An evaluation of cell separation techniques in a model mixed cell population

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Summary

Muscle precursor cells may act not only as a means of inserting normal genes into diseased muscle fibres, in order to correct or alleviate a genetically inherited myopathy, but recent demonstrations have shown they may prove an invaluable tool for the expression of, and systemic dissemination of, non-muscle gene products. If muscle precursor cells are proved to act as such widespread vectors in terms of gene therapy, then it is imperative that methods are properly elucidated to produce large populations of pure viable myogenic cells for such purposes. In the past, many methods of cell separation have been investigated but carry with them the problems of either a lack of myogenic purity of the population or poor percentage recovery of the original cell population.

In the present work we have investigated two methods for segregating myogenic from non-myogenic cells and have critically reviewed the efficiency of separation of the two techniques used. To obtain a quantitative measure of separation efficiency, segregation was carried out on a 1:1 mixture of murine C2 myogenic and murine 3T3 fibroblastic cells. To distinguish between C2 and 3T3 cells, the latter were prelabelled with the fluorescent strain carboxyfluorescein diacetate succinimidyl ester (CFSE). Once incorporated into the cell, CFSE remains there, thus preventing transfer of the label to C2 cells. Both methods of separation used depend on the affinity of myogenic cells for the monoclonal antibody Mab H28, which specifically binds to the mouse neuronal cell adhesion molecule N-CAM, but differ in that one method, "panning", completes segregation by adherence of N-CAM positive cells to a dish precoated with secondary IgG antibody whereas in the other separation proceeds by the use of commercially available IgG-coated magnetic beads. Results indicate magnetic bead separation to be more efficient than panning if the beads are precoated with 0.1% gelatin.

Key words: segregation of myogenic cells, N-CAM, specificity, Dynabeads, carboxyfluorescein diacetate succinimidyl ester labelling.

Introduction

The isolation and cloning of the gene responsible for the condition of Duchenne muscular dystrophy (DMD; Hoffman et al., 1987; Monaco, 1988) has heightened the realization that implantation of normal muscle precursor cells carrying a normal complement of genes may well provide a viable therapeutic method of introducing missing genes and their products into the damaged muscle fibres of patients suffering from primary myopathies (Partridge et al., 1989; Law et al., 1990; Tremblay et al., 1991). Awareness of the value of this method as a therapeutic intervention has received even more credence with the latest finding that muscle precursor cells are capable of expressing introduced genes unrelated to myogenesis and, even more importantly, that they can secrete the products of such introduced genes into the circulation (Dhawan et al., 1991; Barr and Leiden, 1991). This throws open the whole question of using muscle precursor cells as vectors for the introduction of other genes and the distribution of their products systemically round the body.

In contemplating the use of muscle precursor cells as such vectors in gene therapy, for introducing missing gene products, it is therefore important to ensure that the cells that are being used to carry the introduced gene are indeed myogenic for, in case of myopathies, these cells contain the muscle-specific genes that are lacking in the patient's own muscles and, further, only myogenic cells will readily fuse with the host fibre into which they are being introduced. In diseases other than those of muscle, the use of cells that are truly myogenic again has been shown to be imperative (Dhawan et al., 1991; Barr and Leiden, 1991). The source of muscle cells for such gene therapy is human fetal material or
biopsied muscle specimens. The problem with obtaining such material is that it contains a high proportion of other cells, including endothelial cells and fibroblasts, which are neither muscle-gene-specific nor show the characteristics of muscle precursor cells. Further, myogenic cells have been shown to be less immunogenic than other cell types (Karpati et al., 1989; Watt, 1990; Watt et al., 1992), a very important consideration when contemplating the implantation of allogeneic cells into the muscle fibres of young Duchenne dystrophy patients.

