DENDRITIC CELL–LYMPHOID CELL AGGREGATION AND MAJOR HISTOCOMPATIBILITY ANTIGEN EXPRESSION DURING RAT CARDIAC ALLOGRAFT REJECTION

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MHC antigen expression on cell surfaces is not a constant and stereotyped phenomenon, but may be induced or amplified under a variety of conditions (1–10). Immune stimulation of cellular MHC antigen expression has been seen both as a local and a systemic process (2–4, 6, 7, 9, 10), and has been postulated to result from the actions of lymphokines, particularly IFNs (11–17), released by cellular mediators of the immune response (18). Within the context of allograft rejection, modulation of donor MHC antigen expression on indigenous cellular elements of the graft could have a profound influence in facilitating or augmenting both the cellular sites of recipient allosensitization and the cellular targets susceptible to MHC class I and class II allospecific cytotoxicity (19–21). The inbred rat cardiac transplant model has been extensively used for experimental studies of primary vascularized organ allograft rejection. Since the pathogenesis of rejection in this system may be influenced by modulation of MHC antigen expression on cellular components of the rejecting graft, a clear sequential definition of MHC antigenic anatomy during the course of rejection is required. Recent published studies in this area have resulted in conflicting data (22–23). It has been reported (22) that acute cardiac allograft rejection in the rat is associated with massive induction of donor class II antigen expression on graft endothelial cells and cardiac myofibers. In contrast, other data indicate that induction of MHC class II cellular expression is restricted to the endothelium of large vessels during late rejection (23). We have thus reassessed this issue by carrying out a combined immunohistologic and immunoelectron microscopic (IEM)[1] study using a model of acute cardiac allograft rejection in the ACI → Brown Norway (BN) inbred rat strain combination. In this investigation we show that donor MHC class II cellular expression in the rejecting allografts is exclusively confined to interstitial dendritic cells (IDC), and that ongoing rejection is associated with localized IDC–lymphoid cell aggregates that may represent the in vivo counterpart of dendritic cell–lymphocyte clustering in vitro.

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[1] *Abbreviations used in this paper:* DAB, diaminobenzidine; IDC, interstitial dendritic cells; IEM, immunoelectron microscopy.

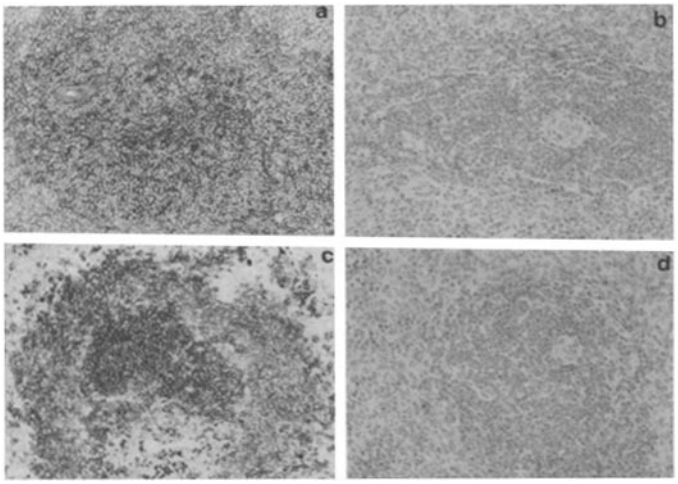

FIGURE 1. Cryostat sections of ACI and BN spleens stained with anti–class I mAb MN4-91-6 and with anti–class II mAb HIS 19, using an indirect immunoperoxidase procedure. (*a*) ACI spleen stained with MN4-91-6; (*b*) BN spleen stained with MN4-91-6; (*c*) ACI spleen stained with HIS 19; (*d*) BN spleen stained with HIS 19. × 120.

Materials and Methods

Animals. All animals used in this study were of the ACI ($RT1^a$) or BN, ($RT1^n$) inbred strains obtained from a commercial supplier (Harlan Sprague Dawley, Inc., Indianapolis, IN) or bred in this laboratory.

Transplantation Groups. Heterotopic intraabdominal cardiac transplantation was carried out by standard methods (24). For allograft studies, the ACI → BN inbred rat strain combination was used (mean survival time, 6–7 d) (25). ACI → ACI syngeneic heart grafts provided appropriate control tissue. Five rejecting ACI cardiac allografts in BN recipients were examined at each daily time period from day 2 to day 6. All allografts were beating and all recipients were alive at the time of graft removal. We examined three ACI → ACI syngeneic heart grafts at day 2, day 3, and day 6 after transplantation. We also used hearts and kidneys from five normal nontransplanted ACI and BN rats for control purposes. Two normal ACI rats received 2.5 ml of 10% solution of shellac-free colloidal carbon (Gunter Wagner Werke, Hannover, Germany), intravenously, 30 min before organ removal for histologic determination of microvascular labeling patterns.

Primary Antibodies. The following mAbs were used in this study: (*a*) MRC OX18, (ascites) reactive with a monomorphic determinant of RT1A, the major immunogenic class I antigen in the rat (26), was obtained from a commercial supplier (Cedarlane, Hornby, Ontario). (*b*) MN4-91-6, (partially purified ascites) reactive with ACI class I MHC antigen ($RT1A^a$) (22) but nonreactive with BN ($RT1A^n$), as assessed on multiple histologic sections of spleen and lymphoid tissues (Fig. 1, *a* and *b*), was provided by Professor J. W. Fabre, Blond McIndoe Centre, East Grinstead, United Kingdom. (*c*) MRC OX6 (ascites), reactive with a monomorphic determinant of rat MHC class II antigens expressed by the RT1B (I-A) region (26), was obtained commercially (Cedarlane). (*d*) 14-4-4S (serum-free supernatant), reactive with a monomorphic determinant of rat class II MHC antigens expressed by the RT1D (I-E) region, was provided by Dr. A. Goldner-Sauvé, McGill University) (27, 28). (*e*) HIS 19, (ascites) reactive with a rat MHC class II α chain determinant expressed by all strains except BN and other stains bearing the $RT1^n$ haplotype (reference 29; Fig. 1, *c* and *d*), was provided by Dr. R. J. M. Stett, University of Groningen, The Netherlands. (*f*) W3/25, (ascites) reactive with the CD4 antigen expressed by rat Th cells and some monocyte/macrophages (30, 31), was obtained from a commercial supplier (Cedarlane). (*g*) MRC OX8 (ascites) reactive with rat T cytotoxic-suppressor cells and NK cells (30) was obtained from a commercial supplier (Cedarlane).

Polyclonal rabbit antibovine Factor VIII–related antigen, an immunologic marker of vascular endothelial cells (32) also used in this study, was obtained commercially (Dimension, Mississauga, Ontario).

