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Antigen processing and presentation by a murine myoblast cell line

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SUMMARY

The ability of non-professional antigen-presenting cells (APC) to process and present antigen to the immune system has been the subject of debate in autoimmunity and tumour immunology. The role of muscle cells in the processing and presentation of antigen to T cells via class I and class II MHC pathways is of increasing interest. Muscle cells are the targets of autoimmune attack in the inflammatory muscle diseases, and direct intramuscular injection of antigen-expressing DNA constructs is under scrutiny as a means of vaccination. Furthermore, the immunological properties of muscle cells are of relevance in attempts to transfer myoblasts as replacement cells in dystrophic diseases or as depot cells for the secretion of certain molecules in deficiency states. Using class I and class II MHC transfectant clones of the C2C12 myoblast cell line, myoblasts have been shown to be capable of presenting antigen to, and stimulating secretion of IL-2 by, T cell hybridomas via both of these pathways. The epitopes which are dominantly presented by professional APC after processing of native antigens were also presented by the myoblast cell line after processing of either ovalbumin (class I) or hen egg lysozyme (class II). Further, antigen processing and presentation via the class II pathway were enhanced by pretreatment of the myoblasts with interferon-gamma (IFN-γ). Up-regulation of invariant chain expression by this treatment may have contributed to this enhanced presentation, but an effect of IFN-γ on the expression of other molecules such as H-2 DM may have also played a role. The demonstration of the antigen-presenting properties of these myoblasts is of relevance to all three areas mentioned above. In each situation myoblasts comprise a significant population within muscle. In the case of inflammatory muscle diseases the process of muscle degeneration and regeneration is on-going, while in the vaccination procedure some muscle damage occurs, and vaccination is more effective when muscle damage has preceded inoculation.

Keywords autoimmunity myositis antigen presentation muscle

INTRODUCTION

An understanding of the role of skeletal muscle in the immune response is becoming increasingly important. Skeletal muscle cells themselves are the targets of autoimmune attack in inflammatory muscle diseases [1–3] and in myasthenia gravis [4], but the mechanisms of induction of autoimmunity and the cells responsible for presentation of autoantigen during the induction phases and the chronic stages of these diseases are unknown. The immunological properties of skeletal muscle also need to be considered in at least two other situations. Myoblast transfer is now under consideration as a means of therapy in certain muscular disorders [5,6], and immunological rejection of the transplanted cells and/or immune responses to the product being replaced (e.g. dystrophin) which is expressed by the transplanted myoblasts are significant considerations [6]. A recent approach to active immunization against various viral antigens has been the direct intramuscular inoculation of DNA constructs consisting of genes encoding the proteins of interest in mammalian expression vectors [7,8]. Such an approach has resulted in very robust cellular and humoral immune responses to certain antigens. It has been assumed that these genes are expressed in skeletal muscle cells, but it is unclear whether the muscle cells themselves, or other cellular components of muscle tissue, are responsible for the presentation of the expressed antigens to the immune system.

The possibility that cells other than the classical antigen-presenting cells (APC) are capable of processing and presenting antigen has been entertained for some time, and aberrant expression of class II MHC and co-stimulatory molecules by non-professional APC has been proposed to contribute to the induction of autoimmunity [9,10]. Some other cell types, such as astrocytes, microglial cells and smooth muscle cells, are

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capable of processing and presenting antigen to sensitized T cells *in vitro* (reviewed in [11]), while others are not, despite being able to express class I and class II MHC antigens on stimulation [12–14] with agents such as interferon-gamma (IFN-γ). The antigen-presenting capacity of skeletal muscle cells has not been well studied, but in one report IFN-γ-treated human myoblasts were shown to be capable of processing tetanus toxoid, and presenting it to sensitized T cells via HLA-DR [11]. In this study we have used a murine myoblast cell line to demonstrate that muscle cells are capable of processing and presenting antigen via both the class I and class II MHC pathways.

