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Childs, Lisbeth Ann

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THE EFFECTS OF MYASTHENIC SERUM ON

SKELETAL MUSCLE CELLS IN CULTURE

Submitted by LISBETH ANN CHILDS

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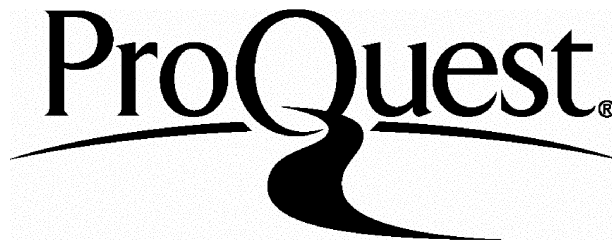
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SUMMARY

Rat muscle cells were grown in culture for use as an experimental
model in which to study the myolytic effects of myasthenic serum in
vitro. Use was made of a procedure which depends upon the selective
uptake of tritium-labelled carnitine by cultured myotubes, loss of
which can be monitored following cytolytic damage. The studies
demonstrated that heat-inactivated myasthenic serum samples caused
myotube-specific lysis in a manner that was dependent on the addition
of complement. The concentration and activity of the complement source
was shown to be a major factor in detecting myotoxicity. Using
optimised assay conditions, a myotoxicity study was carried out using
a range of normal and myasthenic serum samples. In the presence of
guinea-pig complement, heat-inactivated serum samples from 9 out of 13
myasthenic patients showed clear myotoxicity in contrast to 0 out of
12 normal controls and 0 out of 6 polymyositis patients. Neither
heat-inactivated sera alone nor guinea-pig complement alone showed
myotoxicity. A further study defined new conditions under which
previously 'non-toxic' myasthenic serum samples demonstrated
myotoxicity. Removal of anti-AChR antibodies from a myasthenic serum
sample by affinity absorption led to a loss of complement-mediated
myotoxicity. Finally, studies were carried out in which IgG or IgG
depleted of subclass 3, was purified from myasthenic serum samples and
tested for complement-mediated myotoxicity. The IgG fractions caused
myotoxicity in a similar manner to the whole serum.

The studies were extended to human foetal muscle cells
in culture which were shown to be less mature than the cultured rat
muscle cells in this study. Attempts were made to define optimal

growth conditions for the human foetal muscle cells in vitro but these were inconclusive. Comparable complement-mediated myotoxicity by myasthenic serum towards human muscle cultures was not shown. However, manipulation of assay conditions resulted in clear myotoxicity by the 2 myasthenic serum samples tested, relative to normal controls.

The results gained from this work support the suggestion that complement-mediated cell damage, initiated by anti-AChR antibodies, may contribute to post-synaptic membrane degeneration in myasthenia gravis.

PUBLICATIONS

CHILDS,L.A.; HARRISON,R. and LUNT,G.G. (1985).

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Complement-dependent toxicity of serum from myasthenic patients to

muscle cells in culture. J. Neuroimmunol. 9 69-80.

ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
dbcAMP	Dibutyryl adenosine 3',5'-cyclic phosphate
Ara C	Cytosine arabinoside
α-BGT	α-bungarotoxin
BSA	Bovine serum albumin
BSS	Balanced salt solution
CEE	Chicken embryo extract
Cr	Chromium
CPK	Creatine phosphokinase
DMEM	Dulbecco's modified Eagle's medium
cDNA	Complementary deoxyribose nucleic acid
EAMG	Experimental autoimmune Myasthenia gravis
EDTA	Ethylene diamine tetraacetic acid
EPP	End-plate potential
FCS	Foetal calf serum
FDU	Fluorodeoxyuridine
GAHL	Goat anti-human light chain
GM	Growth medium
GPC	Guinea-pig complement
HEPES	N-2-hydroxyethylpiperazine-N'2-ethanesulphonic acid
HS	Horse serum
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MEPP	Miniature end-plate potential
MG	Myasthenia gravis
MIR	Main immunogenic region
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulphonyl fluoride
PPO	2,5-diphenyl oxazole
mRNA	Messenger ribose nucleic acid
SE	Standard error of the mean
SFM	Serum-free medium
Triton X-100	Isooctyl phenoxypolyethoxy ethanol

CONTENTS

INTRODUCTION

"Nevertheless, those labouring with a want of spirits, will use these spirits for local motions as well they can; in the morning they are able to walk firmly, to fling their arms about hither and thither or to take up any heavy thing; before noon the stock of spirits being spent, which has flowed into the muscles, they are scarcely able to move hand nor foot. At this time I have under my charge a prudent and honest woman who for many years has been obnoxious to this form of spurious palsy, not only in her members, but also in her tongue; she for some time can speak freely and readily enough but after she has spoke long or hastily or eagerly, she is not able to speak a word, but becomes mute as a fish, nor can she recover the use of her voice under an hour or two."

<div align="right">Thomas Willis, 1672</div>

The first description of the human disease myasthenia gravis (MG) was probably that above, given by Dr. Thomas Willis in 1672. The disease is clinically characterised by weakness and rapid fatiguability of skeletal muscle. The symptoms of MG are often first noticed following an infection or at a time of psychological stress. The disorder may be selective for a particular group of muscles (eg. ocular, bulbar, limb) or more generalised. Involvement of the respiratory muscles may lead to death. It is now generally accepted that MG is an autoimmune disease, the autoantigen being the acetylcholine receptor (AChR) of the post-synaptic muscle membrane. As a result of antibody-mediated responses, the number of functional AChRs is decreased with consequent

effects on neurotransmission (for comprehensive reviews see Lindstrom, 1979; Vincent, 1980; Newsom-Davis and Vincent, 1982; Harrison and Behan, 1986). The increased understanding of the pathogenesis of MG is closely linked to increasing knowledge of the synaptic organisation, structure, function and turnover of the AChR. Much of this knowledge has been aided by the use of tissue-culture systems. Such systems have proved to be valuable experimental tools for the study of muscle and nerve physiology, biochemistry and immunology.

MG is, in many ways, a model autoimmune disease. Elucidation of the humoral and cellular mechanisms involved will no doubt contribute greatly to our knowledge of immune mechanisms in general and to their breakdown in disease states.

Neuromuscular transmission

In the majority of mammalian muscles, each muscle fibre has a single region of contact with the axon of its controlling motor neurone. This region constitutes the neuromuscular junction (Figure 1). The function of the neuromuscular junction is to transfer the propagated nerve impulse from the motor nerve ending to the muscle fibre resulting ultimately in muscle contraction. A narrow synaptic cleft separates the nerve terminal from the post-synaptic muscle membrane which is thrown up into folds in the region of the end-plate, the AChRs being concentrated at the tips of the folds (Ringel et al., 1975). Impulses are conducted from the nerve cell body along the axon by means of action potentials which involve successive waves of opening and closing ion channels. The nerve impulses can be initiated experimentally by electrical stimulation of the nerve. In the nerve ending, acetylcholine (ACh) is stored in vesicles. Depolarisation of

FIGURE 1

SCHEMATIC REPRESENTATION OF THE NEUROMUSCULAR JUNCTION

Glucose

low affinity uptake
into glia, neuronal
perykarya

Pyruvate

AXON

Citrate

OAA

AcCoA
+
Choline

high
affinity
choline
uptake

choline
acetylase

CoA

ACh
(vesicular)

ACh
(cytoplasmic)

PRE-SYNAPTIC
TERMINAL

SYNAPTIC
CLEFT

choline

acetate

ACh

stimulation–evoked ACh
release (Ca^{2+} dependent)

MUSCLE
MEMBRANE

Nicotinic
AChR

AChE
(membrane bound)

Increased permeability to Na$^+$, K$^+$
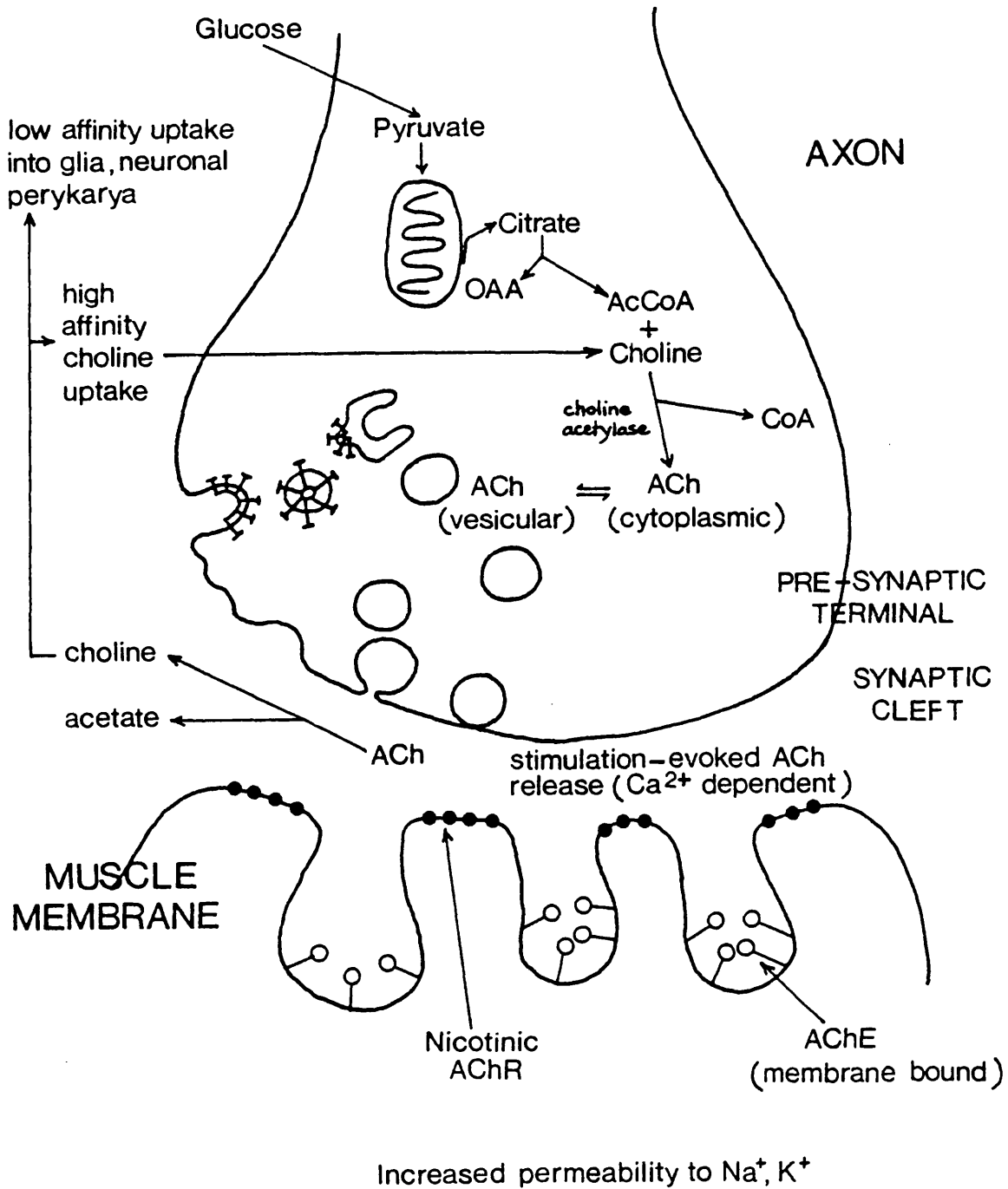
the nerve terminal membrane results in an influx of calcium ions which triggers the release of ACh into the synaptic cleft, probably through fusion of vesicles with the pre-synaptic membrane. The binding of ACh to AChR in the post-synaptic membrane promotes a conformational change which is associated with the brief (1 millisecond) opening of an ion channel. This allows the passage of sodium and potassium ions which flow down their electrochemical gradients. More sodium ions move in than potassium ions move out, resulting in a net influx of positive charge and depolarisation of the muscle membrane, producing an 'end-plate potential' (EPP). If sufficient AChRs are activated, the temporal summation of EPP's causes depolarisation to the threshold value, thereby initiating an action potential. This is propagated along the muscle fibre leading to the activation of the contractile mechanism. The action of ACh is terminated by its dissociation from the AChR and subsequent hydrolysis by acetylcholinesterase (AChE).

Normally, the amount of ACh released and the number of AChRs activated is much larger than the minimum necessary to exceed the threshold value stimulating muscle contraction. This provides a large safety factor ensuring effective neuromuscular transmission. In the absence of a nerve impulse, spontaneous release of a small amount of ACh from the nerve ending occurs, generating small depolarisations (miniature end-plate potentials - MEPPs) in the post-synaptic membrane. By using electrophysiological techniques, the resting membrane potential of muscle, the spontaneous MEPPs and the EPPs can all be measured. With the advent of voltage clamp techniques, it is possible to determine the actual passage of current through sodium channels and the gating currents, which regulate the opening of ion channels. In this way, the mechanisms bringing about the initiation of

an action potential have been elucidated (Figure 2).

Characterisation of the AChR

The AChR at the vertebrate neuromuscular junction is classified pharmacologically as a nicotinic cholinergic receptor, responding to nicotine in a similar manner to ACh. Muscarinic cholinergic receptors, responding to muscarine, are found primarily in the brain and on smooth muscle cells. The pharmacology of nicotinic AChRs has been studied by measurement of the membrane potential or conductance changes induced by ACh and AChR activators (agonists) and by the inhibition of the cholinergic response caused by AChR antagonists which block synaptic transmission. Agonists of the AChR include nicotine, carbamylcholine and decamethonium while characteristic antagonists are benzoquinonium and d-tubocurarine. These ligands resemble ACh in having charged quaternary ammonium groups (Figure 3). The interactions of AChR with the agonists and antagonists mentioned above are readily reversible which precludes their use as ligands to label, isolate and purify the AChR.

Two factors significantly and radically advanced the characterisation of the nicotinic AChRs. The first was the availability of the α-neurotoxins from snake venoms, which bind with high affinity and specificity to the AChR; the second was the discovery of an abundant source of the receptor in the electric organ of several species of electric fishes. The α-neurotoxins were first purified by Chang and Lee (1962) who demonstrated that they produce an anti-depolarising block of the AChR at the neuromuscular junction in a manner similar to that of the cholinergic antagonist d-tubocurarine (Lee, 1972). Although very different in structure from ACh analogues,

FIGURE 2   THE INITIATION OF AN ACTION POTENTIAL

AT A NICOTINIC CHOLINERGIC SYNAPSE
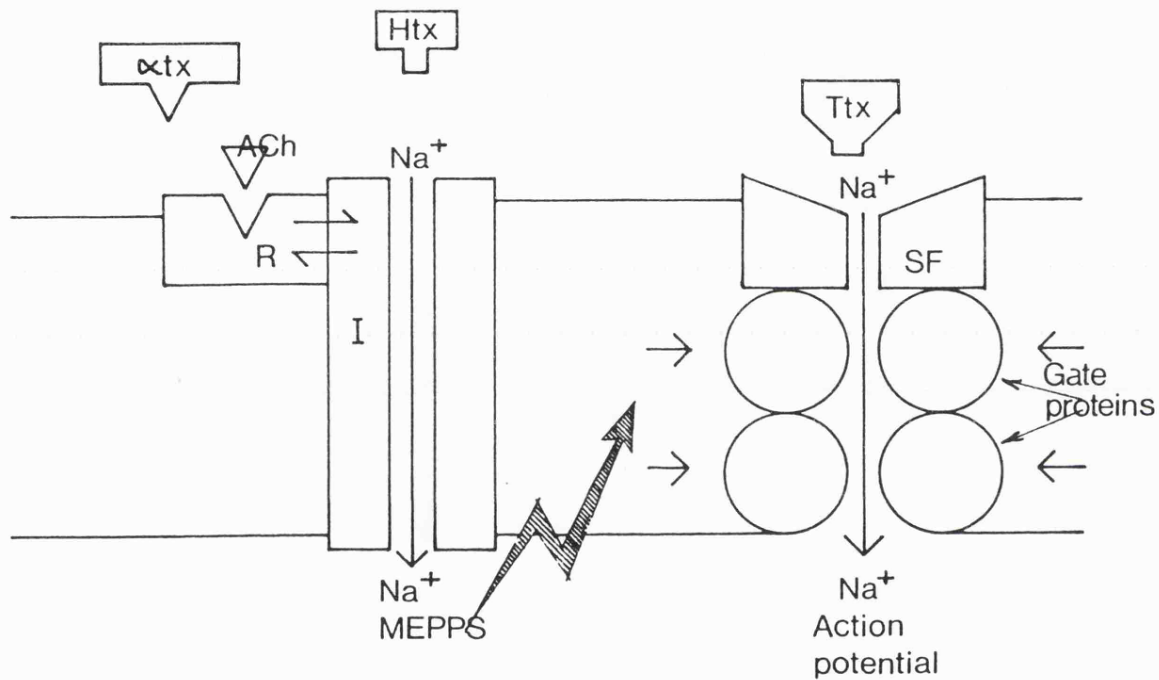
ACh interacts with its receptor R. The interaction results in an opening of the ionophore I, and Na^+ ions enter the cell. This changes the membrane potential and promotes aggregation of the intrinsic gate proteins with the selectivity filter SF, thereby forming a functional sodium channel. The system can be blocked at three distinct sites by toxins: αtoxin, αtx;

histrionicotoxin, Htx; tetrodotoxin, Ttx.

the α-neurotoxins bind to AChRs non-covalently but with high affinity ($K_D \sim 10^{-11}$M) and specificity, at or very near to the ACh binding site. The α-neurotoxins are small compact peptides comprising 61-74 amino acid residues and have a net positive charge. They have been divided into two classes on the basis of their behaviour at the neuromuscular junction. Type I toxins have 60-62 amino acid residues, provide a reversible neuromuscular block and have therefore been exploited as ligands for affinity purification of AChRs (eg. the α-toxins from Naja naja siamensis). Type II toxins have 71-74 amino acid residues and bind almost irreversibly to the AChR. Their usefulness as probes for the AChR has relied upon the finding that they may be radiolabelled to high specific activity with retention of biological activity (eg. α-bungarotoxin (α-BGT) from Bungarus multicinctus).

Radioactive α-BGT, labelled with [^{125}I] is currently the ligand of choice for quantitating the number of AChRs in intact cells, membrane fractions and solubilised extracts of AChRs.

The electric organs of the electric fish are embryonically similar to skeletal muscle but have no contractile elements. The two most studied electric organs are those of the eel, Electrophorus electricus, and various species of the Torpedo genus, the electric ray. The AChR density in electroplaques, the cells of the electric organ, is high, approaching 25% of the total membrane protein. This organ is therefore a rich and homogenous source of AChR which has been used extensively for biochemical characterisations (see Karlin, 1980; Conti-Tronconi and Raftery, 1982 for reviews).

