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The uptake of tritium-labelled carnitine by monolayer cultures of human fetal muscle and its potential as a label in cytotoxicity studies

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SUMMARY

As a novel approach to the investigation of immune responses directed against muscle antigens in inflammatory muscle disease, the use of tritium-labelled carnitine as a selective marker for myotubes in monolayer cultures was investigated. Tritium-labelled carnitine was incubated either with monolayer cultures of human fetal muscle (which contain fibroblasts and myotubes) or with syngeneic monolayer cultures of human fetal fibroblasts. The rate of uptake and loss of tritium-labelled carnitine by muscle cultures was compared with that shown by fibroblast cultures; uptake being five times greater for muscle. Values for K_m and V_{max} were derived for both tissues in culture, the ratio K_m/V_{max} being 3·1 for muscle cultures and 0·46 for fibroblast cultures, indicating the presence of the active transport system for carnitine in the myotube membrane. Freeze-dried radioautographs of muscle monolayers, previously incubated with tritium-labelled carnitine, were made and confirmed the specific intra-tubular localization of the label. Fetal muscle monolayers, previously incubated with tritium-labelled carnitine, were used as targets in long-term cytotoxicity experiments into lymphocyte-mediated myotoxicity. Peripheral blood lymphocytes from patients with inflammatory muscle disease were shown to be myotoxic, but lymphocytes from normal individuals or those with non-inflammatory muscle disease were not. This system is likely to prove much more sensitive than those methods employing chromium-51-labelled cultures. Carnitine-based measures of myotoxicity closely followed the clinical activity of the disease in sequential studies carried out on one patient and the test shows considerable potential as a means of assessing myotube killing by lymphocytes on a per-cell basis.

INTRODUCTION

When skeletal muscle is disaggregated and grown in tissue culture, a monolayer of mixed cell type is formed. Initially, fibroblasts and myoblasts divide, the former adhering to the bottom of the culture vessel a little more rapidly than the latter. The myoblasts differentiate, fusing and forming myotubes, which lie upon and among a bed of fibroblasts. Several methods have been tried as a means of reducing the number of fibroblasts present in these cultures, such as pre-plating (Yaffe, 1968), or the exposure of the cells in the primary cell suspension to cytotoxic drugs (Moss, *et al.*,

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1978) or X-irradiation (Friedlander, Beyler & Fischman, 1978). While these techniques are capable of reducing the numbers of fibroblasts considerably, elimination has not been achieved.

In certain inflammatory muscle diseases, such as polymyositis/dermatomyositis, serological and pathological evidence of immune responses directed against muscle have been described (Currie *et al.*, 1971; Pearson & Bohan, 1977). Lymphocytes from these patients have been shown to adhere to, and to cause disorganization of, cultured muscle monolayers (Currie, 1970). The identification of specific myotube damage by microscopy is handicapped by the difficulty of defining which cells in the culture have been affected by the addition of lymphocytes. The adherence, presumably antigen-specific, of lymphocytes from polymyositis patients to monolayer cultures of chick muscle, has been used as a measure of disease activity (Partridge & Smith, 1976). The numbers of adherent cells present in test lymphocyte populations were compared with those present in populations from patients with non-inflammatory muscle disease. The major disadvantage of this approach is that it gives no information about muscle cell damage. Alternatively, chromium-51 release from labelled muscle cultures, induced by the addition of lymphocytes from patients with inflammatory muscle disease, has been tried as a quantitative test of myotube-specific myocytotoxicity (Dawkins & Mastaglia, 1973). However, as the fibroblasts in the culture also take up chromium-51, the proportion taken up by myotubes is limited. Consequently, when myotube killing is greatest, there is only a small difference between non-specific and specific release with respect to myotube killing, but a much larger difference between specific release and maximum release. Chromium-51-based cytotoxicity studies are not, as a result, very sensitive measures of muscle-specific cytotoxicity.

An alternative approach would be to employ a compound selectively taken up by myotubes and likely to be released rapidly following myotube damage. Uptake by fibroblasts would need to be minimal. Carnitine (γ-amino-β-hydroxybutyric acid·3-methyl-betaine) is a promising candidate. Although it is found in a number of cell types, its concentration in muscle cells is particularly high (Greville & Tubbs, 1968). Furthermore, although cells other than muscle are capable of synthesizing carnitine, both cardiac and skeletal muscle cells depend upon carnitine produced by the liver for their supply, which they take up by means of a membrane-associated active transport system (Rebouche, 1977). The intracellular function of carnitine is to assist in the transport, across mitochondrial membranes, of long chain fatty acids, which are an important source of energy in muscle cell metabolism (Fritz & Marquis, 1965). Physiological studies of carnitine uptake and loss by whole and homogenized animal muscle preparations (Willner, Ginsburg & Dimaure, 1978) and by human myocardial cells from an established cell line (Bohmer, Eiklid & Johsen, 1977), have been published. Carnitine is available commercially in either tritium- or Carbon-14-labelled form.

