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# Dendritic Cells in the Hearts of Spontaneously Hypertensive Rats Treated with Doxorubicin With or Without ICRF-187

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***Histological and immunohistochemical studies using specific monoclonal antibodies were made to evaluate the severity of the chronic cardiomyopathy and the quantitative changes in interstitial dendritic cells (antigen-presenting cells), T helper lymphocytes, T cytotoxic/suppressor lymphocytes, and macrophages in the hearts of spontaneously hypertensive rats (SHRs) treated with doxorubicin at 1 mg/kg per week for 3, 6, 9 or 12 weeks. In addition, an assessment was made of the modifications of the responses of these cell populations by pretreatment of the SHR with ICRF-187, which protects against doxorubicin cardiotoxicity. The number of interstitial dendritic cells/mm² of section of left ventricle was similar in saline-treated control SHRs (76 ± 6) and in those treated with ICRF-187 alone (75 ± 2) but increased markedly (319 ± 33) in animals receiving a total cumulative dose of 12 mg/kg doxorubicin. Treatment with ICRF-187 prior to each administration of doxorubicin attenuated in a dose-dependent manner the increase in numbers of dendritic cells induced by doxorubicin (231 ± 47, 174 ± 11, and 100 ± 16 cells/mm²) after treatment with 6.25, 12.5, and 25 mg of ICRF-187, respectively. Doxorubicin also induced increases in the numbers of T helper lymphocytes and macrophages but not of T cytotoxic/suppressor lymphocytes. These increases were also attenuated by pretreatment with ICRF-187. These data were interpreted as indicating that doxorubicin cardiotoxicity results in the release of substances that initiate immune reactions involving the antigen-presenting cells of the heart and that such reactions are attenuated by pretreatment with ICRF-187. (Am J Pathol 1993, 142:1916–1926)***

It is well known that doxorubicin, a widely used antineoplastic agent, induced a chronic cardiomyopathy characterized by myofibrillar loss and dilatation of the sarcoplasmic reticulum of the myocytes. The protective effect of (+)1,2-bis(3,5-dioxopiperazinyl-1-yl)-propane (ICRF-187) against doxorubicin-induced chronic cardiomyopathy has been documented in humans[1,2] and in experimental animals.[3–8] The usefulness of the spontaneously hypertensive rat (SHR) as a model system for studying this cardiotoxicity is well established.[7] Doxorubicin induces the formation of oxygen free radicals, which damage various cellular components of the heart. Thus, these radicals are considered to be responsible for a major portion of the cardiotoxicity of this agent.[9] ICRF-187 interferes with this process by chelating iron needed for the formation of the oxygen free radicals.[10]

Recent investigations have shown that doxorubicin induces many complex alterations in various immune functions, including cytolytic T-lymphocyte activity.[11,12] Furthermore, doxorubicin has been reported to stimulate CD8-positive cytolytic responses *in vitro*[13] and to enhance the release of interleukin-2 (IL-2) in rats.[14] It has been suggested that the increase in IL-2 production is related to doxorubicin-induced augmentation of cell-mediated cytotoxicity.[15,16] Doxorubicin-activated macrophages have been demonstrated to produce interleukin-1 (IL-1), which may induce T cells to produce IL-2, interferon, or both.[17] Although these observations suggest that alterations induced by immune effector cells contribute to the pathogenesis of doxorubicin-induced chronic

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cardiomyopathy, this hypothesis has not been evaluated in detail. Nevertheless, Huber[13] has suggested that doxorubicin, through free radical effects, damages the plasma membranes of cardiac myocytes, which could serve as a source of antigenic stimulation in doxorubicin toxicity. The initial steps in the development of immune reactions involve the dendritic cells, which function as antigen-presenting cells and present antigens to T lymphocytes.[18] This process is associated with the activation of T lymphocytes and with their production of lymphokines. In this context, it would be of special interest to determine the changes induced by doxorubicin on the immune effector cells of the heart, including dendritic cells, lymphocytes, and macrophages, and to evaluate the extent to which such changes are modified by cardioprotection with ICRF-187. The present study reports the results of quantitative studies of these cell populations in the myocardium of spontaneously hypertensive rats treated with doxorubicin and ICRF-187.

## Materials and Methods

### Animals

Adult male SHRs, 12 weeks old, weighing 250–300 g, were purchased from Charles River Breeding Laboratories (Wilmington, MA). They had free access to Purina rodent chow and water. The experimental protocol was approved by the Animal Care and Use Committees, Division of Intramural Research, National Heart, Lung and Blood Institute, and Division of Research and Testing, Food and Drug Administration. All procedures for animal care and housing were in compliance with the guidelines given in NIH publication 85-23.

### Reagents

Doxorubicin and ICRF-187 were obtained from Adria Laboratories (Columbus, OH). Both drugs were supplied in vials as lyophilized powder. Just before use the drugs were dissolved in normal saline so that the concentrations injected were 1 mg/ml for doxorubicin and 10 mg/ml for ICRF-187.

### Animal Experiments

Fifty-five male SHRs, 14 weeks old, were divided into nine groups, with ten animals in groups 1 and 9 and five animals each in groups 2 to 8. The rats in groups 1, 2, 3, and 4 received 3, 6, 9, and 12 weekly injections of 1 mg/kg doxorubicin, respectively, in the tail vein. The rats in groups 5–7 received injections of 1 mg/kg doxorubicin (intravenous) 30 minutes after pretreatment with 25 (group 5), 12.5 (group 6), or 6.25 (group 7) mg/kg ICRF-187 (intraperitoneal) once weekly for 12 weeks. Control animals received 12 weekly treatments with 25 mg/kg ICRF-187 intraperitoneally (group 8) or saline intravenously (group 9) without doxorubicin.