The nicotinic AChR is an integral membrane protein which can only be released from the membrane by detergent extraction. Use of non-ionic detergent (eg. Triton X-100) results in a soluble form of

FIGURE 3   STRUCTURES OF ACETYLCHOLINE RECEPTOR LIGANDS   8

ACETYLCHOLINE

CARBAMYLCHOLINE

DECAMETHONIUM

D-TUBOCURARINE
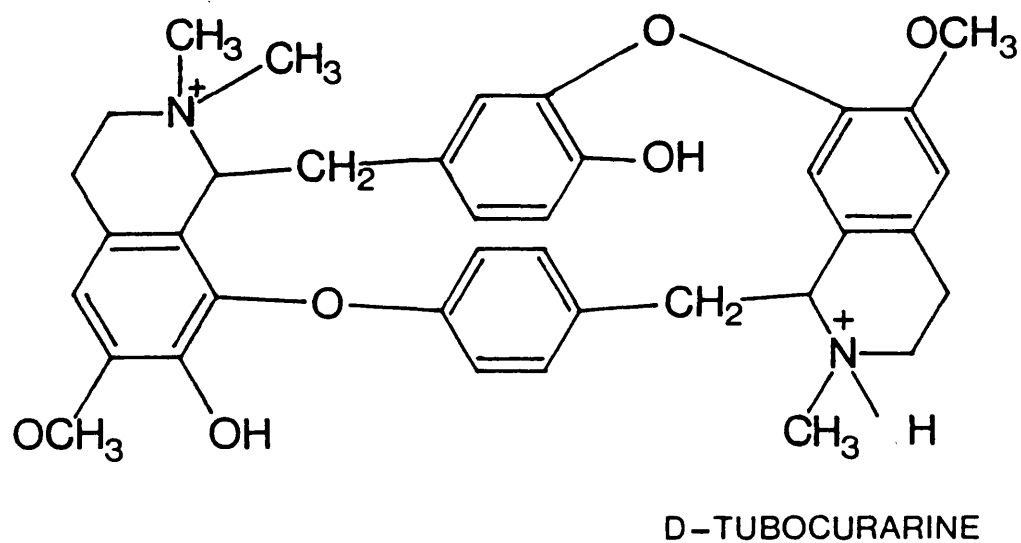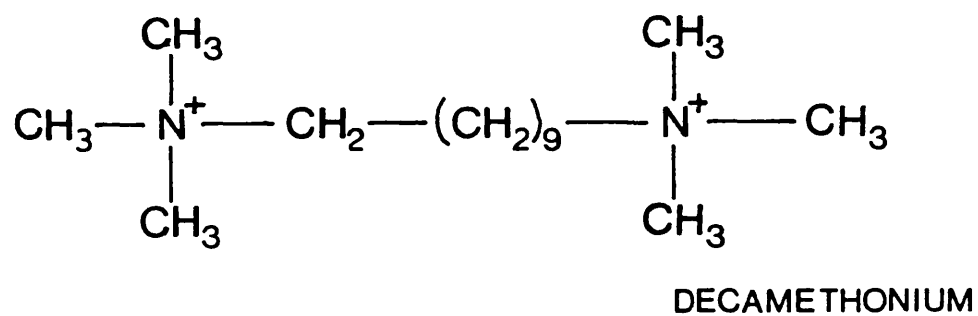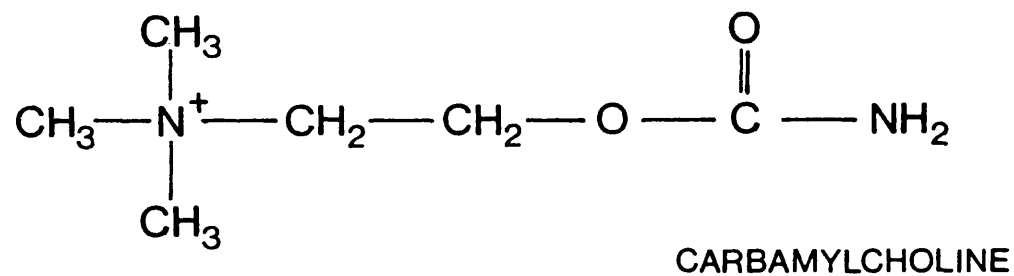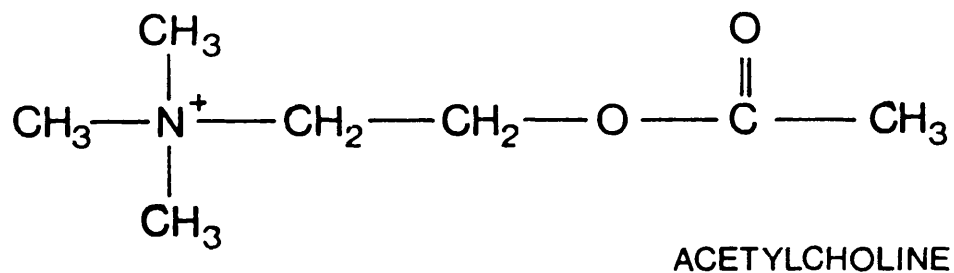
receptor which retains its ability to bind α-toxins and cholinergic ligands. Purification of the _Torpedo_ AChR, after detergent extraction from the membrane and affinity chromatography, showed it to be a glycoprotein comprising 4 subunits designated α, β, γ and δ, in the stoichiometric ratio of 2:1:1:1. The apparent molecular weights of the subunits are 40K, 50K, 60K and 65K respectively. The α-subunit contains the binding site for ACh; these binding sites being on the synaptic surface of the AChR (Kistler et al., 1982). The role of the other three subunits of the receptor is as yet uncertain. Electron microscope studies have revealed that the subunits are very similar in overall structure. The AChR is arranged as a highly symmetrical pentagonal structure, (9nM diameter), around the axis of a central pit, the presumed ion-conducting pore, (2 nM diameter) (Brisson and Unwin, 1985). The AChR spans the membrane, each subunit being exposed on both the cytoplasmic and synaptic surfaces. Identification of the messenger RNA (mRNA) for AChR has allowed the cloning of the complementary DNA (cDNA) which has, in turn, enabled nucleotide sequencing for the polypeptide chains of all four subunits (Noda et al.,1982, 1983ab). These studies have indicated extensive homology between the subunits. Predictions based on the data obtained have suggested a common secondary structure for the portions of the subunits inside the lipid bilayer of the membrane. The arrangement of the polypeptide chains to give a charge lined pore with alternating regions where positive and negative charges predominate (see Stevens, 1985 for review) has led to the first clear picture of the structure of ion channels and of the mechanisms involved in ion translocation.

The experience gained from studies with electric fish has allowed the isolation and characterisation of AChR from vertebrate

skeletal muscle. However, this has proved considerably more difficult

than for the electroplaque AChR, mainly because of the very low

content of AChR in muscle membrane and the high proteolysis that

occurs during purification (see Dolly, 1979 for review). In normal

adult innervated muscle, the AChR comprises less than 1% of the total

membrane protein. After denervation of the muscle, however, the number

of AChRs increases by up to 50-fold providing a useful enriched source

of receptors which has enabled their purification and partial

characterisation from a variety of sources. Initial investigations

suggested that these AChRs had analogous subunit structures to those

of Torpedo AChR (Stephenson et al., 1981; Lindstrom et al., 1979).

Studies in which monoclonal antibodies were raised against individual

Torpedo AChR subunits have shown that these cross-react with mammalian

AChR (Tzartos and Lindstrom, 1980). The additional finding that

immunisation of rats with any of the Torpedo AChR subunits induced an

immune response in the rats to their own AChR suggested that Torpedo

and mammalian AChR shared common antigenic sites (Lindstrom et al.,

1978a). By cloning and sequencing the complementary or genomic DNAs,

Numa and co-workers have elucidated the primary structures of all four

subunits of calf muscle AChR (Noda et al.,1983c; Tanabe et al.,1984;

Takai et al.,1984; Kubo et al.,1985) and of the α and γ subunits of

the human muscle AChR (Noda et al.,1983c; Shibahara et al.,1985). The

nucleotide sequences obtained from these studies show a large degree

of sequence homology with the corresponding Torpedo cDNAs, suggesting

a conservation of the AChR protein between species.

Tissue culture techniques

The founder of modern tissue culture was R.G.Harrison whose paper "Observations on the Living Developing Nerve Fiber" appeared in the 'Proceedings of the 23rd Meeting of the Society for Experimental Biology and Medicine' in 1907. Harrison found that explanted fragments of frog embryo nervous tissue would survive if placed in drops of clotted lymph, and would grow large nerve processes from cells within the explant. Harrison's techniques were soon adapted for the culture of excitable tissue from other species. The first report of cultured skeletal muscle, established from explants of chick embryo leg muscle, was in 1915. These cultures demonstrated spontaneous contractility in the absence of nerves (Lewis, 1915). Since that time, and especially since the development of dispersed cell culture techniques by Konigsberg (1960, 1963), the use of skeletal muscle cultures as a tool for studies encompassing the entire spectrum of cell biology and biochemistry, has flourished.

The four main tissue culture techniques (see Paul, 1975), which have been adapted for skeletal muscle cultures are :-

1) Organ culture in which isolated muscle is maintained intact in an artificial environment. In this way, the characteristics of muscle _in vivo_ may be studied under conditions _in vitro_.

2) Explant cultures in which small pieces of freshly excised muscle are placed into culture dishes, usually under coverslips, from which new cellular outgrowths appear within a few days.

3) Cell cultures in which muscle tissue has been enzymatically dissociated or mechanically dispersed so as to yield a

suspension of single cells. These cells are plated onto the surface of culture dishes where they can be maintained in a uniform environment.

4) Cell lines are a population of cells which have become 'established' enabling them to be maintained in vitro for long periods, sometimes indefinitely. One such example is the clonal cell line L6 (Yaffe, 1968). Many cell lines have, however, properties which differ from those of the original primary cells.

The use of dissociation techniques to establish monolayer muscle cell cultures offers several advantages. The cells grow and develop in a synchronous manner and such cultures are particularly appropriate for the biochemical analysis of myogenesis. The cells can also be maintained under uniformly controlled conditions and may be subjected to a wide range of experimental intervention. Successful monolayer cultures of muscle cells have been established from many species including frog, chick, mouse, rat and human (see Hauschka, 1972; Yaffe, 1973; Konigsberg, 1979 for reviews).

Hauschka and Konigsberg (1966) established that collagen-coated substances promote myogenesis in vitro. This classic study led to the suggestion that a defined molecule serves to trigger or direct muscle differentiation. Hauschka and co-workers went on to show that gelatin was as effective in promoting myogenesis in vitro and that collagen acts by enhancing the attachment of myogenic cells to the culture substrate (see Hauschka, 1972). They also showed that myogenic cells require a protein that mediates this attachment and that this protein binds both to collagen and to the surfaces of myogenic cells. The mediator protein was subsequently shown to be

fibronectin (Chiquet et al., 1979) which is present in the serum used for culturing cells and is possibly also produced by fibroblasts. Muscle is not a homogenous cell type and primary muscle cell cultures are inevitably contaminated by the presence of fibroblasts which arise from connective tissue.

The culture medium for growing muscle in vitro commonly consists of a defined medium, a serum and a chick embryo extract. This extract has been shown to be required for the successful differentiation of avian muscle in vitro although it is apparently not necessary for the growth and differentiation of mammalian muscle in vitro (Hauschka, 1972). Serum has always been included in growth media, but the types used have varied. As there seems to be no requirement for homologous serum (see Paul, 1975), the two types of serum most commonly used are from foetal calf and from horse. The active components in serum and tissue extracts which support muscle growth in culture, have not yet been defined (see "Discussion" section 1.3).

The most routinely grown muscle cultures in many laboratories are those established from embryonic chick or from embryonic and neonatal rat. This muscle is readily available and has known growth characteristics. The growth of human muscle in culture has, however, proved extremely variable (see Witkowski et al., 1976 for review of early work). The techniques employed for human muscle cultures have largely been adapted from those used for chick or rat culture systems. The attraction of cultured human muscle is its potential use to investigate muscle diseases such as the muscular dystrophies. Many studies have thus aimed to compare normal and diseased human muscle in culture (eg. Goyle et al., 1967; Kakulas et

al., 1968; Bateson et al., 1972; Emery and McGregor, 1977; Merickel et

al., 1981; Yasin et al., 1983; Blau et al., 1983); these have

concentrated on differences between normal and abnormal muscle, rather

than the culture conditions required for successful normal muscle

growth. Attempts have been made to standardise the conditions used for

the growth of explant cultures (Witkowski et al.,1976) and new

procedures have been published for the growth of monolayer muscle

cultures from both explant (Askana-s and Engel, 1975) or dissociated

tissue (Yasin et al.,1977; Blau and Webster, 1981). However, although

many investigators base their techniques on these published methods,

modifications have invariably been added and a wide variety of growth

media used (see "Discussion" Tables 32 and 33). The usual source of

muscle for human cultures is from biopsy specimens obtained from

children or adults. There have been relatively few studies carried out

with foetal human muscle (see "Discussion" Table 32) and this may

reflect the difficulty in supply. An additional factor, as many

studies aim to compare normal and diseased muscle, is that foetal

muscle is of an unknown nature. The use of foetal human muscle tissue

has the theoretical advantage that relatively large amounts can be

obtained compared with biopsy material, providing large numbers of

replicate primary cultures, without recourse to the expertise and time

required for cloning techniques and the expansion of cell numbers

(Hauschka, 1974a; Yasin et al.,1981; Blau and Webster, 1981).

Growth of muscle cells in culture

Studies of embryonic skeletal muscle in vitro have demonstrated

that many properties of differentiated muscle in vivo are reproduced

in cell cultures. In both cases, a proliferating pool of mononucleated

cells is the precursor of the multinucleated muscle fibres

(Konigsberg, 1963; Yaffe, 1969). The properties of the cell membranes

of muscle fibres in vivo are known to be strongly influenced by

interaction with motor neurones (see "Introduction" p.18).

Nevertheless, in the absence of neurones, embryonic skeletal muscle

differentiates spontaneously in cell culture where it develops a

striated contractile apparatus and can demonstrate spontaneously

occurring contractions.

The elaboration by chick or rat muscle cultures of the

membrane-bound AChR has been demonstrated electrophysiologically, by

sensitivity to iontophoretically applied ACh (Dryden, 1970; Fambrough

and Rash, 1971) and by the specific binding of radioactively labelled

α-neurotoxins (Patrick et al.,1972; Sytkowski et al.,1973; Prives and

Paterson, 1974; Devreotes and Fambrough, 1975; Prives et al., 1976;

Spector and Prives, 1977; Shainberg and Brik, 1978). AChE, the enzyme

which terminates the action of ACh at the neuromuscular junction, has

also been demonstrated in these cultured muscle cells, appearing

concurrently with AChR (Prives and Paterson, 1974; Prives et al.,1976;

Shainberg and Brik, 1978). As well as these membrane components of

skeletal muscle, the cytoplasmic muscle-specific enzyme creatine

phosphokinase (CPK) has been shown to increase in differentiating

cultured muscle (Shainberg et al.,1971; Morris and Cole, 1972;

Shainberg and Brik, 1978). The substrate for this enzyme is

phosphocreatine, a high energy phosphate compound, generated during

glycolysis, which is required for contractile activity in muscle

fibres. The increases in membrane-bound AChR and AChE and cytoplasmic

CPK proteins are used as markers of muscle differentiation in vitro.

AChRs have also been detected on cultured human muscle.

Sensitivity to iontophoretically applied ACh has been demonstrated in foetal human muscle cultures established by using explant techniques (Harvey et al., 1979) and an indirect immunoperoxidase method was used by Askanas et al. (1977) to detect α-BGT binding to adult human muscle in explant culture. The binding of radioactively labelled α-BGT to human foetal (Adams and Bevan, 1983, 1985) or adult muscle cells in culture (Franklin et al.,1980; Blau and Webster, 1981; Blau et al., 1983) has also been demonstrated. These studies have shown that human muscle cells can develop in vitro to elaborate AChRs on their surface. In addition, an increase in cytoplasmic CPK activity has been observed in adult human dissociated muscle cell cultures, appearing concurrently with AChR (Blau and Webster, 1981; Blau et al.,1983). However, there have been few reports of spontaneously occurring contractions in cultured human muscle cells. Such contractions have been reported in human foetal explant (Harvey et al.,1979) and adult dissociated muscle cultures (Yasin et al.,1977; Blau and Webster, 1981; Blau et al.,1983; Bolhuis et al.,1985).

The distribution of AChRs 'in vivo' and 'in vitro'

The distribution of AChRs in the membrane of vertebrate skeletal muscle has been analysed electrophysiologically, by determining the sensitivity of the membrane to ACh, and by mapping the binding of radiolabelled or fluorescently labelled α-BGT in light and electron autoradiography or fluorescent microscopy studies respectively. At the normal neuromuscular junction, AChRs are highly concentrated on the terminal expansions of the post-synaptic folds. The packing density of these AChRs is 2-6 x 10^7 receptors per junction (Fambrough, 1979). The enzyme AChE is associated with the muscle

basement membrane and is localised over the whole surface of the post-synaptic membrane (McMahan et al., 1978). Extrajunctional receptors, which are barely detectable in normal adult innervated muscle, show a marked proliferation over the entire surface of the muscle fibre following denervation (Axelsson and Thesleff, 1959; Miledi, 1960a; Miledi and Potter, 1971; Hartzell and Fambrough, 1972) and are thought to result from de novo synthesis and direct incorporation into the extrajunctional membrane (Chang and Tung, 1974). If denervated muscle is reinnervated, a reversal of this process occurs with a reduction in the number of receptors outside the end-plate until a normal adult pattern is achieved (Miledi, 1960b). The AChRs of embryonic muscle appear, as in denervated adult muscle, to be evenly distributed over the whole of the post-synaptic membrane, and become concentrated at the end-plate as the animal matures. For rat and chick muscle in vivo, mature neuromuscular junctions are formed by approximately 1-2 weeks after birth (Diamond and Miledi, 1962; Bevan and Steinbach, 1977; Burden, 1977).

Similar techniques to those described above have been used to describe the non-junctional distribution of AChRs in cultured muscle cells, which are grown in the absence of nerves. Differentiated muscle cells established from embryonic chick (Vogel et al.,1972; Fischbach and Cohen, 1973; Sytkowski et al.,1973; Prives et al.,1976), rat (Hartzell and Fambrough, 1973; Axelrod et al.,1976; Land et al.,1977), mouse (Christian et al.,1978), frog (Anderson et al.,1977) and human (Harvey et al.,1979; Askansas et al.,1977; Blau and Webster, 1981; Adams and Bevan, 1983, 1985) sources have AChRs distributed over the entire surface of the cells, in a similar manner to that of embryonic or of adult denervated muscle. However, the distribution of

AChRs is not uniform. Patches of aggregated AChRs are interspersed with diffusely distributed receptors, the number of these AChR clusters increasing with myotube differentiation (Prives et al.,1976; Anderson and Cohen, 1977). The average density of receptor sites differs between species, being highest in chick (Sytowski et al.,1973) and rat embryonic myotubes (Axelrod et al.,1976). These clusters have not been observed, however, in human cultured muscle cells (Askansas et al.,1977; Harvey et al.,1979; Blau and Webster, 1981; Adams and Bevan, 1983, 1985), in rat myogenic cell lines (Vogel et al.,1972; Land et al.,1977) or in embryonic muscle in vivo (Bevan and Steinbach, 1977; Burden, 1977).

Junctional and extrajunctional AChRs

Biochemical studies on affinity purified AChRs have shown that junctional and extrajunctional AChRs have the same subunit composition and the α and β subunits have identical peptide maps (Nathanson and Hall, 1979). The receptors are indistinguishable by gel filtration, sucrose gradient sedimentation and reaction with a rabbit antiserum to electric eel AChR but have slightly different isoelectric points (Brockes and Hall, 1975). However, in the membrane environment, the two types of receptor differ in several of their properties. ACh induced current fluctuation measurements indicate that both conductance and mean channel lifetimes in the junctional region of the membrane differ from those of the extrajunctional region (Cull-Candy et al.,1979, 1982; Schuetze et al., 1985). The two types of receptor also show different sensitivities to d-tubocurarine (Brockes and Hall, 1975). The turnover of junctional AChRs in the membrane is much slower ($T_{\frac{1}{2}} \sim$ 7 days) than that of extrajunctional receptors ($T_{\frac{1}{2}} \sim$ 10-20 hours)

(Berg and Hall, 1974; Chang and Huang, 1975). Extrajunctional receptors have been shown to react preferentially with some anti-AChR antibodies from myasthenic sera (Almon and Appel, 1975; Savage-Marengo et al.,1980; Dwyer et al.,1981; Reiness and Hall, 1981) and with anti-AChR antibodies raised against purified receptor from denervated muscle in experimental animals (Dolly et al.,1983). It has been suggested that the observed differences between the two receptor types may be due in part to carbohydrate residues (Dwyer et al.,1981; Turnbull et al., 1985) or to the degree of phosphorylation (Saitoh and Changeux, 1981).

Non-junctional receptors in aneurally cultured chick and rat muscle cells have an estimated half-life of 22 hours (Devreotes and Fambrough, 1975) or 17 hours (Axelrod et al.,1976; Gardner and Fambrough, 1979), a value similar to that for extrajunctional receptors in embryonic or denervated adult muscle. The aggregates of AChRs that occur on cultured muscle cells resemble the junctional receptors of innervated adult muscle in their concentration and limited membrane mobility (Axelrod et al.,1976). In contrast, the diffusely distributed, non-clustered receptors on cultured muscle cells have a relatively high lateral mobility. However, the diffuse and clustered receptors have similar turnover times (Axelrod et al.,1976; Schuetze et al.,1978) and ACh induced mean channel lifetimes (Schuetze et al.,1978). Cultured muscle cells established from the rat or mouse produce two forms of surface receptors, with isoelectric points indistinguishable from those of junctional and extrajunctional receptors respectively (Sugiyama et al.,1982). This has not, however, been observed with cultured chick muscle AChRs, which have an isoelectric point close to that of extrajunctional receptors alone.

The developmental mechanisms involved in the formation of junctional AChRs at the post-synaptic membrane in vivo are essentially unknown. The mechanisms have been studied in vitro by using cultured muscle cells or co-cultures of differentiated muscle cells and neuronal cells. In these co-cultures, clusters of AChRs are observed in the muscle membrane areas adjacent to neuronal processes. The studies carried out suggest that pre-existing AChRs are redistributed to the area of innervation (Anderson and Cohen, 1977; Anderson et al., 1977; Cohen and Weldon, 1980). Recent evidence has shown that a soluble factor released from neurones may be the agent that induces the aggregation of AChRs in the post-synaptic membranes of muscle cells. A factor in medium conditioned by cultured neurones has been shown to increase the number of AChR clusters in muscle cells in culture (Christian et al.,1978; Podleski et al.,1978; Schaffner and Daniels, 1982). The release of a soluble factor by neurones could therefore be the signal that induces the aggregation of extrajunctional receptors in muscle cells in vivo to form AChRs of the junctional type at the neuromuscular junction.

