Virchows Arch (1995) 426:493–500

ORIGINAL ARTICLE

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A histological study on experimental autoimmune myocarditis with special reference to initiation of the disease and cardiac dendritic cells

Received: 4 January 1995 / Accepted: 1 March 1995

Abstract The precise mechanism of myosin-induced autoimmune myocarditis is unknown. The purpose of the present study was to define the immunohistological and ultrastructural characteristics of the infiltrating cells, especially in the initial phase of the myocarditis. It was demonstrated that OX6-positive dendritic cells first infiltrated the cardiocytes on day 13 after immunization. After day 17, OX6-positive cells, which possessed elongated irregular-shaped processes on the cell surface but contained few phago-lysosomes in the cytoplasm, were located at the margin of an inflammatory field and inserted their processes into the sarcoplasm of cardiocytes. The central portion of the inflammatory field was occupied by ED1-positive inflammatory macrophages, which were rich in phagosomes and which were in contact with degenerating cardiocytes. No evidence was obtained which suggested that lymphocytes directly injured the cardiocytes. These results demonstrated ultrastructural evidence that the type of infiltrating cell that first injures cardiocytes is the cardiac dendritic cell. Inflammatory macrophages thereafter serve as scavengers of degenerating cardiocytes.

Key words Autoimmune myocarditis · Cardiac myosin · Dendritic cell · Macrophage · Ultrastructure

Introduction

The aetiology of idiopathic acute myocarditis is unknown. Some cases of acute myocarditis are caused by viral infection, which are presumed to be the most common pathogens. With recent advances in the immunological analysis, it has been suggested that post-infectious autoimmunity plays important roles in myocardial damage of acute viral myocarditis [5, 9, 16]. Consequently, the mechanism of autoimmune response-mediated myocardial damage has become worthy of note.

Neu et al. [13] reported that immunization of mice with mouse cardiac myosin in susceptible strains caused severe myocarditis and high titres of myosin autoantibodies. Kodama et al. [6] established a novel experimental model of autoimmune myocarditis by immunizing Lewis rats with human cardiac myosin: about 20% of the immunized rats died due to heart failure in this model. In this experimentally induced lethal myocarditis, histological studies revealed extensive myocardial necrosis accompanied by marked cellular infiltration, which mainly consisted of OX42-positive macrophages and W3/25-stained helper T cells [7]. It was suggested that myosin-induced autoimmune myocarditis might be mediated by helper T lymphocytes, and that dendritic cells in the interstitium of the heart might present a cardiac myosin–MHC class II complex as an antigen to the helper T cells [14].

However, the pathohistological mechanism of autoimmune myocarditis has remained obscure owing to insufficient ultrastructural evidence. In particular, the detailed mechanisms as to how the disease is initiated and which cells might first attack cardiocytes are unknown. Furthermore, no reports seem to be available dealing with the detailed features of cardiac dendritic cells, including their ultrastructure, time course of occurrence, and functions in autoimmune myocarditis. Thus, this report aims to investigate the immunohistological and ultrastructural characteristics of infiltrating cells with special reference to dendritic cells at various stages, especially in the initiation phase of the rat autoimmune myocarditis induced by the method established by Kodama et al. [6].

Materials and methods

Male Lewis rats weighing 190–200 g were purchased from Charles River Japan (Atsugi, Japan).

Myosin fraction was isolated from porcine hearts according to Murakami's method [12]. The cardiac myosin fraction was injected into 7-week-old rats at a dosage of 5 mg/kg in an equal volume

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of complete Freund's adjuvant. The immunized rats were killed under ether anaesthesia on days 7, 10, 13, 15, 17, and 20. They were compared with the normal rats as controls.

The hearts were removed immediately after sacrifice, and were cut horizontally at the mid-ventricular level. Samples were embedded in OCT compound (Miles, Elkhart, USA) and rapidly frozen in liquid nitrogen. Cryostat sections were cut sequentially at 10 μm in thickness using a Coldtome CM-41 (Sakura, Tokyo, Japan). After fixation in acetone for 10 min, the sections were incubated with mouse monoclonal antibodies against rat-derived antigens: OX6, OX8, ED1, ED2, W3/25, preparatory to processing according to the indirect enzyme-immunohistochemical method. The antigen-antibody reactions were visualized with diaminobenzidine, or with both 4-chloro-1-naphthol and alkali-phosphatase for double staining.

To identify the types of inflammatory cells, we used mouse monoclonal antibodies, using the following features for each antibody: OX6 antibody (Serotec, Oxford, UK) for recognizing MHC class II-expressing cells including dendritic cells, monocytes, and B lymphocytes; OX8 antibody (Serotec) for detecting cytotoxic/suppresser T lymphocytes (CD8); ED1 (Serotec) and ED2 antibodies (Serotec) for recognizing inflammatory and resident macrophages, respectively; and W3/25 antibody (Serotec), which is specific for CD4 T cells and dendritic cells.