Immunohistology. Multiple left ventricular transverse sections of all allografts and syngeneic heart grafts were immediately embedded in OCT compound (Tissue-Tek, Naperville, IL), snap-frozen in cold isopentane and dry ice, and maintained at −70°C before use. All BN recipients' hearts, kidneys, spleens, parathymic, and cervical lymph nodes were also similarly processed for immunohistologic assessment. The indirect immunoperoxidase method used for examination of 5–6 μm cryostat sections has been previously detailed in another publication (33). Affinity-purified peroxidase-conjugated goat anti–mouse IgG $(Fab)_2$ (Cappel Laboratories, Malvern, PA) was used as secondary antibody for visualization of reactivity of all mAbs. For determination of Factor VIII–related antigen staining patterns, the secondary antibody used was peroxidase-conjugated, affinity-purified goat anti–rabbit IgG (Cedarlane). The peroxidase reaction product was detected using H_2O_2-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO). Negative controls included W6/32, a mouse mAb that does not react with rat tissues; control ascitic fluid where applicable; secondary antibody only; and DAB alone. For positive controls, sections of rat spleens were stained concurrently with all mAb each time the staining procedures were carried out, and the topograpic distribution of cellular staining with each mAb was compared with that previously established for each reagent, and to all of the above negative controls. We used the mAbs MRC OX18, MN4-91-6, MRC OX6, 14-4-4S, and HIS 19 for immuohistologic studies of normal ACI hearts, ACI cardiac allografts, and ACI syngeneic heart graft controls. Successive step sections of all allografts at day 4 were also stained with MAb W3/25 and MRC OX8, as well as again with HIS 19. All BN allograft recipients' lymph nodes and spleens were stained with the donor-specific class I and class II mAbs MN4-91-6 and HIS 19. All BN recipients' own hearts and kidneys were stained with the monomorphic mAbs MRC OX18, MRC OX6, and 14-4-4S. For parallel histologic observations, transverse sections of allografts, syngeneic heart grafts, and recipients' own hearts were fixed in neutral buffered formalin, processed in a routine manner, and H and E–stained sections were examined.

Immunoelectron Microscopy. For IEM, we also used the indirect peroxidase procedure. Primary antibodies studied in all allografts and syngeneic heart grafts were MRC OX18, MRC OX6, and HIS 19. Right ventricular sections of all heart grafts 5 mm in length and 1 mm in thickness were immersed for 4 h in freshly prepared 2% paraformaldehyde in 0.1 M phosphate buffer and 0.6% NaCl at pH 7.2. The tissue fragments were then chopped into 50-μm slices using a Smith-Farquhar Tissue Sectioner (TC-2, DuPont de Nemours/Sorvall Instruments Div., Newton, CT). After three successive 10 min washes in PBS (pH 7.2), the tissue was incubated with the primary mAb overnight at 4°C with agitation. After three successive 10 min PBS washes, the tissues were incubated with affinity-purified goat anti–mouse IgG $(Fab)_2$ for 2 h at 4°C with agitation. After three successive 10 min. washes in PBS, the tissues were incubated in 0.05% DAB in absence of H_2O_2 in 0.05 M Tris buffer at pH 7.8 for 10 min, and with DAB and H_2O_2 (0.01%) for 15 min at 37°C with agitation in the dark. After washing with water, the tissue was fixed in 1% osmium tetroxide for 1 h, dehydrated in graded alcohols, and embedded in Epon. Ultrathin sections selected from 1-μm Epon embedded thick sections were examined in a Philips EM 300 electron microscope. Controls included tissue incubated with secondary antibody alone, and with DAB only.

Interstitial Cell and T Cell Phenotype Quantitation Methods. For numerical assessments of the frequency of interstitial cells reactive with mAb HIS 19 in all normal ACI hearts, in some ACI to BN cardiac allografts, and in all ACI to ACI syngeneic heart grafts, the number of positive cells enclosed by a Leitz reticle at 25 × objective (0.04 mm^2) were enumerated in a random manner. This was based upon the observation that IDC of the normal rat heart show a uniform distribution. We counted positive cells enclosed within a total of 50 reticles from randomly selected areas. The resultant data were expressed as mean positive cell number/mm^2. All allografts at day 4 and some allografts at days 3 and 5 showed conspicuous localized, well-defined areas of HIS 19^+ cell aggregation at a density

far greater than that present in normal ACI hearts, which necessitated a modification in the method of HIS 19 cellular quantitation. Since in this and in other studies, the maximum number of class II–reactive interstitial cells encountered within a single 0.04-mm^2 reticle in the normal ACI heart has been found to be eight cells (A. G. Darden, R. D. C. Forbes, R. D. Guttmann, unpublished observations), we arbitrarily divided the sites for cellular enumeration in these particular allografts into areas of high-density HIS 19 cellular aggregation and into uniform background HIS 19^+ areas, on the basis of the presence or absence of ≥ 12 positive cells enclosed by a single reticle. The uniform background HIS 19 cell density was determined by random enumeration of areas containing <12 positive cells per reticle. Again, positive cells enclosed by 50 reticles were counted and expressed as number of cells/mm^2. The number of areas of high-density HIS 19 cellular aggregation was determined in a single transverse section of each allograft showing the greatest incidence of such areas, and positive cells enclosed by a single reticle in each of these areas were enumerated. For comparative purposes the mean high-density HIS 19 cellular frequency was expressed as number of positive cells/mm^2. Serial sections of all day 4 allografts were also consecutively stained with HIS 19, with W3/25, and with MRC OX8 to determine the relative frequencies of cells reactive with these mAb both in areas of high-density HIS 19 aggregation and in uniform background HIS 19^+ areas. Methods for enumeration of reactive cells in each of these areas were the same as those used for quantitation of HIS 19 cellular positivity. Statistical analysis was carried out by standard methods (Student's t test).

Results

Routine Histologic Studies

All allografts at day 2 showed negligible cellular infiltration and were generally similar to syngeneic heart grafts and to recipients' own hearts. A nonspecific epicarditis, secondary to the technical procedure of transplantation, was present in both allografts and syngeneic heart grafts. Two of five allografts at day 3 were similar histologically to those at day 2. The remainder showed multifocal, localized, generally perivascular mononuclear cell aggregates. There was a variable degree of diffuse subendocardial and interstitial infiltration, which by day 4 became more widened and pronounced. Day 4 allografts also showed minor focal areas of segmental myofiber degeneration and myocytolysis, in some cases associated with local erythrocyte extravasation. All allografts at days 5–6 were characterized by diffuse mononuclear cell infiltration, progressive microvascular disruption with widespread interstitial hemorrhage, and extensive myofiber necrosis. Large arteries showed minimal alterations throughout the course of rejection. There was negligible cellular infiltration of all syngeneic heart grafts, and with the exception of nonspecific epicarditis, these grafts were generally similar in appearance to recipients' own hearts.

