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Demonstration and phenotypic characterization of resident macrophages in rat skeletal muscle

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SUMMARY

Using immunohistochemical techniques and a panel of murine monoclonal antibodies that recognize rat cells of the mononuclear/phagocyte lineage, it has been demonstrated that the cells labelled with these antibodies are widely distributed throughout the perimysial and endomysial sites in the rat skeletal muscle. These cells have a distinctive dendritic morphology and form phenotypically heterogenous populations. Double immunoperoxidase staining with the monoclonal antibody and anti-Von Willebrand factor antiserum, which recognize vascular endothelial cells, revealed that most of these resident macrophages are closely associated with microvasculature. The perivascular location of these cells suggests that at least some of the resident macrophages may be pericytes. These observations indicate that the macrophages form an important component of the non-muscle cell population in the muscle tissue, and may be intimately involved in various immunopathological conditions of the skeletal muscle.

INTRODUCTION

In muscle inflammation, it has been shown that mononuclear infiltrates in the muscle are frequently accompanied by large numbers of macrophages (Arahata & Engel, 1984). Most of these macrophages (so-called inflammatory macrophages) have been considered to be cells entering the tissue from the blood and functioning as scavengers in search of tissue debris and unwanted materials in diseased muscle (Metchnikoff, 1905; Cline & Lehrer, 1968). However, recent studies on the functional properties of macrophages, particularly in local immunological reactions, indicate that their involvement begins with the early events that lead to stimulation of lymphocytes and induction of immune responses and extends to the effector inflammatory reactions (Mackaness, 1970; Adams & Hamilton, 1984). Thus, the macrophages exert a much wider role by modulating immunological and inflammatory responses rather than only acting as scavengers. In view of the fundamental function of macrophages in altered tissue homeostasis such as inflammation, it is important to study their distribution and precise location in the tissue. To date, a wide distribution of tissue-associated (resident) macrophages has been demonstrated in the lymphoid (Dijkstra *et al.*, 1985) and several non-lymphoid organs such as liver, lung, kidney and small intestine (Widmann, Cotran & Fahimi, 1972; Green & Kass, 1964; Hume, Perry & Gordon, 1984). However, the presence of cells of the mononuclear phagocyte system in skeletal muscle is not known. In this study, the presence, localization and phenotypic characteristics of the cells of mononuclear/phagocyte system in the rat skeletal muscle were investigated.

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MATERIALS AND METHODS

Rats

Eight adult (8–10 weeks old) Lewis rats were studied. All rats were obtained from Charles River Laboratories (Boston, MA). Animals were anaesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). The quadriceps femoris muscle was removed and rapidly frozen in liquid nitrogen-cooled 2-methylbutane.

Immunoreagents

Table 1 lists the murine monoclonal antibodies used to localize the antigens, along with their specificities and immunoglobulin subclasses. Peroxidase-conjugated affinity-purified goat anti-mouse IgG $F(ab')_2$ fragment was obtained from Cappel Laboratories (West Chester, PA). Peroxidase-conjugated rabbit anti-human Von Willebrand factor (factor VIII-related antigen) antiserum (Dako Corp., Santa Barbara, CA), which also reacts with the same antigen in rats, was used to label vascular endothelial cells (Mukai, Rosai & Burgdorf, 1980). Normal mouse IgG (Bioproducts Science Inc., Indianapolis, IN) was used in control experiments. Acid phosphatase activity was detected using naphthol AS-B1 phosphate as the substrate at pH 5·0 (Barka & Anderson, 1962).