We describe here experiments on the uptake of tritium-labelled carnitine by monolayer cultures of human fetal skeletal muscle, together with studies of its rate of metabolism and loss, the specificity of labelling in mixed myotube/fibroblast cultures and its potential as a marker for studies of lymphocyte-mediated myotube-specific cytotoxicity.

MATERIALS AND METHODS

Fetal tissue sources. Following approval by our ethical committee, human fetal muscle suitable for culture was made available to us, both from the Medical Research Council Human Tissue Bank at the Royal Marsden Hospital, London, and from the Institute of Obstetrics and Gynaecology at Hammersmith Hospital.

Tissue culture. Human fetal skeletal muscle was removed by dissection and placed in TC 199 medium (Flow), buffered with 0·35% sodium bicarbonate and containing 10% fetal bovine serum (Seralab) and penicillin and streptomycin (Flow). The tissue was finely chopped with a sterile scalpel and incubated at 37°C for 30 min in 0·025% trypsin in 0·5 mM EDTA (trypsin/versene). After digestion, the small muscle pieces were carefully teased apart, the larger clumps of tissue were allowed to sediment and the released cells incubated in 25-cm^2 plastic tissue culture flasks (Falcon). The non-adherent cells were removed and cultured in similar 25-cm^2 flasks. After 1 to 3 days of culture, the cells were detached by incubation with trypsin/versene, split and re-cultured in flasks. This treatment was repeated once or twice and the cells finally dispersed into flat-bottomed 96-well

tissue culture microtitre plates (Nunc) at a concentration of 2–4 × 10^5 cells/well. For radioautographic studies, 1 × 10^6 cells were finally dispersed into 35-mm-diameter plastic dishes (Falcon). Culture was continued for between 1 week and 10 days at 37°C and in 5% carbon dioxide in air, the medium being changed when necessary and the concentration of fetal bovine serum being reduced during the later stages of the culture period to allow myoblast fusion to take place, with the development of myotubes. The maturity of the cultures was judged by microscopy: only those cultures containing a confluent monolayer of healthy cells and an acceptable proportion of myotubes were used in the subsequent experiments. By similar methods, confluent cultures of skin fibroblasts were also obtained from each fetus and established in microtitre trays on the day preceding an experiment.

Labelling with carnitine. Tritium-labelled carnitine (^{3}H-C), specific activity 750 mCi/mmol (the Radiochemical Centre, Amersham), was diluted in TC 199 medium to a final molarity of 5·17 μmol/l. Twenty microlitres of this solution, containing 0·1 μCi of tritium, were added to 80 μl of TC 199 medium in each microculture well, which contained either a fibroblast or a muscle cell monolayer. For radioautographic experiments, 400 μl (containing 2 μCi) of ^{3}H-C solution was added to 600 μl of TC 199 medium in each 35-mm-diameter plastic dish and incubated for 24 hr. When making estimates of K_m and V, 0·1 μCi of ^{3}H-C per well, in different molarities of unlabelled carnitine, was added to parallel cultures of muscle cells, and incubated for varying periods of time.

Harvesting of carnitine-labelled cultures. After monolayers of fetal muscle or fibroblasts had been incubated with ^{3}H-C for an appropriate length of time, the cultures were washed three times in TC 199 medium, 50 μl of 4% sodium dodecyl sulphate were added to each well and the plate allowed to stand overnight at 4°C. The contents of each well were transferred to scintillation vials and each well washed with two 0·2-ml volumes of 0·85% saline, the washings being transferred also to the appropriate scintillation vials. To each vial was added 1·5 ml of Unisolve 100 scintillation fluid (Koch–Light), they were then mixed on a vortex mixer and allowed to stand overnight at 4°C. Emission of β-particles was measured photometrically on an LKB Rack-Beta scintillation counter.