The immunized rats were perfused with a Locke solution via the cardiac apex, followed by administration of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The hearts were removed, chopped into 1-mm cubes, and immersed into the same fixative for 3 h. After washing in the buffer, the tissues were post-fixed in 1% OsO_4 for 2 h. After dehydration through the ethanol-propylene oxide series, they were embedded in Araldite resin. Ultrathin sections were double stained with uranyl acetate and lead citrate shortly before examination in a Hitachi H-7000 electron microscope.

Results

Pathological and immunohistological study

In the normal rat heart, OX6-positive cells were scattered rather evenly in the interstitium of the myocardium, with no concentration near the epicardium or the endocardium. They were mononuclear cells with spindle-shaped profiles, extending elongated cytoplasmic processes among cardiocytes (Fig. 1a). ED2-positive cells were large mononuclear cells with broad and irregular-shaped cytoplasm. They were often located either in the perivascular spaces or among cardiocytes. The ED2-positive cells were approximately twice as frequent as OX6-positive cells. W3/25 antibody also stained elongated cells, which were scattered among cardiocytes (Fig. 1b). They resembled the OX6-positive cells in shape and distribution, but were slightly more numerous than OX6-positive cells. Immunostaining using OX8 and ED1 antibodies demonstrated only rare positive cells in normal hearts.

At 7 days after immunization, there was no evident myocarditis. Immunohistologically, however, OX6-positive cells had slightly increased in number, but they were identical in shape and distribution pattern to the cells seen in the interstitium of normal hearts. There were no differences in the number and shape of cells positive for ED1, ED2, OX8, or W3/25 between the experimental animals and the controls.

By day 10, OX6-positive cells were found to have gathered in the perivascular spaces of the small vessels near the epicardium of the right ventricle (Fig. 1c). In serial sections of this site, groups of OX6-positive cells were shown to be associated with only a few of the ED1- and ED2-positive macrophages. Although a few W3/25-positive cells were located around small vessels with other types of inflammatory cells, they did not increased in number at the stage. The number and distribution of OX8-positive cells were almost same as the normal heart.

At day 13, infiltrating mononuclear cells were scattered in the myocardium, frequently forming clusters in the interstitium. Most of these were immunostained with the OX6 antibody (Fig. 1d). W3/25-positive cells also increased in number among cardiocytes and around the small vessels, whereas the microscopic observation showed no clusters (Fig. 1e). However, ED1-positive cells were detectable in the lumen of small blood vessels near the epicardium of the right ventricle (Fig. 1f). ED2-positive cells had slightly increased in number in the myocardium compared with the normal controls, but cells of neither type formed a cluster. OX8-positive cells had not changed in number.

On day 15, inflammatory lesions were recognized macroscopically as grey discoloured spots on the surface of cardiac muscles. Microscopically, it was easily recognizable that the infiltrating cells formed clusters among cardiocytes, some of which were necrotic. Immunohistological studies using serial sections indicated that the major population of the infiltrating cells were immunostained with the OX6 antibody (Fig. 2a). ED1-positive cells were intermingled with OX6-positive cells at the centres of the clusters, while only a small number of ED2-positive cells were included (Fig. 2b, c). W3/25-positive cells also appeared at the same lesions, but they were less numerous than the OX6-positive cells.

By days 17 and 20, inflammatory lesions had spread transmurally, growing into huge foci, particularly in the right ventricle. In these regions, numerous inflammatory cells infiltrated into the myocardium, which was now undergoing extensive necrosis of cardiocytes. The central region of every necrotic nest was occupied by ED1-positive cells, while in the peripheral region OX6-positive cells predominated (Fig. 3a). In contrast, ED2-positive cells were scarcely detected at the centre of necrosis (Fig. 3b), although they were normal in number in the myocardium outside the inflammatory foci. W3/25-immunostained cells were also gathered in clusters among the cardiocytes. Only a few OX8-positive cells were seen in the central and peripheral regions of the necrotic nests at all stages of the myocarditis.

Electron microscopy

At day 13, monocytes, neutrophils, and lymphocytes were found adhering to the endothelium of small venules and invading the perivascular spaces passing through the endothelium. Some were already located in the interstitium of the myocardium. At this stage, no morphological signs showing a direct connection of inflammatory cells to cardiocytes were recognizable.