### Pathological Study

One week after the last injection, the rats were killed with an overdose of pentobarbital sodium. The entire heart was excised. Prior to fixation, a section was taken from 49 of the 55 hearts for use in the immunohistochemical study (see below). The remaining tissues were fixed in buffered 10% formalin. Blocks of formalin-fixed heart tissue were embedded in glycol methacrylate plastic resin. One-μm-thick sections were stained with alkaline toluidine blue. The severity of the cardiomyopathy was assessed according to Billingham's semiquantitative grading scale of 0 to 3[19] by light microscopic examination. The grading scale was based on the number of muscle cells showing myofibrillar loss and cytoplasmic vacuolization: 0 = no damage; 1 = <5% cells; 1.5 = 6–15% cells; 2 = 16–25% cells; 2.5 = 26–35% cells; 3 = >35% cells. All sections were evaluated without prior knowledge of the treatment given to the SHR. A $\chi^2$ test was used to determine the significance of differences in the severity of cardiomyopathy scores among the different groups.

### Monoclonal Antibodies

Monoclonal antibodies used included OX 6, W3/25, OX 8, W6/32, ED1, and ED2. OX 6 antibody reacts with Ia antigens [class II major histocompatibility complex (MHC) antigens], B lymphocytes, and macrophages.[20] W3/25 antibody reacts with CD4 antigens, T helper lymphocytes, and macrophages.[21] OX 8 antibody reacts with CD8 antigens, T cytotoxic/suppressor lymphocytes, and natural killer cells.[22] W6/32 antibody was used as a negative control for the specificity of immunoperoxidase staining because it reacts with human HLA-A, -B, and -C antigens.[23] These antibodies were purchased from Sera-Lab, Ltd. (Accurate Chemical and Scientific Corporation, San Diego, CA). ED2 antibody recognizes a membrane antigen of fixed tissue macrophages of rats.[24] Monocytes, dendritic

cells, lymphocytes, and granulocytes are negative for ED2 antibody. ED1 antibody recognizes both macrophages and dendritic cells; however, lymphocytes and granulocytes are negative.[24] ED1 antibody and ED2 antibody were purchased from Bioproducts for Science, Inc. (Indianapolis, IN).

### *Immunohistochemical Staining*

The indirect immunoperoxidase procedure of Steiniger et al[25] was used in this study. Blocks of unfixed heart tissue were embedded in Polyfreeze tissue freezing medium (Polysciences, Inc., Warrington, PA) and snap-frozen in isopentane/dry ice. Cryostat sections, 5 μm thick, were cut, air-dried for 20 minutes at room temperature, fixed in absolute ethanol for 10 minutes at 4 C, and washed three times in phosphate-buffered saline (PBS), pH 7.4. The sections were incubated with the appropriate dilution of primary antibody (ranging from 1:40 to 1:200) in PBS containing 1% bovine serum albumin and 0.1% sodium azide. All antibody incubations were carried out in a moist chamber at 4 C for 1 hour. After three washes with PBS, the sections were incubated for 1 hour with peroxidase-conjugated rabbit antimouse IgG (DAKO, Santa Barbara, CA) diluted 1:20 in PBS with 5% inactivated normal rat serum, and the sections were then washed with 0.1 mol/L Tris-buffered saline, pH 7.6. The color was developed with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% $H_20_2$ for 10 minutes at room temperature. The sections were counterstained with 1% methyl green for 2 minutes, dehydrated, and mounted with Permount. For positive controls, frozen sections of normal rat spleen were stained concurrently with the sections of the hearts.

### *Method for Counting Dendritic Cells, Lymphocytes, and Macrophages*

A Zeiss Videoplan 2 (Carl Zeiss, Inc., Oberkochen, Federal Republic of Germany) was used to count the numbers of dendritic cells, lymphocytes, and macrophages in sections of left ventricular myocardium. Only cells that had a dendritic morphology and a clearly positive reaction for OX 6 antibody were included in the count of dendritic cells. In longitudinal sections of myocardium, dendritic cells appeared elongated in shape, with a centrally located nucleus and long cytoplasmic processes. In transverse sections, they had irregular or triangular shapes with relatively large nuclei, little cytoplasm, and long processes. Neither large nor small round cells were included, because such cells represent macrophages and lymphocytes, respectively. The number of dendritic cells was counted in five rectangular high-power fields (×400), each measuring 210 × 200 μm. Because they were present in smaller numbers, macrophages and T helper lymphocytes were counted in 10 rectangular medium-power fields (×250), and T cytotoxic/suppressor lymphocytes were counted in 50 medium-power fields (×250). Each of these fields measured 350 × 320 μm. The resulting data are expressed as mean number of cells (± SD)/mm.$^2$

### *Statistical Analysis*

The significance of differences in cell counts was evaluated using Student's double-tailed *t* test, with $P \leq 0.05$ as the level of significance.