Radioautography. Monolayer cultures of human fetal muscle in 35-mm-diameter plastic dishes, incubated with ^{3}H-C as described, were washed three times in TC 199 medium and snap-frozen by floating the dishes on liquid nitrogen. The frozen monolayers were dried in a freeze-drier (Chem-Lab) to a vapour pressure of 50 mTorr and kept under vacuum at 4°C in a glass desiccator. At radioautography, several 1-cm^2 pieces were cut from the bottom of each dish with a hot scalpel and placed in turn on a 1-cm-diameter stainless-steel post in a darkroom. A thin film of high-plasticizer K2 photographic emulsion (Ilford) was taken up on a 2-cm-diameter platinum wire loop mounted on a glass rod, and placed over each 1-cm^2 piece. The films were dried rapidly in air and exposed in light-proof boxes for 2 weeks. The preparations were developed in D19 developer (Kodak), stopped in 3% acetic acid and fixed in a 1:3 dilution of Amfix (Kodak), before staining the cell monolayers with haematoxylin and eosin.

Statistical comment. There was considerable variation in the number and size of myotubes between muscle cell cultures derived from different fetal tissue sources, although there was little variation between wells plated out from the same tissue source. As a result, it was necessary to normalize data obtained from different experiments on ^{3}H-C uptake, made using different preparations of fetal muscle. This might have been done by relating the total uptake of ^{3}H-C to the amount of DNA/well, but this method would not have allowed for differences in the proportion of myotubes to fibroblasts. It was decided to express the uptake of ^{3}H-C at any one time as a fraction of the uptake of ^{3}H-C at 5 hr in the same experiment. In this way, it was possible to pool information from different experiments.

Lymphocyte-mediated myotoxicity. Monolayer cultures of human fetal muscle were incubated with 0·1 μCi/well of ^{3}H-C as described above and the wells washed three times with culture medium. Purified peripheral blood lymphocyte populations, from patients with active or inactive muscle disease, non-inflammatory muscle disease or normal individuals, were prepared on Ficoll/Hypaque density gradients as previously described (Stern, 1979). Lymphocyte concentrations were adjusted to 10 × 10^6, 5 × 10^6, 2·5 × 10^6, 1 × 10^6 or 0·25 × 10^6/ml and 100 μl of each suspension added to each of five replicate muscle culture wells. These cultures were incubated for 18 hr at 37°C in 5% carbon dioxide in air, washed three times in medium and retained ^{3}H-C harvested as described above.

Lymphocyte-mediated myotoxicity was calculated according to the formula:

$$\left[\left(\frac{TR\ ^3H\ C - ER\ ^3H\ C}{TR\ ^3H\ C}\right) - \left(\frac{TR\ ^3H\ C - CR\ ^3H\ C}{TR\ ^3H\ C}\right)\right] \times 100,$$

where TR ^{3}H-C = retained ^{3}H-C in the absence of lymphocytes, ER ^{3}H-C = retained ^{3}H-C in the presence of test lymphocyte populations, and CR ^{3}H-C = retained ^{3}H-C in the presence of control lymphocyte populations.

RESULTS

Carnitine uptake and loss

In early experiments (not quoted), ^{3}H-C was added to monolayer cultures of human fetal muscle and its disappearance followed. Approximately half the label disappeared immediately from the supernatants and subsequent loss was minimal. However, most of the ^{3}H-C could be recovered from the monolayers by washing. It seemed possible that carnitine, which is hydrophobic, might non-specifically associate with membranes. Fig. 1 shows the results of experiments in which ^{3}H-C was incubated with muscle monolayers for 22 hr and replicate cultures harvested for scintillation counting after increasing numbers of washes. The recovery of ^{3}H-C fell markedly over the first two washes, but succeeding washes did not appreciably lower carnitine recovery from the monolayer.

In Fig. 2 the uptake with time of ^{3}H-C by fetal muscle and fetal fibroblast cultures seen in several different experiments has been pooled in the manner described in the Materials and Methods section. Approximately five times as much ^{3}H-C was recovered from muscle cell cultures as from fibroblast cultures at each interval of incubation. Fibroblasts took up ^{3}H-C steadily, but more slowly and the larger standard error for fibroblast uptake reflects the smaller amount of label recovered. The fractional values for the recovery of ^{3}H-C were subjected to regression analysis, the