Table 1. Murine monoclonal antibodies used in this study*

Monoclonal antibody	Specificity	Immunoglobulin subclass	Reference
ED1	Cytoplasmic antigen monocyte and most macrophage subpopulations	IgG1	Dijkstra *et al.* (1985)
ED2	Membrane antigen on IgG2a tissue macrophage of lymphoid and non-lymphoid organs		Dijkstra *et al.* (1985)
ED3	Membrane antigen on macrophages predominantly confined to lymphoid organs	IgG2a	Dijkstra *et al.* (1985)
OX6	Rat Ia antigen (monomorphic)	IgG1	McMaster & Williams (1979)
W3/25	Rat CD4 antigen	IgG1	Jefferies *et al.* (1985)
OX19	Rat CD5 antigen	IgG1	Williams (1985)

* ED1, ED2, ED3, and W3/25 were obtained from Bioproducts Science Inc. (Indianapolis, IN). OX6 and OX19 were kindly provided by Dr Alan F. Williams, University of Oxford, Oxford, U.K.

Immunohistochemical procedures

Serial 6-m cryostat sections of unfixed muscle were cut in the transverse plane, transferred to poly-L-lysine-coated coverslips, fixed with ether for 5 min and air-dried overnight at 4°. Endogenous peroxidase was eliminated by exposure to 0·09% hydrogen peroxide in methanol for 3 min at 4° (Streefkerk & Van der Ploeg, 1974). The sections were washed three times in phosphate-buffered saline (PBS) and sequentially treated at 4° with: monoclonal antibody at a dilution of 1:500 overnight, PBS rinse for 20 min, peroxidase-conjugated goat anti-mouse IgG $F(ab')_2$ fragment at a dilution of 1:200 for 2 hr, and PBS rinse for 15 min. Subsequently, the sections were developed with 0·05% diaminobenzidine tetrahydrochloride and 0·01% hydrogen peroxide in 0·5 M Tris buffer, pH 7·6, for 10 min at room temperature, rinsed in distilled water, dehydrated, and mounted in Permount. Sections were lightly counterstained with methyl green. For control sections, monoclonal antibody was replaced by normal mouse IgG at a concentration of 2 g/ml. Selected sections of either series were also stained for acid phosphatase before counterstaining with methyl green.

Double immunoperoxidase staining was carried out to reveal the anatomic relationship between cells of mononuclear/phagocyte lineage and vascular endothelial cells. The following procedure was used: after completion of the immunoperoxidase staining with monoclonal antibodies, the sections were washed in PBS for 1 hr at 4°, and incubated with peroxidase-conjugated rabbit anti-human Von Willebrand factor antiserum at a dilution of 1:200 for 2 hr at 4°. After washing, the peroxidase was developed with 0·02% 4-chloro-1-naphthol and hydrogen peroxidase solution for 10 min at room temperature. The coverslips were rinsed in PBS and mounted on glass slides in phosphate-buffered glycerol. In light microscopy, the cells of mononuclear/phagocyte lineage labelled with monoclonal antibody stained brown, while the vascular endothelial cells labelled with the rabbit antiserum stained purple in the same section, so that the anatomic relationship between the cells of mononuclear/phagocyte lineage and the blood vessels was readily observed.

Quantitative analysis of mononuclear/phagocyte subsets

For quantification, 10 randomly selected endomysial and perimysial sites were analysed by counting the number of cells labelled with each monoclonal antibody at a magnification of 200 (0·25 mm^2/field) in each section. For each site, the cells per 250 muscle fibres were counted in each of eight rats. Cells reacting for acid phosphatase were also counted in the same manner.

RESULTS

Distribution of cells of mononuclear/phagocyte lineage in skeletal muscle

Immunoperoxidase staining with ED1, ED2 and ED3 monoclonal antibodies was performed on frozen sections of skeletal muscle, and the results are shown in Fig. 1. There was a striking and unexpected scatter of isolated cells, which appeared to localize in narrow interstitial space between the fibres (Fig. 1a, b). These cells had a remarkable dendritic appearance with small nuclei, little cytoplasm, and several elongated processes. Quantitatively, as shown in Table 2, there was a large number of ED2-positive ($ED2^+$) cells in both endomysial and perimysial sites, whereas $ED1^+$ appeared less frequently and occasional $ED3^+$ cells (Fig. 1c) were seen in the interstitial connective tissue. All of these cells were morphologically indistinguishable and occurred throughout the muscle tissue. There were more of these cells occurring in the perimysial site than in the endomysial site (Table 2). In addition, a large number of $ED1^+$ and $ED2^+$ cells, and a few $ED3^+$ cells in the fascia and subfascial connective tissue, where $ED2^+$ cells predominated, were seen.