## *Results*

### *Incidence and Severity of Doxorubicin-Induced Cardiomyopathy*

Doxorubicin-induced myocardial lesions in SHRs were characterized by cytoplasmic vacuolization and myofibrillar loss (Figure 1). These myocyte alterations have been previously observed in SHRs[7] and other animals[3–6,8] and in humans[1,2] receiving doxorubicin. Data on the incidence and severity of the myocardial lesions are summarized in Table 1. In the rats given doxorubicin alone, the lesions were proportionally more severe as the cumulative dose of doxorubicin increased. A majority of the SHRs (4 of 5) given the lowest cumulative dose (3 mg/kg of doxorubicin) had minimal lesions (lesion score, 1), while at the next higher dose (6 mg/kg) most animals (4 of 5) had a lesion score of 1.5 (Table 1). At 9 mg/kg, 3 of 5 rats had lesion scores of 2.0. SHRs given the highest cumulative dose (12 mg/kg) had the most severe lesions (two animals had a lesion score of 2.5 and three animals had a lesion score of 3).

In animals treated with the combination of doxorubicin and ICRF-187, the severity of the resulting myocardial lesions was inversely proportional to the dose of ICRF-187. No alterations were observed in eight of the 10 animals in the group receiving doxorubicin plus 25 mg/kg ICRF-187; the other two animals in this group had minimal lesions (score = 1) (Figure 2). In contrast, all rats given doxorubicin and the lowest ICRF-187 dose (6.25 mg/kg) had moderate to severe myocardial lesions (lesion

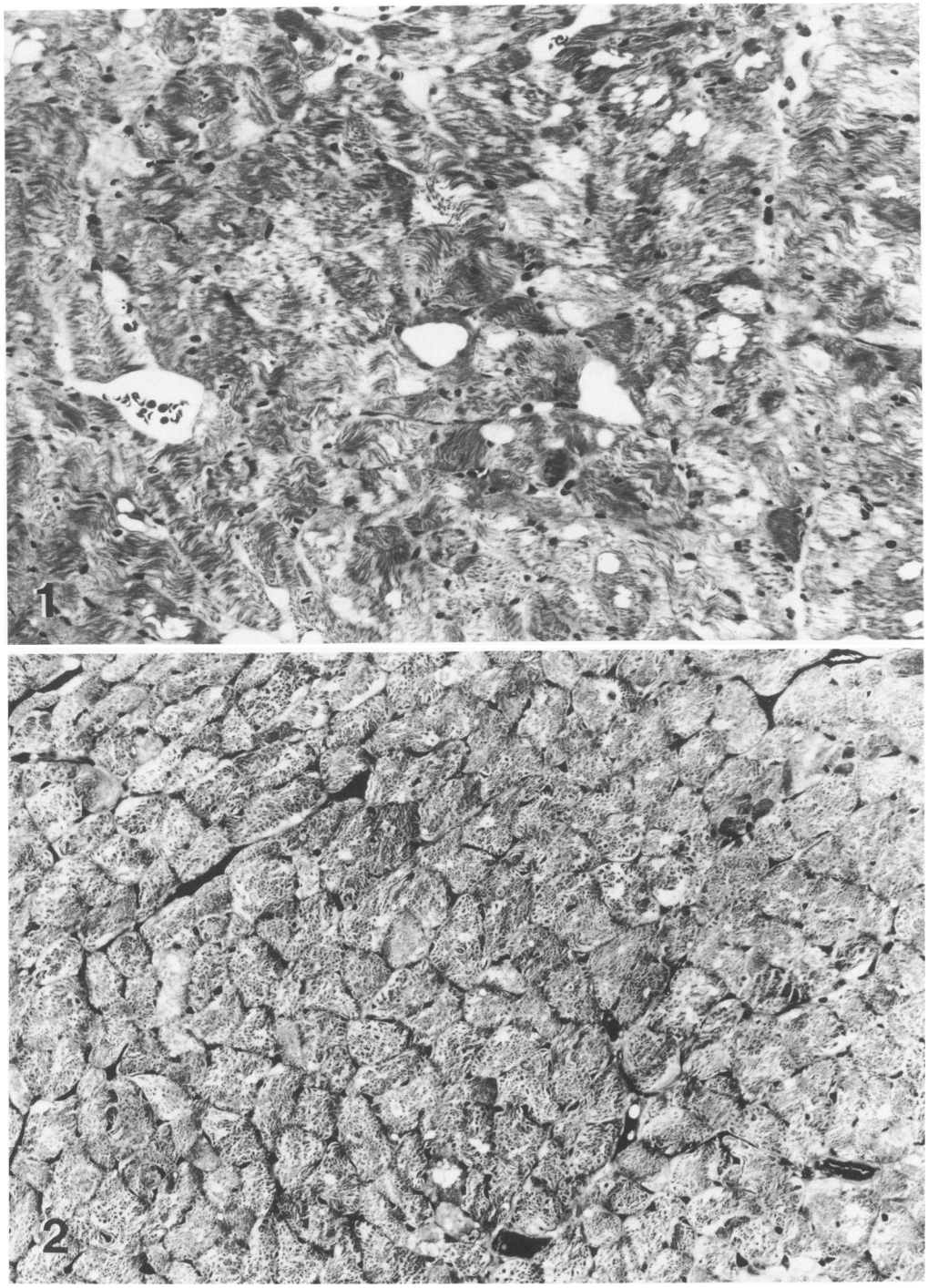


**Figure 1.** *Toluidine blue-stained, 1-μm-thick section of left ventricular myocardium from SHR given 1 mg/kg doxorubicin alone for 12 weeks shows many vacuolated myocytes. ×250.*
**Figure 2.** *Toluidine blue-stained, 1-μm-thick section of left ventricular myocardium from SHR given 1 mg/kg doxorubicin with 25 mg/kg ICRF-187 for 12 weeks shows minimal vacuolization of myocytes. ×250.*

scores, 2 to 3). At the intermediate dose of ICRF-187 (12.5 mg/kg), most animals had mild to moderate lesions (lesion scores, 1.5 or 2.0). No lesions were found in the hearts from rats given saline or ICRF-187 without doxorubicin (lesion score of 0).