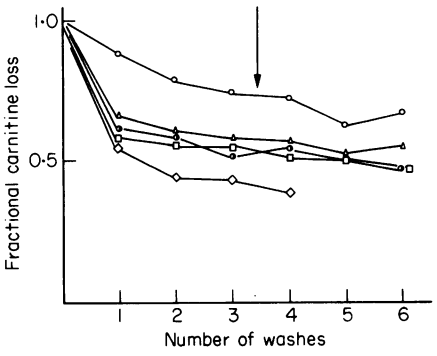

Fig. 1

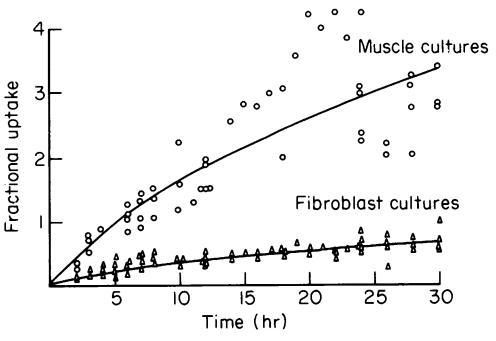

Fig. 2

Fig. 1. The influence of repeated washing with medium of human fetal muscle monolayers on the fractional retention of tritium-labelled carnitine. Each point represents the mean retention in five replicate cells. Five separate experiments are shown. Fractional retention is calculated as described in the text, assuming the retention *ab initio* to be unity. Regression analysis of each curve gave r values greater than 0·92 and standard errors of the mean less than 0·1, where $\log_{10} y = \log_{10} a + b\,x$, and a and b are constants. The arrow indicates the point at which cultures are normally selected for experimental use.

Fig. 2. The fractional retention of tritium-labelled carnitine by fetal muscle cultures (o——o) and by fetal fibroblast cultures (▵——▵) over 30 hr of incubation in eight experiments. The retention of ^{3}H-C in each experiment has been normalized to a value of 1 for muscle culture retention at 5 hr in the same experiment. The uptake of ^{3}H-C followed a power curve (the best fit for both types of monolayer by linear regression is shown) where, for muscle cultures, $y = 0{\cdot}7\ x^{0{\cdot}3}$ ($r = 0{\cdot}92$) and, for fibroblast cultures, $y = 0{\cdot}13\ x^{0{\cdot}49}$ ($r = 0{\cdot}84$). Standard errors of the mean were 0·25 and 0·27 respectively. Each value represents the mean of five replicate wells harvested after increasing intervals of incubation.

best fit obtained being that of a power curve, where $y = 0{\cdot}7\ x^{0{\cdot}3}$ with a correlation coefficient of 0·92 (r), for muscle cultures. Although retention increased progressively over the time period studied, an incubation period of between 8 and 12 hr resulted in sufficient ^{3}H-C retention to keep counting errors below 5%.

Observations on the rate of ^{3}H-C uptake and loss by muscle cell cultures from a single fetus were also made (data not shown). Replicate cultures were incubated with ^{3}H-C for 22 hr, washed once in medium and the culture then continued in medium containing 0·0588 nmol of unlabelled (cold) carnitine, equimolar with the ^{3}H-C solution. Before harvesting, cultures were washed three times in medium. In the presence of equivalent concentrations of cold carnitine, there were two phases apparent in the loss of ^{3}H-C from the monolayers: an early phase (over the first 8 hr), in which loss is slow, and a later phase, during which loss accelerates.

The effect of the presence of increasing molarities of cold carnitine upon the uptake of a constant dose of ^{3}H-C was examined (data not shown). When the muscle cultures were incubated for 2, 4, 5 or 6 hr, there was a linear relationship between $1/v$ (where v = pmol ^{3}H-C retained by cultures) and the ratio of cold to tritium-labelled carnitine, showing that the capacity for carnitine uptake by myotubes was not saturated. However, after 22 hr of incubation, $1/v$ seemed to reach a plateau at cold : tritium-labelled carnitine ratios of between 8 and 11. This suggests that, at this total carnitine dose (1 to 1·5 nmol), saturation may be achieved and, by extrapolation, that ^{3}H-C of less than 200 mCi/nmol specific activity might not be sufficient to label the cultures under our conditions.

Fetal muscle cultures were incubated with 0·0588, 0·1176, 0·1764 or 0·2352 nmol ^{3}H-C for 5, 6 or 22 hr before harvesting and double-reciprocal (Lineweaver–Burke) plots made of ^{3}H-C recovery from these cultures and homologous fibroblast cultures. Regression analysis values, K_m and V_{max}, are shown in Table 1. K_m for muscle remained low at 0·1, while K_m for fibroblasts rose from 0·1 to 0·8. V_{max} for muscle was higher than fibroblast values, but not so much after 22 hr, when they were similar for both types of monolayer. However, K_m/V (1/slope) was consistently seven-fold higher for muscle cultures compared with fibroblast cultures. This shows a remarkable efficiency in carnitine uptake by muscle cultures, even though they contained a minority only of myotubes among a bed of fibroblasts homologous with the cells in the fibroblast cultures.

