

CHARACTERIZATION OF THE TISSUE MACROPHAGE AND
THE INTERSTITIAL DENDRITIC CELL AS DISTINCT
LEUKOCYTES NORMALLY RESIDENT IN THE CONNECTIVE
TISSUE OF RAT HEART

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The leukocytes normally present in the interstitial connective tissues of nonlymphoid organs are likely to play an important role in the physiology of the immune system. They might also be involved in the development of autoimmune responses, and, in transplantation, they make an important contribution to the immunogenicity of grafted organs (e.g., 1, 2). However, these cells are as yet poorly characterized, and have been studied mainly in organs potentially exposed to constant antigenic stimulation, such as liver, lung, and skin (e.g., 3). There have been virtually no studies on the leukocytes normally present in the connective tissues of antigenically secluded organs, such as the heart.

It has long been known that macrophage-like cells are present in the connective tissues of many organs (4). More recently, an intensely class II-positive leukocyte of dendritic morphology, and distinct in many ways from lymphocytes and macrophages, has been described in the connective tissues of all organs studied, except the brain, and named the "interstitial dendritic cell" (5). This cell appears very similar to the highly immunogenic dendritic cells described by Steinman et al. (6) in the organized lymphatic tissues, and is likely to be related to the dendritic Langerhans cell of the epidermis (7). The precise relationship of the interstitial dendritic cell to connective tissue macrophages has been uncertain. The possibility that the description of the interstitial dendritic cell might simply represent a redefinition of the tissue macrophage has led to some uncertainty.

Here we demonstrate that the class II-positive interstitial dendritic cells of the heart can be differentiated in the connective tissues from class II-negative leukocytes that react with a macrophage-specific mAb. In addition to phenotypic differences, there are interesting differences in the distribution of these two cell types within some organs and marked differences in radiation sensitivity. The presence of two distinct types of leukocyte in the connective tissues of quiescent, nonlymphoid organs such as the heart might have important physiological implications and could be important in understanding transplantation phenomena.

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Materials and Methods

Rats. Adult male rats of the DA (RT1^a), BN (RT1ⁿ), WAG (RT1^u), and SHR (RT1^k) strains were obtained from Harlan-Olac U.K. Ltd. (Bicester, UK).

BMAC-5 mAb. This was derived from a BALB/c mouse immunized with homogenates of spleens from DA strain rats. The splenocytes of the immunized mouse were fused with the mouse myeloma line NS-O using conventional techniques. The BMAC-5 cell line was selected on the basis of an interesting pattern of staining when screened on frozen sections of spleen using the immunoperoxidase technique. The antibody was used in the form of immune ascites partially purified by ion exchange chromatography. Ouchterlony tests on culture supernatants established that the BMAC-5 antibody was of the IgG1 subclass.

Other Antibodies. The MRC OX6 and MRC OX17 cell lines, secreting IgG1 mouse antibodies against rat RT1-B and RT1-D class II MHC antigens, respectively (8), were the kind gift of A. F. Williams (Cellular Immunology Unit, Sir William Dunn School of Pathology, Oxford). The mouse IgG1 antibody F15-42-1 to human Thy-1 (9), which does not react with rat tissues, was used as a control for the immunohistology studies. The antibodies were used as immune ascites partially purified by ion exchange chromatography. The ED1, ED2, and ED3 mAbs (10) were purchased from Serotec (Bicester, UK) and used as supplied.

For two-color immunohistological studies, rabbit antisera to the rat leukocyte common (LC)¹ antigen (CD45) and rat class II MHC antigens were used. The rabbit antisera to pure rat LC molecules were the same batches as previously described in detail (11).

Rabbit antisera to pure rat RT1-D class II MHC molecules were raised as follows. The class II MHC molecules were purified from detergent-solubilized DA spleen membranes using a combination of MRC OX17 mAb affinity and sepharose CL 6B gel filtration chromatography, essentially as described previously (e.g., 12-14). The final preparation was in 0.5% sodium deoxycholate (DOC). For the immunizations, the DOC was removed by sequential dialysis against 0.01 M Tris, pH 8.4, at 4°C (pH 8.4 buffer), and then physiological saline (0.15 M NaCl). A rabbit was immunized with 36 µg of class II protein in CFA intramuscularly to the thighs, and boosted at 3 and 7 wk after the first injection with 36 µg of class II antigen in IFA. The rabbit was bled at 7 and 14 d after the final boost. The sera were pooled, the Ig precipitated with sodium sulphate, and then further purified by G-200 gel filtration.