Immunohistologic and Immunoelectron Microscopic Studies of Class I MHC Antigens

Normal ACI Hearts. Normal ACI hearts examined with both donor-specific class I mAb MN 4-91-6 and the monomorphic class I mAb MRC-OX18 showed a narrow, irregularly continuous interstitial staining pattern throughout, with numerous sites of branching and arborization in close proximity to myofibers (Fig. 2*a*). Adjacent sections stained with anti–Factor VIII–related antigen showed a generally similar staining distribution (Fig. 2*b*). Local intraluminal deposition of intravenously administered colloidal carbon was restricted to this

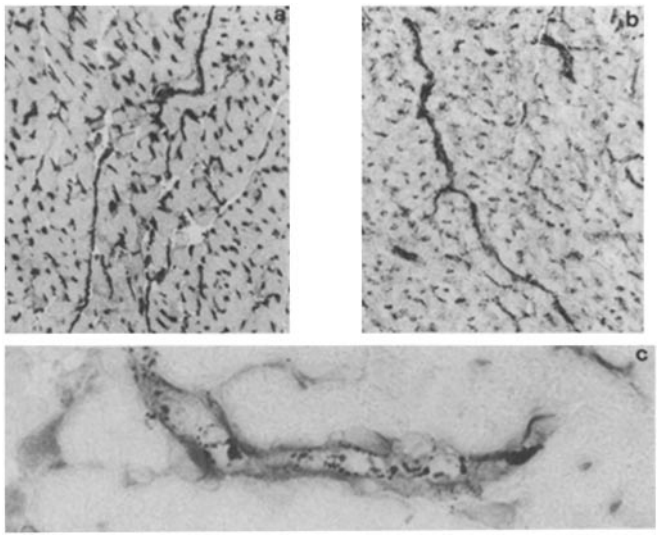

FIGURE 2. Cryostat sections of a normal ACI heart stained with anti-class I mAb MN4-91-6 and with anti-Factor VIII antibody. (*a*) Microvascular-interstitial staining pattern with MN4-91-6, × 120; (*b*) generally similar staining pattern with anti-Factor VIII antibody, × 120; (*c*) endothelial staining with MN4-91-6 of a microvascular segment containing intraluminal colloidal carbon, × 600.

extensive network of interstitial class I and anti-Factor VIII antigen staining (Fig. 2*c*). The microvascular endothelium was thus clearly class I-reactive, as has previously been reported in the normal rat heart (22, 23, 34). The endothelium of large muscular vessels exhibited a similar staining pattern. In addition, both class I mAbs stained isolated interstitial cells, some of which showed morphological characteristics of IDC, which was confirmed by IEM (35). However, when serial-step cryostat sections were examined histologically with anti-Factor VIII antigen, some apparently discrete interstitial cells showed characteristic granular cytoplasmic staining, and thus represented capillary endothelial cells in oblique section. This indicated that meaningful histologic quantitation of IDC, based upon overall interstitial cell reactivity with anti-class I reagents alone, was not possible in the rat heart. Cardiac myofibers showed no histologic evidence of reactivity with either of the class I mAbs, although both mAbs appeared to stain intercalated discs. IEM using MRC OX18 as primary antibody failed to reveal sarcolemmal, cytoplasmic, or junctional myofiber staining.

ACI Cardiac Allografts in BN Recipients. In allografts up to day 5, the vascular/interstitial staining pattern with both donor-specific class I mAb MN4-91-6 and anti-Factor VIII-related antigen was comparable to normal ACI hearts. From days 3–5, an increased intensity of microcirculatory endothelial staining with MN4-91-6 was observed, primarily at sites of focal cellular infiltration. This may represent a localized increase in density of donor class I antigen expression by graft endothelium in these areas. At days 5 and 6, the pattern of interstitial staining with both MN4-91-6 and anti-Factor VIII-related antigen became markedly fragmented and discontinuous. This coincided with the extensive disintegration of the allograft microcirculation seen in routine histologic sections.

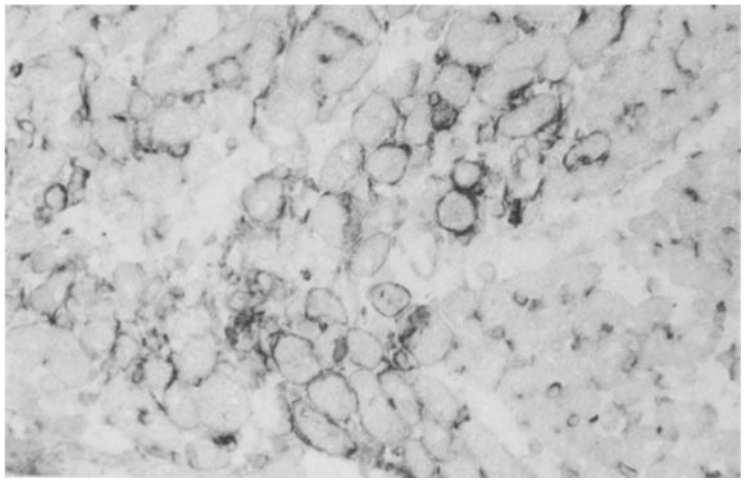

FIGURE 3. Cryostat section of an ACI → BN cardiac allograft at day 4 stained with anti–class I mAb MN4-91-6. Groups of myofibers are present in transverse section, some of which show distinctive circumferential surface staining. × 250.

In contrast, continuous circumferential staining of large vessel endothelium, similar to that seen in normal ACI hearts, was present throughout the course of study. In sections examined with the monomorphic class I mAb MRC OX18, discrete staining of microvascular endothelium became progressively more obscured from day 3 onward because of concomitant reactivity of numerous cellular elements of the interstitial infiltrate. However, large vessel endothelial staining was readily apparent.

Class I reactivity of cardiac muscle cells was not seen by either light or electron microscopy over the first 3 d after transplantation. By days 4–6, scattered groups of myofibers showed surface staining with MN4-91-6, indicative of donor MHC class I expression. These immunohistologic changes could be unequivocally identified by the presence of discrete circumferential surface reactivity in transverse sections of cardiac muscle cells (Fig. 3). In longitudinal sections of myofibers, however, interpretation was somewhat more difficult. Commonly, apparent segmental MN4-91-6$^+$ surface staining of myofibers showed a similar staining pattern with anti–Factor VIII–related antigen in serial-step sections, indicative of anti–class I staining of closely approximated endothelial elements. Myocyte staining with MRC OX18 could not be generally assessed by light microscopy after day 3, because of extensive interstitial accumulation of infiltrating cells reactive with this mAb. However, at days 4–6, IEM studies with MRC OX18 showed the presence of a continuous pattern of sarcolemmal staining of groups of cardiac muscle fibers that were not in immediate proximity to reactive elements of the infiltrate (Fig. 4). There was no histologic or ultrastructural evidence of an overall increase in intensity or degree of class I myocyte surface expression over this time period.