Maintenance of AChRs at the neuromuscular junction

The factors that maintain AChRs at a high concentration on the post-synaptic membrane have been studied by using muscle cells in culture. In culture, the synthesis of new receptors occurs at a constant rate which somewhat exceeds that of degradation; the two processes not being closely coupled (Devreotes and Fambrough, 1975). Newly synthesised AChRs are localised in the Golgi apparatus (Fambrough and Devreotes, 1978) and approximately 3 hours are required before they appear in the plasma membrane (Devreotes et al.,1977;

Fambrough and Devreotes, 1978). In the cultured muscle cells, approximately 20-30% of the receptors are not exposed on the surface membrane (Devreotes and Fambrough, 1975; Devreotes et al.,1977; Patrick et al.,1977). Membrane AChRs appear to be selected randomly for degradation which occurs by an energy-dependent process involving internalisation, proteolysis and the release of degraded amino acid residues into the medium (Devreotes and Fambrough, 1975; Merlie et al., 1975). The receptors at the intact neuromuscular junction are degraded by similar mechanisms (Fumagalli et al.,1982). Cluster formation and turnover of AChRs in cultured muscle is thought to result from both migration of diffusely distributed AChRs and insertion of newly synthesised AChRs (Stya and Axelrod, 1983). Factors increasing the degradation rate of AChRs, such as myasthenic anti-AChR antibodies (see "Introduction" p.33) or non-immunological complexes that exert their effects through cross-linkage of AChRs, have been shown to disperse the receptor clusters before internalisation takes place (Axelrod, 1980; Bursztajn et al.,1983). These observations would indicate that the AChR clusters observed in muscle cultures are not structurally fixed into the sub-membrane cytoskeletal framework as had been previously suggested (Prives et al.,1982).

There is some evidence that electrical activity can regulate AChR synthesis and/or distribution in cultured muscle. AChR levels in chick muscle are decreased by electrical stimulation or depolarisation of the muscle membrane (Shainberg et al.,1976; Betz and Changeux, 1979) and increased by agents such as tetrodotoxin which inhibit muscle activity (Shainberg et al.,1976; Birnbaum et al.,1980). These effects are thought to be mediated by changes in intracellular levels of calcium (Birnbaum et al.,1980; McManaman et al.,1982) and

cAMP (Betz and Changeux, 1979) which in turn affect AChR synthesis. Inhibition of muscle activity in muscle cultures by use of tetrodotoxin or high levels of extracellular calcium have also been shown to increase the size of receptor clusters in chick (Cohen and Pumplin, 1979) and rat muscle (Bursztajn et al.,1984) although not the final levels of AChR in rat muscle (Bursztajn et al.,1984). This lack of effect by tetrodotoxin on AChR synthesis in rat muscle was suggested by the authors to be a species specific phenomenon.

Naturally occurring dense clusters of immobile AChRs on cultured rat muscle have been shown to have half lives similar to those of the laterally mobile diffuse receptors (Axelrod et al.,1976). At the neuromuscular junction in vivo, the junctional receptors are much more stable than the non-clustered extrajunctional receptors (Berg and Hall, 1974; Chang and Huang, 1975). The nature of the factors regulating the maintenance of junctional receptors is therefore still in doubt. However, the blockade of muscle activity in vivo by use of agents such as tetrodotoxin or α-BGT has been shown to cause the appearance of extrajunctional receptors in a similar manner to denervation (Lomo and Rosenthal, 1972; Berg and Hall, 1975; Pestronk et al.,1976). Also, chronic electrical stimulation has been shown to reduce the extrajunctional ACh sensitivity of denervated muscle in vivo (Lomo and Rosenthal, 1972) or in situ (Hall and Reiness, 1977) by mechanisms which inhibited AChR synthesis (Hall and Reiness, 1977). It is probable that at the intact neuromuscular junction, both electrical and neuronal factors play a part in maintaining the level of AChRs at the post-synaptic membrane.

The basic defect in myasthenia gravis

The elucidation of the precise site of the defect in MG was
aided by electrophysiological studies. The muscle of myasthenic
patients has been shown to have abnormally small EPPs, that are either
below the threshold required to initiate muscle contraction or rapidly
become so during repetitive stimulation (Elmqvist et al.,1964). Other
studies have shown that the action potential generated by the nerve is
normal, but that the amplitude of MEPPs in myasthenic patients is
markedly reduced (Elmqvist et al.,1964; Santa et al.,1972; Albuquerque
et al.,1976a). Myasthenic motor end-plates also show reduced
sensitivity to iontophoretically applied ACh in vitro (Albuquerque et
al., 1976a; Rash et al.,1976). The question of whether the
physiological defect in MG is primarily pre-synaptic or post-synaptic
was resolved following the availability of the post-synaptically
acting α-neurotoxins (see "Introduction p. 5). Fambrough et
al.(1973), using iodinated α-BGT to localise AChR, demonstrated
reduced numbers of α-BGT binding sites in myasthenic end-plates,
indicating a reduced number of available AChRs. These reductions were
shown to be as low as 30% of normal. The area of the post-synaptic
membrane that can bind α-BGT is also reduced (Engel et al.,1977b)
which reflects a decrease in the content of AChR rather than blocking
of α-BGT binding to the receptor by some other factor (Lindstrom and
Lambert, 1978).

Morphological changes at the myasthenic end-plates
suggest degeneration and regeneration of the neuromuscular junction
with flattening and simplification of the post-synaptic folds (Figure
4). In some regions, the synaptic cleft is widened and in others, the
post-synaptic membrane lacks any apposing nerve terminal (Engel and

Santa, 1971; Santa et al.,1972). In contrast, the structure of the axon terminals present, has been shown to be retained. However, the terminals have been shown to contain twice the normal concentration of ACh (Ito et al.,1976; Cull-Candy et al.,1978, 1980).

The reduction of AChRs and the morphological changes observed in myasthenic end-plates result in decreased efficiency of neuromuscular transmission. If this is reduced to a point at which the threshold value is not reached, action potentials are not triggered. This will ultimately lead to a reduction of muscle power and hence to the weakness and susceptibility to fatigue characteristic of MG.

The autoimmune nature of myasthenia gravis

The first suggestions that MG might be of autoimmune origin occurred in the late 1950's, following studies by two different groups of workers. Simpson (1960) pointed out the association of MG with other illnesses thought to be autoimmune (eg. Hashimoto's thyroiditis, systemic lupus erythematosus, rheumatoid arthritis). It was suggested by Simpson (1960) that the disease was caused by the presence of antibodies to the ACh receptive substance at the motor end-plate. Nastuk and co-workers observed wide variations in serum complement levels in myasthenic patients (Nastuk et al.,1960) together with the presence of serum complement-fixing antibody binding in vitro to skeletal muscle sections (Strauss et al.,1960). The latter study was the first demonstration of a circulating tissue specific antibody related to MG. These anti-striated muscle antibodies were, however, later shown to be connected with a tumour of the thymus, and are not specific to MG (Oosterhuis et al.,1976; Limburg et al, 1983). Another piece of evidence supporting the hypothesis of an autoimmune origin in

FIGURE 4
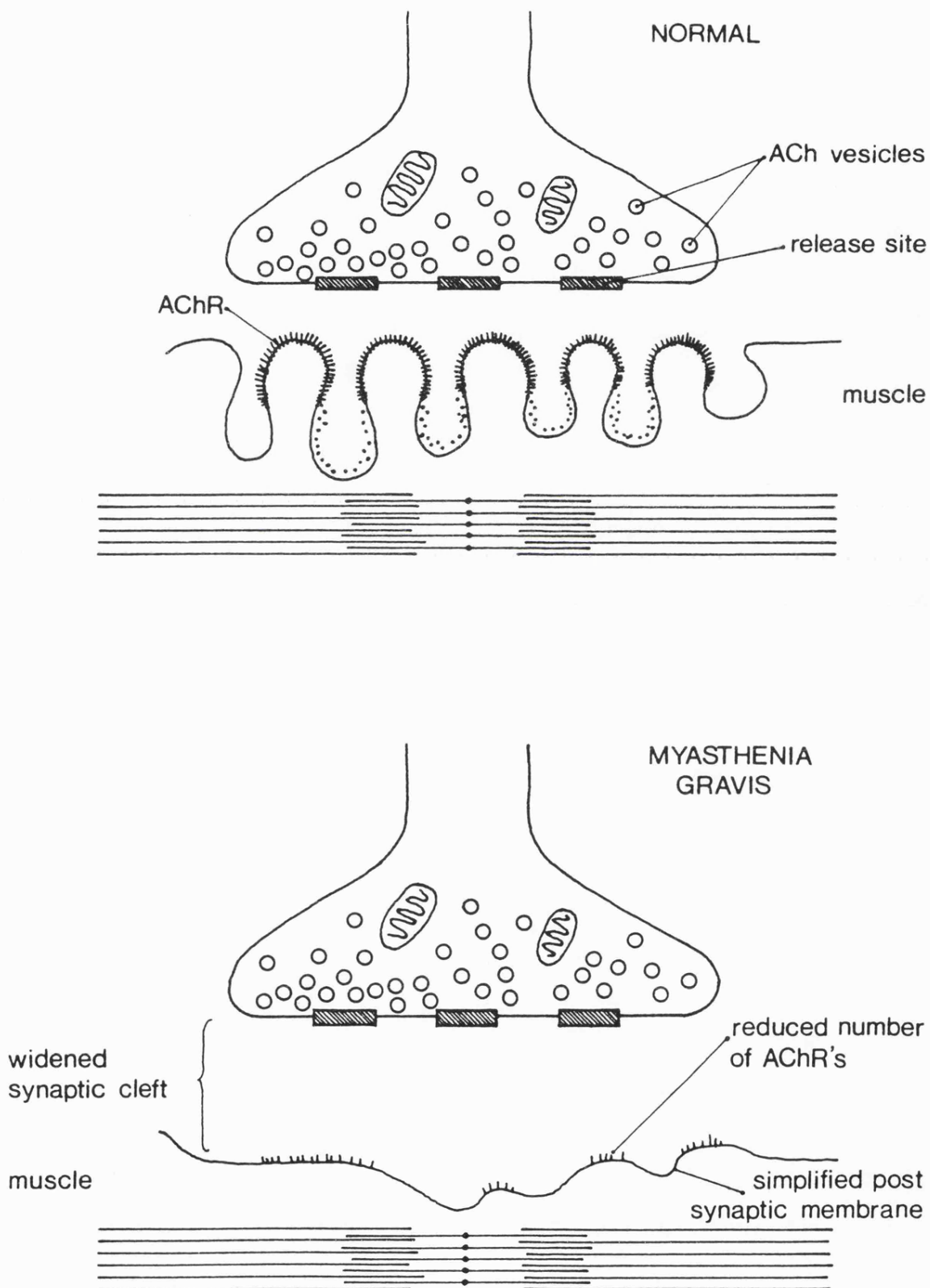
DIAGRAM SHOWING NORMAL AND MYASTHENIC NEUROMUSCULAR
JUNCTIONS

MG was the observation that approximately 75% of patients with MG

demonstrated structural abnormalities of the thymus (Castleman, 1966),

a tissue thought to be involved in immune regulation. Histologically,

the thymus of a myasthenic patient and the thyroid in autoimmune

Hashimoto's disease appear similar (Simpson, 1960). Surgical removal

of the thymus from patients with MG is often associated with a marked

improvement in clinical symptoms (Scadding et al.,1979). Many early

studies, however, failed to show a serum factor that would block

neuromuscular transmission (eg. Nastuk et al.,1959; McFarlin et

al.,1966). The serendipitous experiment by Patrick and Lindstrom

(1973) who immunised rabbits with purified electric eel AChR producing

severe myasthenia-like symptoms in the animals, suggested that the

AChR was the target of an autoimmune attack in MG. This experiment,

and those of other workers (Sugiyama et al.,1973; Heilbronn and

Mattson, 1974) provided an experimental model (experimental autoimmune

myasthenia gravis, EAMG), for the study of the human disease.

Antibodies to skeletal muscle nicotinic AChRs were

found in 1973, after a cross-reacting AChR from rat denervated muscle

was purified. Almon et al. (1974) demonstrated that approximately 50%

of myasthenic sera could inhibit α-BGT binding to the AChR. They later

identified the factor as IgG (Almon and Appel, 1975). Shortly after

this, Bender et al.(1975), using an indirect immunoperoxidase

labelling technique, showed that myasthenic sera blocked the binding

of α-BGT to the motor end-plates of normal human muscle. The ability

of a high proportion of myasthenic serum antibodies to fix complement

in the presence of small quantities of Torpedo AChR was also

demonstrated (Aharanov et al.,1975).

A radioimmunoassay for the quantitation of anti-AChR

antibodies in MG sera soon became a standard diagnostic procedure

(Lindstrom et al.,1976a; Lindstrom, 1977; Monnier and Fulpius, 1977;

Newsom-Davis et al.,1978; Dwyer et al.,1979; Tindall et al.,1981;

Carter et al.,1981). The radioimmunoassay procedure, illustrated in

Figure 5, uses crude detergent extracts of amputated human limb muscle

as the source of AChR antigen. The detection of positive serum

anti-AChR antibody levels is specific for the diagnosis of MG, 70-95%

of patients having elevated levels relative to normal controls

(Lindstrom et al.,1976a; Lindstom, 1977; Mittag et al.,1981, 1984;

Monnier and Fulpius, 1977; Ito et al.,1978; Lefvert et al.,1978).

Anti-AChR antibody characteristics

The serum anti-AChR antibodies present in MG consist of

populations belonging to the IgG fraction, although in the early

stages of MG, antibodies belonging to the IgM type have been reported

(Lefvert et al.,1978). In individual myasthenic patients, the serum

anti-AChR antibodies are found in different subclasses of IgG (Vincent

and Newsom-Davis, 1980; Lefvert et al.,1981; Tindall, 1981; Whiting

et al.,1983). A low proportion of anti-AChR antibodies in MG have been

shown to be directed against the ∝-BGT binding site (Dwyer et

al.,1979; Mittag et al.,1981; Vincent and Newsom-Davis, 1979, 1980, ˙

1982; Whiting et al.,1983). These findings indicate that the

antibodies are polyclonal, being directed to several determinants on

the AChR of skeletal muscle. The diversity of anti-AChR antibodies is

also demonstrated by the variation seen between individual sera in

their cross-reactivity with AChR preparations from different species

(Lindstrom et al.,1978b; Savage-Marengo et al.,1979, 1980; McAdams and

Roses, 1980; Harrison et al.,1981; Vincent and Newsom-Davis, 1982) and

FIGURE 5

DIAGRAMMATIC REPRESENTATION OF THE RADIOIMMUNOASSAY

FOR ANTI-AChR ANTIBODIES

AChR   + $[^{125}I]\,\propto$-BGT

+   ANTI- AChR ANTIBODIES IN MYASTHENIC SERUM

+   ANTI- HUMAN IgG

IMMUNE COMPLEX PRECIPITATES
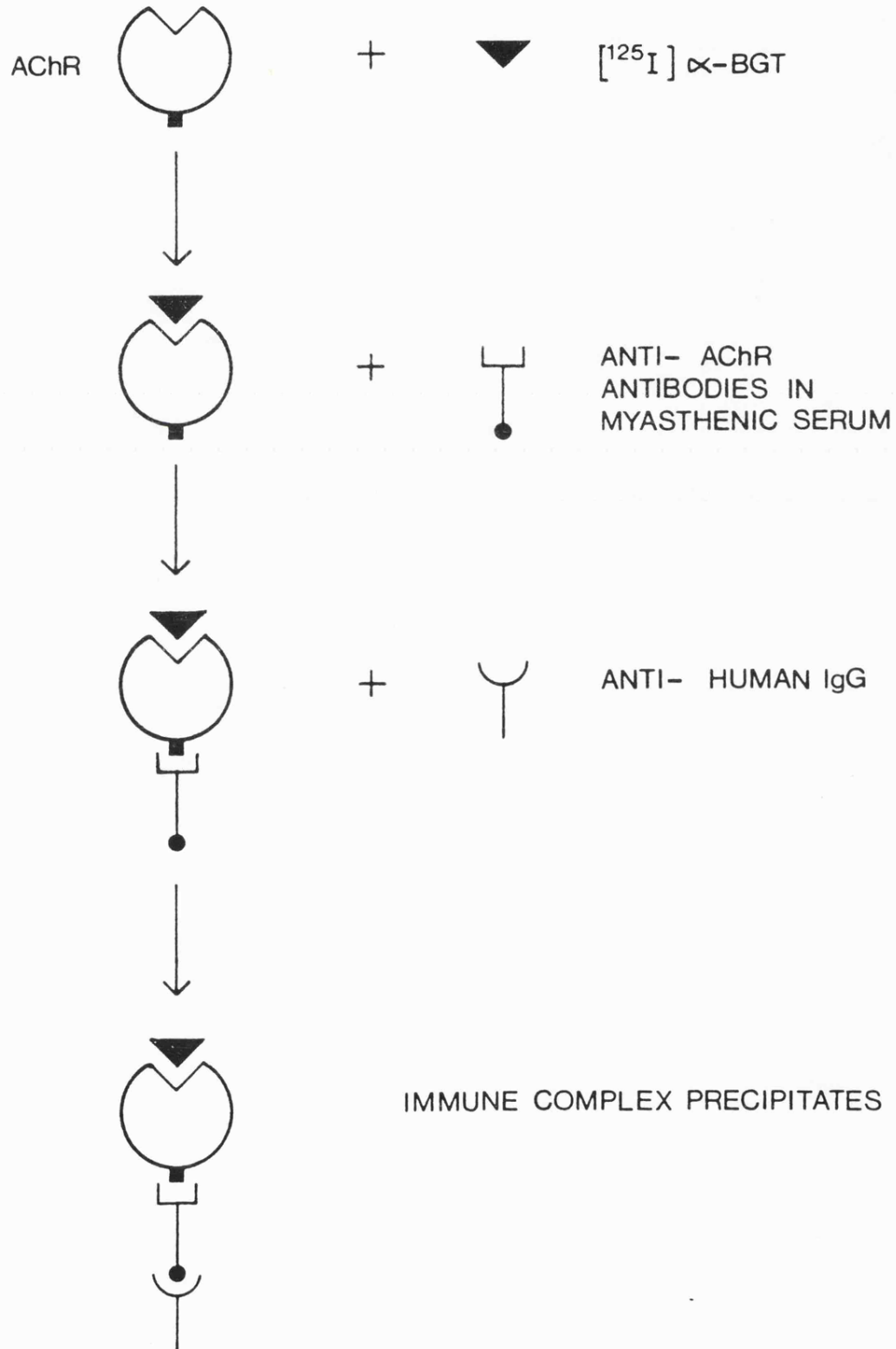
also from different muscles of the same species (Almon and Appel, 1975; Weinberg and Hall, 1979; Vincent and Newsom-Davis, 1979, 1982; Compston et al.,1980). Monoclonal antibody studies have confirmed the polyclonal nature of myasthenic anti-AChR antibodies. Monoclonal antibodies have been produced which bind to a variety of sites on AChRs (Tzartos and Lindstrom, 1980). Most of these, however, bind to a region on the α-subunit distinct from the cholinergic binding site. This region has been termed the main immunogenic region (MIR) of the AChR. By competitive binding studies using subunit specific monoclonal antibodies versus human MG sera binding to human AChR, it has been shown that the majority of human anti-AChR antibodies are directed at the MIR. Other myasthenic antibodies are directed against sites on other AChR subunits, but only a few have been shown to bind to sites on the α-subunit outside the MIR (Tzartos et al.,1982, 1983). The polyclonal nature of the antibodies in MG is most probably the cause of the well documented low correlation between clinical status and serum anti-AChR antibody titres (Lindstrom et al.,1976a; Ito et al.,1978; Lefvert et al.,1978); pathogenicity being confined to specific subpopulations. However, within an individual patient, there appears to be a better correlation between anti-AChR antibody level and disease activity (Newsom-Davis et al.,1978, 1979; Carter et al.,1980).

The pathogenicity of anti-AChR antibodies

The question as to whether circulating anti-AChR antibodies are pathogenic, or merely represent a secondary response to AChR damage caused by some other agent, is critical in understanding the pathogenesis of MG. Several lines of evidence support the theory that

circulating humoral factors act as primary agents in the disease process. Plasma exchange of MG patients is associated with temporary clinical improvement paralleled by a decrease in the concentration of serum anti-AChR antibodies (Pinching et al.,1976; Dau et al.,1977; Newsom-Davis et al.,1978). A subsequent deterioration is often associated with a sharp rise in antibody titre (Carter et al.,1980). Placental transfer of anti-AChR antibodies from a myasthenic mother to the foetus can cause transient neonatal MG, with an anti-AChR antibody level at birth similar to that of the mother (Keesey et al.,1977). The antibody level declines thereafter with a half-life of 8 days, full recovery occurring within three weeks. Toyka et al. (1977) showed that the clinical symptoms of MG could be produced in mice by injection of myasthenic IgG. These mice showed subsequent reductions in MEPP amplitudes and α-BGT binding to the motor end-plates, indicating a reduction in the number of functional AChRs. Recent studies have also shown that the injection of experimental animals with monoclonal antibodies directed against the MIR of the AChR, results in the acute form of EAMG (see "Introduction" p41) in the recipient animals (Tzartos and Lindstrom, 1980; Burres et al.,1981; Gomez and Richman, 1985).

In order to cause synaptic dysfunction, anti-AChR antibodies need to leave the vascular system, diffuse into the extra-cellular space, enter the synaptic cleft and reach the AChRs on the post-synaptic folds. Zurn and Fulpius (1976) used complexes of α-BGT covalently coupled to IgG, to show that molecules the size of antibodies can reach the AChR in situ in the mouse diaphragm. Following this, localisation of IgG at the motor end-plates in myasthenic patients, detected by using peroxidase-labelled

Staphylococcal protein-A, was reported by Engel et al.(1977a).