Much attention in myoblast transfer therapy has been directed towards the production of a pure population of myogenic cells for implantation. In the past, various methods of cell separation have been employed in an attempt to enrich for myogenic cells. Some methods exploited the differences in substratum adhesiveness between the cells to be separated (Yaffe, 1968), and others exploited the differences in cell density when separated in a discontinuous Percoll gradient (Morgan, 1988), but these earlier methods failed to produce pure myogenic cell populations. More success has been achieved using the Fluorescence Activated Cell Sorter (FACS) (Webster et al., 1988). This latter method utilises the high level binding of antibodies to the muscle-specific neuronal cell adhesion molecule (N-CAM). This molecule is present on the surface of myoblasts throughout myogenesis (Moore et al., 1987) and is therefore a useful marker of skeletal muscle. The disadvantage of the FACS method, however, is the loss of up to 50% of the original population of cells (Webster et al., 1988). The recent application of the “Panning method” for separating cells (Wysocki and Sato, 1978; Morhenn et al., 1983; Linge et al., 1989) has resulted not only in the production of an enriched myogenic cell fraction, but also in the recovery of 90-95% of the original population (Jones et al., 1990). To achieve this we utilised the antibody Mab H28, derived from mouse/rat hybrid hybridoma cells. This antibody specifically recognises mouse muscle N-CAM (Gennarini et al., 1984, 1986; Walsh and Moore, 1986), and thus enabled us (Jones et al., 1990) to obtain an enriched myogenic fraction of cells from a mixed cell population prepared by enzymatically disaggregating neonatal mouse muscle (Watt et al., 1982). In this method the myogenic cells are recognised by Mab H28, which is derived from mouse as the antibody Mab H28 is specific to mouse muscle N-CAM (Gennarini et al., 1984, 1986; Walsh and Moore, 1986). As assessment of our results by morphological criteria is difficult due to the often fibroblastic appearance of the C2 myogenic cell, it was necessary to have a means of distinguishing between the two cell types used. In the past we have used antibodies to the intermediate filament proteins desmin and vimentin, to characterise myogenic and fibroblastic cells (Jones et al., 1990). However, although vimentin filaments are found in cultured fibroblasts, they are not exclusive to them and are also present in myogenic cells (Stewart, 1990). In the light of this, in our present work we have chosen to prelabel the 3T3 cells prior to their co-incubation with the C2 cells. The agent used is the vital stain (5- and 6-) carboxyfluorescein diacetate succinimyl ester (CFSE). This has been used for long-term staining of cells in culture without adverse consequence to their morphology, physiology or growth characteristics (Bronner-Fraser, 1985). Two advantages of CFSE are that there is no surface labelling of the cell, which could potentially interfere with the separation procedure, and that it only fluoresces after cleavage by intracellular esterases,
resulting in the chromophore becoming negatively charged and ensuring that it remains localised within the 3T3 cell population. As such it is an ideal label for the experiments described here for it allows easy visual interpretation of our results without the ambiguity sometimes experienced with anti-vimentin immunofluorescence staining.

Materials and methods

Culturing of cells
Cells of the C2 myogenic cell line were cultured in BHK-21 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 IU/ml penicillin, 100 μg/ml streptomycin on a substratum precoated with 0.01% sterile gelatin solution. 3T3 cells were grown in DMEM supplemented with 10% newborn calf serum, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Both C2 and 3T3 cells were maintained at 37°C in 5% CO2.

Prelabelling of 3T3 cells with CFSE
Confluent 3T3 cultures were briefly rinsed in 0.02% ethylene-diaminetetra-acetic acid (EDTA) in calcium- and magnesium-free Hanks’ balanced salt solution (CMF-HBSS), and detached from the growth surface of the flask with a solution of 0.25% trypsin/0.02% EDTA in CMF-HBSS. Following detachment, the action of trypsin was inhibited by the addition of 1 ml of fetal calf serum and the resulting cell suspension centrifuged at 4°C at 350 g for 5 minutes. The resulting cell pellet was washed and resuspended in 5 ml CMF-HBSS to which CFSE (Molecular Probes, Inc, USA) was added to give a final concentration of 500 ng/ml. Cells were incubated with CFSE for 15 minutes at 37°C. CFSE-stained cells were pelleted and washed twice with CMF-HBSS prior to resuspension in growth medium at a density of 2 x 10^5 cells/ml, and plated onto the wells of sterile multitest slides coated with 0.01% sterile gelatin solution.

Several other concentrations of CFSE were tried, ranging from 50 μg/ml to 50 ng/ml. The addition of 500 ng/ml of CFSE to 3T3 cultures resulted in the cells staining intensely at both 3 and 20 hours. Similar trials were performed with C2 cells and unlabelled 3T3 fibroblasts were used. We thus present data using CFSE-labelled 3T3 cells only.