With acid phosphatase stain on muscle sections, large numbers of isolated positive structures were observed. The morphology and distribution of the cells reacting for acid phosphatase (Fig. 2d) strongly suggested that positive structures were almost certainly macrophages rather than particular areas of muscle fibre. The frequency and distribution of the acid phosphatase-positive cells were similar to those seen with ED2 antibody (Table 2), although the acid phosphatase activity was occasionally very faint in some cells with only a few positive granules.

Using OX6 monoclonal antibody, intensely Ia^+ cells with a dendritic appearance could be demonstrated in normal skeletal

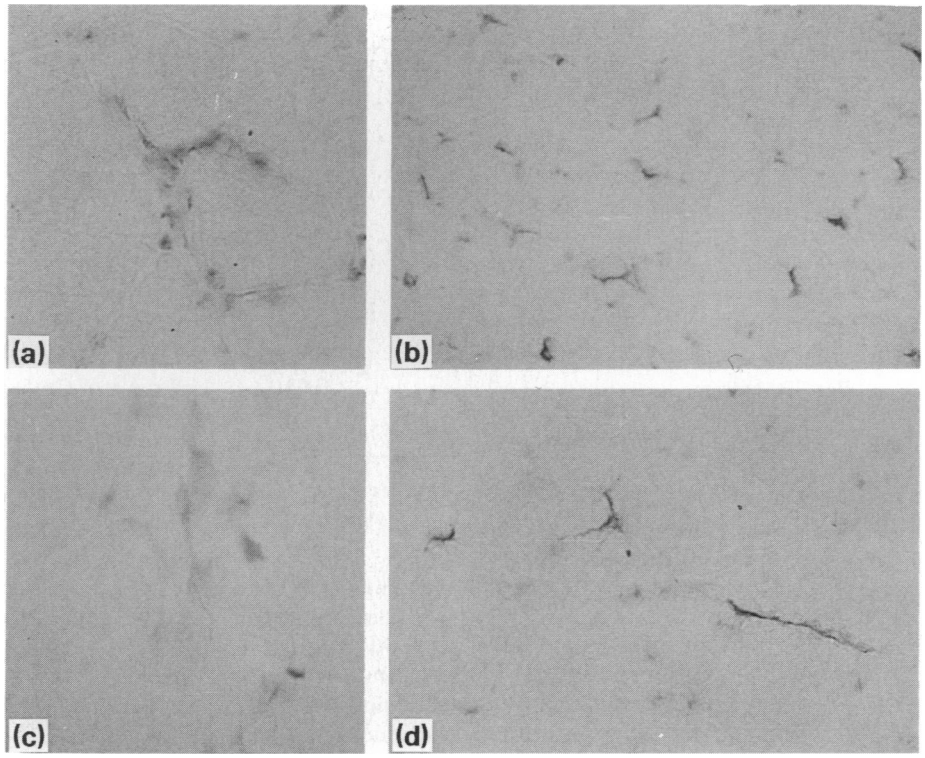

Figure 1. Cells of mononuclear phagocyte system in rat skeletal muscle. Immunoperoxidase staining of cells reacted with ED1 (a), ED2 (b), ED3 (c) and OX6 (d) monoclonal antibodies (a, × 260; b, × 130; c, × 390; d, × 260). Sections were lightly counterstained with methyl green.