In sections stained with alkaline toluidine blue, a stromal response was observed in the groups treated with doxorubicin. This stromal response was manifested by the proliferation of fibroblastlike cells in the interstitium. Some of these proliferating cells

Table 1. *Cardiomyopathy Scores in SHRs Given 12 Weekly Doses of 1 mg/kg Doxorubicin with or without 25, 12.5, or 6.25 mg/kg ICRF-187*

| | No. of animals | Cardiomyopathy scores | | | | | | Scores ≤1.5 |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 1.5 | 2 | 2.5 | 3 | |
| 1 mg/kg doxorubicin alone/week for 3 weeks | 5 | 0 | 4 | 1 | 0 | 0 | 0 | 5/5* |
| 1 mg/kg doxorubicin alone/week for 6 weeks | 5 | 0 | 0 | 4 | 1 | 0 | 0 | 4/5 |
| 1 mg/kg doxorubicin alone/week for 9 weeks | 5 | 0 | 0 | 0 | 3 | 1 | 1 | 0/5† |
| 1 mg/kg doxorubicin alone/week for 12 weeks | 5 | 0 | 0 | 0 | 0 | 2 | 3 | 0/5† |
| 1 mg/kg doxorubicin plus 25 mg/kg ICRF-187/week for 12 weeks | 10 | 2 | 8 | 0 | 0 | 0 | 0 | 10/10‡ |
| 1 mg/kg doxorubicin plus 12.5 mg/kg ICRF-187/week for 12 weeks | 5 | 0 | 0 | 3 | 1 | 0 | 1 | 3/5‡ |
| 1 mg/kg doxorubicin plus 6.25 mg/kg ICRF-187/week for 12 weeks | 5 | 0 | 0 | 0 | 3 | 1 | 1 | 0/5 |
| 25 mg/kg ICRF-187 alone/week for 12 weeks | 5 | 5 | 0 | 0 | 0 | 0 | 0 | 5/5 |
| Saline for 12 weeks | 10 | 10 | 0 | 0 | 0 | 0 | 0 | 10/10 |

* Numerator, number of animals with a cardiomyopathy score of 1.5 or less; denominator, number of animals examined.
† Significant difference when compared with the group given saline ($P < 0.05$).
‡ Significant difference when compared with the group given 1 mg/kg doxorubicin alone for 12 weeks ($P < 0.05$).

were shown to be interstitial dendritic cells on the frozen sections stained with OX 6 and W3/25 monoclonal antibodies. In the toluidine blue-stained sections, it was not possible to distinguish these dendritic cells from other cells. In the group treated with doxorubicin alone, the severity of the stromal response increased with the dose, as indicated by the dendritic cell counts (see below). In the groups treated with the combination of doxorubicin and ICRF-187, the severity of the stromal response was significantly reduced. In contrast to the groups treated with doxorubicin and doxorubicin plus ICRF-187, no stromal response was present in the two control groups (saline or ICRF-187 without doxorubicin).

## *Immunohistochemical and Quantitative Study of Dendritic Cells*

Both OX 6 and W3/25 antibodies identified interstitial dendritic cells of myocardium in all groups of rats. Neither myocytes nor endothelial cells were stained with these antibodies. Morphologically, dendritic cells in the control groups were characterized by a distinct dendritic shape with slender processes (Figure 3). In contrast, dendritic cells in the groups treated with doxorubicin alone appeared more irregular in shape, with longer processes (Figure 4). Moreover, the number of dendritic cells in these groups was greater than in the control groups (Table 2).

The numbers of OX 6-positive dendritic cells/mm$^2$ of tissue section in the different groups of animals are given in Table 2. The hearts of SHRs in the two control groups (saline and ICRF-187) had similar numbers of dendritic cells (76 ± 6 and 75 ± 2/mm$^2$, respectively). The numbers of dendritic cells increased with the total cumulative dose of doxorubicin: 120 ± 19, 162 ± 27, 243 ± 60, and 319 ± 33 cells/mm$^2$ in the groups receiving cumulative doses of 3, 6, 9, and 12 mg/kg of doxorubicin, respectively. Compared to the mean values obtained in SHRs treated with doxorubicin alone, the numbers of dendritic cells were decreased in animals receiving doxorubicin plus ICRF-187. In these animals, the numbers of dendritic cells were inversely proportional to the dose of ICRF-187: 100 ± 16, 174 ± 11, and 231 ± 47 in animals pretreated with 25, 12.5, and 6.25 mg/kg of ICRF-187, respectively. No consistent morphological difference was found between the dendritic cells in the hearts of control SHRs (Figure 3) and those treated with doxorubicin plus ICRF-187 (Figure 5).