At days 5 and 6, coarse cytoplasmic staining of groups of myofibers was detected with all anti–class I and anti–class II mAb, as well as in sections incubated with the horseradish peroxidase–conjugated secondary antibody alone. Concur-

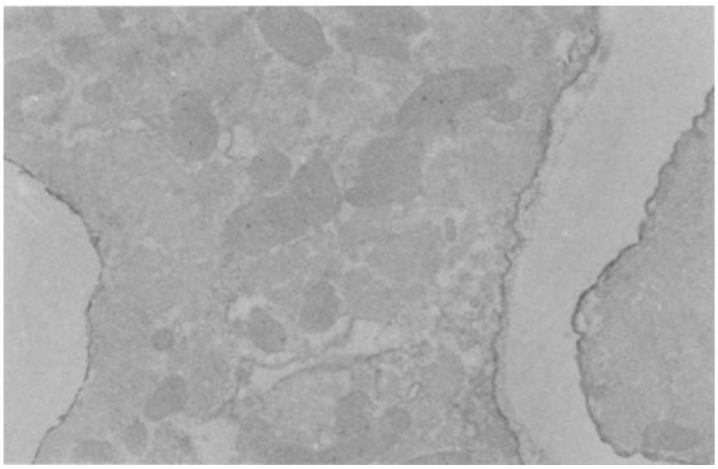

FIGURE 4. IEM of cardiac myofibers of an ACI → BN allograft at day 4 using an indirect immunoperoxidase procedure. There is distinctive surface sarcolemmal staining with anti-class I mAb MRC OX18. × 16,800.

rent IEM examinations using each of these reagents commonly revealed the presence of electron-dense granular material within the sarcoplasm of ultrastructurally altered cardiocytes showing extensive cell membrane discontinuities. Horseradish peroxidase has been used as an electron-dense protein tracer for detection of irreversible sarcolemmal permeability alterations of cardiac myofibers in a variety of experimental conditions (36). The appearance and distribution pattern of the intrasarcoplasmic electron-dense material seen in the current study at days 5–6 were similar to those documented in markedly altered cardiac myofibers after systemic administration of horseradish peroxidase alone. These observations provide evidence that the diffuse cytoplasmic staining of cardiac muscle cells seen with both primary and secondary antibodies in allografts during late rejection was due to nonspecific sarcoplasmic accumulation of the protein reagents as a direct result of myofiber necrosis.

ACI Syngeneic Heart Grafts in ACI Recipients. Syngeneic heart grafts examined at days 2, 3, and 6 showed a staining pattern with mAb MN4-91-6 and MRC OX18 that was indistinguishable from normal ACI heart controls. Cellular infiltration of all the heart grafts was negligible. There was no suggestion of myofiber reactivity with both anti-class I reagents.

Class II MHC Antigens

Normal ACI Hearts. All normal ACI hearts showed the same staining pattern with monomorphic anti-class II mAbs MRC OX6 and 14-4-4S as was seen with HIS 19, which has been shown to be reactive with ACI class II alloantigens, but does not react with BN, as a feature of the $RT1^n$ haplotype (29). Staining was exclusively confined to interstitial cells that were randomly distributed throughout the myocardium without topographic predilection. Intracytoplasmic colloidal carbon was not seen in these positive cells. The vast majority of stained cells showed histologic characteristics of IDC (Fig. 5*a*), and this was confirmed by IEM. The mean result of random enumeration of HIS 19^+ interstitial cells in

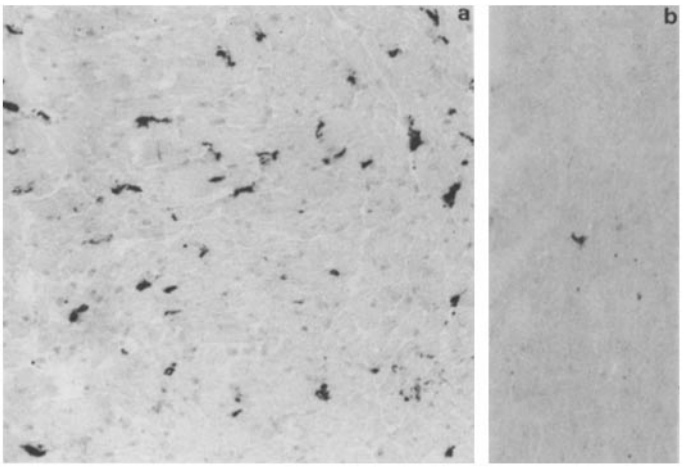

FIGURE 5. Cryostat sections of a normal ACI heart (*a*), and an ACI → BN allograft at day 2 (*b*), stained with anti–class II mAb HIS 19. (*a*) Extensive staining of interstitial cells, many of which exhibit IDC morphology; (*b*) a marked reduction in HIS 19 cellular staining. × 160.

the normal ACI heart controls was 55 ± 6.3/mm^2, as indicated in Table I (time, day 0). There was no staining of vascular elements or of cardiac myofibers.

ACI Cardiac Allografts in BN Recipients. As shown in Table I, ongoing rejection was associated with marked numerical fluctuations in HIS 19 IDC density within allografts as a function of time. All allografts at day 2, and three of five allografts at day 3 showed a significant reduction in HIS 19 cellular staining when compared with normal nontransplanted ACI hearts (Fig. 5*b*). In contrast, two day 3 allografts and five day 4 allografts showed an overall frequency of HIS 19 IDC staining that appeared to exceed that present in normal ACI controls. In addition, the uniform distribution of HIS 19–reactive IDC, which was consistently seen in nontransplanted ACI hearts, was altered by the presence of multiple compact high-density aggregates of HIS 19$^+$ IDC (Fig. 6). These focal areas were associated with dense localized accumulations of unstained lymphoid cells. For histologic quantitation of HIS 19$^+$ IDC density in those allografts where a similar lack of uniform IDC distribution was present, we arbitrarily divided cryostat sections into high density IDC aggregates and into uniform background IDC areas on the basis of the presence or absence of ≥12 HIS 19$^+$ cells per 0.04-mm^2 reticle (see Materials and Methods). As indicated in Table I, the uniform background IDC frequency in two of five allografts at day 3 and all allografts at day 4 was not significantly different from normal ACI heart controls. In addition, there were a total of 43 high-density IDC aggregates distributed among these allografts that showed a highly significant localized increase in HIS 19$^+$ cellular staining when compared with both uniform background areas and normal ACI controls. Serial-step sections through 25 high-density IDC aggregates distributed among day 4 allografts were stained with mAbs HIS 19, W3/25, and MRC OX8, and the density of cellular staining with each of these reagents was compared with that present in uniform background IDC areas (see Materials and Methods). As shown in Table II, the proportion of lymphoid cells in areas of high-density IDC aggregation expressing the W3/25 Th cell class II–reactive marker was

TABLE I

Frequencies of Donor Class II IDC in ACI → BN Cardiac Allografts

Time (d)	Number of grafts	Mean IDC in uniform background* (frequency/mm²)	High density aggregates*	
			Total aggregates‡	Mean IDC (frequency/mm²)
0§	5	55.0 ± 4.3	—	—
2	5	9.5 ± 4.3‖	—	—
3	3	9.7 ± 4.3‖	—	—
	2	60.5 ± 6.3¶	11	377.5 ± 37.5‖
4	5	44.8 ± 13.5¶	32	352.5 ± 52.5‖
5	5	14.8 ± 10.5‖	3	375.0 ± 75.0‖
6	5	1.5 ± 1.0‖	—	—

* Cryostat sections were arbitrarily divided into high-density class II IDC aggregates and into uniform background areas on the basis of the presence or absence of ≥12 cells reactive with donor-specific class II IDC mAb HIS 19/0.04 mm². High-density class II aggregates were seen only in allografts 3–5 d after transplantation.