These facts strongly support the idea that anti-AChR

antibodies are the primary agent in the disease process.

Theoretically, the interaction of anti-AChR antibodies with AChRs

could be pathogenic in one of three ways:-

1) By blocking the ACh binding site on the receptor or

   altering the receptor such that it cannot bind ACh

2) By altering the turnover of AChR in the membrane either

   by increasing the rate of degradation or by decreasing the

   rate of synthesis

3) By causing complement-mediated destruction of the

   post-synaptic membrane

Considerable evidence has been produced supporting the involvement of

AChR blockade and altered AChR turnover in MG. However, less data have

been reported in support of the third mechanism, complement-mediated

lysis of the post-synaptic membrane. The three mechanisms will be

discussed in turn.

Blockade of AChR function

The early discovery by Mary Walker (1934) that treatment of

myasthenic patients with inhibitors of acetylcholinesterase led to a

rapid improvement in symptoms, strengthened the theory that the

disease was due to a 'curare-like' substance which blocked

neuromuscular transmission. However, there are few reports of direct

blockade by myasthenic sera of the electrophysiological response of

AChR. Studies by Albuquerque et al. (1976b) failed to demonstrate the

inhibition of the ACh response by myasthenic sera in rat or human

muscle in vitro. Subsequent reports have shown that myasthenic serum

or immunoglobulins could reduce the sensitivity of cultured human

(Bevan et al.,1977), rat (Anwyl et al.,1977) and chick muscle cells

(Harvey et al.,1978a) to applied ACh. However, these effects could

partly be explained in terms of increased AChR degradation under the

conditions of the assays used (Bevan et al.,1978). A reduction in the

amplitude of MEPPs,in response to myasthenic sera, has been

demonstrated in human myasthenic (Sanders et al.,1981) and normal

intercostal muscle (Ito et al.,1978) and also in rat (Shibuya et

al.,1978) and mouse diaphragm (Lerrick et al.,1983). Interestingly,

the latter two studies reported that the effects of myasthenic sera

were rapid and reversible by washing, and it was subsequently shown by

Lerrick et al. (1983), that the myasthenic serum factor responsible

for reducing the MEPP amplitudes was heat labile and not IgG. It would

thus appear that a heat-labile factor can affect the post-synaptic

AChR independently of antibody.

If blockade of AChR function is an important pathogenic

feature in MG, specific anti-αBGT binding site antibodies or

'anti-site' antibodies would be expected to correlate with clinical

severity of symptoms in myasthenic patients. Immunoglobulins from

myasthenic patients are known to block [^{125}I]α-BGT binding to

detergent solubilised AChR to a varying extent (Almon and Appel, 1975;

Dwyer et al.,1979; Mittag et al.,1981; Vincent and Newsom-Davis, 1979,

1980, 1982; Whiting et al.,1983). These effects have served as a

method of identifying antibodies directed at or near to the

cholinergic binding site. However, good correlation between

'anti-site' antibody levels in myasthenic patients and disease

severity has not been shown (Lefvert et al.,1978; Vincent and

Newsom-Davis, 1979). Myasthenic immunoglobulins have also been shown

to inhibit ∝-BGT binding to AChRs in cultured chick (Fulpius et al.,1980, 1981) and rat muscle cells (Drachman et al.,1982) and it was reported that the degree of blockade correlated with the extent of clinical severity of disease in the patients studied. The blocking effects of myasthenic immunoglobulins on cultured muscle cells are observed independently of complement and under conditions where increased degradation of receptor is unlikely. However, the blockade may not necessarily reflect that of ACh binding in vivo as the small molecular weight of ACh (182 compared to 8000 for ∝-BGT) may allow accessibility to the ACh binding site, even in the presence of antibody. Recent studies in which EAMG was passively transferred to animals by immunisation with monoclonal antibodies directed at AChR antigenic determinants remote from the cholinergic binding site, have confirmed that neuromuscular transmission can be impaired in the absence of functional AChR blockade (Lennon and Lambert, 1980; Richman et al., 1980).

Altered turnover of AChR

AChR degradation has been measured in cultured muscle cells after labelling the surface AChRs with $[^{125}I]$∝-BGT (Devreotes and Fambrough, 1975). As the labelled receptors undergo degradation, the attached $[^{125}I]$∝-BGT is broken down and $[^{125}I]$ tyrosine appears in the culture medium. The addition of myasthenic immunoglobulins has been shown to accelerate the degradation of AChRs in rat (Kao and Drachman, 1977a; Appel et al.,1977) and human muscle cells in culture (Bevan et al.,1977) and in a mouse muscle cell line (Hudgson et al.,1982); the rate increasing 2-3 fold above that of normal controls (Kao and Drachman, 1977a). Significant acceleration of AChR degradation in rat

muscle cultures has been observed in 66% (Conti-Tronconi et al.,1981) and 90% (Drachman et al.,1982) of myasthenic patients studied. These studies also demonstrated a correlation between disease severity and the extent of AChR degradation (Conti-Tronconi et al.,1981; Drachman et al.,1982). The acceleration of degradation in every case was shown to be triggered by immunoglobulins independently of complement. Both the normal AChR degradation process and the accelerated degradation produced by myasthenic immunoglobulin are temperature dependent (Kao and Drachman, 1977a; Appel et al.,1977) and involve energy-dependent processes (Appel et al.,1977). Increased degradation is dependent on the ability of IgG to cross-link receptors (Drachman et al.,1978a, 1980) which is possible because of its divalent nature (see Figure 6). Monovalent Fab fragments are inactive, except when they are cross-bridged by a second antibody (Drachman et al.,1978a, 1980; Prives et al.,1979; Lindstrom and Einarson, 1979). Morphological studies indicate that such cross-linking causes redistribution of AChR in the membrane, followed by enhanced endocytosis. Aggregation of AChRs in muscle cultures following the addition of myasthenic serum or anti-AChR antiserum raised in experimental animals, has been observed by fluorescence microscopy (Lennon, 1978a), autoradiography (Prives et al.,1979) and by freeze-fracture electron miscroscope studies (Pumplin and Drachman, 1983). Myasthenic antibody-induced increased degradation of extra-junctional and junctional AChR in adult muscle in situ has also been demonstrated (Reiness et al.,1978; Stanley and Drachman, 1978; Merlie et al.,1979) indicating that this is not purely a feature of non-junctional receptors on aneurally cultured muscle cells.

Accelerated degradation of AChRs, in response to specific binding by myasthenic IgG in rat cultured muscle cells, has been shown

FIGURE 6 DIAGRAM SHOWING INTER- AND INTRA-

MOLECULAR CROSS-LINKING OF AChR

BY ANTI-AChR ANTIBODIES

INTER-MOLECULAR
CROSS-LINKING

INTRA-MOLECULAR
CROSS-LINKING
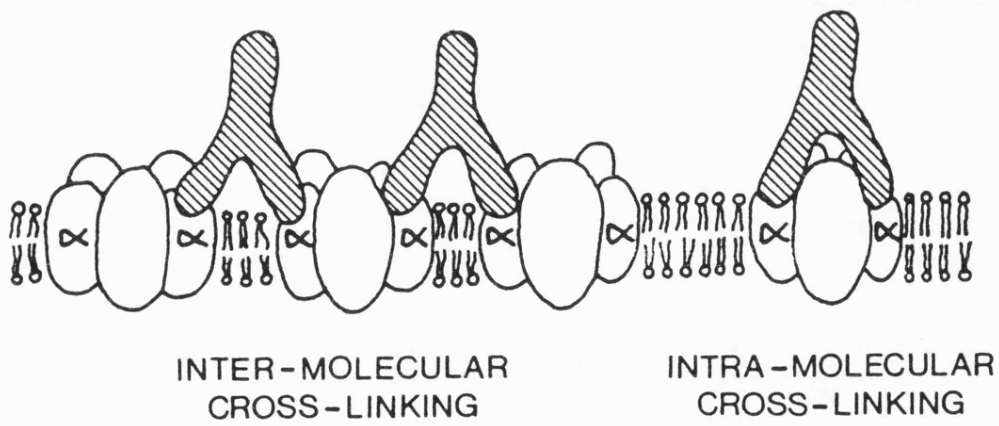
to occur independently of the rate of AChR synthesis, which remains unaffected (Drachman et al.,1978b). In contrast to these results, studies by Fulpius et al. (1980) indicated that myasthenic IgG increased receptor synthesis in chick muscle cell cultures. More recently, it has been reported that myasthenic IgG, after passive transfer to mice, causes an increase in AChR synthesis which partially compensates for the increase in AChR degradation (Wilson et al.,1983ab).

The importance of accelerated degradation as a primary mechanism whereby the number of endplate AChRs is reduced in MG is unclear. Studies by Hudgson et al. (1982) demonstrated that myasthenic serum from pre- and post-thymectomy patients, who retained similar anti-AChR antibody levels in spite of clinical improvement, showed similar effects on AChR degradation in mouse muscle cultures. The results suggested that the observed effects were correlated more with anti-AChR antibody titre than with clinical symptoms. Berman et al. (1981) also showed that sera from both paralysed and asymptomatic immunised mice could increase the degradation of AChR on cultured muscle to the same extent and, moreover, that junctional AChRs from paralysed and non-paralysed immunised mice have similar half-lives. Studies by Drachman et al. (1982) indicated that a better correlation with disease state in MG was given by determining a combined blocking and degradation effect of a range of myasthenic sera. Although both of these mechanisms could lead to a loss of functional AChRs at the neuromuscular junction in MG, it is doubtful that they alone would account for the gross morphological damage observed at myasthenic motor endplates (see "Introduction" p.23). It is possible that the combined effects of these mechanisms could be important in the early.

stages of MG and in EAMG when neuromuscular transmission is impaired

but in the absence of signs of post-synaptic membrane degeneration

(Engel et al.,1977a, 1979; Sahashi et al.,1978; Toyka et al.,1978).

Complement-mediated membrane destruction

The complement system consists of nine protein components

(numbered 1 to 9) which are present in an inactive form in serum (see

Alexander and Good, 1977; Lachmann and Peters, 1982 for reviews).

Several complement components are enzymes which, when activated, act

in sequence to amplify the effects of a small stimulus. There are two

recognised pathways of complement activation. The classical pathway

usually requires an antibody-antigen reaction for activation and is

initiated by the binding of C1q (one of the components of C1) to the

antibody-antigen complex (Figure 7). Activation of the alternative

pathway does not normally require such a reaction; the first component

in the pathway, C3, being activated by a variety of stimuli such as

zymosan, cobra venom factor and properdin. However, activation by

aggregates of IgA has been shown. When C9, the final component of the

complement system, is activated as a result of the classical or

alternative pathways, lesions that resemble small holes are produced in

susceptible membranes. The lesions permit loss of intracellular

constituents, entrance of extracellular ions and cellular swelling

until the cell disintegrates. The regulation of complement mechanisms

is complex. The complement system plays an important role in both

inflammatory and immune reactions including the opsonisation of target

cells (eg. bacteria) coated with antibody for clearance by phagocytes

(as a result of C3 binding) and destruction of such cells by

complement-mediated lysis (as a result of C5-C9 binding).
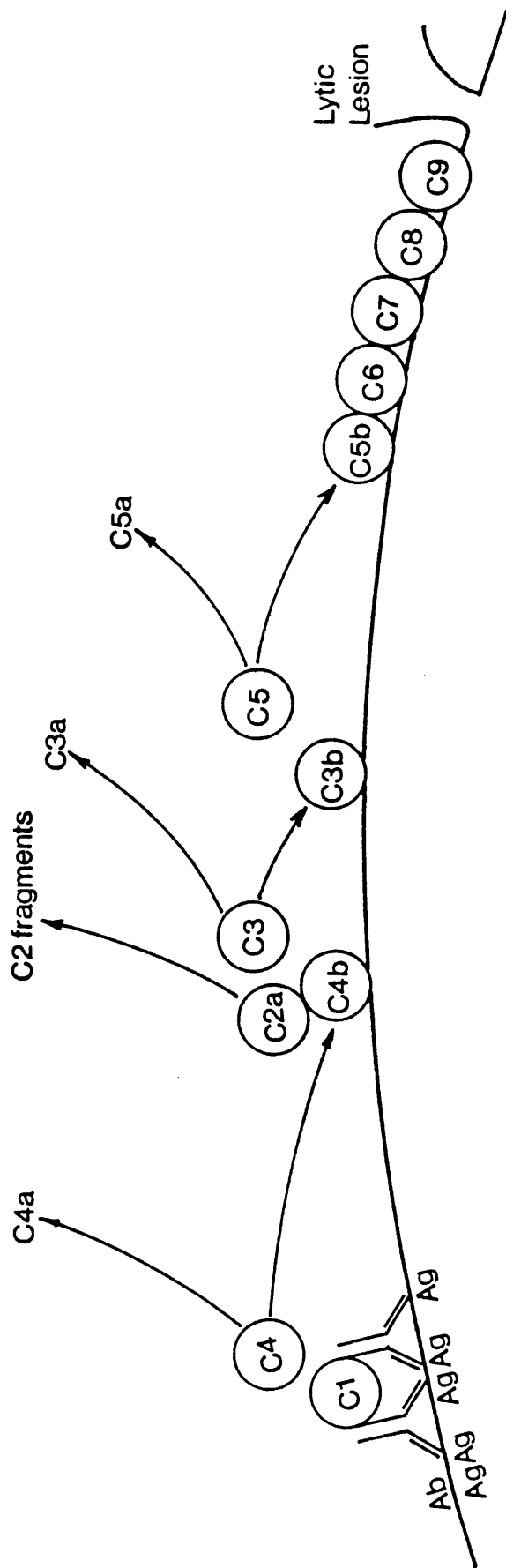
FIGURE 7   DIAGRAMMATIC REPRESENTATION OF THE REACTION SITES OF THE CLASSICAL
COMPLEMENT PATHWAY

A role for complement in the pathogenesis of MG was first suggested by the observation that serum complement levels fell during exacerbation of the disease, and rose to or above normal levels during periods of remission. Nastuk et al. (1960) proposed that complement was bound to antigen-antibody complexes on myasthenic muscle, thereby reducing levels in serum. There has been little direct evidence for the lytic action of myasthenic serum on muscle cells. The study of serum complement levels by Nastuk et al. (1960) followed an earlier observation that 2 out of 22 myasthenic serum samples showed cytolytic activity towards frog muscle in vitro (Nastuk et al.,1959). However, cytolytic effects were also demonstrated by 1 out of 9 normal control sera, although after a much longer lag time. Studies by Liveson et al. (1976) showed that 3 out of 17 myasthenic sera lysed mouse muscle cells in culture, as judged by light microscope examination. This effect was abolished by heating the sera (thus destroying complement activity) but was not restored by the addition of guinea-pig complement. There have occasionally been other reports in the literature referring to a search for direct evidence that myasthenic or EAMG sera can be cytolytic to muscle cells. Harvey et al. (1978b) looked for complement-mediated lysis of chick muscle cells in culture by myasthenic sera and anti-Torpedo AChR antiserum raised in rabbits. However, the occurrence of natural anti-chick antibodies in these sera and in normal control sera hampered any firm conclusions. Lennon (1978b) demonstrated that anti-AChR antiserum from rats with EAMG, in the presence of fresh rat sera as a source of complement, failed to lyse cultured rat muscle cells, despite the fact that IgG and C3 were demonstrated by immunofluorescence to be bound to the cells. Ultrastructural and light microscopic studies by Engel et al. (1977a),

using peroxidase labelled Stapylococcal protein-A and rabbit
anti-human C3, showed the presence of IgG and C3 on the muscle
endplates of myasthenic patients. A continuation of these studies
(Sahashi et al.,1980) showed, in addition, the presence of C9 on the
muscle endplates. IgG, C3 and C9 were located on disintegrating
junctional folds and on debris in the synaptic clefts providing the
first evidence for antibody-dependent, complement-mediated injury to
the post-synaptic membrane in MG. This occurrence would readily
account for the gross morphological damage to the post-synaptic
membrane observed in MG (Figure 4).

The involvement of C3 in EAMG passively transferred to
mice by injection of myasthenic immunoglobulins was investigated by
Toyka et al. (1977). If the animals were first treated with cobra
venom factor to deplete C3, the reduction in MEPP amplitudes and in
$\propto$-BGT binding to muscle endplates was less severe than in the control
animals. C3 depletion was similarly found by Howard and Sanders (1980)
to minimise the effects of passive transfer of EAMG by myasthenic
serum to rats. In contrast to C3, C5 was inferred not to be involved
in EAMG as genetically C5 deficient mice reacted similarly to normal
controls when injected with myasthenic immunoglobulins (Toyka.et
al.,1977).

Niemi et al. (1981) examined the involvement of
complement in EAMG induced by immunisation of rabbits with Torpedo
AChR. In 7 out of 10 rabbits, the levels of complement-fixing
antibodies rose immediately before, or coincident with, muscular
weakness suggesting an involvement in the pathogenic process. Serum
complement levels frequently, but not always, rose as EAMG developed,
which contrasts with the results obtained by Nastuk et al. (1960) for

the human disease. It was also demonstrated by Niemi et al. (1981) that serum samples taken from rabbits with EAMG at the time of onset of muscular weakness and then decomplemented, caused a reduction in MEPP amplitudes when applied to frog muscle fibres in vitro. This reduction was usually accentuated by the addition of guinea-pig complement, suggesting again a direct role for complement in impairing neurotransmission.

The production of EAMG in Lewis rats, by immunisation with purified electric fish AChR in the presence of additional adjuvants such as B. pertussis, produces a unique form of disease in the recipient animals. The first or 'acute' phase is characterised by an invasion of phagocytic cells into the muscle endplates (Engel et al.,1976, 1977b; Lennon et al.,1978). The second or 'chronic' phase of the disease more closely resembles human MG, demonstrating increased levels of anti-AChR antibodies (Lindstrom et al.,1976b), altered morphology of muscle endplates (Engel et al.,1976, 1977b) and decreased numbers of AChRs in the post-synaptic membranes (Lindstom et al.,1976b). The phagocytic invasion of muscle endplates is not generally seen in the 'chronic' phase of EAMG (Engel et al.,1976, 1977b) and has not been implicated as having a role in human MG as the presence of phagocytes at muscle endplates has only rarely been observed (Santa et al.,1972). The 'acute' phase of EAMG is not generally observed in animals immunised with AChR without additional adjuvants (Lindstrom, 1980; Vincent, 1980). Ultrastructural studies similar to those carried out with myasthenic endplates, have demonstrated the presence of IgG and C3 on both intact and degenerating folds in the synaptic cleft of rats with 'chronic' EAMG (Sahashi et al.,1978) but it is not clear why, in this phase of EAMG

or in human MG, the presence of C3 does not opsonise the muscle membrane for phagocytic attack. Injection with EAMG immunoglobulins from rats in the 'chronic' phase of disease produces the 'acute' phase of EAMG in recipient rats (Lindstrom et al.,1976c; Engel et al.,1979). IgG and C3 were detected on the junctional folds of the muscle endplates as early as 6 hours after passive transfer of immunogloblins (Engel et al.,1979). AChR-rich folds coated with IgG and C3 were shed into the synaptic cleft within 24 hours, implying that initial damage took place before the later phagocytic invasion of the motor endplates. It was subsequently shown by Lennon et al. (1978) in similar experiments, that rats depleted of C3 by treatment with cobra venom factor before passive transfer with EAMG immunoglobulins or immunisation with purified AChR, showed no clinical signs of EAMG and no evidence of phagocytic cells at the muscle end-plates. However, over 60% of the AChRs were shown to be bound by antibody indicating that these complexes alone were not pathogenic. Later studies showed that the passive transfer of 'acute' EAMG by monoclonal antibodies to AChR antigenic determinants, was dependent on C3 in rats and C4 in guinea-pigs (Lennon and Lambert, 1981). The latter observation would suggest that activation of complement via the classical pathway was necessary for the production of EAMG in these animals (see Figure 7). It was proposed by Lennon and co-workers (1978) that in 'acute' EAMG, anti-AChR antibody and C3 act synergistically to opsonise the membrane-bound AChR for attack by the inflammatory cells, the consequent loss of AChR at the end-plates being the primary factor in the impairment of muscular weakness. However, as there is little morphological evidence of a direct cellular attack at the end-plate in MG (Santa et al.,1972) the relevance of the 'acute' phase in EAMG is

questionable.

In contrast to the observed effects of complement depletion in EAMG, there is little information concerning complement deficiency in myasthenic patients, although one report (Riggs et al.,1980) has described a single patient with partial C2 deficiency. Another way in which circulating anti-AChR antibodies could be pathogenic is by the formation of circulating immune complexes. Subsequent deposition of such complexes in tissue can lead to complement activation and tissue damage. The presence of low levels of circulating immune complexes in MG has been suggested (Barkas et al.,1981) and C1q binding activity in some myasthenic sera has been shown (Casali et al.,1976; Barkas et al.,1981). Studies on EAMG in rabbits, using Torpedo AChR for immunisation, showed that pretreatment of the animals with immune complexes containing receptor and anti-receptor antibodies, suppressed the disease process. Antibody levels were reduced and synthesis of antibody was terminated (Barkas and Simpson, 1982). However, methods for the detection of immune complexes are largely non-specific, and further information concerning their role, if any, in MG must await the development of specific assay methods. The presence of nicotinic AChR on mononuclear phagocytes has been demonstrated (Whaley et al.,1981), the synthesis of the C2 component of complement by these monocytes being stimulated by cholinergic agonists. This study demonstrated that such synthesis was inhibited in the presence of myasthenic IgG or purified sheep anti-Torpedo AChR antibodies. The biological significance of AChR on monocytes remains to be determined and the relevance of the inhibition of C2 synthesis by these cells in MG is, as yet, unclear.

