THE MONONUCLEAR CELL POPULATION IN RAT LEG MUSCLE: ITS CONTRIBUTION TO THE LYSOSOMAL ENZYME ACTIVITIES OF WHOLE MUSCLE EXTRACTS

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SUMMARY

The mononuclear cell fraction of rat hind-limb muscle was obtained by digestion with clostridial collagenase in the presence of calcium ions, filtration through nylon screens and washing to remove the enzyme. Final traces of contaminant myofibrillar debris were separated by isopycnic centrifugation in a Percoll density gradient. Whole muscle, washed cells and Percoll-fractionated cells were extracted in the presence of non-ionic detergent and the supernatants assayed for the lysosomal enzymes cathepsins B + L, N-acetyl-β-glucosaminidase, β-glucuronidase and protein. The enzyme levels were highest in the muscle from young rats, but the percentage of recovered activity in the mononuclear cell fraction was little altered by the age of the animal. The values obtained were: cathepsin B + L, 2.4–4.0%; N-acetyl-β-glucosaminidase, 4.3–7.6%; and β-glucuronidase, 6.3–10.3%. Because of unavoidable losses in preparation these are minimal values and the actual levels of activity from the mononuclear cell fraction in muscle would be higher. The specific activity values of the cell lysates were raised after isopycnic centrifugation and were nearly constant over the age range 65–180 days. Substantially higher specific activity values were obtained for the cells from rats of 38 days. When grown in culture the mononuclear cell fractions were seen to contain mainly fibroblasts and myoblasts with only few leucocytes. The cultures reached confluence by the second week, at which time numerous myotubes had formed. In addition there were groups of large, circular cells with a prominent centrosphere. The origin of these latter cells is uncertain.

It was concluded that although total lysosomal enzyme activity was higher in young rats there was little effect of age on the distribution of activity between muscle fibres and mononuclear cells in the muscle.

INTRODUCTION

Skeletal muscle contains a large number of different proteolytic enzymes, including most of the known lysosomal cathepsins. Many of these enzymes have been implicated in the normal turnover of myofibrillar components and several recent reviews have provided a detailed account of the properties of each protease (Bird, 1975; Bird & Carter, 1980; Pennington, 1977). Generally, these proteolytic activities in extracts of muscle are low compared to other soft tissues, and for most enzymes there is uncertainty concerning their distribution between the muscle fibres and the other cells that reside or migrate into the tissue. In their cell fractionation studies, Canonico & Bird (1970) demonstrated that muscle lysosomes were not derived exclusively from

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muscle fibres. A second, non-muscle cell type was identified from its phagocytic capacity. By pretreating these cells in vivo with Triton WR 1339, a population of low-density lysosomes was obtained by isopycnic centrifugation. On the basis of these results it was suggested that the swollen and less-dense lysosomes had originated from infiltrating leucocytes. More recently, Pennington and co-workers (Park, Parsons & Pennington, 1973; Edmunds & Pennington, 1981) demonstrated that if mast cells are degranulated in vivo by injecting compound 48/80 then all alkaline proteinase activity is lost from the muscle. Therefore, this enzyme appears not to be a true muscle proteinase and can have no action in the turnover of myofibrillar proteins.

In order to determine which of the proteinases are directly involved in protein turnover, pure myoblast cultures at the pre- and post-fusion stages were examined by Bird et al. (1981). Clearly, in this system only myoblast-derived enzymes can be identified, but under these in vitro conditions the levels of activity were found to be raised by several orders of magnitude. Therefore the proteolytic events observed in vitro may not be truly representative of normal turnover in intact muscle.

The experiments reported here were done in order to assess directly the contribution of the mononuclear cell population to the total lysosomal enzyme activity of whole muscle. This has been achieved by dispersing the tissue enzymically and harvesting the indigenous population of small, mononuclear cells. The different cells that could be recovered by this procedure were subsequently examined for type during short-term culture.

MATERIALS AND METHODS

Male Sprague–Dawley rats (Carworth CFE Strain) were bred in our animal house. Clostridial collagenase (type II), 4-methylumbelliferonyl N-acetyl-β-D-glucosaminide, 4-methylumbelliferoyl β-D-glucuronide and 4-methylumbelliferone were from Sigma London Chemical Co.; N-CBZ-L-phenylalanyl-L-arginine 7-amido-4-methylcoumarin and 7-amino-4-methylcoumarin were from Bachem Feinchemikalien AG, Bubendorf, Switzerland; Eagle's minimal essential medium and Hanks' complete balanced salt solution were from Flow laboratories Ltd, Irvine, Scotland; foetal calf serum was from Gibco Europe Ltd, Uxbridge, England; Percoll and Percoll density marker beads were from Pharmacia (GB) Ltd, Hounslow, England; woven nylon screens were from Plastok, Birkenhead, England.