Immunohistology. Tissue samples for immunohistology were frozen in liquid nitrogen immediately after harvesting. Cryostat sections of 5 µm were cut on to gelatinized slides, air dried, fixed in acetone, and stored at -20°C until used. To stain the sections, the slides were thawed, and the appropriate mouse mAb or rabbit antiserum was put on the sections. These were incubated at room temperature for 30 min and washed twice in Tris-buffered saline. For immunoperoxidase staining, horseradish peroxidase-coupled rabbit anti-mouse Ig (Dako Corp., Copenhagen, Denmark) was used at a 1:7 dilution in 20% normal rat serum to block antibodies crossreacting with rat Igs. The second incubation was at room temperature for 30 min and the slides were then washed twice. The coloration was developed at 37°C with diaminobenzidine (Sigma Chemical Co., Poole, UK) at 0.6 mg/ml containing 3 µl of 100 vol hydrogen peroxide. Sections were counterstained with Harris's haematoxylin.

For two-color immunohistology, fluorescence rather than histochemistry was used. The primary antibodies (BMAC-5, MRC OX6, rabbit anti-LC, rabbit anti-class II) were incubated alone or in various combinations on frozen sections. After washing, the fluorescent anti-Ig antibodies were added, either alone or as a mixture. Rhodamine-conjugated goat anti-mouse Ig and fluorescein-labeled goat anti-rabbit Ig (Tago Inc., Burlingame, CA) were used at a final dilution of 1:10. The anti-Ig antibodies were diluted in 20% normal rat serum as mentioned above.

Nonspecific Esterase Staining. This was performed on unfixed frozen sections essentially as described by Ornstein et al. (15), using 2-naphthyl butyrate as a substrate at pH 6.0. The sections were then fixed in acetone, and immunohistochemistry with mouse mAb and

¹ Abbreviations used in this paper: DOC, sodium deoxycholate; LC, leukocyte common.

peroxidase-labeled rabbit anti-mouse Ig was performed as outlined above. The slides were then counterstained with Harris's haematoxylin.

Phagocytic Studies. Colloidal carbon (India ink; Rowney Kandohar, UK) was administered intravenously (40 μ l in 0.8 ml of saline), and the rats were killed 90 min later. Tissues were removed and processed for immunohistochemistry as outlined above.

Irradiation. Rats were given 1,000 rad of irradiation from ^{137}Cs using a Gammacell 1000 (Isomedix, Whippany, NJ) and killed 7 d later for immunohistological studies.

Results

Initial Immunohistological Studies

The BMAC-5 antibody on initial screening gave strong staining of the red pulp of spleen, but no staining of the white pulp or marginal zone. This suggested that it was a macrophage marker, and so the hybridoma was cloned and the antibody was studied on liver sections. The results were very interesting and are given in Fig. 1, *a* and *b*. Fig. 1 *a* shows that BMAC-5 stained isolated cells in the liver lobules, consistent with the staining of Kupffer cells. However, there was very little staining in the region of the portal triad, where the interstitial dendritic cells have previously been located (5). This was confirmed by staining a consecutive liver section with an anti-class II antibody (Fig. 1 *b*). The pattern was quite different, with many class II-positive cells, as expected, in the triad. This strongly suggested that the BMAC-5 antibody was staining tissue macrophages, but not interstitial dendritic cells, and might therefore be valuable for studies distinguishing these cell types.

The BMAC-5⁺ cells in the liver lobule were positively identified as Kupffer cells by injecting india ink into a rat, harvesting the liver 1 h later, and then staining frozen sections of the liver with the BMAC-5 antibody. All of the BMAC-5⁺ cells had taken up colloidal carbon (data not shown).

The ED series of mouse anti-rat macrophage mAbs are well established (10), and we therefore compared the staining of BMAC-5 with ED-1, ED-2, and ED-3 on sections of spleen, lymph node, thymus, heart, kidney, liver, and pancreas. BMAC-5 gave a pattern identical to ED-2, strongly suggesting that these two antibodies have the same specificity. To our knowledge, the ED series (10) has not been studied in detail on nonlymphoid tissues.