Lymphocytes and thymocytes in Myasthenia gravis

The mechanisms that trigger the sensitisation to AChR and subsequently maintain the levels of circulating anti-AChR antibodies are unknown. As in many other autoimmune diseases, there is interest in the possible role of defective immunoregulation in the development of the autoantibody and autoimmune reaction. The high incidence of thymoma or structural changes in the thymus in patients with MG (Castleman, 1966) has drawn attention to the role of the thymus in MG. The thymus is responsible for the development of immunocompetent T cells, which are involved in both regulatory and effector functions. The presence of thymic lymphocytes which express AChR (Fuchs et al.,1980) and thymic myoid cells, which have been shown to differentiate in culture to express AChR (Wekerle et al.,1975; Kao and Drachman, 1977b) suggests that these cells could provide the initiating stimulus for lymphocyte production of anti-AChR antibodies. The recognition of thymic AChR as 'non-self' could occur as a consequence of either an alteration of the antigenic determinants of the AChR or defective immunoregulatory mechanisms which would otherwise prevent or limit such autosensitisation. Thymic cells from patients with MG can spontaneously synthesise anti-AChR antibody in culture (Vincent et al.,1978) and can enhance anti-AChR antibody production by autologous peripheral blood lymphocytes (Newsom-Davis et al.,1981). Recent studies have suggested that this enhancement could be mediated by antigen-presenting cells rather than by AChR-specific T-helper cells in the thymus (Willcox et al.,1984).

It has been suggested that the relative proportions of T-suppressor and T-helper cells, which regulate antibody synthesis by B lymphocytes, could be of importance in MG. Studies employing

monoclonal antibodies and indirect immunofluorescence as phenotypic markers of T cells have suggested that there are decreased numbers of circulating suppressor cells in MG (Bahir et al.,1981; Skolnik et al.,1982). Decreased suppressor cell activity has also been inferred from the impaired ability of myasthenic T cells, compared to normal controls, to inhibit anti-AChR antibody production by myasthenic lymphocytes (Shinomiya and Yata, 1981). However, other studies on specific T cell populations or their functional activity in MG have proved inconsistent (see Lisak et al.,1985 for review).

There are several reports of increased _in vitro_ proliferative responses by myasthenic peripheral lymphocytes, to purified electric fish or human AChR (Abramsky et al.,1975; Richman et al.,1976; Conti-Tronconi et al.,1977; McQuillen et al.,1983; Hohlfeld et al.,1984). This was originally interpreted as suggesting that a cellular immune response, acting via delayed hypersensitivity or cytotoxic mechanisms, could be important in producing an immunopathologic reaction at the endplate in patients with MG (Lisak et al.,1985). Early morphological studies reported the presence of collections of lymphocytes, termed lymphorrhages, surrounding necrotic muscle fibres in approximately 20% of myasthenic muscle biopsies (Engel and McFarlin, 1966; Woolf, 1966). However, there is no evidence that cell mediated immune mechanisms are directly responsible for the end-plate changes seen in MG. It is probable that the enhanced proliferative response _in vitro_ is indicative of an increased number and/or activity of specific T helper cells which interact with antigen-presenting cells and B cells to produce anti-AChR antibody (Lisak et al.,1985).

Outline and aims of the project

Most of the evidence implicating antibody-dependent complement-mediated destruction of the muscle end-plate in MG is indirect (see "Introduction" p.37). A major reason for the lack of study in this area probably lies in the difficulty of identifying and quantifying muscle lysis. Using skeletal muscle cells in culture, such studies have hitherto depended upon visual assessment or the release of intracellular ^{51}Cr (see "Discussion" section 4.3). However, visual assessment would only detect gross damage, and ^{51}Cr release has been criticised for a lack of specificity (Cambridge and Stern, 1981) in that this radiolabel is taken up not only by the cultured myotubes, but also by the fibroblasts which inevitably accompany them. Cambridge and Stern (1981) have published a procedure for measuring myotube-specific cytotoxicity based upon the use of radiolabelled carnitine. This method, designed to assay the myotoxic nature of polymyositic lymphocytes, depends upon the selective uptake by cultured myotubes of tritium-labelled carnitine which is subsequently released following cell lysis. A major advantage of the procedure is the five-fold slower uptake of carnitine by fibroblasts (Cambridge and Stern, 1981). This method is therefore directly applicable to the study of myotube specific lysis mediated by antibodies.

Preliminary investigations in the laboratory, using the carnitine release method, indicated that myasthenic sera (absorbed with minced chicken liver) caused lysis of chick myotubes in culture whereas normal sera treated similarly did not (M. Bird - unpublished). For the present project, the effects of myasthenic sera on rat skeletal muscle cells in culture were investigated. A comprehensive analysis of the lytic activity of myasthenic sera was carried out and

the studies extended to include human muscle cells in culture. Human
cultures proved less amenable to myotoxicity studies and attempts were
made to improve their growth. The results of the work undertaken are
divided, for the sake of clarity, into two sections : the first
section describing studies on the cultured rat muscle system and the
second being concerned with human muscle cell cultures. The overall
aim of these studies is to clarify the role of humoral antibodies in
the pathogenesis of MG.

MATERIALS

Tissue culture

Dulbecco's modified Eagle's medium (DMEM) and 2.5% (w/v) crude trypsin solution in Hank's balanced salt solution, were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland.

Donor horse serum (HS) and foetal calf serum (FCS) were obtained from Gibco Ltd., Uxbridge, Middlesex, England; and were heat-inactivated (56°C, 30 min) before use. New batches of serum were tested for their ability to promote good myotube growth before routine use.

Penicillin (5000 U/ml) and streptomycin (5000 µg/ml) solution, L-glutamine (200 mM), kanamycin (10 mg/ml) and fungizone (amphotericin B - 250 µg/ml) were obtained from Gibco Ltd., Uxbridge, Middlesex, England.

Deoxyribonuclease, hormones and co-factors were supplied by Sigma Chemical Co., Kingston-upon-Thames, England. Glucose and sucrose (analytical grade) were obtained from B.D.H. Chemicals Ltd, Poole, England. All reagents supplied in a non-sterile form were sterilised before use by passage of stock solutions through sterile filters (0.2 µM) from Sera-Lab, Crawley Down, W. Sussex, England.

24-well tissue culture plates (15.5 mm diameter wells) and all other sterile plastic ware used for tissue culture were supplied by Nunc, Gibco Ltd., Uxbridge, Middlesex, England.

All tissue culture procedures were carried out in an Intermed Pathfinder laminar flow cabinet.

The compositions of the tissue culture growth media (GM) and the balanced salt solutions (BSS) used, were as follows:-

<u>Composition of growth media</u>

<u>GM1</u>   DMEM supplemented with:

Donor horse serum	10.0% (v/v)
Glucose	0.15% (w/v)
Glutamine	2.0 mM
Penicillin	100 U/ml
Streptomycin	100 µg/ml

<u>GM2</u>   DMEM supplemented with:

Donor horse serum	2.0% (v/v)
Glucose	0.15% (w/v)
Glutamine	2.0 mM
Penicillin	100 U/ml
Streptomycin	100 µg/ml

<u>GM3</u>   DMEM supplemented with:

Foetal calf serum	20.0% (v/v)
Glucose	0.15% (w/v)
Glutamine	2.0 mM
Penicillin	100 U/ml
Streptomycin	100 µg/ml

Growth media were stored at 4°C and used within 4 weeks.

Serum-free medium (SFM)

DMEM supplemented with:

Glucose	0.15% (w/v)
Glutamine	2.0 mM
Penicillin	100 U/ml
Streptomycin	100 μg/ml
Hydrocortisone	0.5 μM
Transferrin	5.0 μg/ml
Progesterone	20.0 nM
Putrescine	0.1 mM
Selenite Na	30.0 nM
Thyroxine	3.0 μg/ml
Biotin	1.0 μg/ml
Insulin	0.2 U/ml

Hormones and co-factors were made up as x200 stock solutions and stored at -20°C before use. SFM was stored at 4°C and used within 3 weeks.

Composition of balanced salt solutions

Puck's BSS

NaCl	8.0 g
KCl	0.4 g
Na_2HPO_4	0.024 g
KH_2PO_4	0.03 g
Glucose	6.0 g
Sucrose	15.0 g

Made up to 1 litre with double distilled H_2O containing 0.01M HEPES. Final pH 7.3.

Dulbecco's phosphate buffered saline

NaCl	8.0 g
KCl	0.2 g
$Na_2 HPO_4$	1.15 g
$KH_2 PO_4$	0.2 g
* $CaCl_2 .2H_2O$	0.132 g
* $MgCl_2 .6H_2O$	0.1 g

Made up to 1 litre with double distilled H_2O

pH 7.2

* Only included where BSS used for washing cell cultures

Source of muscle for tissue culture

Rat muscle was obtained from the thigh tissue of neonatal (1-2 days)
white CFHB rats.

Human foetal muscle was obtained from foetuses (approximate age 8-16
weeks) supplied by the Royal United Hospital, Bath following pregnancy
terminations by suction on a vacuum line. As soon as possible after
operation, the foetal limbs were placed in growth medium and stored at
$4^{\circ}C$ for up to 72 h.

Radiochemicals

Carrier-free Na[^{125}I] in dilute NaOH (100 mCi/ml) was from Amersham
International (Amersham, Bucks, U.K.) and was stored at room
temperature for not longer than 3 weeks after its activity reference
date.

D,L-[Me-^{3}H] carnitine hydrochloride (1 mCi/ml, specific activity 2
Ci/mmol) and L-[Me-^{3}H] carnitine hydrochloride (1 mCi/ml, specific

activity 87 Ci/mmol) were purchased from Amersham International,

Amersham, Bucks, U.K.

Ligands

∝-BGT from **Bungarus multicinctus** was purchased from Boehringer Corps,

Mannheim, W.Germany.

Benzoquinonium chloride was a generous gift from Stirling Winthrop

Inc., Renssalaer, New York, USA.

Decamethonium bromide was obtained from Sigma Chemical Co.,

Kingston-upon-Thames, England.

Serum

Serum samples from normal volunteers were obtained from colleagues in

the department. Myasthenic serum samples were obtained from several

hospitals in the U.K. as samples received for the routine assay of

anti-AChR antibodies. Plasma obtained as a result of plasmapheresis of

two myasthenic patients-MG patient 4, sample (ii), and MG patient 13,

sample (v) was also obtained from the Royal United Hospital, Bath and

Southmead Hospital, Bristol. Serum samples were frozen and stored at

$-20^{\circ}C$.

Goat anti-human IgG antiserum was prepared in the department by

repeated intramuscular injection of purified human IgG into a goat.

$[^{125}I]$-labelled goat anti-human light chain antibodies (1.5µg/ml,

specific activity 1µCi/µg) were a generous gift from Dr. Ahmed Jehanli.

Lyophilised guinea-pig complement serum was obtained from Miles

Laboratories, Elkhart, Indiana, USA (stated activity +ve haemolysis at

0.04 ml when the complement was at a 1:10 dilution) or from Flow

Laboratories, Irvine, Ayrshire, Scotland (stated activity $C'H_{50}$/ml =

250, where one $C'H_{50}$ is the amount of complement producing 50% lysis of sensitised sheep red blood cells under stated conditions). The serum was reconstituted in the diluent provided and used immediately. Rabbit anti-foetal calf AChR antiserum and rabbit anti-rat junctional AChR antiserum were the generous gift of Miss Susan Walsh.

Sheep anti-<u>Torpedo</u> AChR antiserum was the generous gift of Dr. Susan Wonnacott.

Normal fresh rat serum and rabbit serum were obtained from white CHFB rats or white New Zealand rabbits respectively.

Source of muscle AChR

Human adult muscle was supplied by the Royal United Hospital, Bath and the Bristol Royal Infirmary and was obtained from lower limb amputations resulting from severe vascular disorders or from road traffic accidents. Within 15 min of operation, calf muscle was crudely dissected free from fat, tendon and skin, transported in ice, solid carbon dioxide or liquid nitrogen and stored at $-80^{\circ}C$ for up to six months.

Chemicals

Standard Laboratory Reagents were from Sigma Chemical Co., Kingston-upon-Thames, England or from B.D.H. Chemicals Ltd., Poole, England.

Gel filtration reagents were supplied by Pharmacia Ltd., Hounslow, U.K.

Ion exchange resins, DEAE-cellulose filter discs and GFC glass fibre filter discs were from Whatman Lab. Sales Ltd., Maidstone, Kent, England.

Counting instruments

[^{125}I] was counted in an LKB Ultrogamma counter.

Tritium was counted in a tritium-specific channel in a Packard

Tri-Carb scintillation counter (model 3255) for a 2 minute counting

period. Correction for quenching was made using the channels ratio

method. The efficiency of tritium counting was 38%.

METHODS

1. Tissue Culture

1.1 Preparation of tissue culture plates

Tissue culture plates (24-well, see "Materials") were pre-coated with photo-polymerised collagen gel as described by Masurovsky and Peterson (1973).

Collagen was prepared from rat tails by the method of Ehrmann and Gey (1956). A rat's tail was washed in 70% (v/v) ethanol for 30 min, the skin was pulled off and the tendons dissected out with bone forceps. The tendons were washed in sterile distilled water and placed in 0.1% (v/v) sterile glacial acetic acid (100 ml) at $4^{O}C$ for 48h to extract the collagen. The resulting solution was centrifuged at 800g for 2h at $23^{O}C$. The supernatant was diluted with sterile distilled water to give a protein concentration of 1 mg/ml and stored at $4^{O}C$.

Collagen solution was mixed with riboflavin (Flavin mononucleotide, 0.05% w/v) at a ratio of 4:1 (v/v). Samples of this mixture (30 μl) were spread in the dark over the surface of each tissue culture well. The gel was photo-polymerised by exposure to fluorescent light in the laminar flow cabinet for 1h and dried overnight at $37^{O}C$. The plates were then washed for 2-3 min with distilled water and incubated with the appropriate growth medium for at least 30 min before the addition of cells.

1.2 Preparation of rat myotube cultures

Rat myotube cultures were prepared from the thigh muscle of newborn rats essentially according to the method of Yaffe (1973). Rats (1-2 days old) were decapitated and the bodies placed in 70% (v/v) ethanol for 10 min. Hind limbs were excised from the bodies and washed in Ca^{2+}- and Mg^{2+}-free balanced salt solution (Puck's B.S.S.- see

"Materials"). Dissection of limbs was then carried out under a Swift
binocular viewer at x10 magnification. After removal of the skin,
tissue surrounding the bone was stripped and placed in a 35mm plastic
dish containing Puck's B.S.S. (1 ml). The tissue was minced with a
pair of fine iridectomy scissors to produce a slurry and transferred
with washing to a sterile plastic tube (final volume 8.5 ml).
Deoxyribonuclease (1 mg/ml, 0.5 ml) was added to prevent cell clumping
induced by deoxyribonucleic acids released from dead cells. Crude
trypsin solution (2.5% w/v, 1 ml) was then added to give a final
concentration of 0.25% (w/v). The tissue suspension was incubated at
$37^{o}C$ for 60 min with intermittent mixing. The suspension was then
centrifuged at 400g for 5 min, the supernatant was discarded and
growth medium (5 ml of GM1, see "Materials") was added to the
resulting pellet. Any residual trypsin action was inhibited by the
serum component of the growth medium. Cells were released from the
tissue fragments by gentle trituration with a glass Pasteur pipette
(15-20 cycles). The mixture was left to stand for 3 min and the
supernatant, containing free cells, removed for filtering. Further
growth medium (5 ml) was added to the remaining tissue and the
trituration process was repeated. The combined cell suspensions were
filtered through two layers of nylon bolting cloth (53 μm aperture) to
remove remaining cell aggregates and tissue clumps. A sample of the
filtrate was added to an equal volume of trypan blue (0.2% w/v), to
visualise dead cells, before cell counting in a haemocytometer. Cells
were added to 24-well (15.5 mm) culture plates at a final density of
2.5×10^5 cells per well (1.3×10^5 cells/cm^2) in GM1 (1 ml). Cultures
were grown in an atmosphere of 10% CO_2/air (later cultures were grown
in a humidified atmosphere of 5% CO_2/air). Growth medium was replaced

by fresh, pre-warmed medium every three days. After 3-4 days growth, when fusion of myoblasts was judged morphologically to be complete (see "Results" section A 1.2), cytosine arabinoside (10 μM) or fluorodeoxyuridine (15 μg/ml) and uridine (35 μg/ml) were added to the cultures for 72h. These mitotic inhibitors reduced the growth of fibroblasts in the cultures. In routine preparations of rat myotube cultures, 6-8 rats were used each time so that dissection, dissociation and plating of cells could be achieved within 2-3 h.

1.3 Preparation of human myotube cultures

Human myotube cultures were established by using muscle tissue from the arms and legs of 8-16 week foetuses. Single cell suspensions were obtained as described for rat neonatal muscle. Variations in methodology and in growth media are described in the "Results" section.

1.4 Repassaging of human muscle cell cultures

For some experiments human muscle cells in culture were repassaged essentially as described by Konigsberg (1979). Single cell suspensions, prepared as described in section 1.3, were incubated in non-collagen coated tissue culture flasks (25ml) at a density of 1 x 10^6 cells/flask at 37°C in an atmosphere of 10% CO_2/air. The growth medium was GM3 (see "Materials"). After 3 days, the cells were washed with Puck's B.S.S. (see "Materials") and resuspended in Puck's B.S.S. containing 0.025% (v/v) trypsin (5 ml). The flasks were gently swirled at 23°C and when approximately half of the cells had become detached from the surface of the flask (3-5 min), the cell suspension was decanted and trypsin action terminated by the addition of growth

medium (10 ml). After centrifugation at 400g for 5 min, the resulting

pellet was resuspended in growth medium (5 ml) by gentle trituration.

The cells were filtered through 2 layers of nylon bolting cloth (53 μm

aperture) and seeded onto 24-well (15.5 mm) culture plates at a

density of 2.5 x 10^5 cells/well in the appropriate growth medium (1

ml) (see "Results" section B 1.5).

1.5 Preparation of fibroblast cultures

Fibroblast cultures were established from newborn rat skin or

from foetal human skin. Single cell suspensions were prepared as

described for rat muscle tissue and the cells were plated in 24-well

(15.5 mm) culture plates, pre-coated with rat tail collagen, at a

density of 1.25 x 10^5 cells per well (0.65 x 10^5 cells/cm^2).The growth

medium used was GM3 containing 20% foetal calf serum (see

"Materials"). After one day in culture, the plates were washed with

growth medium to remove dead cells and fresh medium added. This was

subsequently changed every 3 days. A confluent layer of fibroblasts

was obtained after 6-7 days growth.

1.6 Morphological examination of cultures

Myotube cultures were routinely examined under a Zeiss inverted

phase-contrast microscope before and after all assays so that any

culture wells where the myotubes had become detached from the surface

as a result of the washing procedures could be ignored.

2. Iodination of α-bungarotoxin

2.1 Preparation of [^{125}I]-labelled α-BGT

α-BGT was iodinated by the chloramine-T method (Hunter, 1967)

as modified by Urbaniak et al.(1973). Carrier-free Na[^{125}I] (100

mCi/ml) in dilute sodium hydroxide (20µl) was added to α-BGT (20 µg, 2.5

nmol) in 0.05M potassium phosphate buffer, pH 7.5 (30µl), followed by

chloramine-T (0.5% w/v) in 0.05M potassium phosphate buffer, pH 7.5

(10µl). The mixture was stirred for 1 min at 23°C and the reaction was

terminated by the addition of sodium metabisulphite (0.016% w/v) in

0.05M potassium phosphate buffer (0.75ml), pH 7.5, followed by carrier

potassium iodide (1%, w/v) in 0.05M potassium phosphate buffer (0.2

ml), pH 7.5. Labelled α-BGT was separated from free [^{125}I] by passage

through a column of Sephadex G-25 (25 x 1 cm) previously equilibrated

with 0.01M potassium phosphate buffer, pH 7.5, containing 1% (w/v)

BSA. Fractions (1 ml) were collected and a sample (5 µl) from each

fraction was counted for radioactivity. The peak fractions, containing

[^{125}I]α-BGT, were pooled and the specific radioactivity was

calculated, assuming 100% recovery of protein. [^{125}I]α-BGT was stored

at 4°C for not longer than 3 weeks.

A typical elution profile from the G-25 Sephadex column is

shown in Figure 8. Approximately 91% (range 83.8 - 94.9%) of the

[^{125}I] was incorporated into the protein (mean ± SD,n = 90.9 ± 5.0%, 6

preparations). The specific radioacivity was in the range 671-782

Ci/mmol with a mean value of 732.7 ± 44.2 (6) Ci/mmol (mean ± SD,n).