Separation of a mixed cell suspension into two cell populations by the panning method has been previously described (Pizzey et al., 1988) that this protocol does not damage the calcium-independent adhesion of C2 myoblasts mediated by N-CAM. Each separate culture of C2 or 3T3 cells was then resuspended in its appropriate growth medium and incubated overnight in the wells of sterile multitest slides at a density of 2 x 10^5 cells/ml. Twenty hours later, cells were washed and fixed as described above for CFSE labelling. Following three brief rinses in PBS, 50 μl of a 1:40 dilution of primary antibody in PBS, washed and mounted with 3T3 marked C2 cells and unlabelled 3T3 fibroblasts were used. We subject to separation using one of the two following separation techniques. In some cases, 1:1 mixtures of CFSE-marked C2 cells and unlabelled 3T3 fibroblasts were used. We were not able to quantify a small number of 3T3 cells that were collected by Dynabead attachment (see Table 1, below) as their presence was obscured by the large number of fluorescing C2 myoblasts. We thus present data using CFSE-labelled 3T3 cells only.

Staining of C2 and 3T3 cells for the presence of surface N-CAM
This procedure was undertaken to ensure that at the start of the experiment the C2 cells were capable of binding anti-N-CAM antibody and, in contrast, that the 3T3 cells were fibroblastic and would not respond in this way. Separate cultures of C2 and 3T3 cells were therefore briefly rinsed in warm 0.02% EDTA in CMF-HBSS, detached from the growth surface of the flask with 1 ml of 0.25% trypsin/0.02% EDTA in CMF-HBSS; the action of trypsin stopped with fetal calf serum and the cells pelleted by centrifugation at 4°C for 5 minutes. We have previously shown (Pizzey et al., 1988) that this protocol does not damage the calcium-independent adhesion of C2 myoblasts mediated by N-CAM.

Preparation of 1:1 mixed population of 3T3 and C2 cells
Cultures of 3T3 cells were trypsinised from the surface of the culture flask and incubated for 15 minutes with CFSE at a final concentration of 500 ng/ml. Cells were washed twice in CMF-HBSS, resuspended in 1 ml of growth medium, and a volume of the cell suspension containing 6 x 10^5 cells was transferred to a sterile conical-based centrifuge tube. C2 cells were trypsinised from the surface of the culture vessel, centrifuged and resuspended in growth medium. A volume of the cell suspension containing 6 x 10^5 cells was taken and added to the CFSE-labelled 3T3 cells in the sterile centrifuge tube. The cells were briefly mixed, pelleted and subjected to separation using one of the two following separation techniques. In some cases, 1:1 mixtures of CFSE-marked C2 cells and unlabelled 3T3 fibroblasts were used. We were unable to quantify a small number of 3T3 cells that were collected by Dynabead attachment (see Table 1, below) as their presence was obscured by the large number of fluorescing C2 myoblasts. We thus present data using CFSE-labelled 3T3 cells only.

Cell separation procedures
Separation of cells using the panning method
Separation of a mixed cell suspension into two cell populations by the panning method has been previously described (Jones et al., 1990). Briefly, the pellet of 1.2 x 10^6 3T3/C2 cells was resuspended in 300 μl of anti-N-CAM antibody, mixed with 500 μl of CFSE, incubated on ice for 60 minutes. During this time, the surface of a 60 mm bacteriological grade dish, which does not normally support the attachment and growth of cells, was evenly coated with goat anti-rat IgG at a concentration of 10 μg/ml in 0.05 M Tris buffer, pH 9.5. After 40 minutes, the IgG solution was gently poured from the dish, the latter washed three times with PBS and twice with 5% FCS in PBS. Following incubation with anti-N-CAM, cells were washed, resuspended in 2 ml of 5% FCS/PBS and layered onto the surface of the prepared dish on ice for 80 minutes. After incubation, cells to which the N-CAM antibody had not bound, and had therefore remained in suspension, were removed from the dish by gently pouring off the supernatant, washing carefully three times in 5% FCS/PBS so as not to
disturb any attached cells and pooling the washes with the initial supernatant, to constitute the “non-adherent” fraction. Cells immobilised on the dish surface by the goat anti-rat IgG/anti-mouse N-CAM complex, were removed by adding 2 ml 5% FCS/PBS and agitating in order to detach the cells from the dish. This was repeated twice more and the three washes were pooled to constitute the “adherent” fraction. Both fractions were pelleted, resuspended in BHK-21 growth medium and adjusted to a density of $5 \times 10^4$ cells/ml before plating into the wells of gelatin-coated multitest slides. Subsequent to overnight incubation at 37°C and 5% CO₂, cells were washed in 3% sucrose in PBS, fixed in a 4% paraformaldehyde/3% sucrose solution in PBS for 30 minutes, washed and scored for the presence of fluorescing and non-fluorescing cells.