MATERIALS AND METHODS

Reagents

Ovalbumin (OVA) and hen egg lysozyme (HEL) were purchased from Sigma Chemical Co. (St Louis, MO). The peptides $OVA_{257-264}$ and OVA_{55-62} were manufactured by Chiron Mimotopes (Melbourne, Australia). Murine IFN-γ was purchased from Genzyme (Cambridge, MA) and murine IL-2 from Pharmingen (San Diego, CA).

Cell lines

The cell line C2C12 [15], a murine myoblast cell line derived from a C3H mouse ($H-2^k$) which is capable of fusion to form myotubes under the appropriate conditions, was obtained from ATCC (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS). The cell line I-3 is an L cell transfectant expressing H-2 K^b [16]. The T cell hybridomas 13.13 and 1G8 recognize peptides $OVA_{257-264}$ and OVA_{55-62}, respectively, in the context of K^b [16]. These hybridomas are routinely grown in DMEM plus 10% FCS containing 0·3 mg/ml G418 since they express CD8 encoded on a transfected plasmid which uses neomycin resistance as a selection marker [16]. T cell hybridoma 3A9 [17] recognizes peptide HEL_{46-61} in the context of $I-A^k$.

Flow cytometry

Cells were harvested from monolayers using trypsin in a standard protocol [18] and labelled by direct or indirect immunofluoresence using the following MoAbs. Hybridomas producing MoAbs to $H-2K^k$ (ATCC HB16) and $I-A^k$ (HB15) were obtained from ATCC. The MoAbs to $H-2K^b$, Y3 [19], and 53-6-72 (anti-mouse CD8) were supplied by J. McCluskey (Adelaide, Australia). Culture supernatants were concentrated using ammonium sulphate precipitation and stored at −20°C. The MoAb preparations were diluted in PBS to previously determined optimal concentrations before use and the staining and flow cytometry were as described previously [18] using fluorescein-conjugated $F(ab)'_2$ sheep anti-mouse immunoglobulin (Silenus, Hawthorn, Australia) as the secondary reagent. Flow cytometry was performed using a FACS Analyser (Becton Dickinson, Mountain View, CA) in the Department of Pathology, University of Western Australia or in the Department of Clinical Immunology, Flinders Medical Centre.

Transfections

C2C12 was transfected by lipofection (Lipofectin; GIBCO, Gaithersburg, MD) as previously described [18]. The K^b transfectant was prepared by co-transfection of the gene pI41 (Dr K. M. Hui, Singapore) encoding the complete genomic sequence of H-2 K^b [20] along with a construct encoding resistance to G418 (pWLneo), followed by selection in G418 (GIBCO). The $I-A^k$ transfectant was prepared by co-transfection of constructs encoding the cDNA for $I-A^k\beta$ and $I-A^k\alpha$ in the expression vector pcEXV-3 (supplied by Dr L. Glimcher, Harvard) and pWLneo followed by selection in G418. Transfectant clones were derived by limiting dilution and K^b-5 was further selected using immunomagnetic beads coated with MoAb Y3 to yield a population of cells expressing $H-2K^b$ at a level similar to the I-3 cell line [16].

Peptide presentation

APC (5×10^4) were pulsed with various concentrations of OVA peptides for 60 min at 37°C in 96-well plates, washed three times and then co-cultured with 1×10^5 T cell hybridoma cells. Culture supernatants were collected after 24 h and IL-2 release was determined by bioassay using CTLL cells [21] or by ELISA (Pharmingen). Supernatants from concanavalin A (Con A)-treated T cell hybridoma cells were used as positive controls.

Antigen processing and presentation

For analysis of processing and presentation by the class I pathway, native OVA at various concentrations was introduced into