Detailed Immunohistological Studies

Staining of many nonlymphoid organs with the BMAC-5 antibody demonstrated staining of isolated cells in the connective tissues of most organs examined (Fig. 1, *c-g*). In the heart (Fig. 1 *c*), the pattern of staining resembled very closely that seen with anti-MHC class II antibodies (5), and this pattern could easily be misinterpreted as staining of the interstitial dendritic cells. In the pancreas (Fig. 1 *e*), it was interesting to note that the BMAC-5⁺ cells were seen throughout the organ, but only rarely in the islets of Langerhans. However, class II-positive cells were frequently seen in the islets, as previously reported (16). BMAC-5 also gave staining of isolated cells in the cerebrum (data not shown), which perhaps represented the microglia.

In striking contrast to the numerous BMAC-5⁺ cells seen in the connective tissues of liver, heart, pancreas, and intestine, such cells were virtually completely absent from both the cortex and medulla of kidney (Fig. 1 *f*). Very rarely, a few BMAC-5⁺ cells could be seen in the connective tissues surrounding an arteriole in the kidney, but this was exceptional. We were careful to compare the kidney with the

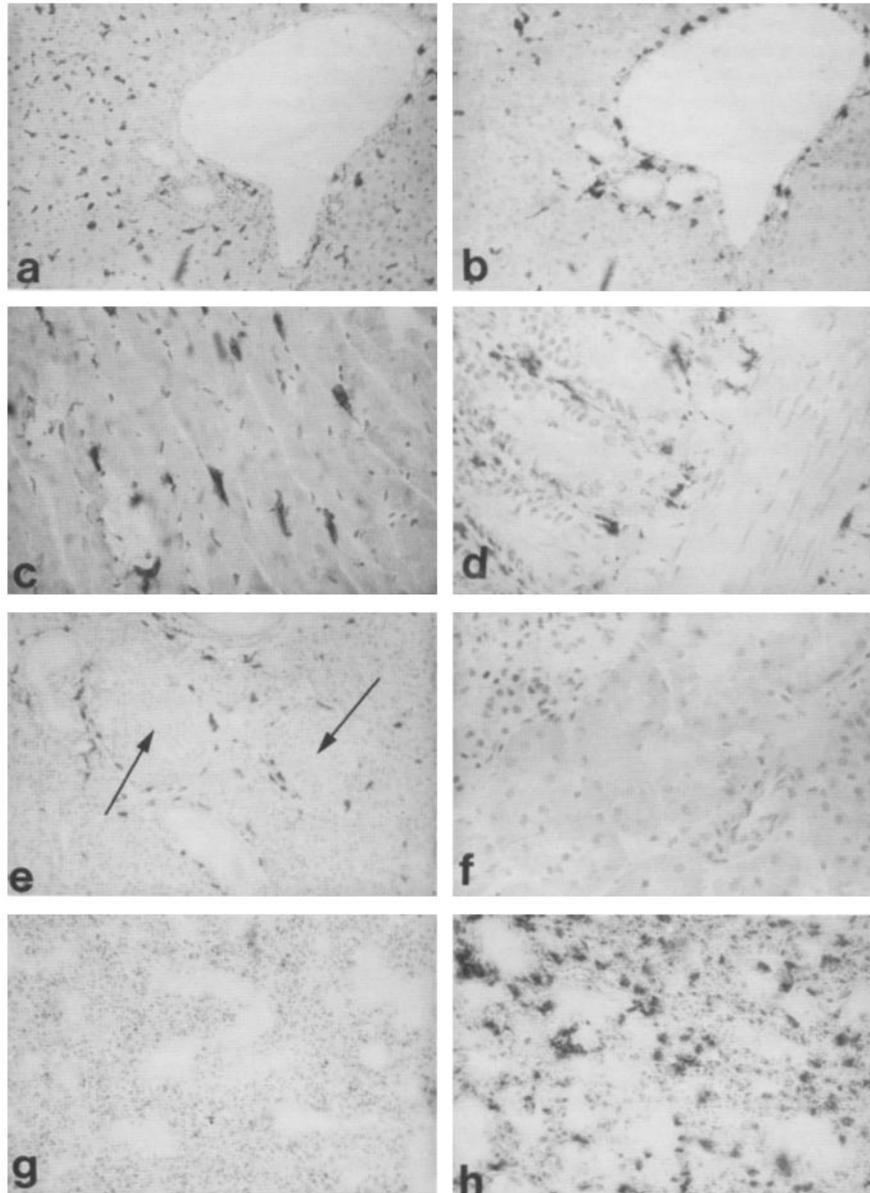


FIGURE 1. Frozen sections of tissues from the DA rat strain were stained with the mouse anti-rat macrophage mAb BMAC-5 or the mouse anti-rat MHC class II antibody MRC OX6, using the immunoperoxidase technique. (a) Liver, BMAC-5 antibody; (b) liver, MRC OX6 antibody; (c) heart, BMAC-5 antibody; (d) small intestine, BMAC-5 antibody; (e) pancreas, BMAC-5 antibody. The islets of Langerhans are arrowed. (f) Kidney, BMAC-5 antibody; (g) lung, BMAC-5 antibody; (h) lung, MRC OX6 antibody. (a, b, e, g, and h) $\times 100$. (c, d, and f) $\times 250$. The control F15-42-1 antibody did not give any staining of these tissues.

other tissues from the same rat, to show that the discrepancy between kidney and heart existed in the same animal. Moreover, several different strains were also examined (DA, WAG, BN, and SHR), and all gave the same result. In all cases, the kidneys had numerous MHC class II-positive interstitial dendritic cells present, but no BMAC-5⁺ cells.

It was also interesting that the lung parenchyma did not have BMAC-5⁺ cells (Fig. 1 *g*), although there were numerous MHC class II-positive cells present (Fig. 1 *h*).