## *Immunohistochemical and Quantitative Study of Lymphocytes and Macrophages*

In W3/25 antibody-stained preparations, T helper lymphocytes were identified in two patterns: 1) cells making contact with dendritic cells in clusters consisting of one or two dendritic cells and several T helper lymphocytes (Figure 6) and 2) cells not mak-

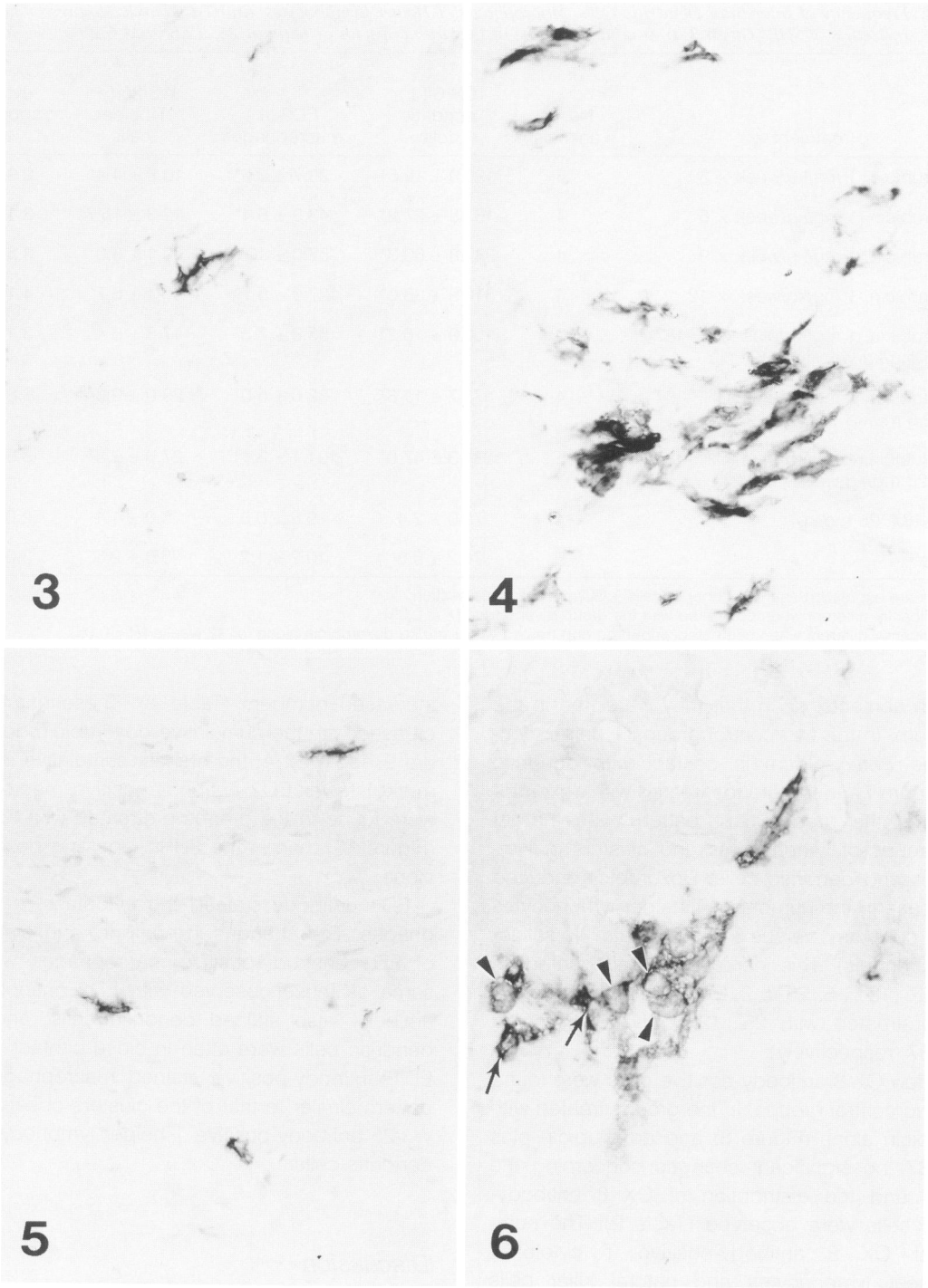


**Figure 3.** *Section incubated with OX6 monoclonal antibody. Left ventricular myocardium from SHR given 25 mg/kg ICRF-187 alone for 12 weeks shows normal appearing dendritic cell. ×400.*
**Figure 4.** *Section incubated with OX6 monoclonal antibody. Left ventricular myocardium from SHR given 1 mg/kg doxorubicin alone for 12 weeks shows marked increase in the numbers of OX6 antibody-positive dendritic cells. ×400.*
**Figure 5.** *Section incubated with OX6 monoclonal antibody. Left ventricular myocardium from SHR given 1 mg/kg doxorubicin with 25 mg/kg ICRF-187 for 12 weeks shows a significant reduction in the numbers of OX6 antibody-positive dendritic cells (compare with Figure 4). ×400.*
**Figure 6.** *Section incubated with W3/25 monoclonal antibody. Left ventricular myocardium from SHR given 1 mg/kg doxorubicin alone for 12 weeks shows a cluster of W3/25 antibody-positive dendritic cells (arrows) and T helper lymphocytes (arrowheads). ×630.*