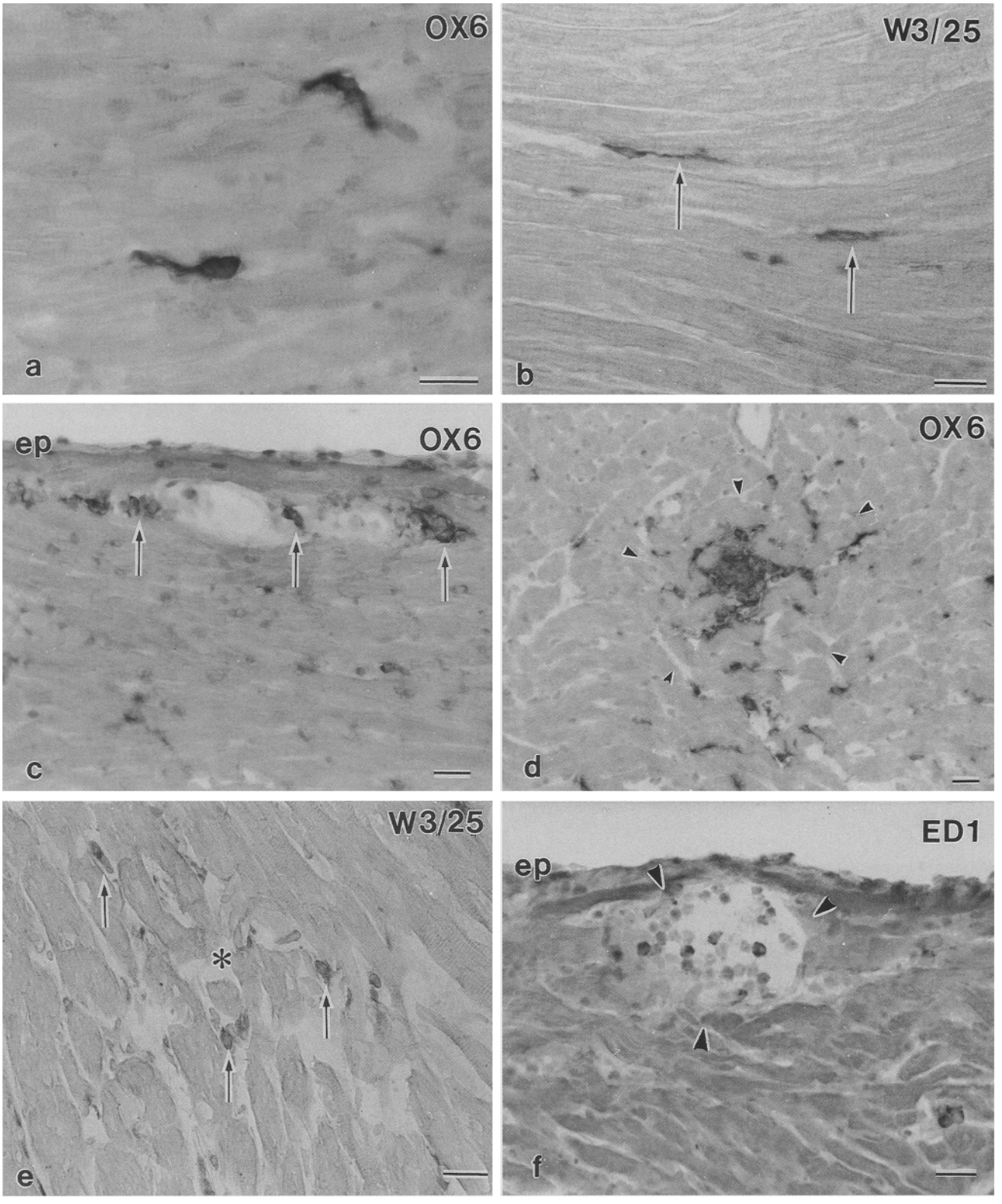

Fig. 1a–f Immunohistochemical reaction of rat cardiac tissue in the normal condition and in the initial phases of the experimental autoimmune myocarditis. **a** OX6-positive cells in a normal rat heart. They extend elongated cytoplasmic processes in the interstitial spaces between cardiocytes. ×400. **b** W3/25-positive cells (*arrows*) in a normal heart. They also possess elongated processes among cardiocytes. ×400. **c** Day 10 after immunization. OX6-positive cells (*arrows*) gather in the perivascular spaces of small blood vessels beneath the epicardium (*ep*) of the right ventricle. ×250. **d** Day 13. OX6-positive cells infiltrate in the myocardium to form clusters in a forming inflammation focus (*arrowheads*). ×160. **e** Day 13. W3/25-positive cells (*arrows*) aggregate at the inflammatory lesion (*asterisk*), but they do not form a cluster. ×250. **f** Day 13. ED1-positive cells are seen in the lumen of a small blood vessel (*arrowheads*) near the epicardium (*ep*) of the right ventricle. ×250, *bar*=20 mm