Whole muscle extracts

A portion of blotted, chopped muscle from each animal was homogenized in 9 vol. of 50 mM-sodium citrate buffer (pH 5.0) containing 0.2 % (v/v) Triton X-100, stirred at 4 °C for 18 h and then centrifuged at 30000 g for 20 min.

Isolation of mononuclear cells

Rats were killed by cervical dislocation, the hind-leg muscles removed and washed several times in Hank's complete balanced salt solution (HCS). The muscle was trimmed free of fat and epimysium and chopped finely. The tissue fragments were again washed with HCS and then blotted on filter paper. A portion (5 g) was suspended in 50 ml of HCS, containing 2 mg/ml of clostridial collagenase, previously clarified by centrifuging at 20000 g for 20 min, and passed through a 0.2 μm filter. The muscle suspension was agitated in an incubator at 37 °C for 4 h. At the end of this period the dispersed cells were collected by filtering out coarse pieces of residual connective tissue and muscle fibres using a nylon filter with 200 μm pores.
and then passing the cells through a second filter with 35 μm pores. The filters were rinsed through with a small volume of HCS and the cells in the filtrate were collected by centrifuging at 750g for 5 min and resuspending in 10% (v/v) bovine serum in Hank's calcium- and magnesium-free salt solution (HIS) to inactivate residual collagenase. The cells were centrifuged at 250g, washed three times in HIS and finally collected into two equal pellets.

One pellet was taken up in 4 ml of citrate-buffered Triton X-100, and held at 4°C for 18 h to lyse the cells. The cellular debris was removed by centrifuging at 2000g for 10 min. The second pellet was resuspended in 30 ml of 30% (v/v) Percoll made iso-osmotic with HIS and centrifuged at 2000g for 30 min to separate the cells from residual myofibrillar debris. Marker beads were added to a replicate tube of Percoll to indicate the density in each layer. Density gradient fractions of 1.5 ml were collected and diluted with 8 vol. of HIS. The cells in each fraction were then pelleted by centrifuging at 17000g for 15 min and lysed in 2 ml of buffered Triton X-100.

Cell culture
Muscle tissue was collected using aseptic techniques and dispersed using a sterile solution of collagenase in HCS. The flask was shaken at 37 °C and samples removed hourly up to 4 h. The cells were harvested and washed as before in HIS. In some experiments the cells were collected from Percoll gradients after separation from the final traces of myofibrillar debris. The separated cells were cultivated on glass coverslips at 37 °C in Eagle's minimal essential medium with Earle’s salts (MEM), containing 4 mM-glutamine, 2 mg/ml NaHCO3, 100 international units per ml penicillin, 100 μg/ml streptomycin and 10% (v/v) foetal calf serum in an atmosphere of 5% CO2/95% air. Each 35 mm dish contained 2.5 ml of medium with an initial cell density of 2.5 x 10^6 to 5 x 10^6 cells/ml. The cell layer was washed free of debris after 36 h and the medium subsequently replaced at 48 h intervals. Coverslips were removed at selected times up to 14 days, the cells fixed for 20 min in methanol and then stained with Giemsa.

Enzyme assays
Lysosomal cysteine proteinases, mainly cathepsins B and L, were estimated fluorimetrically at pH 6.0 and 37 °C using 10 μM-N-[2-carboxy-2-phenylacetyl]-L-arginine 7-amido-4-methylcoumarin as substrate in a reaction volume of 3.0 ml (Barrett, 1980). The released 7-amino-4-methylcoumarin was monitored continuously using a recorder attached to a Perkin Elmer 3000 fluorescence spectrometer fitted with a thermostatically controlled cell holder. The instrument was set to an activation wavelength of 340 nm and emission wavelength of 460 nm. The β-N-acetyl-glucosaminidase activity was estimated fluorimetrically after the method of Leaback & Walker (1961) using 10 μM-4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as substrate in 30 mM-sodium citrate buffer (pH 4.5). The reaction mixture included 0.1% (v/v) Brij 35, which was also used as diluent for the enzyme, as recommended by Barrett (1980), in the cathepsin assay. The released 4-methylumbelliferone was monitored continuously in the reaction medium (1.2 ml) with the fluorimeter set at an activation wavelength of 320 nm and an emission wavelength of 450 nm. The β-D-glucuronidase activity was estimated similarly with 10 μM-4-methylumbelliferyl-β-glucuronide in 50 mM-sodium acetate buffer (pH 4.5). Standards of pure 7-amino-4-methylcoumarin and 4-methylumbelliferone were prepared in the respective assay buffers containing 0.1% (v/v) Brij 35. Alkaline proteinase activity was determined with casein as substrate (Edmunds & Pennington, 1981). For these assays the muscle and cell preparations were extracted in 10 mM-potassium phosphate buffer (pH 7.7), containing 50 mM-KCl as described.