‡ Multiple cryostat sections of each allograft were stained with mAb HIS 19. The number of high-density aggregates was determined in that single transverse section of each allograft that showed the greatest number of aggregates. Total aggregates represents the sum of all aggregates present in the selected transverse sections of all allografts at each daily time period or subdivided time period 3–5 d after transplantation.

§ Grafts designated day 0 represent normal nontransplanted ACI heart controls.

‖ Significant difference when compared with normal nontransplanted ACI heart controls ($p < 0.01$).

¶ No significant difference when compared with normal nontransplanted ACI heart controls ($p > 0.05$).

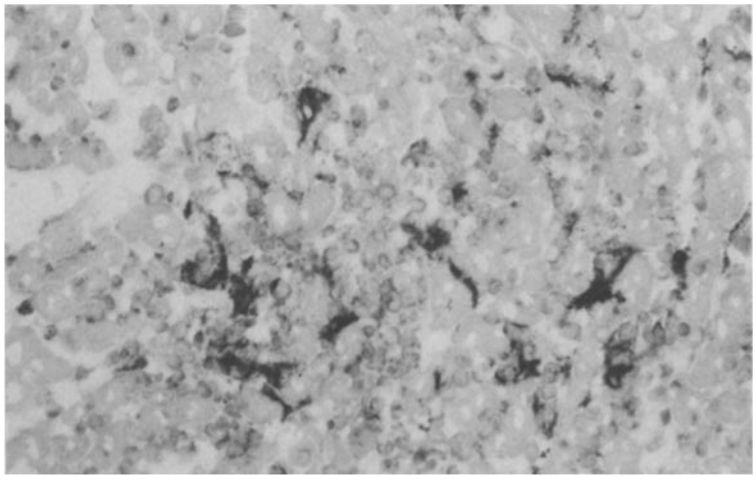

FIGURE 6. Cryostat section of an ACI → BN cardiac allograft at day 4 stained with anti-class II mAb HIS 19. There is prominent local aggregation of HIS 19⁺ cells with IDC morphology in an area of extensive local sequestration of smaller lymphoid cells unreactive with the antibody. × 250.

TABLE II

Mean Frequencies of W3/25- and MRC OX8–reactive Cells in ACI → BN Cardiac Allografts at Day 4

Allografts (day 4)	IDC*	W3/25‡	MRC OX8‡	Ratio of W3/25 to MRC OX8
		cells/mm²		
High density IDC aggregates§‖	363 ± 48	1,216 ± 334	547 ± 150	2.29 ± 0.50
Uniform background IDC areas§‖	46 ± 6	76 ± 16	140 ± 18	0.53 ± 0.12

* Donor class II IDC were enumerated on the basis of cellular staining with mAb HIS 19.

‡ The mAb W3/25 is reactive with rat CD4 antigen and is a marker of the class II–reactive Th cell subset (30). The mAb MRC OX8 is a marker of the class I–reactive T cytotoxic/suppressor subset (30).

§ Cryostat sections were arbitrarily divided into high-density IDC aggregates and uniform background IDC areas, as outlined in Table I.

‖ A total of 25 high density IDC aggregates among five allografts 4 d after transplantation were evaluated.

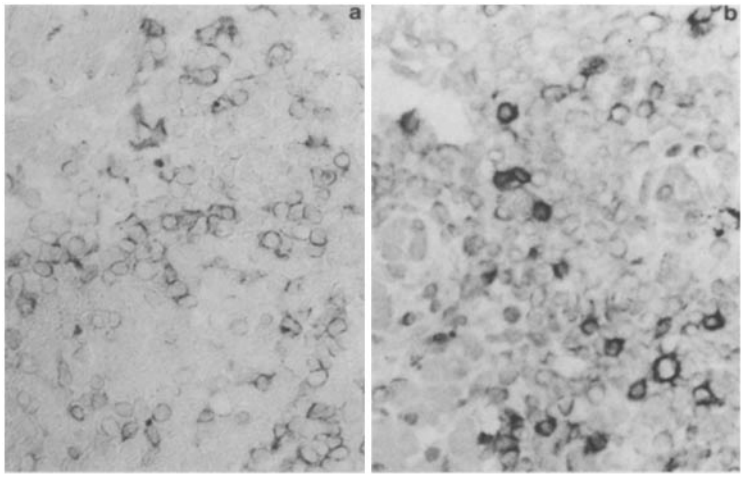

FIGURE 7. Successive cryostat sections of an ACI → BN cardiac allograft at day 4 in an area of high-density IDC aggregation. (*a*) Cellular staining with W3/25; (*b*) cellular staining with MRC OX8. There is a greater proportional cellular staining with W3/25 than with MRC OX8, and all stained cells resemble lymphocytes. × 250.

approximately twice that which stained with MRC OX8, a marker of the rat T cytotoxic/suppressor class I–reactive subset (Fig. 7, *a* and *b*). In contrast, MRC OX8–reactive cells significantly exceeded those stained with W3/25 in uniform background IDC areas, and the W3/25 to MRC OX8 ratio was essentially reversed when compared with the high-density aggregates. The vast majority of cells reactive with each of these reagents resembled lymphocytes. By IEM using HIS 19 as primary antibody, positive cells in the high-density aggregates were characterized by numerous elongate irregular processes that lay in close complex apposition to clusters of unstained mononuclear cells showing the fine structural characteristics of lymphocytes. The ultrastructural features of the HIS 19^+ cells were those of IDC (Fig. 8). As shown in Table I, there was a significant fall in uniform background HIS 19 IDC by day 5, and only three high-density IDC