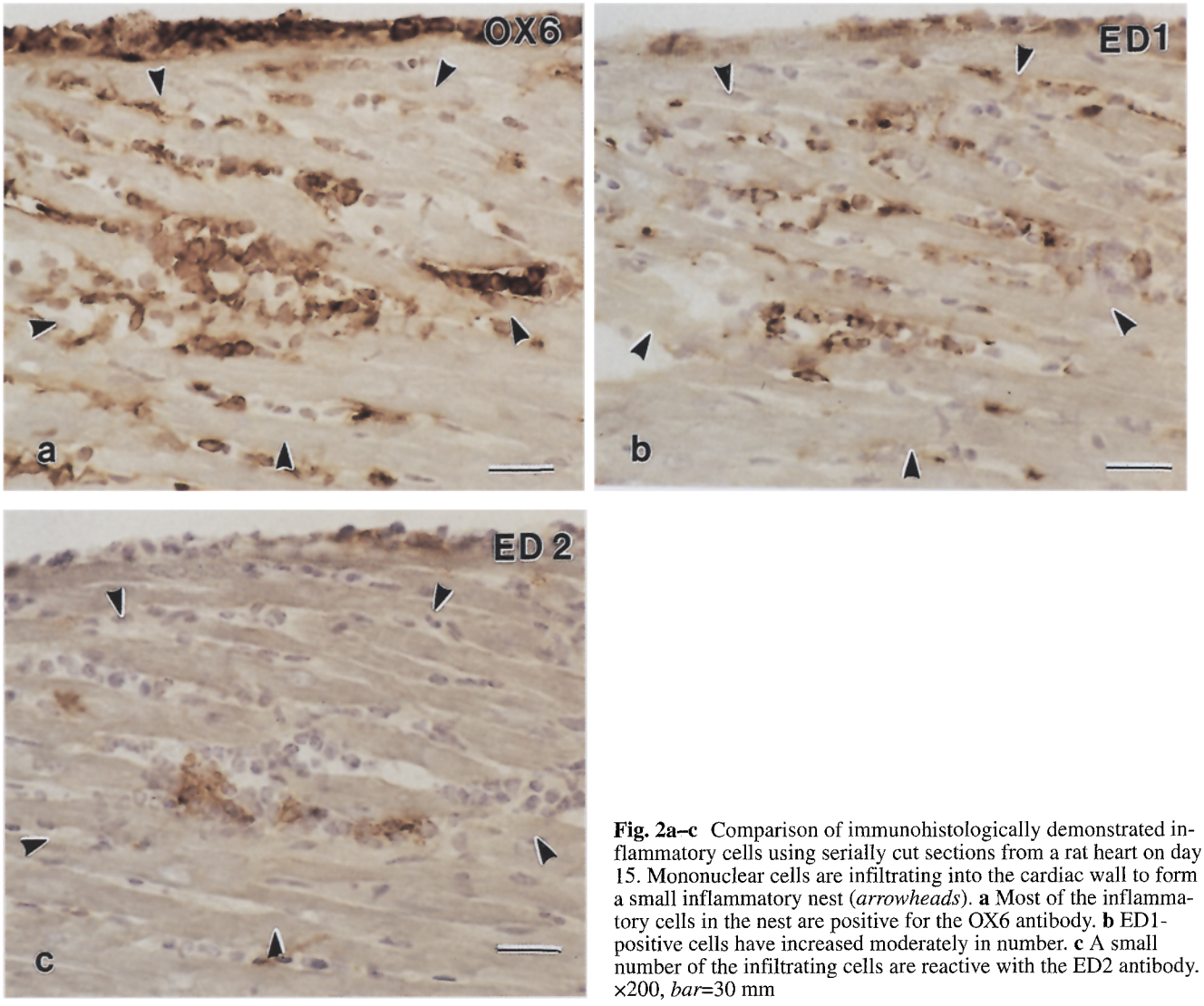

Fig. 2a–c Comparison of immunohistologically demonstrated inflammatory cells using serially cut sections from a rat heart on day 15. Mononuclear cells are infiltrating into the cardiac wall to form a small inflammatory nest (*arrowheads*). **a** Most of the inflammatory cells in the nest are positive for the OX6 antibody. **b** ED1-positive cells have increased moderately in number. **c** A small number of the infiltrating cells are reactive with the ED2 antibody. ×200, *bar*=30 mm