Table 2. Quantitative analysis of cells positive for acid phosphatase and six monoclonal antibodies in normal rat skeletal muscle*

Cells positive for:	No. of positive cells	
	Endomysial site	Perimysial site
Acid phosphatase	60·4 ± 10·8	80·7 ± 11·2
ED1	10·1 ± 3·0	19·5 ± 6·0
ED2	62·4 ± 13·2	80·6 ± 14·9
ED3	1·5 ± 1·3	4·6 ± 1·5
OX6	41·8 ± 10·9	34·4 ± 10·5
W3/25	36·2 ± 12·2	50·9 ± 11·4
OX19	0·6 ± 0·6	0·9 ± 0·5

* Quantitative analysis was performed in 6-m cryostat section of quadriceps femoris muscle.

Ten randomly selected endomysial or perimysial sites were analysed by counting the number of cells per 250 muscle fibres in each of eight adult Lewis rats. All values were expressed as mean ± SD of cells per 250 muscle fibres.

Table 3. Ia and CD4 antigens, and acid phosphatase activity on cells positive for ED1, ED2 and ED3 antibodies in rat skeletal muscle*

Marker	ED1$^+$	ED2$^+$	ED3$^+$
Ia antigens	+†	−	−
CD4 antigen	±	+	±
Acid phosphatase	±	+	±

* Adjacent 6-m sections were used to judge the reactivity on each of ED1$^+$, ED2$^+$ and ED3$^+$ cells for either of OX6 (anti-Ia antigen), W3/25 (anti-CD4 antigen) antibodies or acid phosphatase.

† Staining was graded as : + positive; ± slightly positive; − negative.

Some ED2$^+$ cells were faintly stained with W3/25 antibody.

muscle (Fig. 1d). As seen in the sections stained with ED1, ED2 and ED3 monoclonal antibodies, there were many Ia$^+$ dendritic cells similarly interspersed among muscle fibres. However, in contrast to the ED1$^+$, ED2$^+$ and ED3$^+$ cells, the Ia$^+$ dendritic cells tended to occur more frequently in the endomysial site than in the perimysial site (Table 2). These cells could be also seen in the fascia and subfascial connective tissue. Furthermore, using W3/25 antibody to localize CD4 antigen, which has been demonstrated on macrophage subpopulations (Jefferies, Green & Williams, 1985), it could be shown that there was a large number of CD4$^+$ cells in the muscle tissue (Table 2, Fig. 3d). These were almost identical in morphology and distribution with the cells detected by ED1, ED2 and ED3 antibodies. T-lymphocyte marker OX19 was not detected on mononuclear/phagocyte. Round cells labelled with OX19, presumably T lymphocytes, rarely occurred within the muscle (Table 2).

Ia and CD4 antigens, and acid phosphatase activity on mononuclear/phagocytes in the muscle

To determine the localization of Ia and CD4 antigens, and acid phosphatase activity on the cells positive for monocyte/macro-