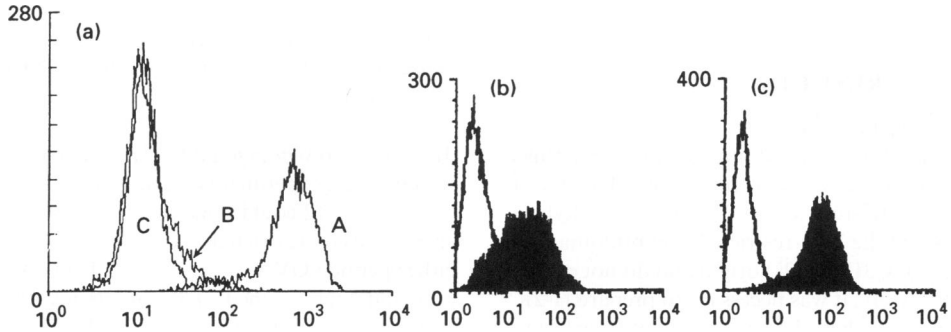

Fig. 1. FACS analysis of transfectant clones. The ordinate represents cell number, and the abscissa fluorescence intensity. (a) K^b-5 cells stained with MoAb Y3, directed at $H-2K^b$ (trace A) or MoAb 53.6.72 directed at CD8 (trace B). C2C12 cells were stained with Y3 (trace C). (b) AK-1 cells stained with MoAb HB15 directed at $I-A^k$ (solid peak) or MoAb to CD8 (open peak). (c) IFN-γ-treated AK-1 cells stained with MoAb to $I-A^k$ (solid peak) or to CD8 (open peak).

the cytoplasm of APC by electroporation [21] using 250 μF at a voltage of 0·45 kV. The washed, electroporated cells (1×10^5) were then co-cultured with 1×10^5 T cell hybridoma cells. For class II antigen processing HEL was co-cultured at various concentrations with 5×10^4 APC in the presence of 1×10^5 T cell hybridoma cells. In each assay supernatants were collected after 24 h for IL-2 assay. The effect of IFN-γ on class II antigen presentation was determined by treating the I-A^k transfectant with 500 U/ml of recombinant mIFN-γ (Pharmingen) for 48 h before use as APC for HEL.

Immunoprecipitation of invariant chain

Transfectant C2C12 cells were grown to 60% confluence in DMEM 10% FCS, IFN-γ was added at 200 U/ml, and these cultures, as well as control cultures, were incubated for a further 48 h. At 43 h medium was changed in all cultures to DMEM without methionine, 5% FCS for 1 h and then this medium was replaced with DMEM without methionine, 5% FCS and 200 μCi ^{35}S-methionine (Trans^{35}S-label; ICN Biomedical, Sydney, Australia) for a further 4 h. The MoAb In-1 [22] was used to precipitate ^{35}S-labelled invariant chain from I-A^k transfectants and untreated NSO myeloma cells. Cells were harvested in trypsin/versene solution and washed three times in PBS. The cell pellet was resuspended in 1 ml of lysis buffer (50 mM Tris–HCl pH 8·0, 150 mM NaCl, 5 mM EDTA, 0·5% NP40, 0·5% deoxycholate, 5 mM iodoacetamide, 2 mM phenylmethysulfonyl fluoride, 1 mM NEM, 1 mM aprotinin) and left on ice for 30 min. After centrifugation at 12 400 *g* for 20 min the lysate was removed and pre-cleared by rotation with a monoclonal mouse anti-human IgG (10 μg) for 3 h at 4°C followed by two sequential incubations with 10 μl of protein G Sepahrose (Sigma) at 4°C (3 h and overnight). The precleared lysate (200 μl) was then incubated with 5–10 μg of one of various MoAbs (anti-class I MHC, In-1, anti-human tumour necrosis factor-alpha (TNF-α)) or without MoAb for 3 h at 4°C followed by addition of 10 μl of protein G Sepharose and overnight rotation at 4°C. Each preparation was then washed four times in lysis buffer and the final pellets were resuspended in SDS–PAGE sample buffer (0·1 M Tris–HCl pH 6·8, 4% SDS, 4% 2-mercaptoethanol, 2% sucrose, 0·001% bromophenol blue), boiled for 5 min and subjected to SDS–PAGE through 10% polyacrylamide. Gels were stained in coomassie blue R, destained and then incubated in Amplify (Amersham, Aylesbury, UK) for 30 min before drying under vacuum at 80°C for 3 h and exposure to autoradiographic film for 2–5 days at −70°C.