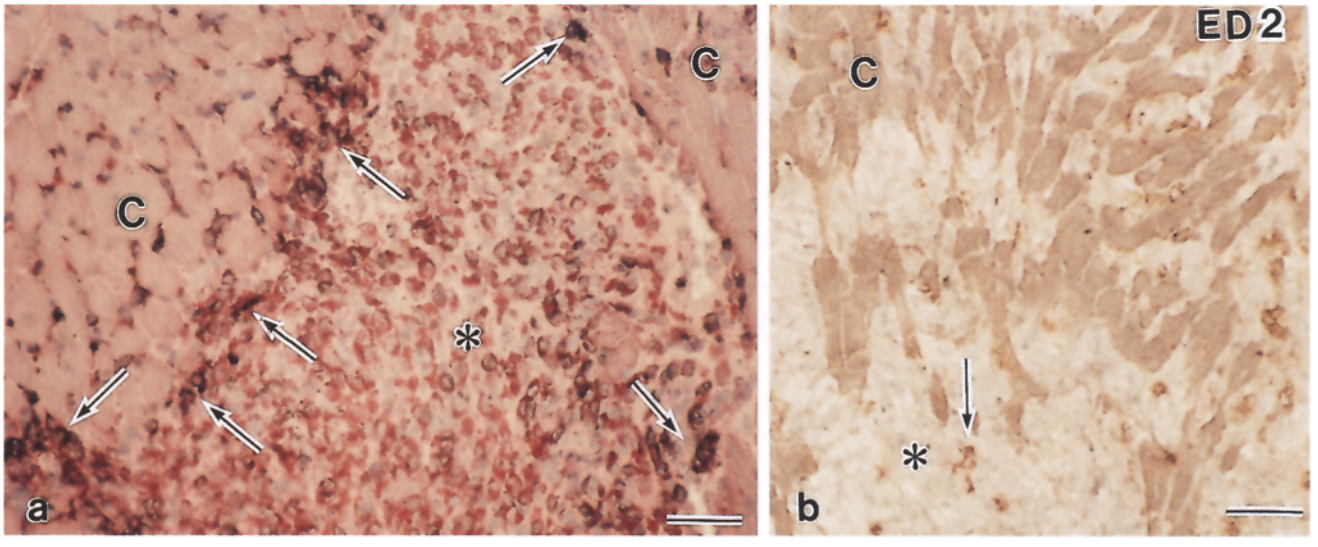

Fig. 3a, b The effector phase of myocarditis on days 17. **a** Double staining using ED1 and OX6 antibodies. The major part of a necrotic lesion (*asterisk*) is occupied by ED1-positive cells (*red*). OX6-positive cells (*black arrows*) are located in the peripheral site of the inflammatory focus. **b** ED2-positive cells (*arrow*) are rare in the inflammatory lesions (*asterisk*). ×160, *bar*=40 mm. (*C* cardiocytes)

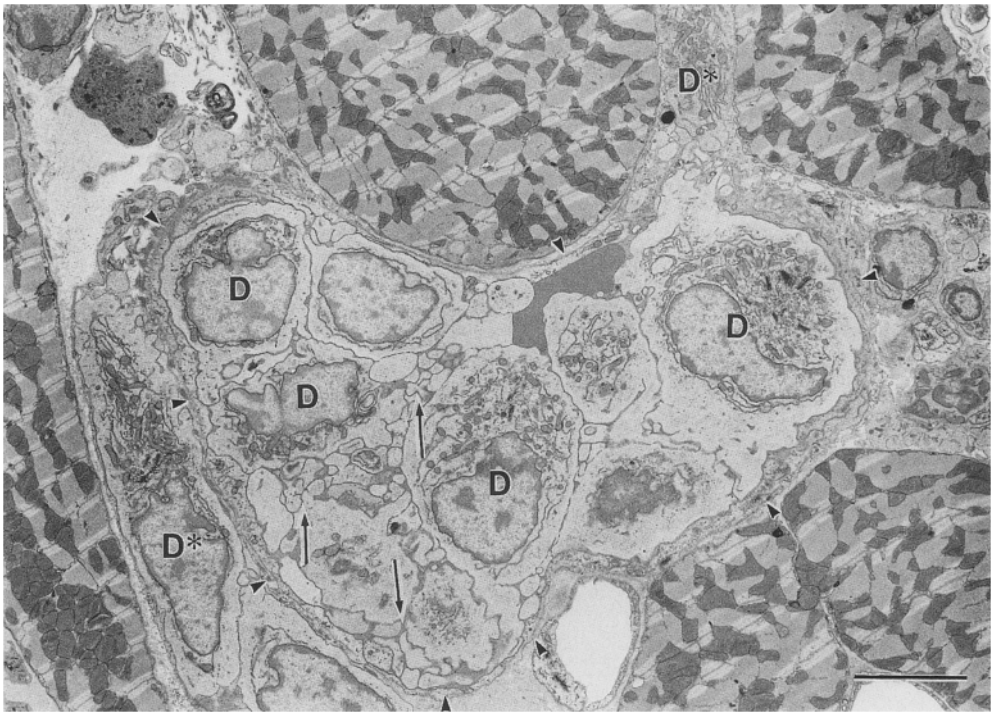

Fig. 4 Day 15. Cardiac dendritic cells (*D*) provided with complicated interdigitating processes (*arrows*) on the cell surface occupy the lumen of a small vessel (*arrowheads*). Cardiac dendritic cells (*D* + *asterisk*) also exist in the perivascular space. ×1500, *bar*=5 mm