PBMC and blood polymorphonuclear leukocytes were completely negative with BMAC-5 when examined both by flow cytometry and by immunoperoxidase staining of cytospin sections. Among lymphoid organs, BMAC-5 gave the following pattern. In spleen, the red pulp was strongly stained, but the white pulp and marginal zone were negative. In lymph node, there were scattered positive cells in the medulla, but no positive cells in the cortex or paracortex. In the thymus, there were isolated positive cells in the cortex, but no positive cells in the medulla. The Peyer patches were completely negative.

Neither ED1 nor ED3 gave any staining of interstitial structures in heart and pancreas. However, as previously noted (10), ED1 (but not ED3) stained the Kupffer cells in liver.

Double Labeling Studies

Nonspecific Esterase. It has previously been demonstrated that heart contains numerous nonspecific esterase-positive structures in the connective tissues (5), as illustrated in Fig. 2 *a*. These were shown to be distinct from the class II-positive interstitial dendritic cells (5). Although we anticipated that the nonspecific esterase would be found in the BMAC-5⁺ cells, this was not the case. Double-labeling studies clearly showed that the BMAC-5⁺ structures and the nonspecific esterase-positive structures were quite distinct (Fig. 2 *b*). The identity of the nonspecific esterase-positive structures, and in particular whether or not they represent leukocytes of some kind, remains unknown.

Class II MHC Antigens. The failure of BMAC-5 to stain the class II-positive cells in the portal triad (Fig. 1, *a* and *b*) initially raised the possibility that the BMAC-5⁺ cells and the class II-positive interstitial dendritic cells were distinct. Double-labeling studies were therefore performed with rat heart, and the results are illustrated in Fig. 2, *c-f*. The great majority of BMAC-5⁺ cells were class II negative, as illustrated in Fig. 2, *c* and *d*. However, a small proportion (<10%) gave weak staining for class II, as illustrated in Fig. 2, *e* and *f*.

LC Antigens. All normal leukocytes express the LC antigen (CD45) (17). In our previous study, the majority of LC-positive structures identified in rat heart were identified as class II positive (5). This suggested that the BMAC-5⁺ tissue macrophages defined above might be LC negative, which would be unusual for normal leukocytes. Double-labeling studies were therefore performed with rabbit anti-LC sera and the BMAC-5 mAb. These experiments clearly illustrate that the BMAC-5⁺ cells are in fact LC positive, as illustrated in Fig. 2, *g* and *h*. However, it is likely that the LC expression by the BMAC-5⁺ cells is weak. With lower concentrations of fluorescein-labeled anti-rabbit Ig, many of the BMAC-5⁺ cells appeared LC negative.