Separation of cells using magnetic beads

The pellet of C2/CFSE-labelled 3T3 cells, prepared as described above, was resuspended in 300 µl of anti-N-CAM antibody, and incubated on ice for 60 minutes. During this time the magnetic beads were prepared. Precocating of beads with 0.1% Bovine Serum Albumin (BSA) is recommended by the bead manufacturer (Dynal U. K. Ltd) in order to prevent non-specific binding of cells to the beads. In some experiments we used beads that had not been precoated in any way, in others we precoated by incubation at 4°C for 60 minutes with 0.1% BSA and in a final series 0.1% gelatin was used as the precoating agent. All precoating and subsequent use of the Dynabeads was carried out at pH 7.4, as recommended by the manufacturer. Subsequent to precoating, beads were washed twice in PBS and resuspended in 5% FCS/PBS prior to their use in separation of the cells types.

Following incubation of the cell population with anti-N-CAM antibody, cells were washed to remove any unbound N-CAM from their surface and resuspended in 300 µl of 5% FCS/PBS in a sterile Eppendorf tube to which 10.5 µl of magnetic Dynabeads covalently precoated with sheep anti-rat IgG was added. The resulting suspension was incubated on ice for 2 hours and gently vortexed for 10 seconds every 15 minutes to prevent settling out of the beads. After the 2 hour incubation, tubes were transferred to a Dynal Magnetic Particle Concentrator (MPC) for 5 minutes, after which the supernatant was removed and stored in a separate sterile tube. Pellets were resuspended and washed twice in 5% FCS/PBS; each time the tubes were returned to the MPC to reconcentrate the beads. Following each reconcentration step, the supernatant was removed and added to that removed after the first concentration step. These pooled supernatants thus contained the “non-adherent” fraction of cells. The pellet remaining in the tubes in the MPC were resuspended in 5% FCS/PBS, this suspension constituting the “adherent” fraction of cells. Both fractions were centrifuged at 350 g for 5 minutes and resuspended in BHK-21 growth medium at a density of $5 \times 10^4$ cells/ml. Samples (50 µl) of each cell fraction were plated into the wells of multitest slides, incubated overnight, washed and fixed 24 hours later, as described for the panning method.

Results

Detection of CFSE within 3T3 cells

Following either 3 or 20 hours of incubation with CFSE, the 3T3 cells remained viable, their morphology was consistent with that of healthy fibroblasts in monolayer culture and when viewed using fluorescence microscopy all cells stained intensely (Fig. 1).

Anti-N-CAM staining of individual cultures of C2 and 3T3 cells

Intense staining was observed when individual cultures of C2 myogenic cells were subjected to indirect immunofluorescence labelling with anti-N-CAM antibody, Mab H28 (Fig. 2). Following the same procedure with 3T3 cells, this antibody yielded totally negative staining on cultures of such cells. This therefore ensured that in the cell separation techniques to be employed only the C2 cells should bind the antibody and hence segregate in the adherent fractions.

![Fig. 1. Culture of 3T3 cells stained with CFSE for three hours. Cells show an overall intense staining when viewed under fluorescent light. Bar, 10 mm.](image-url)
Assessment of the fractions achieved on separation of the mixed cell populations

Following separation of the mixed C2/3T3 cell populations using both the panning and magnetic bead separation techniques, cells segregated into two fractions, a non-adherent and an adherent fraction.

Adherent fractions

Using the panning method of separation the adherent fraction contains cells that bind anti-N-CAM antibody, and therefore attach to the surface of the IgG-coated bacteriological grade dish. Although morphological analysis does not allow us to distinguish fully between 3T3 and C2 cells in culture (Fig. 3A), the prelabelling of the 3T3 cells with CFSE does make this distinction possible. The majority of cells in the panned adherent fraction are not labelled with CFSE (Fig. 3B). Thus these non-fluorescing cells are C2 cells. The few fluorescing cells observed in this fraction are therefore 3T3 cells that are non-myogenic and regarded as "contaminating" cells, in that they do not bind anti-N-CAM antibody and should segregate with the non-adherent fraction. It is likely that these fluorescing cells had not attached to the IgG coated dish, but were present in the supernatant and had not been effectively removed during the washing step to remove the non-adherent fraction.