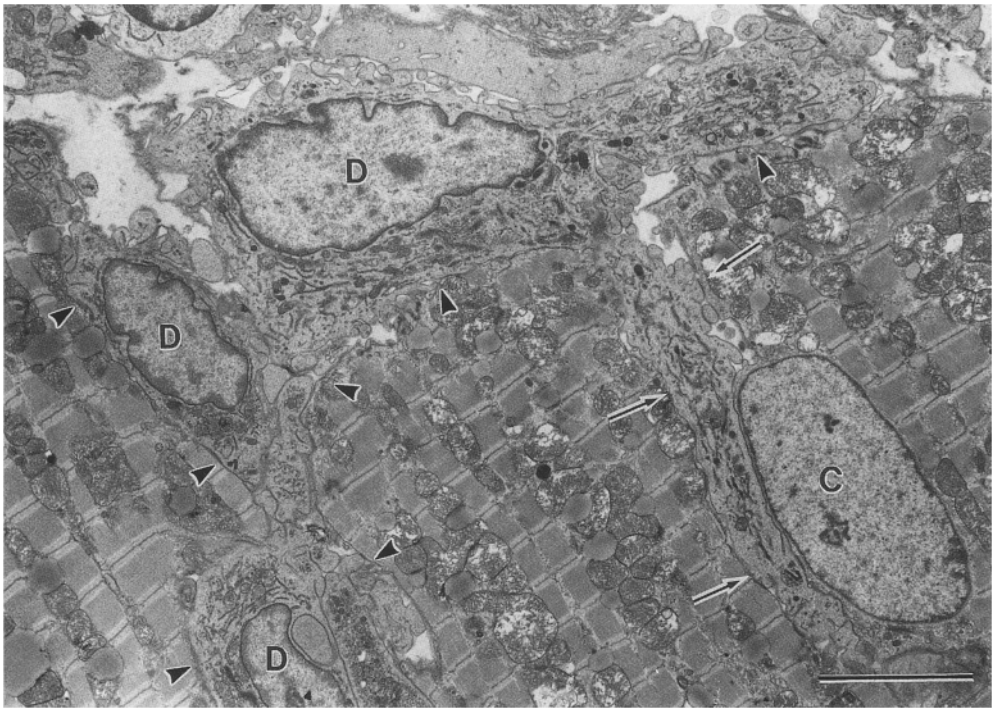

Fig. 5 Day 20. An electron micrograph showing dendritic cells (*D*) at the margin of an inflammatory lesion. The cells insert their long processes (*arrows*) into cardiocytes (*C*) through the basement membrane (*arrowheads*). They possess developed Golgi apparatus, but only a few lysosomes. ×2000, *bar*=5 mm

By day 15 the cells infiltrating into the perivascular spaces had increased conspicuously in number. While several types of inflammatory cells, including monocytes, neutrophils and lymphocytes, were identifiable in both the luminal and the perivascular spaces, cells provided with complicated undulating processes on their cell surface were much in evidence (Fig. 4, indicated by D). These cells resembled monocytes or macrophages to some extent ultrastructurally, but were clearly distinguishable by their electron-lucent processes with complicated contours and by their sparseness in lysosomes and phagosomes. It was thus reasonable to identify them as dendritic cells.

On days 17 and 20 numerous mononuclear cells were found among the cardiocytes, forming cell-rich regions. The dendritic cells were predominant in the periphery of the inflammatory foci, forming clusters in contact with normal cardiocytes. Interestingly, at the margin of the in-