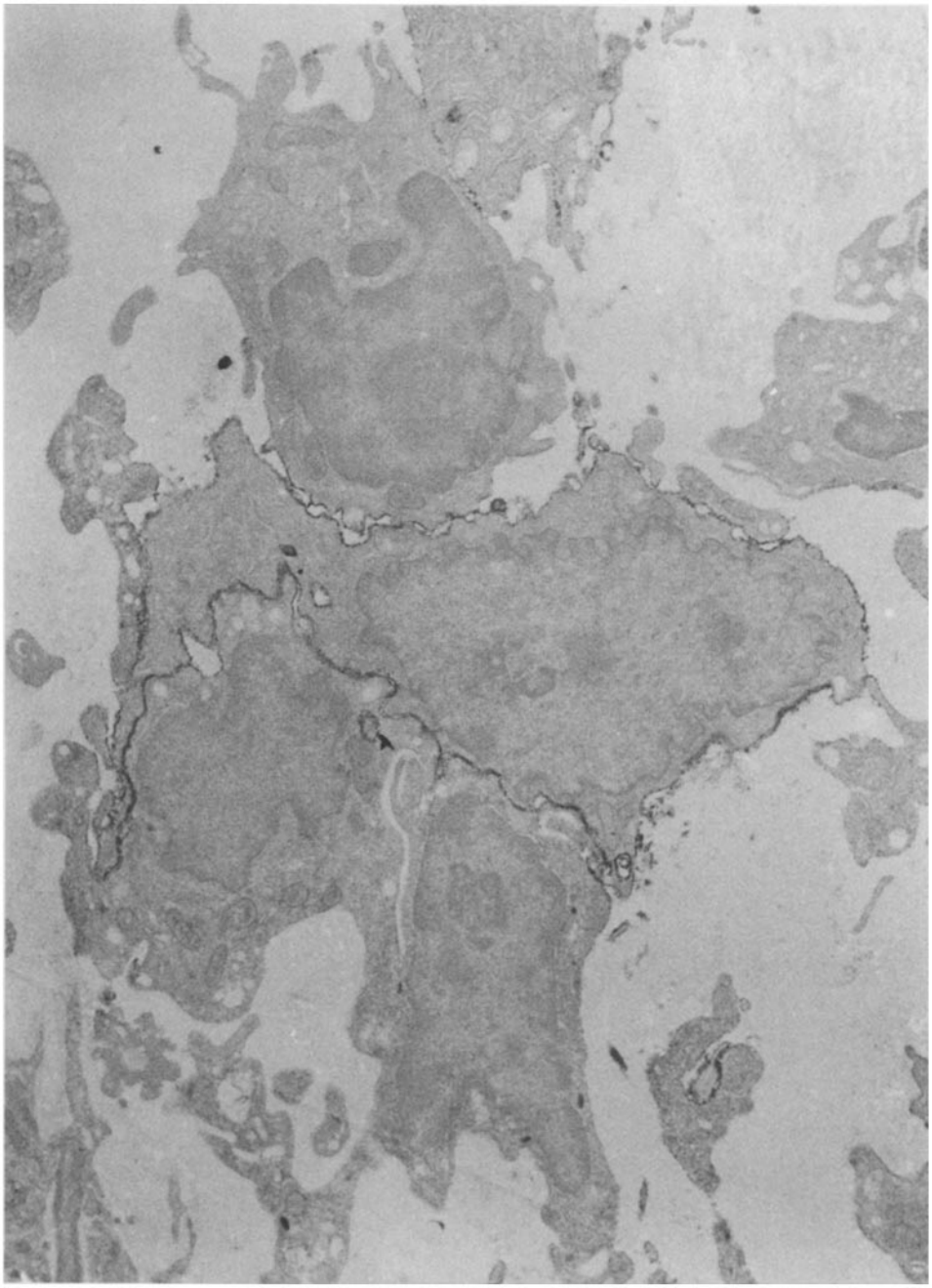

FIGURE 8. IEM of an ACI → BN cardiac allograft at day 4 stained with anti-class II mAb HIS 19. Unstained lymphocytes are clustered in close apposition to a HIS 19$^+$ cell showing complex processes characteristic of IDC. × 11,000.

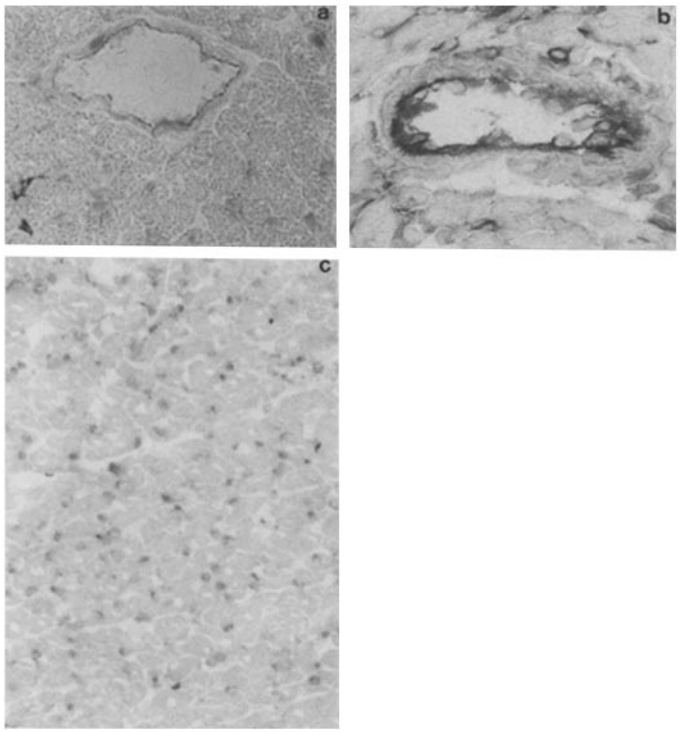

FIGURE 9. Cryostat sections of an ACI → BN cardiac allograft at day 5. (*a*) Section stained with anti-class II mAb HIS 19 showing absence of endothelial staining of a large vessel. The internal elastic lamina is visible. There is staining of adjacent interstitial cells. Cardiac myofibers are unstained. (*b*) Section stained with anti-class I mAb MN4-91-6 shows distinctive endothelial staining of a large vessel, as well as staining of adjacent interstitial cells. (*c*) Section of myocardium stained with HIS 19 shows no staining of myofibers or microvascular elements, although rare positive interstitial cells are present. × 250.

aggregates were seen among the five allografts. At day 6, high-density IDC aggregates were not encountered, and there was a profound depression in HIS 19^+ IDC frequency as compared with normal ACI controls. This progressive loss of class II–reactive IDC directly coincided with extensive overall cellular destruction of the allografts seen in routine histologic sections.

Donor class II reactivity within the allografts was exclusively confined to IDC throughout the course of rejection. The endothelium of the microcirculation and large blood vessels showed no staining with HIS 19, MRC OX6, and 14-4-4S mAb (Fig. 9*a*). This was in contrast to the definite donor class I staining of vascular endothelium in both normal hearts and rejecting cardiac allografts (Fig. 9*b*). There was no surface staining of cardiac muscle cells with HIS 19 (Fig. 9, *a* and *c*), nor was there convincing evidence of surface reactivity with the monomorphic class II mAbs MRC OX6 and 14-4-4S, despite staining of a significant component of the cellular infiltrate. This persistent absence of myofiber class II reactivity in histologic sections was further substantiated by serial IEM studies, which also consistently failed to show myofiber surface staining with HIS 19 (Fig. 10). In parallel examinations using the monomorphic mAb MRC OX6, sarcolemmal staining of cardiac myofibers was localized, segmental, sharply demarcated,

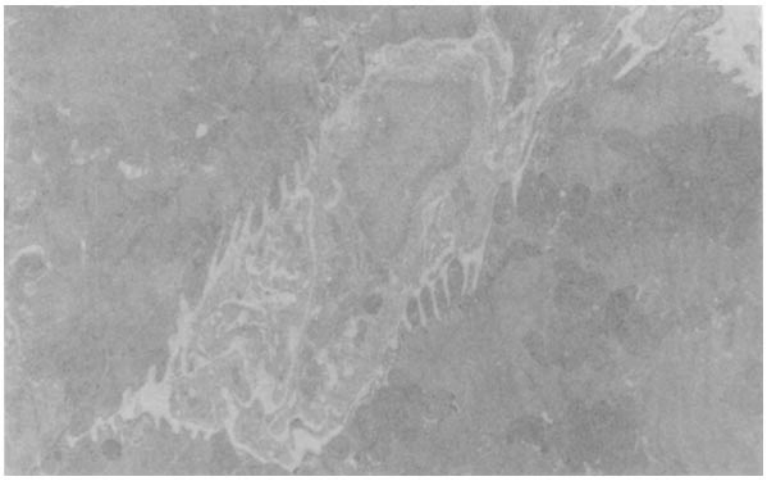

FIGURE 10. IEM of an ACI → BN cardiac allograft at day 6 stained with anti–class II mAb HIS 19. There is no cardiac myofiber staining, as compared with the positive sarcolemmal staining with anti–class I mAb MN4-91-6 in Fig. 4. A mononuclear cell in the adjacent interstitium is also unstained. × 4,200.

and exclusively confined to immediate sites of close apposition to MRC OX6$^+$ mononuclear cell components of the infiltrate. This represents a previously described (37, 38) diffusion staining artifact of the ultrastructural immunoperoxidase method which was also occasionally seen in normal nontransplanted ACI hearts where class II–reactive IDC processes lay immediately adjacent to sites of highly selective, localized, nonspecific sarcolemmal staining.