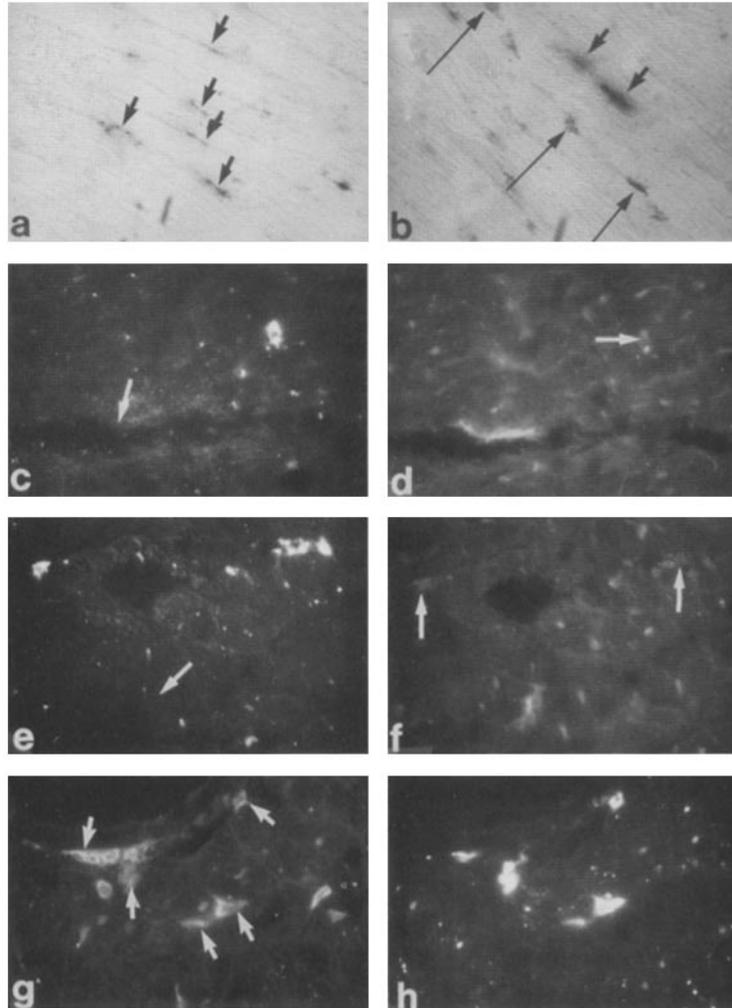


FIGURE 2. Double-labeling studies. Frozen sections of heart from the DA rat strain were stained as follows. (a) Nonspecific esterase. Positive cells are arrowed. (b) Nonspecific esterase followed by the BMAC-5 mAb and the immunoperoxidase technique. The short, thick arrows show the nonspecific esterase positive cells, while the long, thin arrows show the BMAC-5⁺ cells. In c and d, the same section was exposed to the BMAC-5 mouse mAb and rabbit anti-rat MHC class II antibodies, followed by rhodamine GAM and fluorescein GAR. In c, the rhodamine staining of class II-positive cells is shown. In d, precisely the same field as in c shows fluorescein staining of BMAC-5⁺ cells. In c, the position of the class II positive cell is arrowed; and in d, the position of the BMAC-5⁺ cell is arrowed. In e and f, the same section was stained with BMAC-5 and rabbit anti-class II, precisely as in c and d. In e, rhodamine staining of the BMAC-5⁺ cells is shown, with the position of the class II-positive cell arrowed. In f, fluorescein staining of the class II-positive cell is shown, with the position of the BMAC-5⁺ cells arrowed. The BMAC-5⁺ cell at the left of e appears to be weakly class II positive. In g and h, the same section was exposed to the BMAC-5 mouse mAb and rabbit anti-rat LC antibodies, followed by rhodamine GAM and fluorescein GAR. In g, fluorescein staining of LC-positive cells is shown. In h, precisely the same field as in g shows rhodamine staining of BMAC-5⁺ cells. The BMAC-5⁺ cells in g are arrowed. It is clear that all BMAC-5⁺ cells are LC positive. Also, some LC-positive cells are BMAC-5⁻. All $\times 400$.