Table 2. *Frequency of Interstitial Dendritic Cells, Macrophages, T Helper Lymphocytes, and T Cytotoxic/Suppressor Lymphocytes in Hearts of SHRs Given 3, 6, 9, and 12 mg/kg of Doxorubicin with or without 25, 12.5, and 6.25 mg/kg ICRF-187*

| Treatment | No. of animals | OX 6-(+) dendritic cells | ED2-(+) macrophages | W3/25-(+) T helper cells | OX 8-(+) T cytotoxic/ suppressor cells |
|---|---|---|---|---|---|
| Doxorubicin, 1 mg/kg/week × 3 | 5 | 120.0 ± 19.5* | 29.7 ± 3.9* | 10.8 ± 4.4* | 2.9 ± 0.3 |
| Doxorubicin, 1 mg/kg/week × 6 | 4 | 161.8 ± 27.2* | 43.3 ± 6.4* | 22.3 ± 15.7* | 3.1 ± 0.5 |
| Doxorubicin, 1 mg/kg/week × 9 | 4 | 242.8 ± 60.3* | 57.0 ± 10.0* | 30.1 ± 6.0* | 3.5 ± 0.8 |
| Doxorubicin, 1 mg/kg/week × 12 | 4 | 318.9 ± 33.0* | 127.7 ± 5.7* | 37.1 ± 6.7* | 4.1 ± 0.6* |
| Doxorubicin, 1 mg/kg plus ICRF-187, 25 mg/kg weekly × 12 | 10 | 100.0 ± 16.2† | 55.8 ± 7.3† | 17.8 ± 5.4† | 3.1 ± 0.9 |
| Doxorubicin, 1 mg/kg plus ICRF-187, 12.5 mg/kg weekly × 12 | 4 | 173.7 ± 11.3† | 82.0 ± 5.0† | 25.0 ± 2.3† | 3.8 ± 1.5 |
| Doxorubicin, 1 mg/kg plus ICRF-187, 6.25 mg/kg weekly × 12 | 5 | 231.3 ± 47.0† | 101.1 ± 3.1† | 27.0 ± 2.8† | 3.9 ± 0.9 |
| ICRF-187, 25 mg/kg | 4 | 75.0 ± 2.4 | 9.8 ± 0.8 | 5.0 ± 1.1 | 2.0 ± 0.8 |
| Saline | 9 | 76.2 ± 5.8 | 10.2 ± 1.2 | 5.0 ± 2.5 | 3.0 ± 0.7 |

All data are expressed as the number of cells ± SD/mm$^2$ of tissue section.
* Significantly different when compared with the group given saline ($P < 0.05$).
† Significantly different when compared with the group treated with 1 mg/kg doxorubicin alone for 12 weeks ($P < 0.05$).

ing such contacts, such that they were free in the interstitium. In the two control groups T helper lymphocytes were seldom in contact with dendritic cells (Figure 7). In the groups treated with doxorubicin alone, the predominant pattern was that of small groups of lymphocytes and clusters of lymphocytes and dendritic cells. Doxorubicin induced an increase in the number of T helper lymphocytes (37 ± 7 cells/mm$^2$ versus 5 ± 2 cells/mm$^2$ in saline-treated controls). This increase was attenuated by ICRF-187 (18 ± 5, 25 ± 2, 27 ± 3 cells/mm$^2$ in animals pretreated with 25, 12.5, and 6.25 mg/kg ICRF-187, respectively).

Very few OX 8 antibody-positive cells were found in the two control groups. In the groups treated with doxorubicin alone (Figure 8) and doxorubicin plus ICRF-187, no significant changes concerning the number and the distribution of OX 8 antibody-positive cells were observed (Table 2). The numbers of OX 8 antibody-positive T cytotoxic/suppressor lymphocytes and natural killer cells were much smaller than those of W3/25 antibody-positive T helper lymphocytes and of ED1 antibody-positive and ED2 antibody-positive macrophages. ED2 antibody stained the membranes of macrophages, which appeared as large, round or oval-shaped cells. In the control groups few ED2 antibody-positive macrophages were observed. However, in the groups given doxorubicin alone, ED2 antibody-positive macrophages were found in increased numbers (Table 2). These numbers increased with the cumulative doxorubicin dose (Figure 9, Table 2). At the highest cumulative dose (12 mg/kg) fewer ED2 antibody-positive macrophages were found in the groups pretreated with ICRF-187 (Figure 10) than in the SHRs receiving doxorubicin alone.

ED1 antibody stained the cytoplasm of macrophages. The changes in numbers and distribution of ED1 antibody-positive macrophages were the same as those observed with ED2 antibody. ED1 antibody also stained dendritic cells, and these dendritic cells were often in close contact with the ED1 antibody-positive stained macrophages in a pattern similar to that of the clusters observed with W3/25 antibody-positive T helper lymphocytes and dendritic cells.

## Discussion

The results of the present study confirm and extend previous observations concerning the protective effect of ICRF-187 against the chronic cardiomyopathy produced by the administration of doxorubicin to SHRs.[7] The attenuation of the cardiomyopathy by ICRF-187 is dose dependent, as demonstrated by the semiquantitative cardiomyopathy scores obtained in animals pretreated with 6.25, 12.5, and 25 mg of ICRF-187 (Table 1). In addition, the present