ACI Syngeneic Heart Grafts in ACI Recipients. All three syngeneic heart grafts at days 2, 3, and 6 showed minimal cellular infiltration and were generally comparable in appearance to recipients' own hearts. Cellular staining with all MHC class II mAbs was confined exclusively to discrete interstitial cells that commonly displayed IDC characteristics. There was no evidence of vascular or myocardial staining in the grafts. The absence of specific myofiber surface reactivity was confirmed by IEM using both MRC OX6 and HIS 19 as primary antibodies. All syngeneic heart grafts at day 2 and one graft at day 3 showed a marked reduction in IDC cellular staining with all anti–class II mAbs. The density of HIS 19 cellular reactivity was 9.8 ± 1.3/mm^2, which was significantly less than normal ACI controls ($p < 0.01$), but was similar to ACI cardiac allografts examined at the same time period ($p > 0.05$). In two syngeneic heart grafts at day 3 there was a mean HIS 19 cellular frequency of 45.8 ± 8.8, which was also comparable to normal ACI hearts ($p > 0.05$). Syngeneic heart grafts at day 6 showed a HIS 19 frequency of 51.8 ± 6.8/mm^2, which was also similar to nontransplanted ACI controls ($p > 0.05$). Most of these cells exhibited IDC characteristics.

MHC Antigen Expression In Tissues of BN Recipients Of ACI Cardiac Allografts

Multiple sections of spleens, parathymic, and cervical lymph nodes of all BN allografts recipients were stained with donor-specific mAbs MN4-91-6 and HIS

19 to assess possible migration of donor heart–derived class I– and/or class II–reactive interstitial cells to these sites during the course of rejection. Despite extensive immunohistologic examination, there was no cellular reactivity with either of the mAbs in recipient lymph nodes and spleens over the study period. There was thus no histologic evidence for peripheral recipient cellular sensitization by donor graft-derived IDC.

For comparative immunohistologic assessment of class I and class II expression in BN recipients' own hearts and kidneys, we used the monomorphic mAbs MRC OX18, MRC OX6, and 14-4-4S. The staining distribution of all recipients' own hearts with these reagents was similar to normal ACI and BN heart controls. The pattern of MHC class I and class II cellular staining of recipient kidneys was also similar to normal controls and correlated with previously published (39) immunohistologic observations of normal rat kidney. These observations exclude the possibility of systemic immunologic stimulation of MHC antigen expression (7) during the course of allograft rejection in this model.

Discussion

This study has shown that acute rat cardiac allograft rejection in the ACI → BN strain combination was associated with induction of donor MHC class I antigen expression on the surface of allograft cardiac myofibers, as well as with possible intensification of class I expression by the microvascular endothelium. Donor class II cellular expression was exclusively confined to allograft IDC, which are believed to represent the principal class II alloantigen presenting cells in the rat heart (40, 41). However, there were significant fluctuations in the relative frequency of IDC class II expression during the course of rejection. At days 3–4, when overall IDC class II cellular density was numerically greatest and exceeded normal controls, distinctive local compact IDC-lymphoid cell aggregates were present. Lymphoid cells in these focal areas showed a predominant proportional reactivity with mAb W3/25, a marker of the rat class II–reactive Th cell subset (30). These IDC–lymphoid cell aggregates may represent the in vivo counterpart of dendritic cell–lymphocyte clustering, which have been shown to be required for in vitro primary class II allosensitization in the mouse and rat (42–44). It is thus concluded that acute cardiac allograft rejection in the rat can occur in the absence of MHC class II expression by allograft vascular endothelium and cardiac myofibers. Since the IDC are the sole class II–reactive cellular elements in the rejecting grafts, it is difficult to conceive of a critical role for class II alloreactive cytotoxic T cell effectors in the pathogenesis of rejection in this setting. Specific elimination of allograft IDC by class II–allospecific cytotoxic T cells in the absence of concomitant vascular or myocardial damage would not alter cardiac function, and therefore, as an isolated effector mechanism would result in indefinite graft survival. This clearly does not occur.

Ongoing rejection of ACI cardiac allografts in BN recipients was associated with a localized increase in anti–donor class I staining intensity of microvascular endothelial elements, primarily at sites of significant cellular sequestration. This was particularly apparent before the onset of widespread graft necrosis. These observations were similar to those made by Milton and Fabre (22) in another rat cardiac allograft model. At days 4–6, there was unequivocal evidence of donor

class I surface staining of groups of allograft myofibers. By IEM, we also saw anti–class I sarcolemmal staining of cardiocytes over the same time period, using the monomorphic class I mAb MRC OX18. Although this mAb does not distinguish MHC class I antigens of donor and recipient origin, the IEM data were highly consistent with parallel immunohistologic observations made with the donor-specific class I mAb. MHC class I induction on cardiac allograft myofibers during the course of rejection has been reported in all inbred rat models previously studied (22, 23), as well as in human heart transplants (45). It has been suggested that this phenomenon may be related to the action of lymphokines, particularly IFNs, released by elements of the cellular infiltrate (22). As has been previously pointed out by others (22, 23, 34, 45), modulation of class I surface expression on cardiac muscle cells in the rejecting allografts could be quantitative rather than qualitative, since there may be a normal constitutive low-density class I expression on cardiac myofibers, which is below the limit of sensitivity of the in situ immunodetection methods used. However, a variety of lines of evidence indicate that the quantitative degree of cellular MHC antigen expression may play an important role in regulation of the immune response (19, 21). Thus, the induction and/or amplification of MHC class I expression seen on cardiac muscle cells and the microvascular endothelium in the rejecting allografts could serve both to augment overall sensitization of class I–reactive T cell precursors and to facilitate cellular destruction by direct allo–class I–specific cytotoxic mechanisms. The significance of these changes with respect to rejection tempo and ultimate graft outcome remains unclarified.