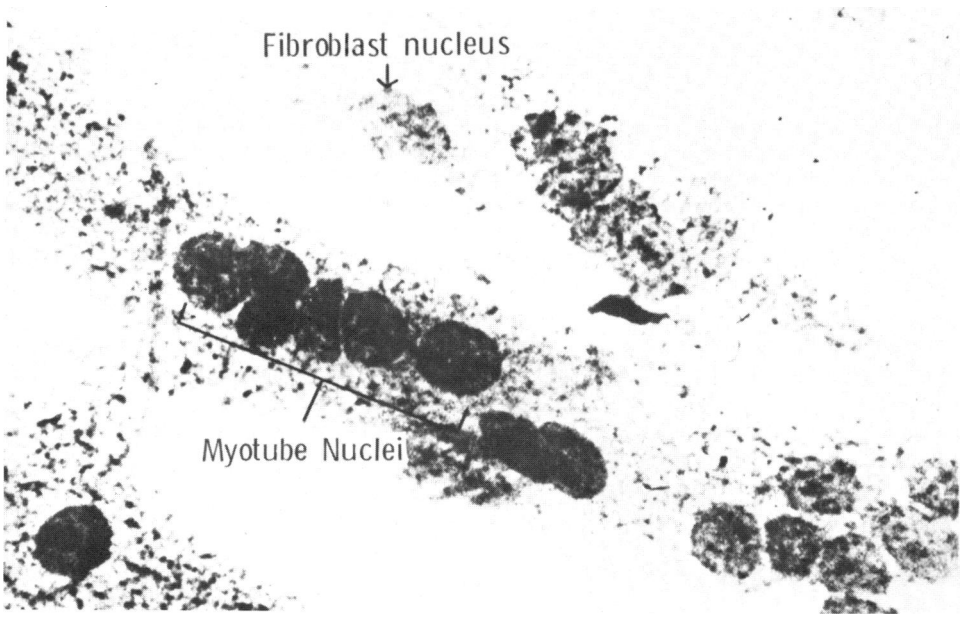

Fig. 3. Photomicrograph of a radioautograph of a freeze-dried fetal muscle monolayer after incubation with ^{3}H-C. The original print was made from a colour transparency. Silver grain deposition is closely related to myotube surfaces: the inter-myotubular spaces contain fibroblasts, as shown, and are almost free of precipitated silver.

Table 1. Linear regression parameters and values for K_m and V_{max} for double reciprocal plots of ^{3}H-C retention by fetal muscle and fetal fibroblast cultures, from the data shown in Fig. 5

	Hours of fibroblast culture			Hours of muscle culture		
Parameters	5 hr	6 hr	22 hr	5 hr	6 hr	22 hr
a_0	1·76	1·86	0·38	1·21	1·26	0·38
a_1	0·34	0·27	0·33	0·13	0·07	0·04
K_m/V $(1/a_1)$	2·94	3·70	3·03	7·69	14·29	25·00
s.e.m.	0·47	0·57	0·36	0·21	0·10	0·03
K_m	0·1932	0·1452	0·8684	0·1074	0·0555	0·1143
V_{max}	0·5682	0·5376	2·6316	0·8264	0·7937	2·8571
r	0·96	0·92	0·98	0·95	0·96	0·99
X-intercept $(1/K_m)$	5·18	6·89	1·15	9·31	18·02	8·75
Y-intercept $(1/V_{max})$	1·76	1·86	0·38	1·21	1·26	0·35

Values for linear regression are shown for the formula $y = a_0 + a_1x$, r = correlation coefficient and s.e.m. = standard error of the mean.

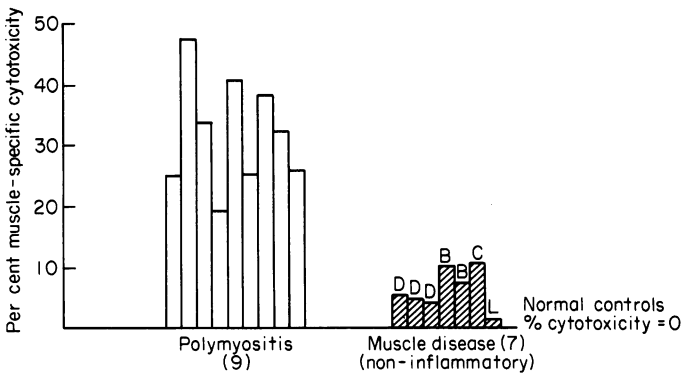

Fig. 4. Myotoxicity mediated by lymphocytes from 11 patients with clinically active polymyositis (□) and seven patients with non-inflammatory muscle disease (▨), compared with cells from normal individuals. Per cent myotoxicity was calculated as described in the Materials and Methods section: values for populations from normal individuals were therefore zero. The data given are restricted in each case to five replicate wells to which 5×10^5 lymphocytes were added. Mann–Whitney rank sum analysis, comparing active with non-inflammatory groups, gave $P \leqslant 0{\cdot}0025$. D = Duchenne muscular dystrophy, B = Becker dystrophy, C = congenital dystrophy (undiagnosed), L = limb girdle dystrophy.