2.2 Biological activity of [^{125}I]-labelled α-BGT

The biological activity of [^{125}I]-labelled α-BGT

preparations was determined by measurement of the proportion of

radiolabelled α-BGT that could be bound by a large molar excess of

purified AChR from Torpedo marmorata (kind gift from Mr. Ian

Richards). Triplicate samples of Torpedo AChR (30 pmol, 100μl) in 0.01M

potassium phosphate buffer, pH 7.4 containing 0.1% (w/v) BSA, 1% (v/v)

Triton X-100 and 0.01% (w/v) sodium azide, were incubated with the

test radiolabelled α-BGT (0.5 pmol, 50 μl) for 90 min at 23°C in the

presence and absence of excess unlabelled α-BGT (3 μM). Bound [^{125}I]

α-BGT was separated from free toxin on DEAE-cellulose filter discs

essentially as described by Schmidt and Raftery (1973). Each sample

was applied to 2 DEAE-81 cellulose filter discs (diameter 24 mm)

premoistened with the above assay buffer and left to stand for 2 min.

Samples were then vacuum filtered in a Millipore filter unit. The

filters were washed (3 x 1 ml) with the above buffer and counted for

radioactivity. Biological activity was calculated as follows:-

Biological activity = $\dfrac{\text{Specific cpm bound (sample)} \times 100}{\text{Total cpm added}}$

(%)

The biological activity of [^{125}I]α-BGT was in the range 58.5 - 71.9%

with a mean value of 65.3 $\pm$ 4.4 % (6), (mean $\pm$ SD,n).

FIGURE 8

GEL FILTRATION OF [^{125}I]α-BGT ON SEPHADEX G-25
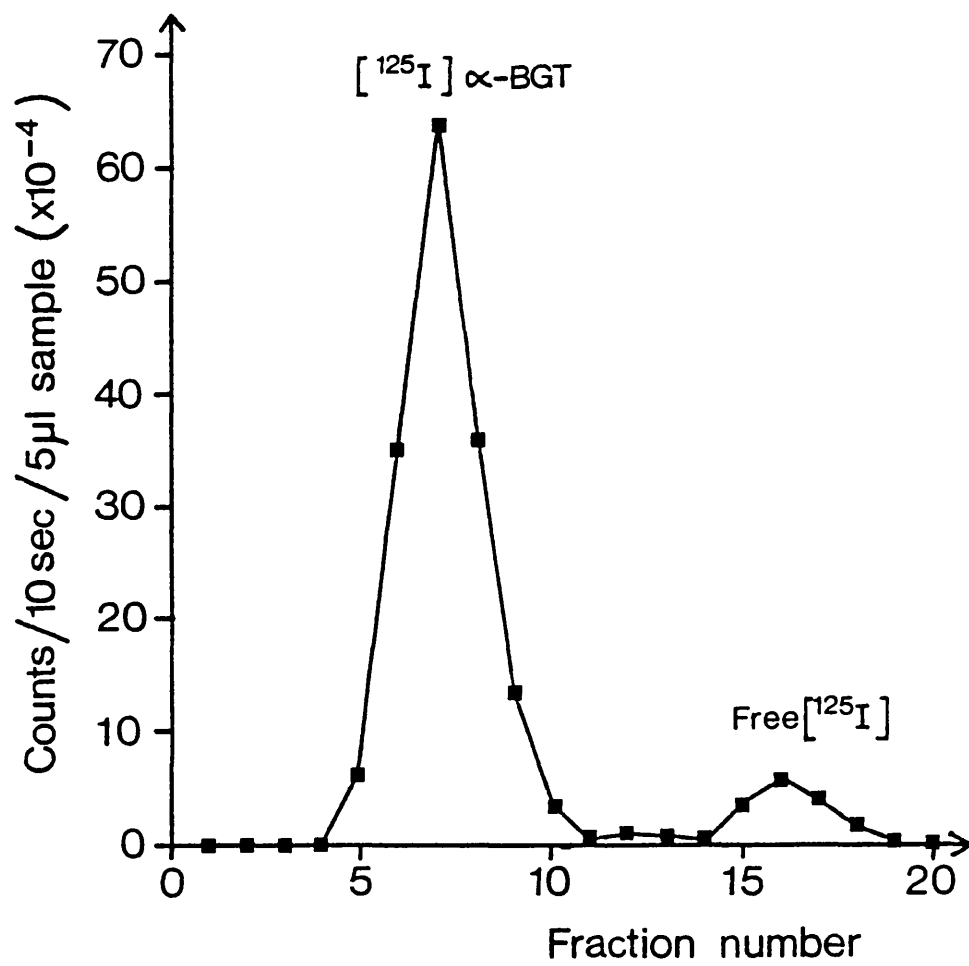
3. [^{125}I]α-BGT binding to myotube cultures

Replicate myotube cultures were washed twice with growth

medium and incubated for 30 min at 23°C in fresh medium with or

without 1mM decamethonium bromide (0.275 ml). An appropriate dilution

of [^{125}I]α-BGT (see "Results" section) was added to the cultures (25µl)

which were then incubated for 60 min at 23°C. Each dilution of toxin

was added to 8 replicate culture wells, 4 of which had been

preincubated with 1mM decamethonium bromide. At the end of the

incubation time, the cultures were washed 3 times with growth medium

(0.75 ml, 2-3 min) followed by 2 brief washes with phosphate buffered

saline* (0.75 ml) (see "Materials") before solubilisation as described

in section 9. Radioactivity was measured in a LKB Ultrogamma counter

and specific [^{125}I]α-BGT binding to the cultures was calculated from:-

$$\frac{\text{total cpm} \quad - \quad \text{non-specific cpm}}{\text{specific radioactivity}}$$

total cpm (-DBr) — non-specific cpm (+DBr)

specific radioactivity

(cpm/pmol [^{125}I]α-BGT)

* Repeated washings of the cultures with balanced salt solutions were

found to detach myotubes from the collagen surface. Cultures were

therefore routinely washed with growth medium, final washes with

phosphate buffered saline being included if the protein content of

cultures was to be estimated.

4. Determination of creatine phosphokinase activity

Creatine phosphokinase (CPK : EC 2.7.3.2) activity was

determined by using a CPK assay kit obtained from Sigma Chemical Co.,

Kingston-upon-Thames, Surrey, England (procedure no. 45-UV). Culture

wells (6-12 wells) were washed twice with growth medium (0.75ml, 2-3

min) and twice with phosphate buffered saline (0.75ml, 2-3 min) before

scraping the cells off the bottom of the culture wells into a small

volume of 20mM potassium phosphate buffer, pH 7.4 (0.2 - 0.5ml) by

using a teflon-coated spatula. The resulting suspension was

homogenised in a Potter glass homogeniser and stored at -20°C until

use.

The CPK assay method depends on the reaction scheme:-

$$ADP + \text{phosphocreatine} \xrightarrow{\quad CPK \quad} ATP + \text{creatine}$$

$$ATP + \text{glucose} \xrightarrow{\quad \text{hexokinase} \quad} ADP + \text{glucose-6-phosphate}$$

$$\text{glucose-6-phosphate} + NADP \xrightarrow{\quad \text{* G6P DH} \quad} \text{6-phosphogluconate} + NADPH$$
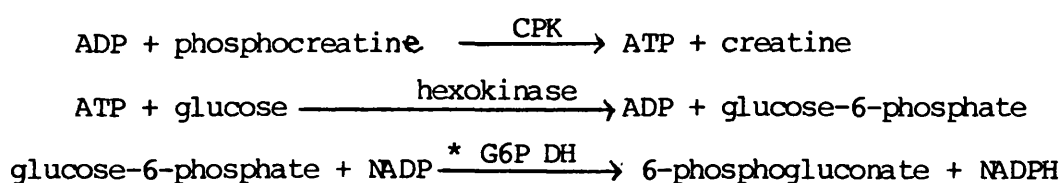
* G6P DH is glucose-6-phosphate dehydrogenase

When NADP is reduced to NADPH the A_{340} sharply increases and is

proportional to the CPK activity.

5. Determination of acetylcholinesterase activity

Acetylcholinesterase (AChE : EC 3.1.1.7) activity was

determined according to the method of Ellman$_\wedge$(1961) as modified by
et al.

Wilson et al. (1973). Culture wells (12-24 wells) were washed twice

with growth medium (0.75ml, 2-3 min) and twice with phosphate buffered

saline (0.75ml, 2-3 min) before scraping the cells off the bottom of

the culture wells into a small volume of 20mM potassium phosphate

buffer, pH 7.4 (0.2 - 0.5ml), using a teflon-coated spatula. The

resulting suspension was homogenised in a Potter glass homogeniser and

stored at -20°C until use. The homogenate was diluted (1:1) with 20mM

potassium phosphate buffer, pH 7.4, containing 0.5% (w/v) Triton X-100

and left to stand for 30 min at 4°C. After centrifugation at 10,000g

for 15 min, the supernatant, containing the solubilised enzyme, was

assayed for AChE activity.

Non-specific cholinesterase activity was determined in the

presence of the specific AChE inhibitor BW 284C51, and deducted from

total cholinesterase activity to give a specific value for AChE.

6. Protein measurements

Protein was determined by the method of Lowry et al. (1951)

using BSA diluted in the appropriate buffer system for the sample as a

standard. The protein content of cell cultures was determined from

solubilised extracts (section 9). The protein content of culture wells

with no added cells was taken as a blank value and deducted from all

test values.

7. Determination of uptake of [Me-^{3}H]-carnitine by cultures

DL-[Methyl-^{3}H] carnitine hydrochloride (specific activity 2

Ci/mmol, radioactive concentration 1 mCi/ml) was diluted under sterile

conditions in growth medium to a final concentration of 10 μM. Further

dilutions were made as appropriate (see "Results" section) before

addition of each concentration (0.75 ml) to 4 replicate culture wells.

After incubation for 18h at 37°C in an atmosphere of 10% CO_2/air, the

cultures were washed 3 times with growth medium (0.75 ml, 2-3 min),

immediately solubilised in 0.1M sodium hydroxide (see section 9) and

mixed with scintillation fluid (30% Triton X-100 in toluene, 5 g/l

PPO, 5 ml). The radioactivity of each culture well was determined by

counting in a Packard liquid scintillation counter.

Towards the end of this project, the L-isomer, L-[Methyl-^{3}H]

carnitine hydrochloride, of high specific activity (87 Ci/mmol,

radioactive concentration 1 mCi/ml) was used (after dilution in growth

medium to a final concentration of 0.23 μM).

8. Determination of myotoxicity by serum

Myotube cultures were loaded with [Me-^{3}H]-carnitine as in the

previous section. After washing, fresh medium was added to each

culture well followed by addition of test serum in appropriate amounts

(see "Results" section). The cultures were then incubated at 37°C in

an atmosphere of 10% CO_2/air, for the appropriate length of time,

before washing 3 times with growth medium (0.75 ml, 2-3 min) and

solubilising and counting as before. Control cultures to which no

additions of test serum were made (equivalent volumes of growth medium

alone added) were run simultaneously. Myotoxicity was expressed as the

percentage loss of radioactivity compared with controls according to

the formula:-

$$\frac{CRC - CRT}{CRC} \quad x \quad 100\%$$

where CRC = counts retained in control cultures to which no

additions of test serum were made

CRT = counts retained in test cultures

9. Solubilisation of cultures

Cultures were solubilised by incubation with 0.1M sodium hydroxide (0.3 ml) for 30 min at 23°C. The contents of each culture well were then transferred to appropriate tubes, (LP3 tubes for [^{125}I] counting or scintillation vials for [^{3}H] counting). Each culture well was then further incubated with 0.1M sodium hydroxide (0.2 ml) for 15 min at 23°C and the washings transferred to the appropriate tubes for counting.

10. Binding of myasthenic serum components to myotube cultures

The binding of myasthenic serum components to myotube cultures was assessed by 2 different methods.

10.1 Immunoradiometric assay to detect immunoglobulin binding

Replicate myotube cultures were washed twice with growth medium (0.75 ml, 2-3 min) and fresh medium was added (0.25 ml). Normal or myasthenic serum (25µl) was added to each of four culture wells and incubated for 1 h at 37°C. Cultures were then washed 3 times with growth medium (0.75 ml, 2-3 min) and fresh medium was added (0.25 ml). [^{125}I]-goat anti-human light chain (GAHL) antibodies (10µl) were added to each culture well and the cultures were incubated for 2 h at 23°C, washed 3 times with growth medium (0.75 ml, 2-3 min) and solubilised in 0.1M sodium hydroxide (0.5 ml) as described in the "Methods" section 9, before counting in a LKB Ultrogamma counter. Cultures with no additions of test sera were run simultaneously to determine non-specific binding of [^{125}I]-GAHL to the cultures.

10.2 Loss of [^{125}I]α-BGT binding

Replicate myotube cultures were washed twice with growth medium (0.75 ml, 2-3 min) and fresh medium containing myasthenic or normal serum (various amounts - see "Results" section) was added to each of 8 culture wells. The cultures were incubated for 1 h at 37°C, washed 3 times with growth medium (0.75 ml, 2-3 min) and assayed for [^{125}I]α-BGT binding as described in section 3. Control cultures, with no additions of test serum, were run simultaneously. Results are expressed as the percentage [^{125}I]α-BGT binding sites remaining relative to the control.

11. Absorption of sera with rat liver homogenate

For some experiments, human serum was absorbed with rat liver homogenate in an attempt to remove any non tissue-specific components present which could bind to rat muscle cultures. A rat liver was washed in phosphate buffered saline, diced and homogenised in an equal volume of phosphate buffered saline. After centrifugation at 10,000g for 15 min, samples of the pellet (2.5 ml) were stirred with serum (5 ml) for 2h at 23°C. The serum was recovered by centrifugation at 10,000g for 30 min.

12. Preparation of human AChR

12.1 Preparation of crude muscle extract from human adult muscle

Detergent extracts of muscle were prepared from human adult amputated legs according to the method of Stephenson et al. (1981). Muscle (200g) was coarsely chopped and homogenised in a Waring blender

at maximum speed for 1 min in 4 volumes of Buffer A comprising 20mM

potassium phosphate buffer, pH 7.4, 1mM EDTA, 0.1mM PMSF, 0.1mM

benzethonium chloride, 2mM benzamidine hydrochloride, bacitracin (500

μg/ml) and 0.01% (w/v) sodium azide. The homogenate was centrifuged

(20,000g, 60 min, 4°C) and the resulting supernatant was decanted and

discarded. The pellet was resuspended and homogenised as before in

Buffer A (1 volume) containing additionally 2.5% (v/v) Triton X-100

(extraction buffer), stirred for 3 h at 23°C and centrifuged

(100,000g, 60 min, 4°C). The resulting supernatant was filtered

through glass wool. This crude muscle extract was used in the

radioimmunoassay (see section 13) for anti-(AChR) antibodies in human

sera and IgG. It was also used for coupling to Sepharose 4B-∝-BGT

complexes (see section 14.2).

12.2 Determination of AChR content of muscle extracts

The AChR content of crude detergent extracts of muscle was

determined by a method adapted from that of Meunier et al. (1972).

Triplicate samples of the extract (100μl) were incubated with 10 nM

[^{125}I]∝-BGT in extraction buffer (50 μl) for 45 min at 23°C. Specific

binding of [^{125}I]∝-BGT was blocked by parallel incubations containing

1 mM benzoquinonium chloride. Saturated ammonium sulphate was added to

give a final concentration of 40%. After further incubation for 16 h

at 4°C, the precipitates were collected on Whatman GFC glass fibre

filter discs, washed 3 times with 40% saturated ammonium sulphate (1

ml) by vacuum filtration on a Millipore filter unit and counted for

radioactivity. Serial 2-fold dilutions of the receptor extract were

assayed to ensure sufficient excess of [^{125}I]∝-BGT. The AChR content

of the crude extract was calculated in terms of [^{125}I]∝-BGT binding

sites as follows:-

$$\text{AChR (pmol/ml extract)} = \underline{\text{specific cpm sample}} \quad x \quad \text{dilution}$$

$$\text{specific radioactivity} \quad \text{factor}$$

$$\text{(cpm/pmol } [^{125}I]\alpha\text{-BGT)}$$

Detergent extraction of human muscle gave yields of 0.45 - 1.3 pmol $[^{125}I]\alpha$-BGT binding sites / ml extract equivalent to 0.61 - 1.54 pmol per gram of frozen muscle tissue (1.14 $\pm$ 0.48, 4 preparations, mean $\pm$ SD).

13. Radioimmunoassay of anti-(AChR) antibodies

A radioimmunoassay procedure similar to that previously described (Lindstrom et al. 1976a; Carter et al. 1981) was used to determine the anti-(AChR) antibody content of fractionated serum samples. This procedure detects anti-(AChR) antibodies not directed at the α-BGT binding site.

Crude human muscle extract (0.05 pmol specific α-BGT binding sites) was labelled by incubation with excess $[^{125}I]\alpha$-BGT (0.5 pmol) for 45 min at 23°C. Specific binding of $[^{125}I]\alpha$-BGT was blocked in parallel incubations with 1 mM benzoquinonium chloride. The resulting solutions were incubated in triplicate with serum or serum fractions (5 µl) appropriately diluted with normal human serum or IgG for 2 h at 23°C (or 16 h at 4°C). The labelled AChR-Ab complex was precipitated by the addition of goat anti-(human IgG) antiserum (35 µl, the volume was chosen to ensure maximum precipitation of complex) and incubation

for 2 h at 23°C (or 16 h at 4°C). The resulting precipitates were

collected by centrifugation (3000g, 10 min) at 4°C and the pellets

were washed twice with 10 mM potassium phosphate buffer, pH 7.4

containing 0.15 M NaCl, 1% (v/v) Triton X-100 and 0.1% (w/v) sodium

azide (RIA buffer) by alternate suspension and centrifugation and

counted for radioactivity. For each sample, maximal formation of

[125I]α-BGT-AChR-Ab complex was ensured by repetition of the assay

using serial 2-fold dilutions of sample in order to obtain a linear

relationship between the volume of undiluted sample and precipitated

radioactivity.

The anti-(AChR) antibody titre of serum or serum fractions

is expressed as moles of specific α-BGT binding sites

precipitatedper litre of sample as follows:-

$$\text{titre} = \frac{\text{specific cpm sample}}{\text{specific radioactivity}} \times \frac{\text{dilution}}{\text{factor}}$$
$$(\text{cpm/pmol } [^{125}I]\alpha\text{-BGT})$$

14. Depletion of anti-(AChR) antibodies from myasthenic serum

14.1 Preparation of the α-BGT affinity column

α-BGT (2mg) was covalently linked to cyanogen

bromide-activated Sepharose 4B (March et al., 1974) according to the

method of Lindstrom et al. (1981). Sepharose 4B (50 ml packed beads)

was washed with 0.1 M sodium chloride (1L), followed by distilled

water (500 ml). The beads were resuspended in cold 1M sodium carbonate

solution (200 ml) and cooled to 4°C. Cyanogen bromide (2.5 g)

dissolved in acetonitrile (2 ml) was added to the Sepharose 4B

solution and stirred for 2 min at 4°C. The mixture was rapidly

filtered and washed with ice cold distilled water (500 ml). ∝-BGT (2

mg) was dissolved in 0.2 M sodium hydrogen carbonate, pH 9.4 (100 ml)

and added to the activated Sepharose beads. After stirring overnight

at 4°C, the affinity beads were collected by filtration and the eluant

was retained for protein estimation. The beads were then washed with

distilled water (400 ml), resuspended in 2 M glycine, pH 9.0 (200 ml)

and stirred overnight at 4°C to block unreacted groups. The affinity

beads were again filtered and washed sequentially with 0.1 M acetate

buffer, pH 4.0, containing 1 M sodium chloride (150 ml) and 0.1 M

borate buffer, pH 8.0, containing 1 M sodium chloride (150 ml). This

washing process was repeated 3 times, after which the beads were

equilibrated in 10 mM potassium phosphate buffer, pH 7.4 before

coupling to human AChR. The final density of ∝-BGT covalently linked

to activated Sepharose 4B was calculated to be 0.04 mg/ml beads.

14.2 Coupling of AChR to the ∝-BGT-affinity column

Human AChR was coupled to the ∝-BGT affinity column by using

an adaptation of the method of Lang et al. (1982). AChR, prepared as a

crude detergent extract of human leg muscle (see section 12) was

applied as a batch (49.5 - 210 pmol [^{125}I]∝-BGT binding sites at a

concentration of 0.45 - 1.3 pmol/ml) to ∝-BGT-Sepharose 4B (25 ml

packed volume) and stirred gently overnight at 4°C. The affinity beads

were then washed extensively on a scintered glass funnel with

phosphate buffered saline, pH 7.2, containing an additional 0.5 M

sodium chloride (500 ml) followed by phosphate buffered saline alone

(500 ml). The beads were then packed in a column (1.7 x 30 cm) and

equilibrated with phosphate buffered saline, pH 7.2, before

application of myasthenic sera. A control column, consisting of

ɑ-BGT-sepharose 4B to which no AChR had been coupled, was similarly

prepared.