Protein determination
The Wang & Smith (1975) modification of the Lowry method was used to determine the concentration of protein in the Triton X-100-containing solutions. Standards were prepared from vacuum-dried bovine serum albumin dissolved in Triton X-100.
RESULTS

Extracts were prepared from the hind-leg muscles of rats aged from 38–180 days. The levels of the lysosomal enzymes, cathepsins B + L, N-acetyl-β-D-glucosaminidase and β-D-glucuronidase obtained per g of tissue are shown in Fig. 1. The concentration of each enzyme in the tissue declined with age up to 120 days and was then fairly stable.

Preliminary experiments were done to dissociate the muscle tissue, according to previously published methods. Trypsin (Jones, 1977) appeared to cause extensive destruction of the released mononuclear cell fraction during incubation and was not tested further. The incubations with clostridial collagenase were made in the calcium- and magnesium-free medium as recommended by others (Bischoff, 1974; Bekoff & Betz, 1977), but under these conditions the tissue was dispersed very slowly. Collagenase requires free Ca\(^{2+}\) for activity and therefore cannot function adequately if calcium salts are omitted. When the collagenase was dissolved in a calcium-containing medium (HCS) the muscle was very rapidly broken down and this method promoted the release of much larger numbers of mononuclear cells. With incubation times shorter than 4 h the quantity of cells obtained was less, as judged from the enzyme assays on the lysed washed pellets: 54–82 % of the 4 h value after 2 h and 79–96 % after 3 h. Therefore, in all of the experiments the incubation time was fixed at 4 h, although this is somewhat longer than usual for the enzymic dispersion of tissues. During the incubation

![Fig. 1. Lysosomal enzyme activities in extracts of whole muscle. The values are given as units of muscle, where 1 unit is defined as 1 μmol of substrate hydrolysed/min at 37 °C. Experimental details are given in the text. (O—O) Cathepsin B + L; (△—△) N-acetyl-β-D-glucosaminidase; (□—□) β-D-glucuronidase.](image-url)
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period the muscle fibres were rapidly and extensively degraded with mostly only short fragments remaining after 4 h.

The recovery of the three enzyme activities in lysates of the washed cell pellets was determined, and in Table 1 these results are presented as the percentage of the activity found in the corresponding whole muscle extracts. The specific activity values for each of these lysates are also given. At 38 days the animals were in a very rapid phase of growth, which declined steadily to an exceedingly low value at 180 days. However, the changing growth pattern appeared to have very little influence on the actual percentage of activity recovered in the cell pellets. In these assays the proteinase activity showed the lowest, and β-D-glucuronidase the highest, recoveries at all age points. This probably reflects differences in the composition of the lysosomal hydrolases of the mononuclear cell fraction compared to the muscle fibres.

Table 1. Recovery of lysosomal enzyme activities in the mononuclear cell fraction obtained by collagenase digestion of rat hind-leg muscles

<table>
<thead>
<tr>
<th>Age of rats (days)</th>
<th>Cathepsin B + L</th>
<th>N-acetyl-β-D-glucosaminidase</th>
<th>β-D-glucuronidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Recovery</td>
<td>Specific activity (× 10^4)</td>
<td>% Recovery</td>
</tr>
<tr>
<td>38</td>
<td>3.6</td>
<td>10.1</td>
<td>7.6</td>
</tr>
<tr>
<td>58</td>
<td>3.5</td>
<td>6.7</td>
<td>4.3</td>
</tr>
<tr>
<td>65</td>
<td>4.0</td>
<td>6.4</td>
<td>6.1</td>
</tr>
<tr>
<td>120</td>
<td>2.4</td>
<td>3.5</td>
<td>4.7</td>
</tr>
<tr>
<td>132</td>
<td>3.9</td>
<td>2.2</td>
<td>5.0</td>
</tr>
<tr>
<td>180</td>
<td>4.0</td>
<td>4.2</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Specific activity values are given in μmol substrate hydrolysed/min per mg protein. Data are the averages of two animal experiments at each age.

