in ultrastructural studies of delayed type hypersensitivity,[25] as well as in skin graft rejection.[5,6]

The ultrastructural alterations observed in HRP-permeated cardiocytes in this study have been shown by others to represent manifestations of nonspecific irreversible injury[11] but are similar to those known to occur as a direct result of microcirculatory hypoperfusion.[30] Although the current morphologic data do not exclude the possibility of cell-mediated destruction of cardiocytes, strong evidence has been presented to indicate that progressive primary destruction of heart-muscle cells by direct immunologic mechanisms is not the critical determinant of graft outcome in unmodified rat cardiac allograft rejection.

Fibrin, a natural tracer of increased vascular permeability, was diffusely dispersed throughout the allograft interstitium during the intermediate and late phases of rejection, which coincided with the occurrence of all forms of microvascular endothelial injury documented in this study. Extravascular fibrin accumulation is a cardinal feature of the delayed hypersensitivity reaction and has been implicated as the principal cause of skin induration in that setting.[31] It has been consistently observed in this laboratory and well documented by other workers[32] that rat cardiac allografts become progressively more firm or "indurated" during the intermediate and late phase of the rejection process (Day 4 to Days 6 and 7). It is thus possible that extravascular fibrin formation could contribute to "induration" of the myocardium, leading to reduced cardiac compliance with secondary alterations in coronary hemodynamics. Such a functional derangement could be a significant factor in the ultimate rejection of cardiac allografts. The identification of the cellular elements, or their products, which mediate fibrin formation in this setting requires further study.

Exudate macrophages, defined as mononuclear cells with dense peroxidatic cytoplasmic granules and the presence of HRP reaction product within membrane-bound vesicles and larger vacuoles,[17,18] constituted a significant component of the cellular infiltrate at all time periods, although no attempts at quantitation were made. This study has demonstrated the presence of HRP reaction product diffusely dispersed throughout the cytoplasm of some exudate macrophages, the proportion of which markedly increased during the late preterminal phase of allograft rejection. The random distribution of these functionally altered cells strongly suggests that intracytoplasmic HRP sequestration was not a reflection of cellular injury related to overall tissue hypoxia, but was an expression of individual cell dysfunction and degeneration. Given these findings, it is highly possible that cell membrane permeability to HRP could reflect a terminal degenerative stage of the macrophage cell cycle following a transient period of macrophage activation within the allografts, similar to that described in high-turnover granulomas associated with certain delayed hypersensitivity reactions.[33,34] Recent studies by other investigators have suggested that activated macrophages or their products[35] may play an effector role in rat cardiac allograft rejection.[8,36,37] However, this issue requires further clarification.

This study has yielded highly detailed sequential

data that define the nature and progression of target-organ destruction in unmodified first-set rat cardiac allograft rejection. Strong evidence is presented to indicate that the allograft microcirculation is the critical target of immunologic injury and that primary destruction of cardiac muscle cells is not a central determinant of graft failure. The overall morphologic features of cardiac allograft rejection in this model are somewhat similar to those observed in certain forms of delayed-type hypersensitivity[20,25] as well as in skin graft rejection,[5,6] where immunologically nonspecific lymphokine-mediated phenomena have been strongly implicated as the final common pathway of tissue damage.[38-41] This controlled serial ultrastructural tracer study provides a detailed basis for further comprehensive morphologic analysis of the nature of the critical effector mechanisms involved in this complex from of lethal organ injury.

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