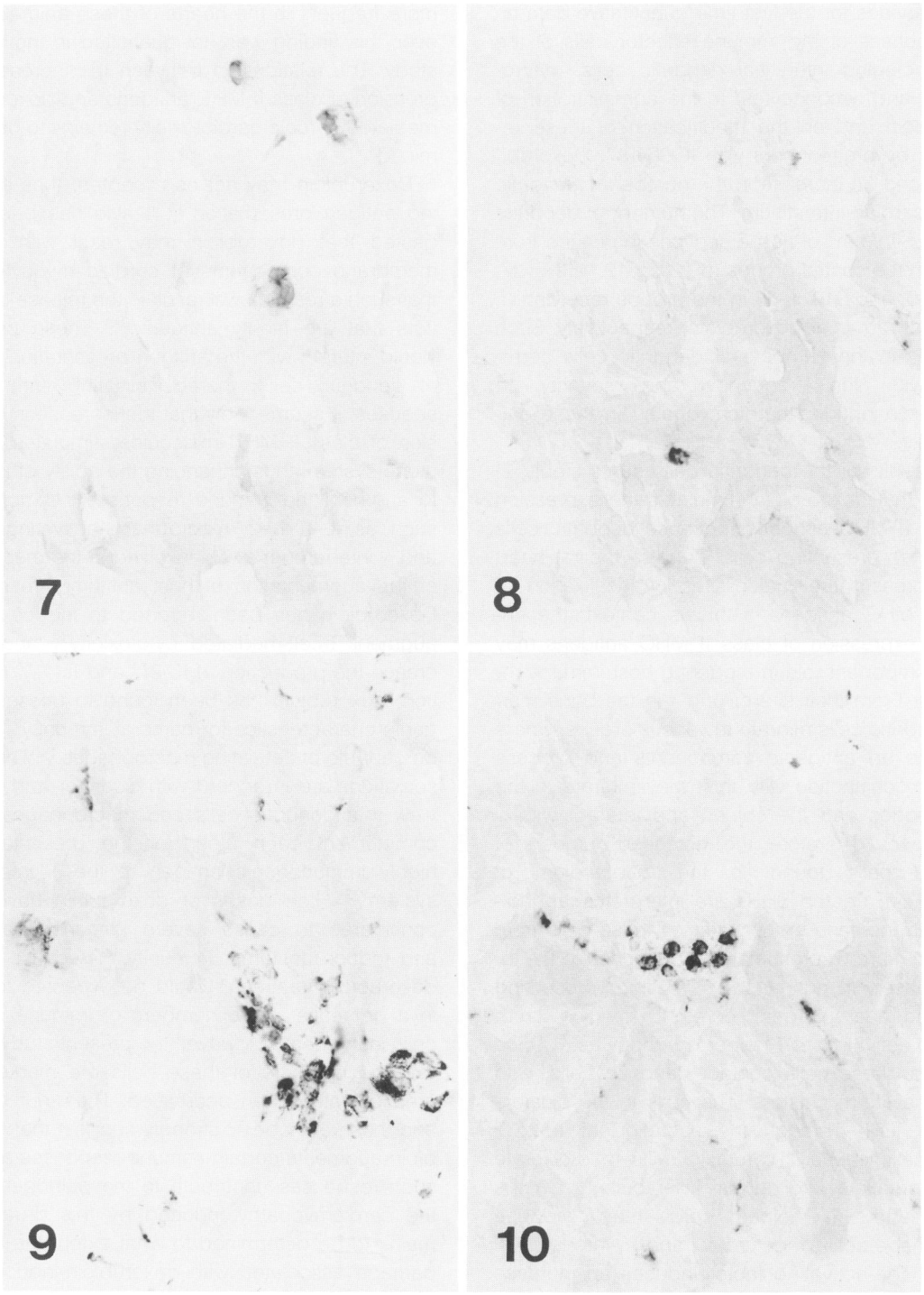


**Figure 7.** *Section incubated with W3/25 monoclonal antibody. Left ventricular myocardium from SHR given saline for 12 weeks shows W3/25 antibody-positive T helper lymphocytes. ×400.*
**Figure 8.** *Section incubated with OX8 monoclonal antibody. Left ventricular myocardium from SHR given 1 mg/kg doxorubicin alone for 12 weeks shows few OX8 antibody-positive T cytotoxic lymphocytes. ×400.*
**Figure 9.** *Section incubated with ED2 monoclonal antibody. Left ventricular myocardium from SHR given 1 mg/kg doxorubicin alone for 12 weeks shows marked increase in the numbers of ED2 antibody-positive macrophages. ×400.*
**Figure 10.** *Section incubated with ED2 monoclonal antibody. Left ventricular myocardium from SHR given 1 mg/kg doxorubicin with 25 mg/kg ICRF-187 for 12 weeks shows a significant reduction in the numbers of ED2 antibody-positive macrophages (compare with Figure 9). ×400.*

study provides for the first time quantitative data on the responses of the immune effector cells of the heart, including interstitial dendritic cells, macrophages, and lymphocytes, to the administration of doxorubicin and on the modification of these responses by pretreatment with ICRF-187. Doxorubicin induced an increase in the number of dendritic cells in cardiac interstitium. The number of dendritic cells (± SD)/mm$^2$ of tissue sections increased from 76 ± 6 in the control groups to 120 ± 19, 162 ± 27, 243 ± 60, and 319 ± 33 in the groups receiving 3, 6, 9, and 12 mg of doxorubicin, respectively. Such increases in the numbers of dendritic cells correlated with the frequency and severity of doxorubicin-induced cardiomyopathy in these animals.