The specific activity values showed a progressive fall with age, indicating that the enzymes were diluted with more non-enzymic protein at the older age points. Much of this protein had probably originated from the myofibrils and thus the total recovered protein in each of the lysed cell pellets remained fairly constant over the age range.

Half of the washed cell pellet from each preparation was freed of contaminant myofibrillar debris by isopycnic centrifugation in a Percoll gradient. The cells were collected in a layer with an average density of 1.05, whereas the debris settled above this at a density of 1.02. The specific activity values of the lysosomal enzymes in the cell fraction lysates were determined and these are listed in Table 2. These values were again higher at the earliest time point, but otherwise showed a reasonable constancy from 65 days onward. The total increases in specific activity over the corresponding whole muscle extracts are also shown in Table 2 and, as expected, the highest values were obtained for the older rats.

A preliminary attempt was made to estimate the level of the mast-cell alkaline
proteinase in the cell pellets and a separate experiment was done for this assay using phosphate-buffered KCl (pH 7.7), to extract the enzyme. A recovery of only 9% was obtained for the 120-day-old rats, which clearly indicated that there had been a disproportionate loss of enzyme during incubation with collagenase. Therefore, the result did not appear to be representative of the recovered mononuclear cell fraction.

The mononuclear cells when first harvested were small and rounded, and retained 95% viability as estimated by Trypan blue exclusion. The cells adhered rapidly to the coverslips and regained their morphology within 24–36 h, exhibiting a plating efficiency of approximately 30%. No significant differences were noticed in the cells from any of the different age points, nor did there appear to be any noticeable effect of incubation time during dispersion on the subsequent observations of cell type or distribution. Furthermore, it was confirmed that the layer of myofibrillar debris from the Percoll gradient retained very few viable cells.

### Table 2. Fractionation of the mononuclear cells pellets in a Percoll density gradient to remove contaminant myofibrillar debris

<table>
<thead>
<tr>
<th>Age of rat (days)</th>
<th>Cathepsin B+L</th>
<th>N-acetyl-β-D-glucosaminidase</th>
<th>β-D-glucuronidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity ($\times 10^4$)</td>
<td>Increase in specific activity (fold)</td>
<td>Specific activity ($\times 10^4$)</td>
</tr>
<tr>
<td>38</td>
<td>2.1</td>
<td>1.6</td>
<td>9.7</td>
</tr>
<tr>
<td>65</td>
<td>1.6</td>
<td>3.5</td>
<td>5.7</td>
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<tr>
<td>120</td>
<td>1.0</td>
<td>5.6</td>
<td>5.8</td>
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<tr>
<td>132</td>
<td>1.0</td>
<td>6.2</td>
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<tr>
<td>180</td>
<td>1.3</td>
<td>4.8</td>
<td>5.6</td>
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</tbody>
</table>

Specific activity values (in $\mu$mol substrate hydrolysed/min per mg protein) of the lysosomal enzymes and increases compared to the whole muscle extracts are given. Data are the averages of two animal experiments at each age.

A typical picture from the early, 3-day cultures is shown in Fig. 2. Identification of cell types was based on morphology, fibroblasts having a characteristic 'kite-shaped' appearance at low density whilst myoblasts were spindle-shaped and refractile under phase-contrast microscopy. These two cell types predominated in the cell pellets obtained from these dispersions using clostridial collagenase. In addition some small round cells were found, which may have been leucocytes, but generally these were relatively few in number. We were unable to distinguish any mast cells in these cultures even before the first medium change at 36 h when unattached cells were washed away with the tissue debris and residual erythrocytes.

After a period of 4–6 days in culture the fibroblasts had multiplied rapidly and fusion of myoblasts was seen at many sites. Extensive myotube formation had occurred
Fig. 3. Muscle mononuclear cells maintained in culture for 14 days. The cell layer had reached confluence at 6–8 days. A. The myoblasts have fused and formed myotubes, which are still increasing in size and number. Interspersed with the myotubes are numerous fibroblasts. B. Small groups of large, rounded cells of unknown origin are now prominent in these cultures. Each cell shows a stellate cytoplasmic structure extending to the plasma membrane Bar, 100 μm.
by day 14 and the cells obtained from the oldest rats in this study were equally effective in generating myotubes (Fig. 3A). During the second week another cell type became evident, either singly or in small clones, and was easily recognized by its large size and prominent centrosphere. This cell type multiplied less rapidly than the fibroblasts but many clones could easily be seen within the cell sheet by day 14 (Fig. 3B).