RESULTS

MHC expressing myoblast transfectants

The intention of this study was to investigate the antigen processing and presenting capacity of myoblasts. The T cells to be used were the H-2 K^b-restricted OVA-reactive T hybridomas 13.13 and 1G8 and the I-A^k-restricted T hybridoma 3A9. Since C2C12 cells are of C3H ($H-2^k$) origin and do not express class II MHC constitutively, it was necessary to prepare H-2K^b and I-A^k transfectants. Further, the availability of transfectant myoblast clones constitutively expressing either H-2 K^b or I-A^k allowed us to investigate antigen presentation in the absence of prior stimulation with agents such as IFN-γ which might be expected to alter the expression of a variety of molecules apart from the MHC antigens of interest. Figure 1 depicts the H-2K^b expression by transfectant clone K^b-5 and I-A^k expression by transfectant clone AK–1.

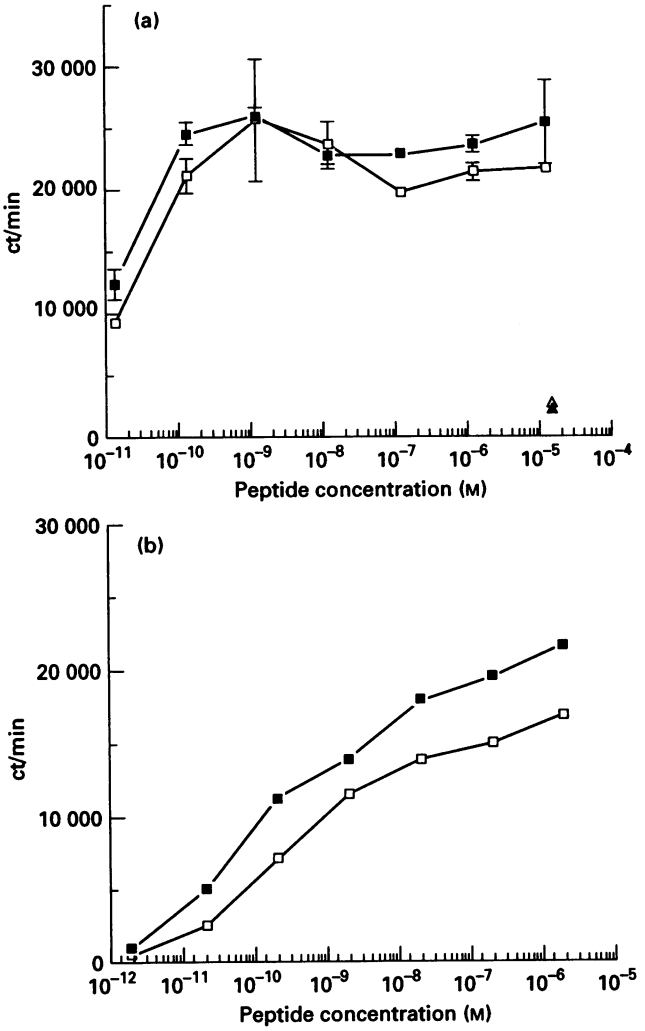

Fig. 2. Peptide presentation by C2C12 myoblasts. (a) Presentation of peptide OVA_{55-62} to the K^b-restricted T cell hybridoma 1G8 or the L cell transfectant I-3. Stimulation by C2C12 cells pulsed with 2×10^{-5} M OVA_{55-62} (▲) or K^b-5 pulsed with the same concentration of $OVA_{257-264}$ (△) is also shown. IL-2 was measured in the culture supernatants after 24 h co-culture and results are expressed as ct/min of ^{3}H-thymidine incorporated by the CTLL cell line in a standard bioassay. Values are shown as the mean of triplicate assays. ■, I-3/OVA_{55-62}; □, K^b-5/OVA_{55-62}. (b) Presentation of peptide $OVA_{257-264}$ to the K^b-restricted T cell hybridoma 13.13. ■, 13.13/I-3; □, 13.13/K^b-5.