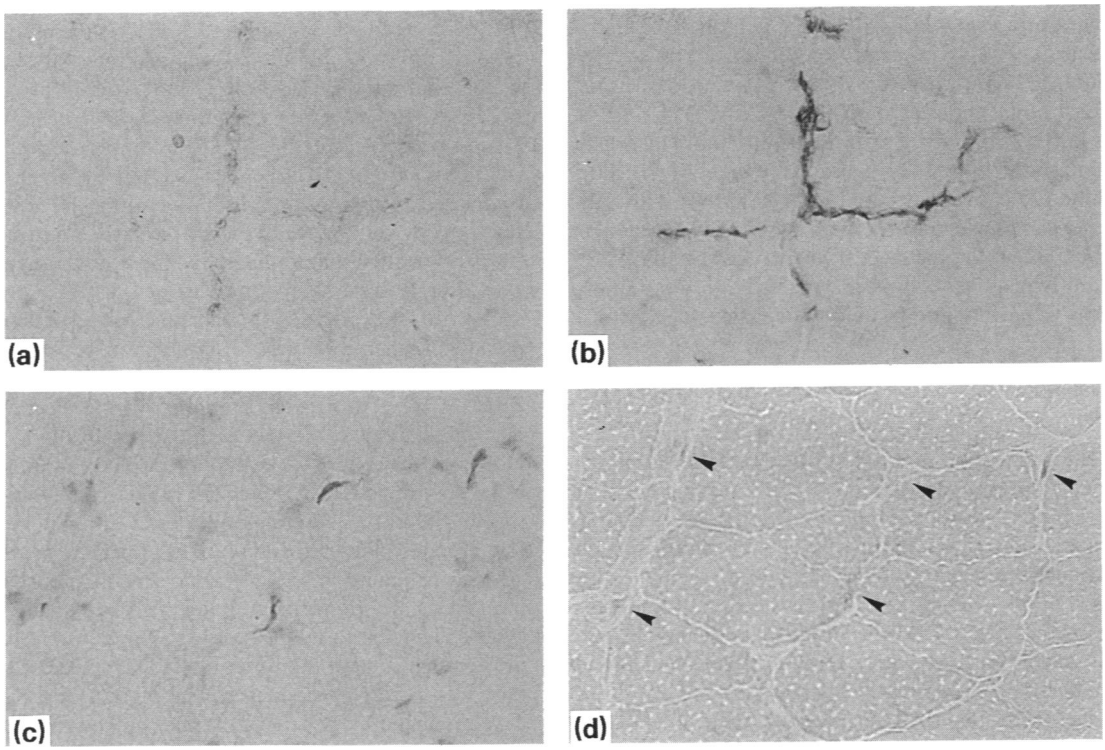

Figure 2. Ia antigens and acid phosphatase on cells of mononuclear phagocyte system in rat muscle. 6-micro thick adjacent sections (a and b, c and d) lightly counterstained with methyl green. ED1$^+$ cells (a) are positive for Ia antigens (b) (a, b, × 390). Most ED2$^+$ cells (c) are positive for acid phosphatase (d, arrow head), but a few ED2$^+$ cells appeared to be negative for this enzyme activity (c, d, × 260).