Fig. 3 shows a photomicrograph made from a colour print of a radioautograph of a fetal muscle culture which had been incubated with ^{3}H-C. The inter-myotubular spaces, which contain fibroblasts, are almost free of silver deposition. Quantitation was not attempted, firstly because the multicellular nature of myotube origin made it difficult to relate myotube uptake to fibroblast uptake, and secondly because the designation of representative areas of the specimen for comparative counting might have introduced an element of bias.

Lymphocyte-mediated myotoxicity

Fetal muscle monolayers were incubated with ^{3}H-C and used as targets in studies of lymphocyte-mediated myotoxicity.

Fig. 4 shows the results of myotoxicity experiments, comparing patients with active polymyositis with those with non-inflammatory muscle disease. The difference in lymphocyte-mediated myotoxicity between these two groups was significant (Mann–Whitney rank sum, $P \leqslant 0{\cdot}0025$). Fig. 5 displays the change in lymphocyte-mediated myotoxicity sequentially in a single patient with

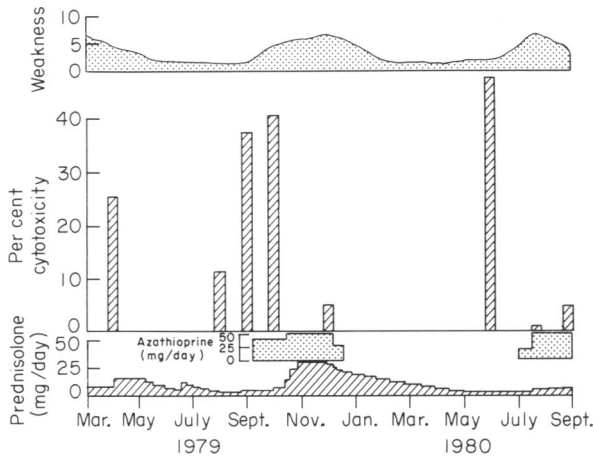

Fig. 5. Myotoxicity mediated by lymphocytes from a patient (C.S.) with dermatomyositis, tested on eight separate occasions and correlated with changes in muscle strength, according to a standard scale. Myotoxicity was calculated, at a dose of 5×10^5 cells/well, according to the method described in the text, from five replicate wells.

dermatomyositis, correlated with her clinical state. Myotoxicity closely mirrored the clinical activity of her disease, as judged by her weakness.

DISCUSSION

These experiments were undertaken in a search for a suitable radiolabelled molecule that might be taken up by myotubes in mixed muscle cell cultures and released rapidly after immunological myotube damage. The washing experiment (Fig. 1) was carried out to see whether the release of ^{3}H-C from damaged myotubes could be detected in culture supernatants. The study showed that there was a tendency for extracellular ^{3}H-C to remain associated with the monolayer, probably in cell membranes. Because this ^{3}H-C can be removed effectively by three washes, in subsequent cytotoxicity experiments, including those reported here, ^{3}H-C retention by the monolayers was measured. All cultures were washed three times before solubilization with sodium dodecyl sulphate for scintillation counting, or before radioautography. We found that it is necessary to employ culture medium as the wash fluid, as the use of other isomolar solutions can lead to rapid detachment of myotubes during the washing process, and ^{3}H-C loss.

When ^{3}H-C was added to parallel cultures of either fetal muscle or fetal fibroblasts, muscle cultures retained the label to a greater extent (Fig. 2). Although myotubes represent only a minority of the cells in cultures of muscle, their presence was sufficient to create this substantial difference in ^{3}H-C retention. The true difference in the uptake of ^{3}H-C between myotubes and fibroblasts in the same monolayer culture will be greater than that shown in our experiments.