14.3 Depletion of anti-AChR antibodies from myasthenic serum

Myasthenic serum (2 ml, containing 84 pmol of anti-AChR

antibodies) was applied to the AChR-ɑ-BGT-Sepharose 4B column or to

the control ɑ-BGT-sepharose 4B column at a rate of 30 ml/h, and

allowed to circulate for 2 h at 23°C. The columns were then washed

with phosphate buffered saline, pH 7.2, and fractions (3 ml)

collected. Fractions showing absorbance at 280 nM were pooled and

concentrated on an Amicon B15 concentrator to the original volume (2

ml) of applied serum. The anti-AChR antibody content of the serum

samples, before and after passage through the affinity columns, was

determined as described in section 13. Anti-AChR antibody depleted

serum samples were stored at -20°C until use.

14.4 Purification of anti-AChR antibody

After application of myasthenic serum to the AChR-

ɑ-BGT-Sepharose 4B column and collection of the effluent, the column

was washed with phosphate buffered saline, pH 7.2, containing

additionally 0.5 M sodium chloride (100 ml) followed by phosphate

buffered saline, pH 7.2, alone (100 ml). The adsorbed antibody was

eluted with 2 M potassium iodide in phosphate buffered saline, pH 7.2,

and fractions (1 ml) collected. Fractions showing absorbance at 280 nM

were pooled and immediately dialysed against phosphate buffered

saline, pH 7.2, (4 x 4L) for 48 h. The non-dialysable material was

concentrated on an Amicon B15 concentrator to a final volume of 1 ml and the anti-AChR antibody content was determined as described in section 13. Purified antibody was stored at $-20^{\circ}C$ until use.

15. Preparation of IgG

15.1 Purification of IgG

IgG was prepared from normal and myasthenic serum by the method of Stevenson and Dorrington (1970). A solution of saturated ammonium sulphate (6 ml, saturated in 0.2 M TRIS-HCl, pH 8.0) was added dropwise to normal or myasthenic serum (10 ml) with stirring at $23^{\circ}C$. The solution was stirred for a further 30 min at $23^{\circ}C$ and the precipitate was sedimented by centrifugation (500 g, 15 min). The precipitate was dissolved in 30 mM potassium phosphate buffer, pH 7.3 (10 ml) and dialysed overnight at $4^{\circ}C$ against the same buffer (4 L). The non-dialysable material was applied to a column (2.9 x 13.2 cm) of DE-52 cellulose pre-equilibrated with 30 mM potassium phosphate buffer, pH 7.3. The column was eluted with the same buffer and fractions (2 ml) collected. Fractions showing absorbance at 280 nM were pooled and concentrated on an Amicon B15 concentrator to the original volume of serum (10 ml). The column was washed with 30 mM potassium phosphate buffer, pH 7.3, containing 1 M sodium chloride before re-use. The anti-AChR antibody content of purified IgG was determined as described in section 13. Purified IgG was stored at $-20^{\circ}C$ until use.

15.2 Depletion of IgG subclass 3 from IgG

Removal of IgG subclass 3 from normal and myasthenic IgG was performed by affinity chromatography on protein A-Sepharose 4B, based

on the method described by Whiting et al. (1983). Purified IgG (5 ml

containing 8.75 - 20 mg) was applied to a column of reconstituted

protein A-sepharose 4B (5 ml), pre-equilibrated with 30 mM potassium

phosphate buffer, pH 7.3. The column was eluted with the same buffer

and fractions (1 ml) collected. Fractions showing absorbance at 280 nM

were pooled and concentrated on an Amicon B15 concentrator to a final

volume of 1 ml. This fraction of IgG consisted of IgG subclass 3. The

other IgG subclasses (IgG subclasses 1, 2 and 4) were eluted from the

column with 0.1 M citrate, 0.1 M sodium phosphate buffer, pH 3.0 and

fractions (1 ml) collected. Fractions absorbing at 280 nM were pooled

and dialysed for 24 h at 4 $^{\circ}$C against 30 mM potassium phosphate

buffer, pH 7.3 (2 x 4L). The non-dialysable material was concentrated

on an Amicon B15 concentrator to a final volume of 5 ml. The protein

A-Sepharose 4B column was washed with 30 mM potassium phosphate

buffer, pH 7.3, containing 1 M sodium chloride (100 ml) before re-use.

The anti-AChR antibody content of the separated fractions was

determined as described in section 13. The separated fractions were

stored at -20°C until use.

15.3 Measurement of IgG

IgG was measured in serum or serum fractions by a radial

immunodiffusion assay using antiserum to human IgG (Immunostics RID

plate, Seward Laboratories, London).

RESULTS

A. RAT SKELETAL MUSCLE CELLS IN CULTURE

1. Growth and characteristics

1.1 Preparation of cultures

Single cell suspensions were prepared from neonatal rat

muscle as described in the "Methods" section 1.2. In initial

experiments, dissociation of tissue was achieved by incubation with

0.1% (w/v) trypsin for 30 min at 37°C. The yield of cells obtained was

$1.63 \pm 0.22 \times 10^6$ per limb (mean $\pm$ SD, 7 preparations). Use of 0.25%

(w/v) trypsin for 60 min at 37°C improved dissociation of tissue, as

judged by ease of subsequent trituration, and routinely gave yields of

$2.5 - 3.0 \times 10^6$ cells per limb. Cell viability, as judged by exclusion

of trypan blue dye, was greater than 95%.

The optimal plating density for cultures was 2.5×10^5 cells per

15.5mm culture well (Table 1). Higher plating densities resulted in

earlier myoblast fusion but similar myotube densities and $[^{125}I]\alpha$-BGT

binding, when estimated on the seventh day of culture. Lower plating

densities resulted in a sparser population of myotubes and lower

levels of $[^{125}I]\alpha$-BGT binding; the total protein content of these

cultures was not lowered to the same extent as $[^{125}I]\alpha$-BGT binding

(Table 1) and this may be a reflection of the presence of fibroblasts.

1.2 Morphology of cultures

After several hours in culture, most of the cells were

attached to the collagen substratum. The mononucleated cells were

initially round in shape. By 1-2 days in culture, spindle-shaped,

TABLE 1

EFFECTS OF INITIAL CELL DENSITY ON SUBSEQUENT RAT MYOTUBE GROWTH

Replicate muscle cultures were initiated at increasing cell densities and $[^{125}I]$ α-BGT binding (10nM) determined on the 7th day in culture, as described in the "Methods" section 3. Each result is the mean $\pm$ SD of 4 culture wells.

Initial cell density $(x10^{-5}/\text{well})$	$[^{125}I]$ α-BGT binding			
	Total (cpm/well)	Non-specific (cpm/well)	Specific (fmol/well)	Protein (μg/well)
0.50	4263 $\pm$ 473	341 $\pm$ 37	5.2	55 $\pm$ 4
1.25	13806 $\pm$ 978	607 $\pm$ 57	17.5	83 $\pm$ 5
2.50	31200 $\pm$ 854	1560 $\pm$ 163	39.3	92 $\pm$ 5
3.75	32644 $\pm$ 957	3984 $\pm$ 163	38.0	102 $\pm$ 9

refractile cells could be distinguished from flat, multi-polar,

non-refractile cells (Figure 9 A). The morphology of the former is

typical of that attributed to myoblasts, while that of the latter

typical of fibroblasts. After 2-3 days in culture, the mononucleated

cells had increased in number, the myoblasts becoming aligned in an

end-to-end manner. After this stage, the cultures entered a period of

rapid cell fusion resulting in the formation of a network of rapidly

growing multinucleated fibres termed myotubes (Figure 9 B). After 6-7

days in culture, myotubes covered the surface of the culture well and

were often highly branched (Figures 9 C and 10). Many of the myotubes

could be seen to contract spontaneously in a steady rhythmical

fashion. These contractions often led to detachment of the myotubes

from the collagen substratum by the ninth day in culture.

1.3 Inhibition of fibroblast growth

After 3-4 days growth, when fusion of myoblasts was judged

morphologically to be complete, mitotic inhibitors were added to

restrict fibroblast growth. The effect of fluorodeoxyuridine (15 μg/ml)

and uridine (35 μg/ml) (FDU) or of cytosine arabinoside (10 μM) (Ara C)

on myotube growth and [^{125}I]α-BGT binding was observed (Table 2). When

no inhibitor was added, the myotube cultures were completely overgrown

with fibroblasts, and this was reflected in the higher protein

content. Addition of either inhibitor checked fibroblast overgrowth

but specific [^{125}I]α-BGT binding was reduced by 22% (+ FDU) and 17% (+

Ara C) respectively. This probably resulted from myoblast fusions not

being complete on addition of inhibitor. Ara C appeared to be the most

effective inhibitor of fibroblast growth, as judged by morphological

examination, and was subsequently used as the inhibitor of choice.

FIGURE 9

LIGHT PHOTOMICROGRAPHS OF RAT MUSCLE CELLS IN CULTURE

A) 1 day after plating the single cell

suspension at 2.5 x 10^5 cells/culture well

(x 80 magnification)

B) The appearance of a 3 day culture after

the onset of myoblast fusion

(x 40 magnification)

C) 7 days in culture showing the dense network

of branched myotubes

(x 40 magnification)
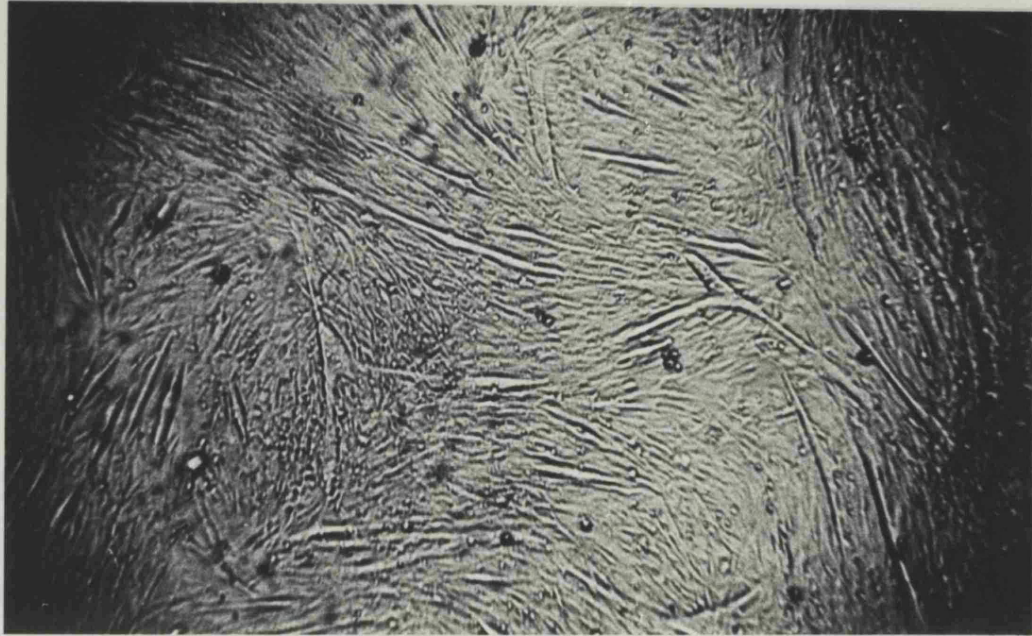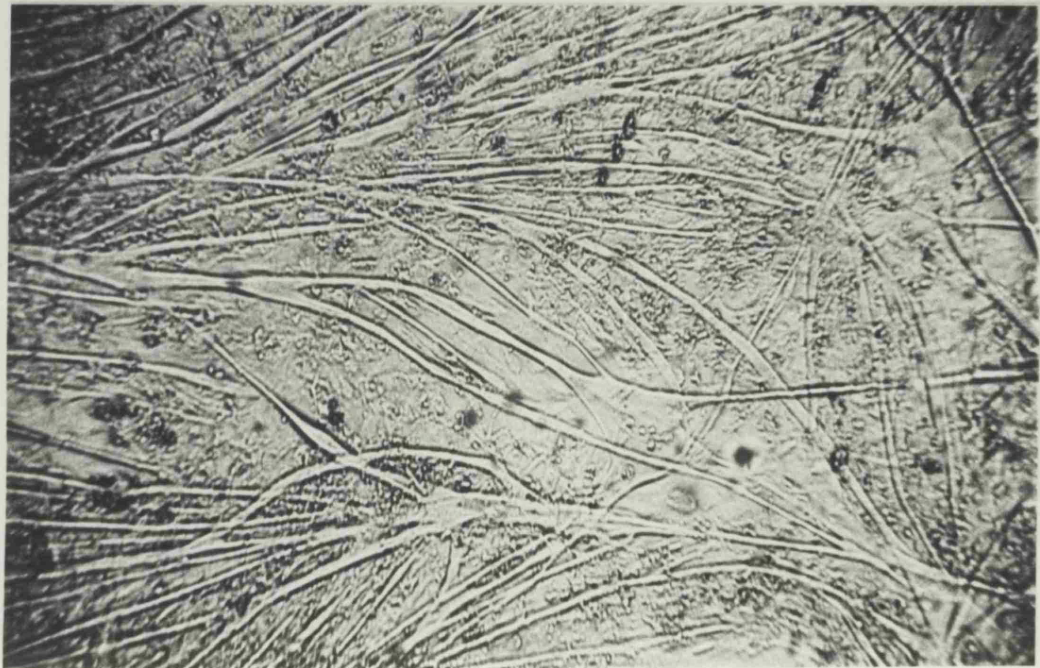
FIGURE 10

PHASE CONTRAST PHOTOMICROGRAPHS OF RAT MYOTUBE CULTURES

The appearance of rat muscle cell cultures, 7 days
after plating the single cell suspension at
2.5×10^5 cells/culture well (x 80 magnification)

A
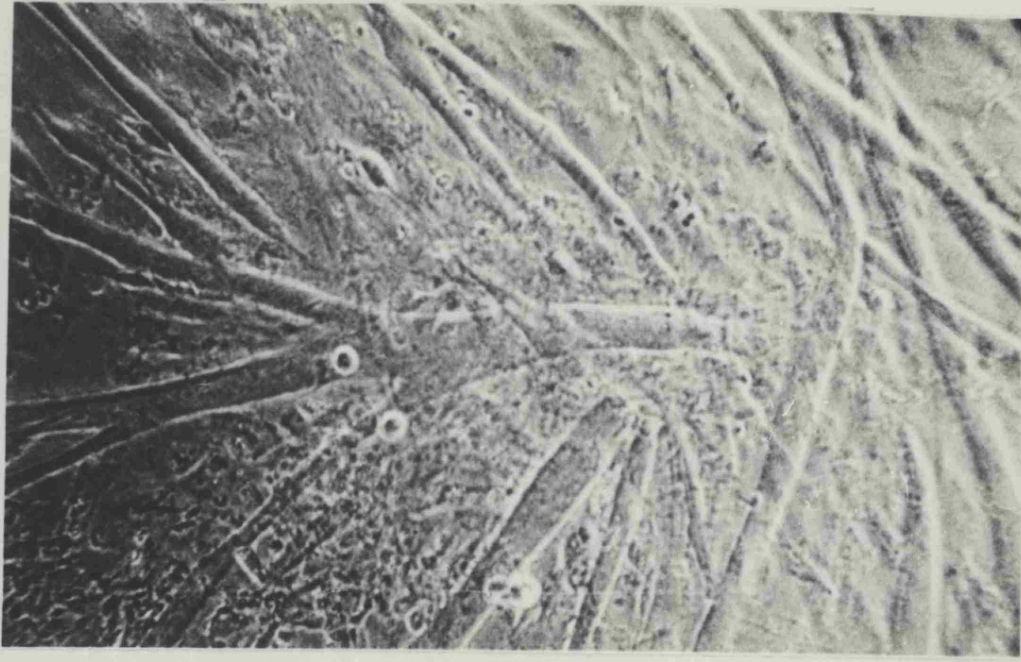
B

TABLE 2

EFFECTS OF MITOTIC INHIBITORS ON SUBSEQUENT RAT MYOTUBE

GROWTH

Fluorodeoxyuridine (15µg/ml) and uridine (35µg/ml) (FDU)

or cytosine arabinoside (Ara C, 10µM) were added to

replicate muscle cultures, on the 3rd day in culture,

for 72h. On the 7th day in culture, $[^{125}I]\alpha$-BGT binding

(10nM) was determined as described in the "Methods"

section 3. Each result is the mean $\pm$ SD of 4 culture wells.

| Test | $[^{125}I]\alpha$-BGT binding | | | |
| | Total | Non-specific | Specific | Protein |
	(cpm/well)	(cpm/well)	(fmol/well)	(µg/well)
No addition	29787 $\pm$ 2417	2740 $\pm$ 187	47.3	160 $\pm$ 14
+ FDU	22773 $\pm$ 840	1741 $\pm$ 197	36.8	92 $\pm$ 8
+ Ara C	24318 $\pm$ 180	1881 $\pm$ 148	39.2	92 $\pm$ 4

A commonly employed method of reducing the number of fibroblasts, with consequent enrichment of myoblasts, is the preplating of initial cell suspensions for 20 min at 37°C in non-collagen coated tissue culture flasks (Yaffe, 1968). Fibroblasts preferentially adhere to plastic surfaces, and subsequent decanting reduces their number in the cell suspension. When this method was tested, there was no apparent morphological difference in the myotube cultures obtained, with or without preplating, and this procedure was not routinely adopted.

1.4 Growth media

In initial culture preparations, a saline extract of whole chick embryos (kind gift from Dr. Ahmed Jehanli) was added to the growth medium (5% v/v). This extract is an essential component for the growth of chick muscle cells in culture (Hauschka, 1972) and is added by many workers to growth media for muscle cells of other species (see Hauschka, 1972; Yaffe, 1973; Witkowski et al., 1976). Cultures grown with or without this extract showed no differences in either the growth or the number of myotubes present and it was accordingly not routinely used. The principal factor in growth medium which affected myotube growth was the donor horse serum. One batch of serum appeared to deteriorate in quality, giving rise to poor, less dense myotube cultures (e.g. culture C used for equilibrium [^{125}I]α-BGT binding studies - see Figure 13b). Use of a new batch of donor horse serum immediately restored the quality of myotube cultures to that routinely expected.

Serum-free chemically defined growth media of various compositions have been used in other laboratories for the growth

and/or fusion of chick, (Kumegawa et al., 1980; Dollenmeier et al., 1981) rat (Florini and Roberts, 1979) and human muscle cells (Yasin and van Beers, 1983). Such a medium (see "Materials") is routinely used in this department for the growth of embryonic rat (Digby et al., 1985) and foetal human neuronal cells (unpublished) and was therefore tested on myotube cultures. After 3 days growth in GM1, cultures were continued in serum-free medium without addition of mitotic inhibitors. In comparison to replicate cultures maintained in GM1, growth was poor, the myotubes appearing thinner and less branched. Binding of [^{125}I]α-BGT was considerably reduced (42% of control - Table 3). However the cultures were remarkably free of fibroblast growth.

1.5 Substrate used for cell attachment

As an alternative to photo-polymerised collagen, gelatin (2mg/ml, 30 µl/culture well) was tested as a substrate for cell attachment and myotube growth (Hauschka, 1972). There was no morphological difference between cultures grown on either substrate but as there was a tendency for myotubes to detach from the gelatin surface during the frequent washes required for biochemical analysis, photo-polymerised collagen was routinely used.

1.6 Protein content of cultures

In one representative myotube culture, protein content was 94.4 ± 7.2 µg/culture well (mean ± SD, 24 culture wells). Between different myotube culture preparations, protein content varied from 84.1 - 123.6 µg/culture well with a mean value of 103.3 ± 14.4 µg/culture well (19 different preparations) tested on the seventh day in culture. As cultures originate from a mixture of cell types giving rise

TABLE 3

EFFECTS OF SERUM-FREE MEDIUM ON SUBSEQUENT RAT MYOTUBE
GROWTH

Replicate muscle cultures were grown for 3 days in GM1
before changing to serum-free medium (see "Materials")
for a further 4 days growth. At this time $\left[^{125}I\right]\alpha$-BGT
binding (20nM) was determined, as described in the
"Methods" section 3, and compared to replicate cultures
maintained in GM1 plus Ara C (10uM). Each result is the
mean $\pm$ SD of 4 culture wells.

	$\left[^{125}I\right]\alpha$-BGT binding			
Growth medium	Total (cpm/well)	Non-specific (cpm/well)	Specific (fmol/well)	Protein (ug/well)
GM1	54884 ± 4898	3820 ± 144	77.0	124 ± 12
GM1/SFM	23198 ± 2115	1694 ± 247	32.4	63 ± 7

to both myoblast and fibroblast growth, each of which contribute to total protein levels, all experimental results are expressed as per culture well rather than per mg protein.

1.7 Time course of the appearance of [^{125}I]α-BGT binding sites in cultures

Rat myotube cultures were tested daily over a period of 10 days for [^{125}I]α-BGT binding and total protein content (Figure 11). Before myoblast fusion (2 days in culture) specific [^{125}I]α-BGT binding was discernible (0.62 fmol/culture well). On fusion of the myoblasts (3 days in culture) this had increased to 17.5 fmol/culture well and thereafter increased daily to a maximum level of 55.4 fmol/culture well on the seventh day in culture. Total protein levels increased rapidly over the first 4 days of growth. This increase was halted after addition of mitotic inhibitor to the growth medium. As culture time increased, progressive detachment of myotubes from the collagen surface of the culture wells, as a result of vigorous spontaneous contractions, led to a loss in [^{125}I]α-BGT binding sites and protein content. Myotube cultures were therefore routinely used for biochemical assays on the seventh day in culture.