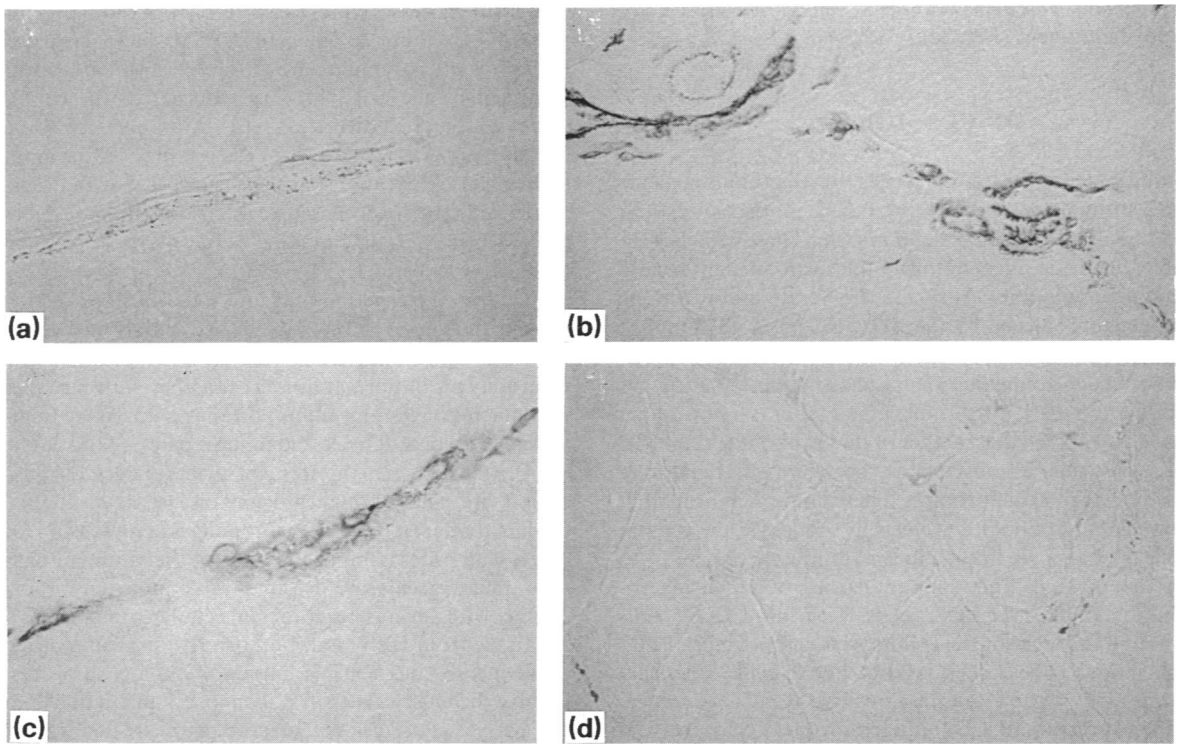

Figure 3. Double immunoperoxidase staining of rat skeletal muscle. Cells labelled with monoclonal antibodies stained brown by using diaminobenzidine as the chromagen. Vascular endothelial cells labelled with anti-Von Willebrand factor antiserum stained purple by using 4-chloro-1-naphthol as the chromogen. Note close association of cells positive for ED1 (a), ED2 (b), OX6 (c) and W3/25 (d) with capillaries or small vessels (purple granular staining in the cytoplasm) in the endomysial and perimysial sites (a, × 390; b, × 130; c, × 390; d, × 260).

phage-specific antibodies ED1, ED2 and ED3, phenotyping of these cells was done on adjacent frozen sections of muscle. As shown in Table 3, Ia antigens were expressed on most of ED1$^+$ cells (Fig. 2a, b), but undetected on ED2$^+$ and ED3$^+$ cells. In contrast to Ia, CD4 antigen was present on almost all ED2$^+$ cells, although some showed very weak expression. Most ED1$^+$ cells and some ED3$^+$ cells appeared to be slightly positive for CD4 antigen. Acid phosphatase activity was readily detected on a majority of ED2$^+$ cells (Fig. 2c, d), whereas ED1$^+$ and ED3$^+$ cells were faintly positive for this enzyme activity. In addition, most Ia$^+$ cells were shown to be acid phosphatase-negative.

Anatomic relationship between monocytes/macrophages and blood vessels

To confirm the initial observation that the cells of the mononuclear/phagocyte lineage were frequently found close to or around the blood vessels, double immunoperoxidase staining was performed. In the rat muscle, rabbit anti-human Von Willebrand factor antiserum specifically labelled vascular endothelial cells. Double-stained sections revealed that the cells positive for ED1, ED2 and ED3 were found with their elongated processes closely associated with capillaries and small blood vessels (Fig. 3a, b). This was also the case with Ia$^+$ or CD4$^+$ cells in the muscle (Fig. 3c, d). Thus, despite their phenotypic differences, most of the tissue-associated macrophages, including Ia$^+$ dendritic cells, appeared to be located at perivascular sites. However, this was not the case with some macrophages with round nuclei and extensive cytoplasm, which were found in the fascia and perivascular space of the relatively large blood vessels. In addition, double immunoperoxidase staining also demonstrated that vascular endothelial cells of rat muscle are negative for Ia antigens (Fig. 3c).

DISCUSSION

These studies demonstrate that cells of the mononuclear/phagocyte lineage are widely distributed in the interstitial connective tissue of the rat skeletal muscle. These cells have a remarkable dendritic morphology and form phenotypically heterogenous populations. Although the nature and functions of the antigens recognized by the ED1, ED2 and ED3 monoclonal antibodies have yet to be determined, they have been reported to recognize distinct macrophage subpopulations in the rat (Dijkstra *et al.*, 1985). Studies at our laboratory on the rat tissues have also shown that neither of these antibodies label the other cell types, such as muscle fibre, nerve, vascular endothelium, lymphocyte or skin fibroblast. These findings indicate that these monoclonal antibodies exclusively recognize the cells of mononuclear/phagocyte lineage in the rat muscle tissue.