Because there was considerable variation in the size and proportion of myotubes to fibroblasts between cultures established from different fetal sources, direct comparison between the retention of ^{3}H-C in different experiments was not possible. A chemical estimate of the myotube mass present in each well might have been made, for example, by measuring the quantities of myosin or 3-methyl-histidine per well and expressing these values as ratios with respect to total DNA/well. However, as these microwell cultures were small, such techniques were not suitable owing to their insensitivity and the time required to carry them out. Normalization of ^{3}H-C retention to the 5-hr value, as described in the Materials and Methods section, was the chosen manipulation. Because there was little variation in the proportion of myotubes present in wells cultured from the same fetal source, the fractional retention of ^{3}H-C at different times reflected the activity of the membrane-associated active transport system for carnitine at different extra- and intracellular molarities of

carnitine. At the same time, these values were independent of the quantity of myotube plasma membrane present in different experiments. When results from different experiments were normalized in this way, ^{3}H-C uptake was shown to be similar for each culture. It was therefore possible to establish and label fetal muscle in a reproducible way with ^{3}H-C.

The values for K_m and K_m/V for muscle cultures compared with fibroblast cultures (Table 1) reflect the presence of an active transport system for carnitine in myotube membranes. In our experiments, ^{3}H-C uptake became more efficient as the period of incubation increased (K_m/V rose) but, as V_{max} for fibroblasts also increased at 22 hr, optimal differences in ^{3}H-C uptake between myotubes and fibroblasts at the molarity and specific activity used were achieved after 8 to 12 hr of incubation. Double-reciprocal regression analysis provides a convenient way of examining the efficiency of ^{3}H-C transport in these monolayer cultures. Such studies could prove helpful both in the standardization of ^{3}H-C-labelled cultures for cytotoxicity experiments in the investigation of hereditary muscle disease and in the other aspects of muscle physiology.

Radioautography was carried out on mixed muscle monolayer cultures to discriminate between the retention of ^{3}H-C by myotubes and the retention by fibroblasts. Because carnitine is a small, rapidly diffusing molecule, it was necessary to freeze-dry the labelled monolayers after washing. The morphology of the monolayers was well preserved, although crystals of culture medium, used as the wash fluid, remained on the surface and led to distortion of the photographic emulsion with faint, non-specific silver precipitation. However, tritium-induced silver grain deposition, by ^{3}H-C, was largely confined to the myotubes. This experiment provided technically independent confirmation of the preferential uptake of ^{3}H-C by the myotubes in these cultures.

The aim of these studies was to increase the sensitivity of measurements of lymphocyte myotoxicity in peripheral leucocyte populations from patients with inflammatory muscle disease. The representative cytotoxicity experiments shown (Figs 4 and 5) demonstrate that the method is sensitive enough for the purpose, and we have found it capable of showing lymphocyte myotoxicity on a per-lymphocyte basis. These studies have been made using 18-hr cytotoxicity experiments, but a standard 6-hr short-term killer cell (STKC) assay is under evaluation. Lymphocytes from some patients with dermatomyositis cause ^{3}H-C loss from muscle cultures in a fashion related to lymphocyte dose, while cells from others damage myotubes at all cell doses. Paradoxically, carnitine retention at high lymphocyte doses probably represents the redistribution of ^{3}H-C into lymphocytes adhering to the monolayer.

Although all myotoxicity experiments were carried out employing a range of lymphocyte doses, the small study in which lymphocytes from inflammatory and non-inflammatory muscle diseases were compared was analysed at a dose of 5×10^5 lymphocytes/well only. This dose gave the clearest significant differentiation between the two groups, but comparison of disease activity between patient samples and sequentially in the same patient will require myotoxicity to be measured on a per-lymphocyte basis, so that comparison by parallel-line assay may be performed (Michie, 1973). Adjustments for variations in the proportion of myotubes between experiments may be done by measuring the retention of ^{3}H-C at a given time for all cultures and normalizing the values as described here.

One patient (C.S.) was studied over an 18-month period (Fig. 4). She presented with typical clinical features of dermatomyositis and investigations revealed elevation of serum creatine phosphokinase and myopathic features on electromyography. Initial treatment with prednisolone led to remission, but her course began to show the fluctuations typical of this condition. Deterioration began after 5 months and initially continued, in spite of increasing doses of prednisolone and the later addition of azathioprine. There followed 6-month remission, when the dose of steroids was reduced. A subsequent myotoxicity test showed marked muscle killing and anticipated her relapse by 2 to 3 weeks. A recent increase in prednisolone dose and the addition of azathioprine has led to a further improvement. The clinical assessment of the activity of her disease was mainly subjective, but with respect to this new measure of myotube damage, it is encouraging to note the independent correlation between the severity of her muscle disease and the degree of myotoxicity shown by her lymphocytes.