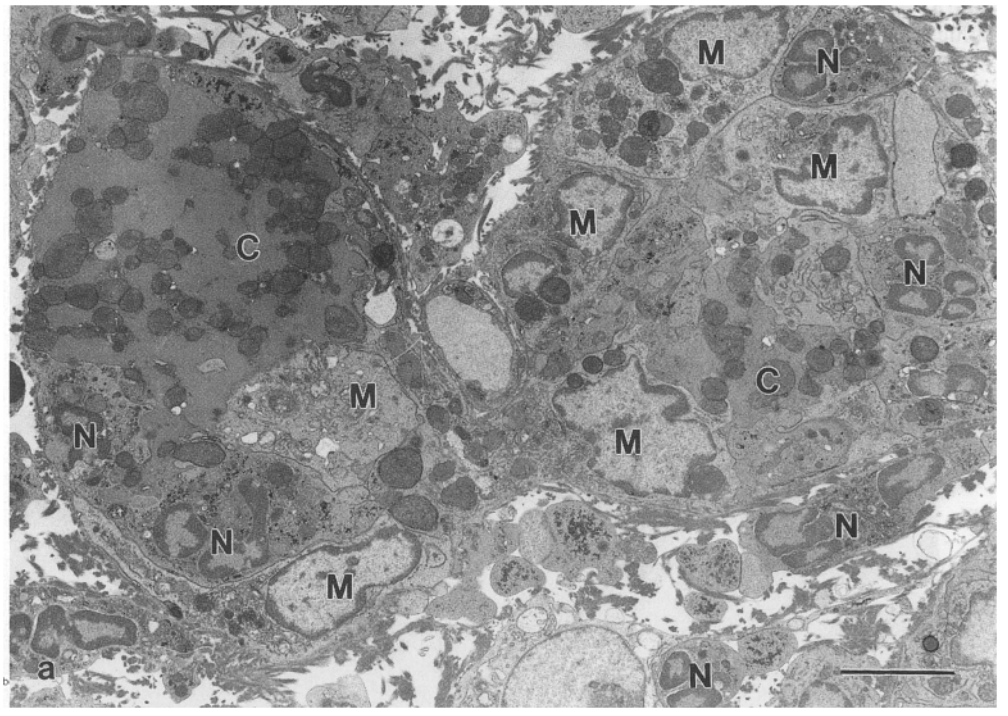

Fig. 6 Day 20. Centre of an inflammatory focus. Macrophages (*M*) and neutrophils (*N*) are eroding degenerated cardiocytes (*C*). These macrophages and neutrophils are rich in phagosomes. ×2000, *bar*=5 mm

flammatory lesions, some of the dendritic cells protruded their long processes into cardiocytes, piercing their basement membrane and dividing their cytoplasm (Fig. 5). Small numbers of lymphocytes were found to be intermingled with the large mononuclear cells in the inflammatory area, but they did not directly connect with the cardiocytes. Most of the lymphocytes were free of electron-dense granules, which are known to occur in cytotoxic lymphocytes and natural killer cells. At the central region of the inflammation, macrophages had greatly increased in number over time, becoming the predominant cell type. Neutrophils had also increased in number in the inflammatory sites.

The macrophages and neutrophils were frequently located in surrounding degenerative and necrotic cardiocytes, which had been phagocytosed by them (Fig. 6). The macrophages in the lesion were large mononuclear cells, rich in lysosomes, lipid droplets, and phagosomes; they varied in size but lacked dendritic processes on the cell surface (Fig. 6).

Discussion

Infiltrating cells in the initiation phase

In the initiation phase of myocarditis OX6-positive mononuclear cells first infiltrate around the small vessels, and at subsequent stages form primary lesions among cardiocytes. Consequently they seemed to play an important role in the initiation of myocarditis. The OX6-positive infiltrating cells were reactive to neither ED1 nor ED2 antibodies and also clearly differed in distribution from the ED1- and ED2-positive macrophages. Ultrastructurally, the large mononuclear cells which corresponded to the OX6-immunostained cells in shape and distribution pattern were characterized by complicated undulating processes and an undeveloped phagolysosome system, thus being differentiated from typical macrophages. These immunohistochemical and ultrastructural findings suggest that the OX6-positive cells present in the early phase of autoimmune myocarditis are cardiac dendritic cells. The dendritic cells in the interstitial regions of the normal rat heart are characterized by strong immunopositivity with OX6 and by possession of elongated cytoplasmic processes [2, 4, 20], but their detailed ultrastructure has not been reported. This is the first ultrastructural record of the cardiac dendritic cells in autoimmune myocarditis. Spencer and Fabre [19] discriminated immunohistochemically between the tissue macrophages and the dendritic cells in the normal rat heart. The present results, in the pathological heart, extend their findings by adding the ultrastructural distinction between the two types of cell.

Characteristics of cardiac dendritic cells

Dendritic cells in lymph nodes are known to express an MHC class II antigen and have an antigen-presenting function to helper T cells [21]. Cardiac dendritic cells also play an important part in antigen presentation in the rejection response of cardiac allografts [1, 8, 10]. Smith and Allen [18] showed that interstitial MHC class II-positive cells obtained from the normal mouse heart expressed a cardiac myosin–MHC class II complex on the cell surface.