The results of the dendritic cell counts (Table 2) suggest that doxorubicin stimulates the expression of class II MHC antigens, because dendritic cells are antigen-presenting cells[18] and in the rat heart they represent the major sites of localization of class II MHC antigens.[18] In this context, the enhanced expression of class II MHC antigens may play an important role in triggering host immune responses. From their site of origin in the bone marrow, dendritic cells migrate to various organs, where they take up antigenic components and process them. In conjunction with this, they migrate to the lymph nodes and the spleen and interact with T lymphocytes. Therefore, the increased numbers of dendritic cells found in the myocardium of doxorubicin- treated SHRs are interpreted as indicating an increase in the traffic of these cells from the bone marrow, presumably in response to the local release of antigens.[18,25] Antigen-presenting cells (and macrophages) secrete IL-1, which stimulates the activation of T helper lymphocytes.[26] Once activated, these cells contact dendritic cells, with which they form clusters. The cells in the clusters produce IL-2. Through the secretion of IL-2, T helper cells cause T cytotoxic cells to proliferate and differentiate into natural killer cells.[27] On the other hand, T helper cells may produce macrophage-activating factor and γ-interferon.[26] Macrophage-activating factor induces an increase in the numbers of macrophages,[25] while γ-interferon is a strong activator of macrophages and regulates the expression of class II MHC antigens in these cells.[28,29] The present study also demonstrates that T helper lymphocytes and macrophages increase in numbers in the hearts of SHRs treated with doxorubicin. Clusters of T helper lymphocyte-dendritic cells also appeared to be more frequent in the hearts of these animals; however, this finding was not quantified in the present study. The relationship between the increased expression of class II MHC antigens and doxorubicin-mediated chronic cardiotoxicity remains to be determined.

Doxorubicin may act as a hapten, thus enhancing antigen presentation.[13] It also has been suggested that doxorubicin may react with plasma membrane components of cardiac myocytes and that such a reaction will result in the release of products that are highly antigenic.[13] These products would interact with the antigen presentation system of dendritic cells, thereby initiating immune responses. It seems likely that the increase in expression of class II MHC molecules stimulates the immune system, thus enhancing the ability of the host to augment the immune response.[30] Lymphokines such as IL-1, IL-2, macrophage-activating factor, and γ-interferon may be involved in the mechanism of the augmentation of host antitumor responses. Doxorubicin has been reported to induce and to augment T-cell-mediated cytotoxicity[12] and to increase the production of IL-2[14] and IL-1.[17] In addition, doxorubicin has been found to possess antigenic characteristics for humoral immunity[31] and to be capable of activating macrophages.[32] These observations are in accord with Spencer and Fabre's view that dendritic cells and macrophages would complement each other for the presentation of highly immunogenic antigen to the T-lymphocyte system.[33] High doses of doxorubicin have been considered to cause severe immunosuppression and reduce antitumor immunity.[34] Because of this, doxorubicin treatment would be expected to result in a decrease in the numbers of immune effector cells in the heart. However, the present study shows that the numbers of these cells are markedly increased rather than decreased. Thus, our findings and those of Huber[13] strongly suggest that doxorubicin augments certain immune responses and that such responses contribute to the pathogenesis of the cardiomyopathy induced by this drug. It remains to be determined to what extent the cardiac damage associated with doxorubicin-induced cardiomyopathy is mediated by oxygen free radicals and by immune mechanisms.

An important finding of the present study is that pretreatment with ICRF-187 attenuated the doxorubicin-induced increases in the numbers of dendritic cells, T helper lymphocytes, and macrophages in the heart. Such reductions were dose dependent and occurred in parallel with the degree

of cardioprotection by ICRF-187. This agent alone did not alter the numbers of dendritic cells, macrophages, and T helper lymphocytes in the heart, thus excluding the possibility of a direct effect on the immune system of the hearts. As mentioned previously, the protection by ICRF-187 against doxorubicin-induced chronic cardiotoxicity is attributed to the fact that ICRF-187 chelates the iron necessary to catalyze the formation of oxygen free radicals that damage cardiac myocytes, particularly their membrane systems. It seems reasonable to believe that the abrogation of this toxicity would lead to a decrease in the release of potentially antigenic materials from cardiac myocytes damaged by oxygen free radicals and that this in turn would lead to a decrease in the response of the dendritic cells. Such a decrease would be followed by a corresponding attenuation in the responses of T helper lymphocytes and macrophages. This concept was in part validated in the present study: reduced numbers of T helper lymphocytes, macrophages, and clusters of T helper cells and dendritic cells were found in the hearts of rats treated with both doxorubicin and ICRF-187.

Huber[13] reported that doxorubicin induces antigenic alterations capable of stimulating cytolytic T lymphocyte responses in cultured myocyte preparations, and that the cytolytic effector cell(s) belong to the CD8-positive T-cell population instead of CD4-positive T cells. The present study showed that CD4-positive T helper lymphocytes, identified by staining with W3/25 antibody, constitute the predominant T-cell population in the hearts of doxorubicin-treated SHRs. The reason for this apparent discrepancy is unknown.

In conclusion, the present study demonstrates that the administration of doxorubicin to SHR results in chronic cardiomyopathy and in a fourfold increase in the number of interstitial dendritic cells of the heart. Macrophages and T helper lymphocytes also increase in number, but T cytotoxic/suppressor lymphocytes do not. These increases in the cell numbers and in the severity of cardiomyopathy are attenuated by pretreatment of the SHR with ICRF-187. These results are interpreted as indicating that doxorubicin treatment triggers immune reactions in which interstitial dendritic cells function as the antigen-presenting cells of the heart.

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