Myoblasts present peptide to class I MHC-restricted T cells

In our initial experiments we determined whether the myoblast cell line C2C12 could present peptide to, and activate, antigen-specific MHC-restricted T cells. K^b-5 or I-3 were pulsed with either peptide $OVA_{257-264}$ or peptide OVA_{55-62} at 37°C for 60 min and then washed. The pulsed myoblasts were then co-cultured with either 13.13 or 1G8 T cell hybridomas for 24 h (Fig. 2). Hybridoma 1G8 was stimulated to secrete IL-2 by K^b-5 cells pulsed with OVA_{55-62}, but not $OVA_{257-264}$, while 13.13 was stimulated by K^b-5 pulsed with peptide $OVA_{257-264}$ (Fig. 2). This stimulation was equivalent to that of the I-3 cell

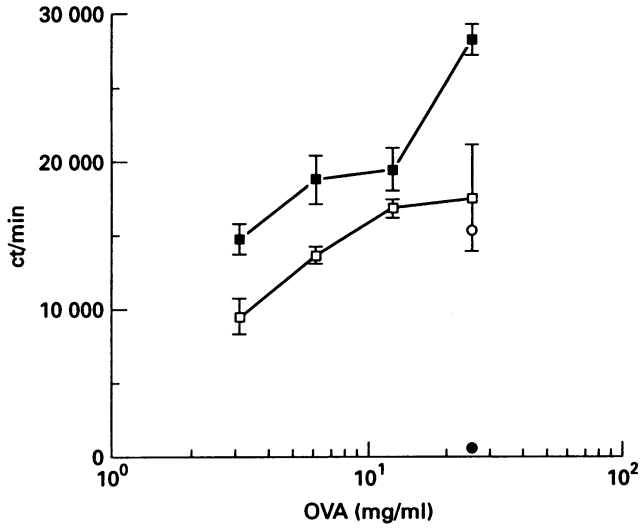

Fig. 3. Processing and presentation of native OVA, by K^b-5 and I-3, to T cell hybridoma 1G8 after electroporation. I-3 cells electroporated with 25 mg/ml OVA and then fixed in 1% paraformaldehyde immediately (●) or after 2·5 h (○) were included to exclude the possibility that peptides contaminating the OVA preparation were responsible for stimulation of IL-2 secretion. IL-2 was measured after 24 h using the CTLL bioassay. ■, I-3; □, K^b-5.

line which expresses an equivalent level of K^b and which has previously been shown to be as effective as splenic mononuclear cells in its capacity to present OVA to these hybridomas (J. McCluskey, unpublished).

Myoblasts process and present OVA to class I MHC-restricted T cells

The transfectant myoblast line K^b-5 and the I-3 cell line were electroporated in the presence of graded concentrations of native OVA, washed and then co-cultured with 1G8 cells for 24 h. Both of the electroporated cell lines stimulated IL-2 secretion by this hybridoma (Fig. 3). To exclude the possibility that contaminating peptides in the OVA preparation had contributed to the antigen presentation, I-3 cells were electroporated with 25 mg/ml OVA and then fixed with 1% paraformaldehyde immediately (0 h), or after 2·5 h, before culture with 1G8 cells. Only those cells fixed after 2·5 h stimulated 1G8 to produce IL-2, indicating that active processing of the OVA sample was necessary for T cell activation (Fig. 3). These data demonstrate that the myoblast cell line was capable of processing native antigen to generate a peptide which represents one of the epitopes recognized by class I-restricted antigen-specific T cells.

Myoblasts process and present HEL via the MHC class II pathway

The myoblast transfectant AK-1 was incubated with native HEL and co-cultured with the I-A^k-restricted T cell hybridoma 3A9. IL-2 secretion was detectable after 24 h (Fig. 4). 3A9 cells incubated in the presence of C2C12 cells plus HEL did not secrete IL-2. When the transfectant cell line was preincubated with IFN-γ for 48 h before assay its antigen-presenting potency