Irradiation Experiments

After lethal irradiation, the class II-positive interstitial dendritic cells disappear virtually completely from rat heart by the fifth day, probably because the bone marrow stem cells are destroyed. They are rapidly reconstituted if the irradiated rats are given syngeneic bone marrow cells (5). This suggests that the interstitial dendritic cells are turning over fairly rapidly in the tissues, with a half-life of the order of 2 or 3 d. It was therefore of interest to see the effects of lethal irradiation on the BMAC-5⁺ macrophages. Rats given 1,000 rad of γ irradiation were killed 7 d later and the hearts stained for BMAC-5 and for class II MHC antigens. The results are given in Fig. 3 and demonstrate that the hearts had lost virtually all of the class II-positive cells, but that the BMAC-5⁺ cells were present in normal density.

Discussion

These studies help to define and distinguish what are likely to be two distinct leukocyte populations present in the interstitial connective tissues of normal, antigenically secluded, nonlymphoid organs. The interstitial dendritic cell (5) constitutively expresses large quantities of class II MHC antigens, is BMAC-5 (ED-2-like) negative, LC (CD45) positive, and circulates fairly rapidly through the tissues, perhaps to carry foreign antigen in a highly immunogenic form to the organized lymphatic tissues. If this cell behaves like the dendritic cell of the organized lymphatic tissue (6), it will have potent antigen-presenting capacity, but no phagocytic activity (for review, see reference 18). The second leukocyte of the connective tissues is identified by the antimacrophage mAb, BMAC-5. The large majority of these cells are class II negative and appear to reside in the connective tissue for prolonged periods, certainly much in excess of 1 wk. This cell probably confers phagocytic capacity to the nonlymphoid organs. If so, its function might be to provide processed antigen fragments to the intensely MHC class II-positive and much more mobile interstitial dendritic cell. These two cell types would thereby complement each other for the provision of highly immunogenic antigen to the T lymphocyte system. This second

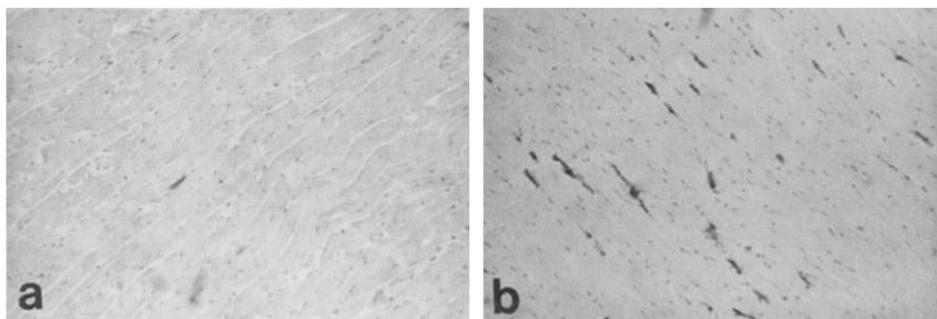


FIGURE 3. Irradiation studies. A DA strain rat was given 1,000 rad of γ irradiation, and 7 d later, its heart was removed and frozen for immunohistological studies using the peroxidase technique. Frozen sections of the heart were stained with (a) the MRC OX6 antibody to class II MHC antigens and (b) the BMAC-5 anti-macrophage antibody. One class II-positive cell is seen in a, although most fields had no class II-positive cells.

cell probably represents the fixed tissue macrophage, which has long been known to be present in connective tissues (4). A summary of the characteristics of the interstitial dendritic cell and the tissue macrophage is given in Table 1.

Steiniger et al. (19), using sequential double labeling with anti-CD4 and anti-class II MHC mAbs, have identified CD4⁺ class II-positive and CD4⁺ class II-negative cells with dendritic morphology in several organs of the rat. The precise overlap between the CD4 marker and our antimacrophage antibody is unknown, but it is likely that their CD4⁺ class II-negative cells correspond at least in part to the tissue macrophages we have defined. Interestingly, Steiniger et al. (19) found CD4⁺ class II-negative cells in the kidney. Wacker et al. (20), using a different anti-rat macrophage antibody, could identify positive cells in the rat kidney. Although one cannot be certain which leukocytes are being stained without double-labeling studies, it is likely that tissue macrophages exist in the rat kidney, but that local hormonal or other factors inhibit expression of the BMAC-5 marker or influence the state of maturation of these cells.