The present study demonstrated that the cardiac dendritic cells, which contained few phagosomes in the cy-

toplasm, were in close contact with cardiocytes and inserted their processes deep into the cytoplasm of the latter in the early phases of inflammation. This morphological finding suggests that cardiac dendritic cells might exert cell-destructive effects, but have insignificant phagocytotic activity, for cardiocytes. Zhang et al. [22] reported that in the rat ischaemic heart dendritic cells migrated to the border zone of myocardial infarction, and they suggested that dendritic cells were involved in the activation of lymphocytes. In the present myocarditis model, cardiac dendritic cells also occurred in the periphery of the inflammatory foci, and strongly expressed the MHC class II antigen. It is most likely that they also present altered cardiac myosin molecules released from injured cardiocytes to helper T cells as antigen.

In addition to OX6 antibody, previous studies reported that W3/25 antibody also detected cardiac dendritic cells [3, 20]. In the present study, W3/25-positive dendritic cells were more numerous than OX6-positive dendritic cells in normal heart, but the latter cells increased more sharply and were always more numerous than the former after immunization. This finding suggests that OX6 antibody is more useful in clarifying the characteristics of cardiac dendritic cells in experimental autoimmune myocarditis.

The functions of macrophages

At the centre of the inflammatory foci where degeneration of cardiocytes proceeded, ED1-positive cells were the predominant type, possessing many lysosomes and phagosomes and lacking in dendritic processes. These cells, apparently inflammatory macrophages, surrounded degenerated cardiocytes and revealed electron microscopic images indicating that they were phagocytosing cardiocytes. These images are of importance because they are evidence of the function of macrophages in scavenging cardiocytes. ED2-positive cells, corresponding to resident macrophages, were rarely seen at the centre of inflammatory regions. McLennan [11] reported that ED1-positive macrophages were abundant within damaged rat skeletal muscle and active in phagocytosing their cell debris, while ED2-positive macrophages were not present within the necrotic lesion. The findings obtained in cardiac muscle in the present study are compatible with those in skeletal muscle.

The functions of lymphocytes

Using anti-CD4 and anti-CD8 antibodies, Neu et al. [14] reported that CD4-positive T cells mediated autoimmune myocarditis in the induction phase while CD8-positive T cells mediated the myocardial injury. In the present study, however, lymphocytes were found to be scarce at the initiation phase when sought by immunohistochemical study and electron microscopy. This investigation failed to demonstrate signs indicating direct damage of cardiocytes by lymphocytes in the developing inflammatory lesions. The types of infiltrating cell that injured cardiocytes in the present model were cardiac dendritic cells, and the ones that phagocytosed necrosing cardiocytes were inflammatory macrophages and neutrophils. Since there is general agreement that activated T cells release several lymphokines that activate macrophages and dendritic cells [15], the role of lymphocytes in autoimmune myocarditis is probably not to injure cardiocytes directly but to induce the release of several lymphokines and thus allow inflammation to develop. In a murine model of acute myocarditis caused by Coxsackie virus B3, first natural killer cells infiltrated and cytotoxic T cells then damaged myocytes in a later phase [17]. It is suggested that the mechanisms of initiation and attacking cardiocytes in the autoimmune myocarditis are different from those in viral myocarditis; cytotoxic T cells and natural killer cells observed in the present myocarditis did not show any sign indicating their initial involvement in the lesions of myocarditis.

In spite of the recent advances in the immunological analysis of several autoimmune diseases, the morphological aspects of the autoimmune myocarditis are insufficiently understood. Cardiac dendritic cells have been thought to have only an antigen-presenting role to helper T lymphocytes, which mediate the induction of the myocarditis. However, the present study demonstrated that the cardiac dendritic cells are initially and decisively involved in the process of destruction of cardiocytes in the early phase of autoimmune myocarditis. This study also demonstrated that the cells scavenging the cardiocytes that had been destroyed were ED1-positive inflammatory macrophages, which had strong phagocytotic activity. We have provided ultrastructural evidence indicating the functions of the infiltrating cell types, particularly cardiac dendritic cells, in autoimmune myocarditis.

Acknowledgements This study was supported in part by a grant for scientific research from the Ministry of Education Science and Culture of Japan (no. 06670700) and by a grant to the Research Committee for Epidemiology and Aetiology of Idiopathic Cardiomyopathy from the Ministry of Health and Welfare of Japan. The author wishes to thank Dr. T. Izumi, T. Iwanaga, and H. Takahasi-Iwanaga, Prof. T. Fujita, and Prof. A. Shibata, Niigata University School of Medicine, for their valuable advice and encouragement throughout this study.

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