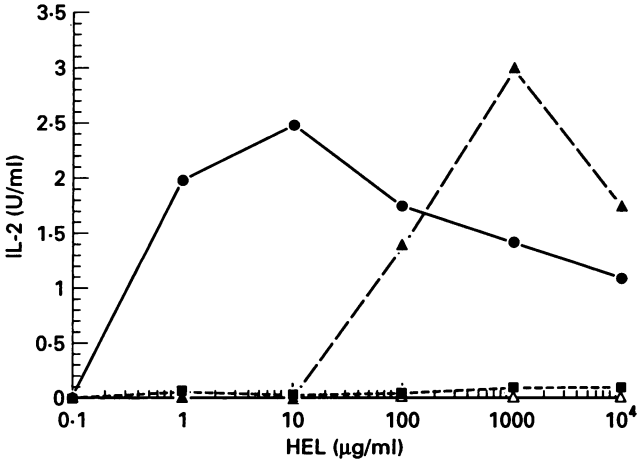

Fig. 4. Processing and presentation of hen egg lysozyme (HEL) to 3A9 cells by AK-1 or C2C12 cells either untreated or after culture with 500 U/ml murine IFN-γ for 48 h. Supernatants were collected after 24 h of antigen presentation and IL-2 concentrations were measured by ELISA. Supernatants from spleen cells (5×10^4) derived from CBA mice which were used as positive controls for HEL presentation contained 30 U/ml of IL-2 after 24 h. ●, C2C12 I-A^k IFN-γ; ▲, C2C12 I-A^k; ■, C2C12 IFN-γ; △, C2C12.

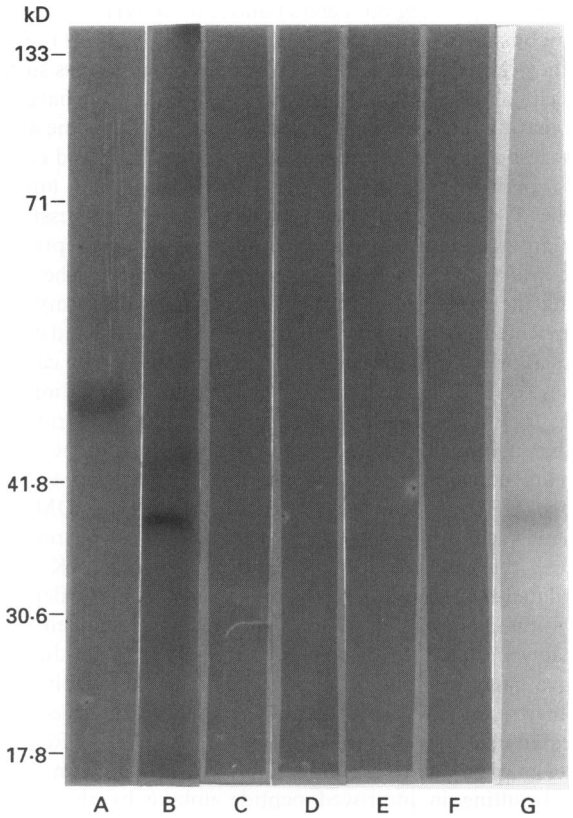

Fig. 5. Immunoprecipitation of invariant chain from transfectant AK-1. Lanes A–D show immunoprecipitates from IFN-γ-treated AK-1 cells prepared using MoAb to murine class I MHC α chain (A), In-1 (B), a negative control consisting of anti-human tumour necrosis factor-alpha (C), and no primary antibody (D). Untreated AK-1 cells were also treated with antibody to class I MHC (E) and In-1 (F). Untreated NSO murine myeloma cells were also precipitated with In-1 as a positive control (G).

was increased 100-fold (Fig. 4). Mouse spleen cells (5×10^4) served as positive controls for antigen presentation and secreted 30 U/ml IL-2 after 24 h.

Expression of invariant chain

One possible explanation for the increased antigen-presenting capacity of IFN-γ-treated myoblasts was the induction of invariant chain expression. Invariant chain was undetectable in the unstimulated AK-1 cells, but was readily demonstrable in IFN-γ-treated cells (Fig. 5). Class I MHC antigen was also more readily precipitable from the IFN-γ-treated cells. IFN-γ treatment of AK-1 cells produced a marginal increase in membrane I-A^k expression (Fig. 1c).

DISCUSSION

In order for a cell to be capable of acting as an APC it must display several properties. First, it must be capable of processing antigen and delivering the appropriate peptides into the antigen presentation pathways; second, it must express the appropriate MHC molecules; and third, it must express adequate adhesion and co-stimulatory molecules [23].