As regards separation using Dynabeads, the majority of cells in the adherent fraction have magnetic beads attached to them, most of these cells attaching many such beads to their surface (Fig. 4a). When examined by fluorescence microscopy, some Dynabead-bearing cells in this fraction do fluoresce (Fig. 4b), conflicting with our expectation that only myogenic, N-CAM-expressing cells should segregate in this fraction. The proportion of "contaminating" 3T3 fluorescing cells that segregate with the adherent fraction depends on the nature of magnetic bead pretreatment. Initial observations of cultures suggested that there were more cells of fibroblastic appearance (Fig. 5a) and more fluorescent cells (Fig. 5b) in the adherent fraction following separation with untreated Dynabeads, as compared to the same fraction achieved after separation with BSA- or gelatin-treated beads. This was not unexpected, given that the beads had not been pretreated to prevent non-specific binding of cells. However, to ascertain if there was an association between the degree of 3T3 cell contamination and the method of separation, the proportions of fluorescing versus non-fluorescing cells in adherent fractions achieved by both panning and Dynabead separation techniques were evaluated (see below).

Non-adherent fractions

In contrast to adherent fractions, all non-adherent fractions contain a large proportion of cells that are fluorescently labelled with the CFSE stain (Figs 6b and 7b). Thus the majority of cells in these non-adherent fractions, regardless of the method of separation used, are 3T3 cells, which have not bound anti-N-CAM antibody. Further, in accord with these cells not having bound anti-N-CAM antibody, where separation was carried out employing Dynabeads, the "non-adherent" cells also failed to bind the magnetic beads (Fig. 6b).

Quantification of cell types within adherent fractions

In order to quantify the percentage of the two cell types present in the adherent fractions, the numbers of 3T3, i.e. fluorescent, and C2, i.e. non-fluorescent, cells present in 10 randomly selected microscope fields of each adherent fraction were counted. The percentages of fluorescing versus non-fluorescing cells segregating in the adherent fractions are shown in Table 1 and Fig. 8. The results show that separation with 0.1% gelatin-
pretreated anti-rat IgG Dynabeads yielded the purest myogenic population, i.e. 95% purity, with panning effecting the next most efficient separation at 90% purity. Pre-treatment of the Dynabeads with 0.1% BSA yielded a less-pure adherent fraction than was obtained with panning or gelatin-treated beads, for only 89% of the cells in this fraction were myogenic. The efficiency of separation was further decreased when untreated magnetic beads were used to separate the two cell populations, for here only 84% of the cells present in the adherent fraction were myogenic.

Fig. 3. (A) Phase-contrast view of a culture of mixed C2 and 3T3 cells 24 hours after plating out. Although some cells (arrowed) appear to take on the stellate shape more characteristic of fibroblasts, and others a more bipolar shape (*) typical of myogenic cells, such morphological criteria are not sufficient to distinguish between these two cell types in vivo. Bar, 40 mm. (B) Fluorescent view of the same field of the mixed population of C2 and 3T3 cells shown in A. Only some of these cells stain positively when viewed under fluorescent light, these being those of more typical fibroblastic stellate shape compared to bipolar myogenic cells. Positive cells are CFSE-prelabelled 3T3 cells. Bar, 40 mm.

Discussion

The beneficial effects of implanting muscle precursor cells into diseased muscle fibres in human myopathic diseases in order to introduce missing gene products has been realised for several years (Watt et al., 1984; Partridge et al., 1989; Law et al., 1990; Tremblay et al., 1991). However, the very recent discovery that such precursor cells are capable of expressing introduced genes unrelated to myogenesis, and furthermore secreting such gene products into the circulation (Dhawan et al., 1991; Barr and Leiden, 1991), has heightened the awareness of the value of these cells in the whole concept of their use as vectors in gene therapy. For both
Cell separation techniques