DISCUSSION

Previous uncertainty as to the distribution of individual enzymes between muscle cells and ‘non-muscle’ cells prompted this investigation to estimate the recovery of several enzyme activities in a non-muscle cell preparation made under conditions to provide an optimal yield. The muscle tissue was incubated with clostridial collagenase, which released these cells by digesting away the intramuscular connective tissue. Preliminary experiments were done to determine the conditions for maximal recovery of activity in this cell fraction. Previously, collagenase has been used in a calcium-free medium (Bekoff & Betz, 1977; Jockusch & Jockusch, 1980), but this is an unsatisfactory condition for an enzyme that requires free Ca\(^{2+}\) for activity and the omission may totally prevent the release of myoblasts (Bischoff, 1974). The commercial preparations of collagenase contain a variable amount of contaminating proteinase activity as determined with azocasein as substrate at pH 7.5 (unpublished observation). In these earlier studies the contaminant proteinase may have contributed more to the disruption of the extracellular matrix than the collagenase itself during dispersion of the muscle cells.

In the experiments reported here the collagenase was fully activated by using HCS for the incubation medium. However, under these conditions the muscle fibres were substantially degraded. The reason for this appears to be autolysis of the fibres by the calcium-activated neutral proteinase. Muscle is a rich source of this enzyme, which is believed to exist preferentially in the muscle fibres (Dayton et al. 1975; Spacenko, Kay, Goll & Otsuka, 1981). There was no reason to believe that activation of this enzyme was causing undue damage to the mononuclear cells as there was a steady increase in yield over the 4 h incubation period. A more serious problem was found to result from the extensive contamination of the cell pellets with the myofibrillar debris. Although some of this debris could be removed by extensive washing of the cell pellets in HIS, it was necessary to fractionate each pellet by isopycnic centrifugation in a Percoll gradient to obtain a clean cell preparation.

The efficiency of this technique for preparing mononuclear cells remains uncertain as the losses could not be measured. However, from the findings in the preliminary experiments and since the muscle was almost totally dispersed by the use of fully active collagenase, the recoveries were probably higher than in previously published methods. It was not feasible though to make accurate counts of cell numbers in the crude pellets, due to the presence of particulate myofibrillar debris.

When the mononuclear cell fraction was assayed for the three lysosomal enzymes it was found that the cell pellet from the muscle of young rats possessed more of each activity per unit weight of tissue than the cell pellets prepared from the muscle of
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older animals. This finding is consistent with past observations on the histology of development, which have shown that the proportion of stromal tissue is higher in the muscle of young animals (Chiakulas & Pauly, 1965). Furthermore, many satellite cells are associated with the developing muscle fibres during early life, declining in numbers towards maturity (Goss, 1978). However, since the level of each activity present in extracts of the whole muscle tissue was similarly increased, the proportion that was derived from the mononuclear cell fraction remained approximately constant. Therefore, when these data are expressed as a percentage of total activity in each muscle sample they do not reveal the effect of age on the decline in mononuclear cell number per unit weight of muscle during growth. The corresponding decline in the levels of activity of the muscle fibres follows a slowing in the growth rate. However, it is well documented that a high concentration of muscle proteinases in early life is associated with a correspondingly faster rate of turnover of the myofibrillar proteins during this most rapid period of growth (Millward, 1980).

The main cell types to be identified in the early cultures of the mononuclear cell fraction were fibroblasts and myoblasts. The fibroblasts may provide the main supply of connective tissue components, which are intimately associated with muscle fibres. The myoblasts, on the other hand, as precursor (myogenic) cells of the multinucleate muscle fibre, are still comparatively abundant in mature rat leg muscle. They probably remain in a latent or quiescent state, fusing together and forming myotubes only when triggered to repair the muscle following damage (Bischoff, 1979). The presence of substantial numbers of myoblasts in our cell pellets has therefore precluded the description of non-muscle cell as favoured by some investigators to cover this minor population of small cells in muscle. Very few leucocytes were observed and no mast cells were identified in these early cultures.

The recovery of the mast cell alkaline proteinase was low, although it is not known if this was due to degranulation or to loss of viability during dispersion. Therefore, contrary to our original expectation, it was not possible to use this assay as an indicator of the overall recovery of mononuclear cells from whole muscle tissue.

The identity of the large round cells in our mixed cell cultures remains uncertain, although it is feasible that they may have been fibroblastic in origin (Dr Marion Ecob, personal communication).

REFERENCES


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