The work described in this study, taken together with data presented by others, suggests that myoblasts can fulfil many of these criteria. They have been demonstrated previously to be capable of expressing both class I and class II MHC molecules, either constitutively or after treatment with IFN-γ [11,24,25], and they can be induced to express adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) [26]. We have now demonstrated that the myoblast cell line C2C12 has the ability to process and present antigen via both the class I and class II MHC pathways and to activate MHC-restricted antigen-specific T cell hybridomas. Furthermore, the processing of these antigens results in the generation of similar peptides to those which are generated by professional APC when processing the same antigens. The use of transfectant myoblast clones which constitutively expressed specific class I and class II alleles allowed us to investigate the antigen-presenting capacity of these cells without the necessity for prior stimulation with IFN-γ [11]. These data indicate that these cells are intrinsically capable of antigen processing and presentation, and the class II data are consistent with the previous observation that the epitope HEL_{46-61} can be presented in an HLA-DM-independent fashion by some cell types [27]. IFN-γ treatment reduced the threshold dose of antigen at which AK-1 cells stimulated IL-2 secretion by the hybridoma 3A9. The degree of activation, as measured by total IL-2 release, remained markedly lower than that of spleen cells. This may be due to a relative paucity of co-stimulatory molecule expression by the myoblasts compared with the APC present in the spleen. The increased efficiency of antigen presentation by AK-1 following IFN-γ may be due to the induction of invariant chain expression, resulting in improved peptide uptake by the class II molecules. Alternative explanations would include the induction of endogenous class II expression, although membrane I-A^k was only slightly increased by IFN-γ, or the induction of other molecules important to the antigen-presentation process such as the murine equivalents of the HLA-DM genes [28].

An immunological function for myoblasts may be of theoretical and practical importance in a number of situations. The data presented here, and by others [11], do not address the role of these cells in the induction of an immune response, but suggest that they may have a role in the maintenance of an ongoing response. This may be of particular relevance in the inflammatory muscle diseases. In these conditions there is persistent degeneration and regeneration of skeletal muscle [29], which necessarily entails the generation of myoblasts before fusion. Furthermore, there is expression of MHC class I and class II molecules on muscle cells [30,31], presumably including myoblasts, and the chronic inflammatory response leads to the local release of cytokines such as IFN-γ [32]. Therefore myoblasts may provide a stimulus for T cells to maintain the autoimmune response. In genetically susceptible individuals [33] the ability of myoblasts to present antigen may also be of importance in the initiation of an anti-muscle response. Damage to muscle induced by viral infections, for example, could induce myoblast proliferation. The local secretion of cytokines as part of the anti-viral response may then stimulate the expression of MHC and other co-stimulatory molecules appropriate for antigen presentation. Further, some viruses have been demonstrated to induce MHC antigen expression in muscle cells [34].

Administration of antigens by direct intramuscular inoculation of the genes in expression vectors has proven an effective means of immunization [7,8]. It is unclear which cells are responsible for antigen presentation in these responses, but the data presented here suggest a role for myoblasts. The immune response is enhanced if the construct is administered at the height of muscle regeneration, for example following mechanical injury or the application of myotoxins such as snake venom [35]. Enhanced uptake and incorporation of the plasmid construct into rapidly dividing myoblasts presumably contributes to this immune response, but the antigen-presenting abilities of the abundant myoblasts present during this time may also be important.

If myoblasts are effective APC then the need to ensure histocompatibility between donor and recipient in myoblast transfer situations [5,36] becomes of paramount importance. Furthermore, these observations raise the possibility that the deficient protein which is to be reconstituted by myoblast transfer (e.g. dystrophin in Duchenne muscular dystrophy) can be presented to the immune system of the recipient by histocompatible myoblasts or by host MHC antigens after myoblast fusion. Immune responses to dystrophin have been reported after histocompatible myoblast transfer [6]. The antigen-presenting capacity of myotubes has not been explored. If these cells also are capable of antigen presentation then the use of transfected myotubes as sources of production in deficiency conditions such as parkinsonism [37] may also require careful matching.

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