Fig. 5. (A,B) Two cells (*) with fibroblastic morphology that have not attached to magnetic beads, are evident in the centre of the field of this phase-contrast view (A) of an adherent fraction produced following separation with untreated magnetic beads. Under fluorescent light (B), these two cells are seen to fluoresce, as are three other cells in view, two of which have attached magnetic beads. The fluorescent cells are therefore 3T3 cells that have not segregated with the non-adherent fraction. Bar, 40 mm.

these uses therefore, a pure population of myogenic cells is necessary if the muscle precursor cell is to be used to its maximum efficiency. In relation to alleviation of myopathic disease it is the precursor cells, as opposed to any other cell type, that fuse readily with the muscle fibres of the recipient into which they are to be introduced (Watt et al., 1982; Partridge et al., 1989), appear less likely to raise a normal immune response in the host (Karpati et al., 1989; Watt, 1990; Watt et al., 1992) and have the added advantage of expressing muscle-specific proteins that may not be expressed within myopathic muscle fibres (Hoffman et al., 1987; Monaco, 1988). Thus for both implantation into recipient muscle and use of precursor cells as vectors for the

Table 1. Percentages of myogenic and fibroblastic cells within adherent fractions following separation of the mixed cell population by panning and magnetic bead separation

<table>
<thead>
<tr>
<th>Method of cell separation</th>
<th>% of C2 cells contained in adherent fraction</th>
<th>% of 3T3 cells contained in adherent fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panning</td>
<td>90.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Untreated Dynabeads</td>
<td>83.78</td>
<td>16.22</td>
</tr>
<tr>
<td>BSA-treated Dynabeads</td>
<td>88.89</td>
<td>11.11</td>
</tr>
<tr>
<td>Gelatin-treated Dynabeads</td>
<td>94.91</td>
<td>5.19</td>
</tr>
</tbody>
</table>

Data from an individual experiment; counting 10 fields per sample in which 200 cells were scored for each of the four conditions. The experiment was repeated four times, the variance from the values given above being less than 5% on each occasion.
Fig. 7. (A,B) As with separation using Dynabeads, the non-adherent fraction of cells achieved after panning of the mixed culture consisted of cells that, as shown by phase-contrast microscopy, assumed a fibroblastic shape (A). It was confirmed that the cells in this fraction were indeed CFSE-prelabelled 3T3 fibroblasts when they were examined under fluorescent light (B). Bar, 20 mm.