There are three distinct subpopulations of tissue macrophage, ED1$^+$, ED2$^+$ and ED3$^+$ cells, with more ED2$^+$ cells predominating throughout the endomysial and perimysial sites of the rat muscle. These cells, mostly ED2$^+$ cells, have acid phosphatase activity and are also positive for CD4 antigen. Indeed, the expression of CD4 antigen on these cells provides further evidence that they are of mononuclear/phagocyte lineage (Jefferies *et al.*, 1985). It was demonstrated that there are large numbers of Ia$^+$ cells with a dendritic appearance scattered in the interstitium of the rat muscle. The Ia$^+$ dendritic cell was first described by Steinman *et al.* (1979) in the mouse lymphoid organs, and has been also demonstrated in various non-lymphoid tissues (Hart & Fabre, 1981). In contrast to the human muscle where capillary endothelium has been reported to express major histocompatibility complex class II antigens (Karpati, Pouliot & Carpenter, 1987), the present study shows that vascular endothelial cells in the rat muscle are negative for Ia antigens. Heretofore, such a widespread distribution of tissue-associated macrophages (resident macrophages) has not been described in skeletal muscle.

As for the origin of tissue macrophages in muscle, the precursor cell, a monocyte of bone marrow origin, is likely to migrate into the tissue by the blood (Van Furth, Raeburn & Van Zwet, 1979). If this is so, it might be expected that the tissue macrophages share the same antigens with blood monocytes. However, the subpopulation of macrophage that predominates in adult muscle is ED2$^+$ and is phenotypically different from that in the blood, where monocytes are predominantly ED1$^+$ (H. Honda, personal observations; Dijkstra *et al.*, 1985). A possible explanation for this phenotypic difference between the monocytes and tissue macrophages is that some ED1$^+$ monocytes emigrating to the muscle tissue are converted into ED2$^+$ or, in much smaller numbers, into ED3$^+$ cells as they differentiate into tissue macrophages. The present observation of the developing muscle, where ED1$^+$ macrophages predominate and where ED2$^+$ and ED3$^+$ cells are rarely seen (H. Honda, H. Kimura and A. Rostami, unpublished data) is in support of this hypothesis.

This study on the anatomic relationship of the tissue macrophages to blood vessels has demonstrated that despite their phenotypic differences, most of these cells are found with elongated processes closely associated with capillaries and small vessels. This intimate association of tissue macrophages with blood vessels is reminiscent of the 'pericyte', which has been identified as the cell lying just external to the capillaries in various tissues (Fawcett, 1986). Although the origin and function of pericytes have not been established, the immunohistochemical observations strongly suggest that the cells closely associated with microvasculature are of mononuclear/phagocyte lineage and that at least some of these cells might be identical with pericytes.

The precise function and inter-relationships of the mononuclear/phagocyte subsets in the muscle are unknown. However, the fact that there is a widespread distribution of resident macrophages in normal muscle, together with recent evidence that macrophages play an important role in tissue homeostasis as well as in local defence and tissue repair (Adams & Hamilton, 1984), suggests that the resident macrophages of muscle may exert a vital role in both health and disease states. In this regard, the fact that most resident macrophages in muscle are located at perivascular sites is important because their intimate association with microvasculature is favourable not only for making contact with various humoral and cellular components entering the tissue from the blood, but also for disposing of unwanted tissue metabolites. During immunopathological processes that involve the muscle, such as polymyositis and dermato-myositis, the perivascular resident macrophages are likely to play an important role in cell-mediated immune response by communicating with the immunocompetent cells and various humoral factors that enter the tissue.

Another point of interest is the observation that in rat

muscle CD4 antigen is expressed on a large number of resident macrophages located in the endomysial and perimysial sites. Although the CD4 glycoprotein may be involved in signalling to the interior of the cell after binding of antigen to the T-cell receptor, and has been shown to interact directly with class II antigens to function as a cell-surface adhesion molecule (Doyle & Strominger, 1987), the functional property of CD4 antigen on macrophages is uncertain.

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