No specificity controls were included in the myotoxicity studies reported here, such as the measurement of lymphocyte-mediated cytotoxicity against other cell types, because the principal

aim was to increase the sensitivity of the measurement of myotube damage in mixed cell cultures. The most interesting alternative cell for cytotoxicity studies is myocardial, and experiments on such cultures have begun. However, a more direct approach would be to define the molecule recognized by myotoxic lymphocytes on the myotube membrane. We have adapted the shearing technique developed by Crumpton and his colleagues (Crumpton & Snary, 1974; Crumpton *et al.*, 1978) to produce reasonably pure preparations of muscle cell membrane. We hope to purify muscle membrane components, such as glycoproteins, from these preparations and examine their antigenic role in inflammatory muscle disease.

This cytotoxic test, based upon the retention of ^{3}H-C by myotubes, has proved both reproducible and helpful in the assessment of disease activity in patients with polymyositis/dermatomyositis. The introduction of STKC tests promises to allow a more accurate definition in the study of disease groups and sequentially in the same patient. Finally, the identification of the target antigen recognized by myotoxic lymphocytes, by the use of blocking experiments, will be an important step in the understanding of the aetiology of inflammatory muscle disease.

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REFERENCES

BOHMER, T., EIKLID, K. & JOHSEN, J. (1977) Carnitine uptake into human heart cells in culture. *Biochim. Biophys. Acta*, **465**, 627.

CRUMPTON, M.J. & SNARY, D. (1974) Preparation and properties of lymphocyte plasma membranes. *Contemp. Top. Mol. Immunol.* **3**, 27.

CRUMPTON, M.J., SNARY, D., WALSH, F.S., BARNSTABLE, G., GOODFELLOW, J. & BODMER, W.F. (1978) Molecular structure of the gene products of the human HLA systems: isolation and characterisation of HLA, B, C and Ia antigens. *Proc. R. Soc. Lond. (Biol.)*, **201**, 1146.

CURRIE, S. (1970) Destruction of muscle cultures by lymphocytes from cases of polymyositis. *Acta Neuropathol. (Berl.)*, **15**, 11.

CURRIE, S., SAUNDERS, M., KNOWLES, M. & BROWN, A.E. (1971) Immunological aspects of polymyositis. *Q. J. Med.* (new series XL) **157**, 63.

DAWKINS, R.L. & MASTAGLIA, F.L. (1973) Cell-mediated cytotoxicity to muscle in polymyositis. *N. Engl. J. Med.* **288**, 434.

FRIEDLANDER, M., BEYLER, E. & FISCHMAN, D.A. (1978) Muscle development *in vitro* following X-irradiation. *Dev. Biol.* **66**, 457.

FRITZ, I.B. & MARQUIS, N.R. (1965) The role of acyl-carnitine esters and carnitine palmitoyl transferase in the transport of fatty acyl groups across mitochondrial membranes. *Proc. Natl. Acad. Sci. USA*, **54**, 1226.

GREVILLE, G.D. & TUBBS, P.K. (1968) The catabolism of long chain fatty acids in mammalian tissue. *Essays Biochem.* **4**, 155.

MICHIE, D. (1973) The biometrics of the spleen weight assay. In *Handbook of Experimental Immunology* 2nd edn (ed. by D. M. Weir), Chap. 30. Blackwell, Oxford.

MOSS, M., NORRIS, J.S., PECK, E.J. & SCHWARTZ, R.J. (1978) Mobility of ^{125}I-labelled proteins in myogenesis. *Exp. Cell Res.* **113**, 445.

PARTRIDGE, T.A. & SMITH, P.D. (1976) A quantitative test to detect lymphocytes sensitised against the surface of muscle cells. *Clin. exp. Immunol.* **25**, 139.

PEARSON, C.M. & BOHAN, A. (1977) The spectrum of polymyositis and dermatomyositis. In *Symposium on Rheumatic Diseases. Med. Clin. North Am.* **61**, 439.

REBOUCHE, C.J. (1977) Carnitine movement across muscle cell membranes. *Biochim. Biophys. Acta*, **471**, 145.

STERN, C.M.M. (1979) Changes in lymphocyte subpopulations in the blood of healthy and sick newborn infants. *Pediatr. Res.* **4**, 781.

YAFFE, D. (1968) Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc. Natl. Acad. Sci. USA*, **61**, 477.

WILLNER, J.H., GINSBURG, S. & DIMAURE, S. (1978) Active transport of carnitine into skeletal muscle. *Neurology (Minneap.)*, **28**, 721.