Donor MHC class II expression was confined exclusively to IDC in all rejecting cardiac allografts. However, there were marked variations in the relative frequency of class II–reactive IDC in allografts as a function of time. A generally similar fluctuating pattern of IDC class II cellular frequency was observed in subjective assessments of a rat cardiac allograft model by Steiniger et al. (23). In the current study, all allografts at day 2 and some allografts at day 3 showed a marked reduction in class II–reactive IDC to ~15% of that seen in normal nontransplanted controls. Similar observations were made in syngeneic heart grafts, although IDC density in recipients' own hearts showed no apparent variation. These data indicate that the early numerical fall in class II–reactive IDC in both allografts and syngeneic heart grafts was directly related to the transplantation procedure and not to the immunological process of rejection. Whether this represents a transient reduction in overall IDC class II surface expression or a net cellular IDC loss has not been clarified. Although it is theoretically possible that the reduction in allograft class II IDC population could temporarily impede recipient cellular sensitization to graft alloantigens, this would appear unlikely in light of the data of McKenzie et al. (41), which indicate that a level of only 5% of the class II–reactive donor IDC population are required to maintain the normal tempo of rat cardiac allograft rejection.

At day 4, and less frequently at day 3, there was an absolute increase in donor class II IDC density in the allografts as compared with normal controls. This was associated with a highly distinctive nonuniform cellular distribution, which was characterized by the presence of large numbers of donor class II–reactive IDC closely aggregated at sites of extensive local accumulation of recipient-derived

lymphoid elements. By IEM, these areas showed numerous unstained lymphocytes in close apposition to complex processes of donor class II–reactive IDC. Immunohistologic studies of the cellular infiltrate at sites of high-density IDC aggregation indicated that cells stained with W3/25, a marker of the class II–reactive Th cell subset, were approximately twice as frequent as those stained with MRC OX8, a marker of the class I–reactive T cytotoxic/suppressor cells. This was in contrast to findings in the uniform background IDC areas, where MRC $OX8^+$ cells predominated and the W3/25 to MRC OX8 ratio was essentially reversed when compared with the high-density IDC aggregates. It has been reported (46) that rat heart IDC may themselves be reactive with W3/25. However, by immunohistology virtually all $W3/25^+$ cells in the high-density IDC aggregates lacked evidence of cellular processes and resembled lymphocytes. Some $W3/25^+$ cells in the dense aggregates could also represent monocytes/macrophages (31), although the vast majority of nondendritic cells seen in these areas by IEM exhibited ultrastructural characteristics of lymphocytes. The morphologic data thus raise the strong possibility that these foci of high-density IDC–lymphocyte aggregation may be the in vivo counterpart of dendritic cell–lymphocyte clustering, which has been shown (42–44) to be absolutely required for primary allosensitization of class II–reactive Th cells in vitro. These compact areas of extensive IDC–lymphocyte localization could therefore represent a highly significant functional compartment within the rejecting allografts, where class II–reactive Th cells in close apposition to IDC proliferate, produce IL-2 and other lymphokines, and provide the drive to effector function of sensitized, allospecific class I–reactive cytotoxic T cell precursors (47). In addition, the predominance of MRC $OX8^+$ cells in nonaggregated uniform background IDC areas is consistent with data indicating that cytotoxic T cell precursors do not require specialized alloantigen presenting cells, but may be sensitized by any donor class I–bearing cellular element (47). Although it is possible that the $W3/25^+$ cells in the uniform background IDC areas may preferentially accumulate in relation to isolated IDC, no such direct relationship could be clearly shown in this study.

The origin of the increased population of class II–reactive IDC in allografts at days 3–4 remains unclear. In situ proliferation of class II–reactive IDC could occur, although we did not see mitotic figures. Alternatively, there could be induction of class II expression on in situ IDC precursors as a result of the actions of lymphokines or related agents produced by elements of the cellular infiltrate. It has been recently shown that class II IDC increase numerically in normal nontransplanted mouse hearts after systemic administration of recombinant IFN-γ (17), which is known to be released by activated T cells (18). Although a significant subpopulation of putative IDC with a $W3/25^+$, class II–negative phenotype has been described in normal rat hearts (46), such potential class II IDC precursors clearly could not be identified in an allogeneic system on the basis of expression of this monomorphic phenotype. At days 5 and 6 during the late preterminal phase of rejection, there was an abrupt numerical fall in donor class II IDC, which was associated with progressive overall cellular destruction of the allografts. Neither donor class I– nor class II–reactive cells were identified in recipient spleens or lymph during the course of rejection. There was thus no

histologic evidence for peripheral recipient cellular sensitization by donor graft-derived IDC.

There was no donor MHC class II surface expression on either vascular endothelium or cardiac muscle cells in the rejecting cardiac allografts. These findings are at variance with those of Steiniger et al. (23), who saw induction of donor class II staining restricted to large vessel endothelium during the late stage of rat cardiac allograft rejection, although donor class II staining of heart muscle cells and microvascular elements was not detected. It is also of interest that anti–class II myofiber staining has not been encountered in rejecting human heart transplants, despite possible amplification of constitutive endothelial class II expression (48). Our data are in particularly sharp contrast to those of Milton and Fabre (22), who reported massive MHC class II induction of both vascular endothelium and cardiac muscle cells during the course of rejection in an inbred rat cardiac model. We cannot account for their observations, as there was no suggestion of similar changes in the strain combination that we have used. Whatever the reasons for the discrepancies in results, the current study has clearly shown that acute rat cardiac allograft rejection can procede at a fulminant tempo in the absence of donor class II antigen expression by both allograft vascular endothelium and cardiac myofibers.

Summary

To determine the pattern of cellular expression of donor MHC class I and class II antigens during the course of rat cardiac allograft rejection, ACI cardiac allografts transplanted to BN recipients were examined from day 2 to day 6 using immunohistologic and immunoelectron microscopic methods. We used both monomorphic and donor-specific mouse anti–rat MHC class I and class II mAbs in this study. In normal ACI hearts, MHC class I reactivity was confined to the vascular endothelium and to interstitial cells. Ongoing rejection was characterized by an increased donor MHC class I staining intensity of microvascular endothelium and induction of donor class I surface reactivity on cardiac myofibers. Donor MHC class II reactivity was exclusively confined to interstitial dendritic cells (IDC) in both normal ACI hearts and in rejecting allografts, although rejection was associated with marked fluctuations in class II IDC frequency. An early numerical depression in class II IDC present in both allografts and syngeneic heart grafts was attributed to a direct effect of the transplantation procedure. By days 3–4, allografts showed an absolute overall increase in donor class II IDC frequency, which was associated with the presence of multiple localized high-density IDC–lymphocyte aggregates. The lymphocytes present in the focal areas were predominantly of the class II–reactive Th cell subpopulation. These aggregates may thus represent the in vivo homologue of dendritic cell–lymphocyte clustering, which has been shown to be required for primary class II allosensitization in the rat and mouse in vitro. During the late phase of rejection, there was a marked numerical fall in donor class II IDC, which correlated with extensive overall graft destruction. This study has shown that acute rat cardiac allograft rejection can occur in the absence of donor MHC class II expression by allograft vascular endothelium and cardiac myofibers. The IDC, which are believed to represent the principal class II alloantigen presenting

cells in the rat heart, remain the sole class II–expressing cellular constituents of the graft throughout the course of rejection.

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