The precise relationship between the interstitial dendritic cell and the tissue macrophage is unknown. Our radiation experiments suggest that the turnover of the tissue macrophage is much slower than that of the interstitial dendritic cell. Therefore, unless the tissue macrophages are actively dividing, it would seem unlikely that the interstitial dendritic cell is derived from the tissue macrophage.

Once the interstitial dendritic cell had been identified and characterized (5), its similarity to the highly immunogenic dendritic cells of lymphoid tissues (6) made it highly likely that it represented the immunogenic passenger leukocyte. This was consistent with the inability of blood leukocytes to substitute for passenger leukocytes of grafts (1). The finding that lymphoid dendritic cells could substitute for passenger leukocytes (2) was further evidence that the interstitial dendritic cell represented the immunogenic passenger leukocyte, but formal proof of this point has not yet in fact been provided.

McKenzie et al. (21) have demonstrated that immunogenic passenger leukocytes

TABLE I
*Summary of the Characteristics of Interstitial Dendritic
Cells and Tissue Macrophages*

Cell type	Class II MHC antigens	BMAC-5 (ED-2) antigen	Radiation sensitivity*	Leukocyte common (CD45) antigen
Interstitial dendritic cells	+ + +	-	Yes	+ +
Tissue macrophages	- †	+ +	No	+ + ‡

* In the sense of continued presence of the tissues at 1 wk after a dose of irradiation that destroys bone marrow stem cells.

† A small minority of tissue macrophages express low levels of class II MHC antigens.

‡ Tissue macrophages might express lower levels of LC antigen when compared with interstitial dendritic cells.

are lost from donor organs within 7 d of lethal irradiation of the donor. At this time, the interstitial dendritic cells are lost but the content of tissue macrophages is undiminished. It is therefore unlikely that the tissue macrophages play any important role as conventional passenger leukocytes in transplantation. However, the possible long-term residence of allogeneic tissue macrophages within transplants could be important. In the first few weeks, months, or perhaps longer after grafting (depending on the period the tissue macrophages remain in situ), class II induction on these macrophages as a result of any inflammatory stimulus might play an important role in provoking a rejection reaction.

Finally, the presence of two distinct leukocytes within the connective tissues has important implications for many studies. For example, the administration of IFN- γ to rodents has been reported on several occasions to increase the number of dendritic cells in the connective tissues, usually interpreted as an IFN-induced migration of dendritic cells into the tissues (e.g., 22). However, an alternative and perhaps more likely explanation is that the IFN- γ has induced class II antigens on the resident tissue macrophages. While the class II-positive cells in the connective tissues of normal, undisturbed organs can be taken as interstitial dendritic cells, this is not the case after any disturbance or inflammation in the tissue.

Summary

Immunohistological studies with a mouse anti-rat macrophage mAb (BMAC-5) demonstrated the presence of numerous positive cells in the interstitial connective tissues of many organs. The pattern resembled that seen with anti-MHC class II antibodies, with the striking exception that BMAC-5⁺ cells were rare or absent in the portal triad, the islets of Langerhans, and the kidney. Double-labeling fluorescence studies were therefore performed in rat heart using the BMAC-5 mAb in combination with rabbit antisera to pure rat class II MHC antigens and pure rat leukocyte common (CD45) antigens. The tissue macrophages in heart were identified as BMAC-5⁺, MHC class II-negative, leukocyte common antigen-positive cells. They could be distinguished from the BMAC-5⁻, MHC class II-positive, leukocyte common antigen-positive interstitial dendritic cells. Moreover, 7 d after lethal irradiation, the class II-positive interstitial dendritic cells had completely disappeared from heart, whereas the BMAC-5⁺ macrophages were present in undiminished numbers. These studies strongly suggest that the interstitial dendritic cell and the tissue macrophage represent two distinct populations of leukocytes within the connective tissues of antigenically secluded organs such as the heart. They have potentially important implications for the physiology of the immune system, as well as for autoimmunity and transplantation.

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