expression and dissemination of gene products, pure populations of myogenic cells are essential. In the past various techniques have been used to remove non-myogenic cells from primary muscle cultures. Although several of these methods have indeed improved the myogenicity, this has sometimes only been by a narrow margin (Yaffe, 1968; Morgan, 1988) and in the one case where separation was highly effective, 50% of the original population was lost (Webster et al., 1988). We recently described a method by which we obtained a very pure myogenic cell population from a mixed primary culture derived from neonatal murine skeletal muscle (Jones et al., 1990). This ‘panning’ method had the advantage of recovering up to 95% of the original population and producing a very enriched population of myogenic cells. However, in addition to producing very pure populations of myogenic cells for vectors in gene therapy, in cases where cells are to be implanted into the recipient’s muscle fibres, large numbers of cells are required for alleviation or correction of the disease in any one subject. In those cases so far attempted in humans, cells have been injected via multiple injection sites into one single muscle, each single injection employing between $3 \times 10^6$ and $10 \times 10^6$ cells (Law et al., 1990). For such large-scale production of myogenic cells the panning method of separation would not yield such high numbers readily, and a more commercially adaptable method of separation would be desirable. In the light of these considerations, we have been comparing the purity of cells achieved following separation of cells using the panning technique and, on the same principle, using magnetic Dynabeads covalently pre-coated with the secondary antibody, anti-rat IgG. Preliminary work carried out using primary cultures obtained by the enzymatic disaggregation of neonatal mouse muscle indicated that this system, where the magnetic bead replaces the bacteriological dish as the solid phase to which the anti-rat IgG is coupled, does facilitate the separation of myogenic from non-myogenic cells (Coleman et al., 1991b). In order to quantify further the efficacy of panning versus bead separation of cells to obtain a pure myogenic cell population, in the present work we have separated myogenic cells from fibroblasts in a ‘model’ mixed cell population, where, unlike primary cultures of murine muscle, the precise
initial proportions of each type of cell is known. In our earlier study (Jones et al., 1990) we routinely found that the proportion of myogenic cells present in standard muscle cultures lay between 40% and 60% of the total cell population. As a result, we have chosen to use a 50:50 mixture of C2 and 3T3 cell types in this study, which reflects the percentages encountered in unseparated muscle cultures. On occasion, we did repeat the separation studies reported here on C2:3T3 ratios of 25:75 and 75:25, but found no evidence of unusual cell panning or Dynabead attachment as a result of these changes, and so these studies were not pursued. Second, when attempting separation of a cell population using the panning method (Jones et al., 1990), the methods of cell characterisation we used to distinguish between fibroblasts and myogenic cells was based on detection of intermediate filament proteins. However, using such markers to compare the efficiency of two separation techniques leads to difficulties in that the intermediate filament protein vimentin used to indicate the presence of fibroblastic cells is not confined to this cell type alone, being detected in other cell types, including myogenic cells (Stewart, 1990). Thus when dealing with a known mixture of myogenic and fibroblastic cells, we chose to prelabel one cell with the fluorescent marker CFSE, which remained within one cell population, so that any slight variations in efficiencies of the two methods of separation could be more critically monitored. The results show that gelatin-precoated magnetic Dynabeads gave the best separation in terms of enrichment of myogenic cells. We chose to use the protein gelatin to precoat the beads as this is the routine substrate used for in vitro growth of muscle cells. Potentially, serum fibronectin in the cell suspension might be sequestered by the gelatin-coated beads via non-antibody-mediated binding. Our choice of fetal calf serum in the growth medium removes this potential anomaly as it is essentially fibronectin-free. The major adhesive glycoprotein of fetal calf serum is vitronectin (Izumi et al., 1989), which does not interact with gelatin (or BSA). Furthermore, we find preferential attachment of myogenic cells over fibroblasts in our assays; the opposite result would be expected if fibronectin played any role in this system as fibroblasts attached preferentially to fibronectin when compared with myogenic cells in vitro, which prefer laminin (Goodman et al., 1989).

We found that separation of cells using the magnetic beads produced several technical disadvantages not encountered with the panning method. Using the panning method, cells to be separated have a large surface area of contact with the anti-rat IgG antibody, for it is evenly spread over the surface of the dish. In incubating the cells with the magnetic beads following their exposure to anti-N-CAM antibody, it is imperative that cells and beads be continually agitated, for if they are allowed to settle in the tube fewer cells are in contact with the beads, thus resulting in a poorer segregation of cells, in comparison to the panning method, into myogenic and non-myogenic fractions. Although magnetic beads can be removed by brief exposure in medium at pH 4.0 during subculture, under normal conditions we found that they can mark the cells for several passages in culture. On one hand this may be a disadvantage, especially when further immunostaining of the cells is required, for they interfere with such subsequent experimental procedures; on the other it may be advantageous, for the beads certainly mark cells for prolonged periods after their segregation with them. Further, the fact that we have been able to decrease gradually the proportion of contaminating 3T3 cells using such agents suggests that some non-specific binding of cells to beads does indeed occur. However, it is also possible that a low level of fibroblast contamination will always occur when using anti-N-CAM antibody to segregate myogenic cells, for some authors have found low levels of surface N-CAM on fibroblastic cells associated with peripheral nerve (Martini and Schachner, 1988; Seilheimer and Schachner, 1988) and on those that proliferate near denervated synaptic sites in skeletal muscle (Gatchalian et al., 1989), although this has not been the case with fibroblasts associated with skeletal muscle during the development of this tissue (Moore and Walsh, 1985).

However, when magnetic beads are pretreated with gelatin prior to their incubation with anti-N-CAM-bearing and non-bearing cells, the separation of C2 myogenic cells from 3T3 fibroblasts is enhanced to produce even more enriched myogenic populations compared to that achieved on panning. Although the differences in percentages of purity seem small at only 5% when comparing panning with gelatin-pretreatment of beads, this difference has to be seen in the context of fibroblast overgrowth, which may result from even this minimum increase in the number of fibroblastic cells. Thus separation using gelatin-pretreated magnetic beads is a very attractive alternative to panning, having the advantage that it is a method amenable to scaling up for segregating large numbers of myogenic cells where an exceedingly high percentage of the original cell population is returned in a viable state.

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References


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