1.8 Equilibrium binding of [^{125}I]α-BGT binding to cultures

After incubation of myotube cultures with [^{125}I]α-BGT, 3 washes with growth medium were sufficient to remove all free radiolabel. Counting errors (SE) of four replicate culture wells were usually less than 5%. Pre-incubation of cultures with decamethonium bromide inhibited [^{125}I]α-BGT binding in a concentration dependent manner (Figure 12). Addition of 1 mM decamethonium inhibited toxin

FIGURE 11

INCREASE OF $[^{125}I]\alpha$-BGT BINDING SITES IN RAT MUSCLE
CELL CULTURES

Replicate muscle cell cultures were tested daily for
specific $[^{125}I]\alpha$-BGT binding (20nM) as described in the
"Methods" section 3. The total protein content of the
cultures was also determined.

(●————●) Specific $[^{125}I]\alpha$-BGT binding/culture well.
Each point is the difference between
total and non-specific binding, each
determined from 4 culture wells.

(●— — —●) Protein content/culture well. Each
point is the mean $\pm$ SD of 8 culture wells.

[a] At this time point, many of the myotubes had become
detached from the surface of the culture wells.
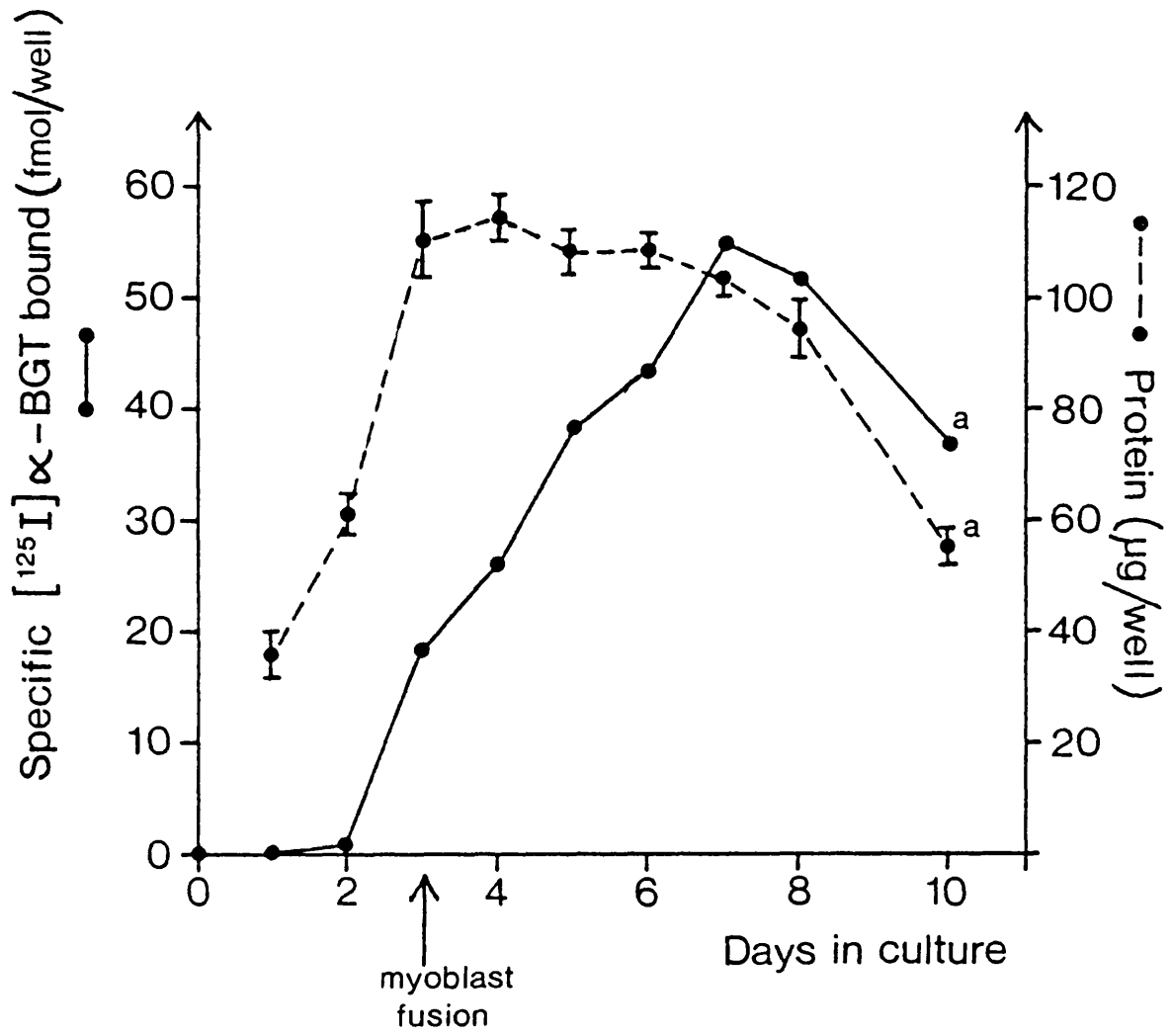
FIGURE 12

INHIBITION OF $[^{125}I]$ α-BGT BINDING TO RAT MYOTUBE

CULTURES BY DECAMETHONIUM BROMIDE

The binding of $[^{125}I]$α-BGT (20nM) to replicate myotube

cultures (7 days in culture) was determined as described

in the "Methods" section 3, after pre-incubation of the

cultures (30 min, $23^{\circ}C$) with increasing concentrations

of decamethonium bromide. Each point is the mean $\pm$ SD

of 4 culture wells.
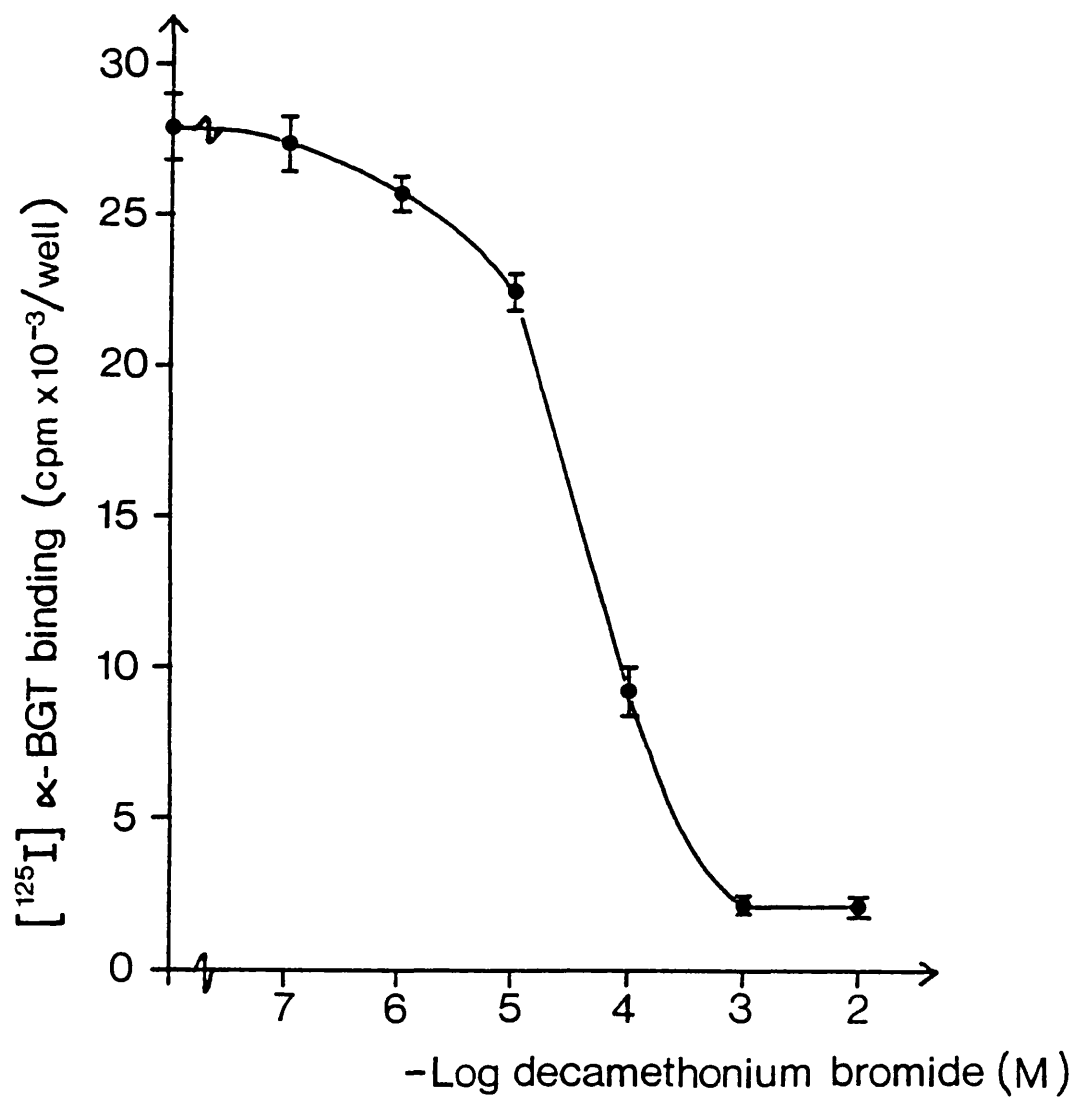
binding by 92%.

The binding of $[^{125}I]\alpha$-BGT to myotube cultures was studied over a range of toxin concentrations. Representative saturation curves are shown in Figure 13. Non-specific binding, measured in the presence of 1mM decamethonium, was linear and accounted for 5-8% of the total binding. Specific binding approached saturation and described a rectangular hyperbola. This was used to estimate K_D and B_{max} (per culture well). Figure 14 shows Scatchard plots obtained from the data in Figure 13. Different B_{max} values (47.2 - 99.3 fmol $[^{125}I]\alpha$-BGT bound per culture well) reflect differences in growth between the 3 cultures tested. Culture C (Figure 13 b) exhibited poor growth, as judged by final density and spontaneous contractility of myotubes. This was reflected in the reduced number of $[^{125}I]\alpha$-BGT binding sites per culture well. Scatchard analysis, however, gave similar K_D values for all 3 cultures amounting to 4.03 $\pm$ 0.56 pmol $[^{125}I]\alpha$-BGT added per culture well, mean $\pm$ SD (3 different cultures), equivalent to 13.4 $\pm$ 1.9 nM $[^{125}I]\alpha$-BGT in the assay conditions used (see "Methods" section 3).

Routine determination of $[^{125}I]\alpha$-BGT binding to different myotube cultures gave values in the range 32.1 - 77.0 fmol/culture well, with a mean value of 55.5 $\pm$ 9.9 fmol/culture well, mean $\pm$ SD (19 different cultures, assayed at 20 nM toxin concentration).

1.9 Acetylcholinesterase and creatine phosphokinase activity

Studies were carried out on the time course of the appearance of AChE and CPK activity in the muscle cultures (Figure 15). Following the onset of myoblast fusion (day 3 in culture), there was a rapid increase in AChE and CPK activity. AChE activity increased steadily from day 4 to day 8 in culture, the level of activity on day 7 being

FIGURE 13

EQUILIBRIUM BINDING OF $[^{125}I]\alpha$-BGT TO RAT MYOTUBE

CULTURES

The binding of $[^{125}I]\alpha$-BGT to replicate myotube

cultures (7 days in culture) was determined as described

in the "Methods" section 3.

a) Binding data from one representative culture showing:-

● Binding in the absence of decamethonium bromide
   (total binding)

o Binding in the presence of 1mM decamethonium
   bromide (non-specific binding)

□ Specific binding

Each point is the mean $\pm$ SD of 4 culture wells

b) Specific binding data from 3 different myotube cultures
   assayed as above.

● Culture A Protein = 110 $\pm$ 9 µg/culture well

o Culture B Protein = 96 $\pm$ 3 µg/culture well

□ Culture C Protein = 84 $\pm$ 6 µg/culture well

(mean $\pm$ SD, 8 wells)

a)
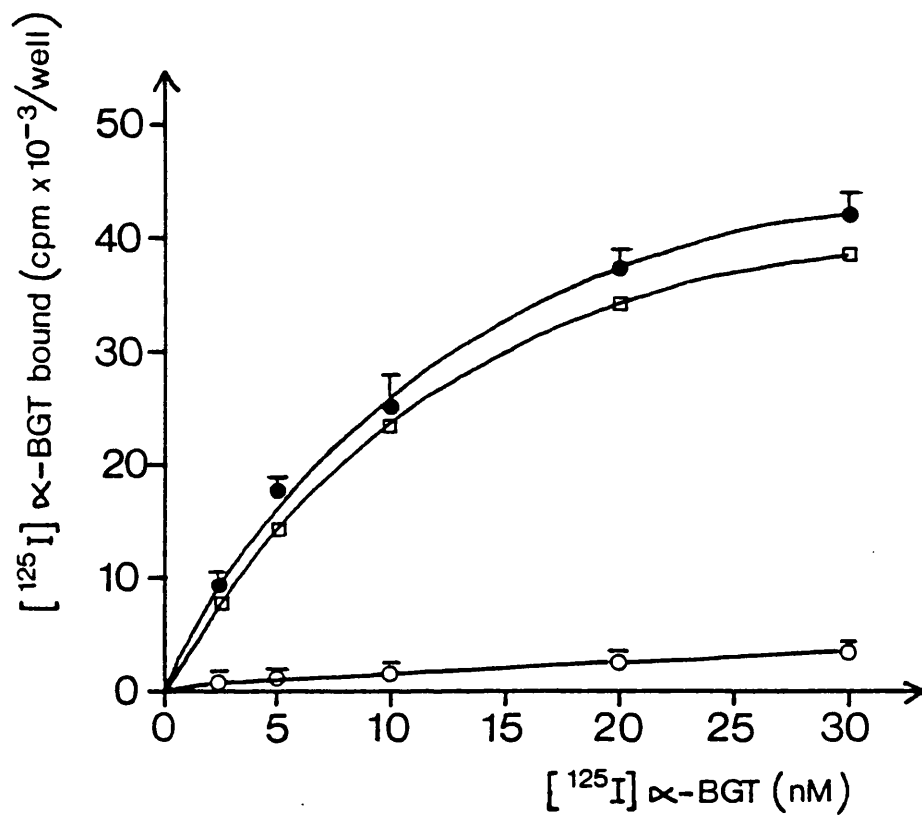
b)
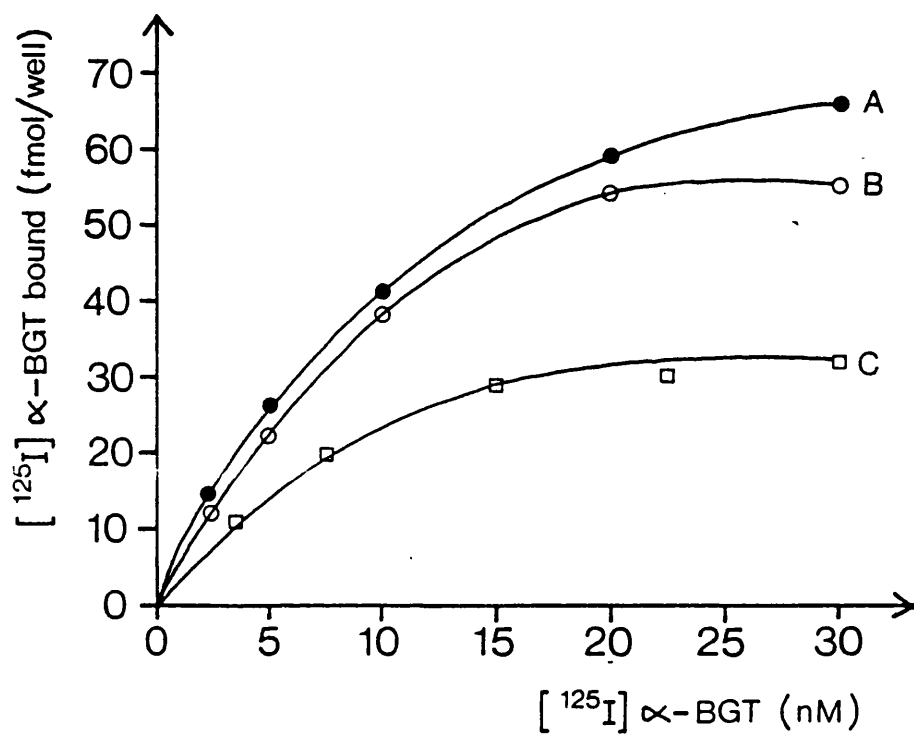
FIGURE 14

SCATCHARD ANALYSIS OF THE BINDING DATA IN FIGURE 13 (b)

Lines were constructed by linear regression by using the data from Figure 13 (b).

Culture	K_D	B_{max}	r^2
● A	14.2 nM	99.3 fmol/well	0.98
O B	14.8 nM	89.8 fmol/well	0.93
□ C	11.3 nM	47.2 fmol/well	0.88

Mean K_D = 13.4 nM
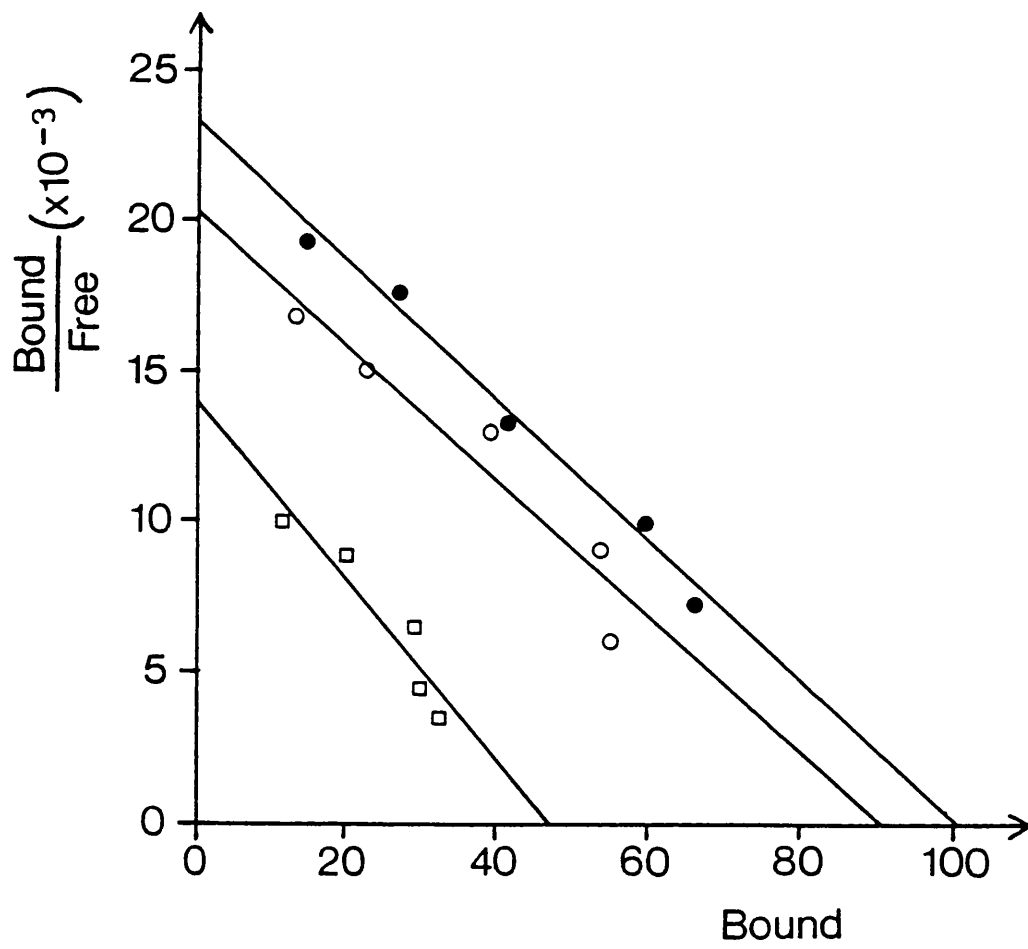
0.23 ± 0.06 nmol substrate converted/min/culture well (mean ± SD, 5 experiments). CPK activity reached a peak value on day 5 in culture after which a plateau was observed. The level of activity on day 7 in culture was 18.1 ± 2.07 nmol substrate converted/min/culture well (mean ± SD, 5 experiments.

1.10 Uptake of [Me-^{3}H] carnitine

Rat myotube cultures and skin fibroblast cultures were tested (over the 6th and 7th day in culture) for uptake of radioactivity after incubation with [Me-^{3}H] carnitine as described in the "Methods" section 7. At the end of the incubation time, 3 washes with growth medium were sufficient to remove all free radiolabel. Figure 16 shows the uptake of [Me-^{3}H] carnitine by 3 similar dense myotube cultures. Uptake of radioactivity was not saturable over the concentration range tested (0.067 - 1.33 µM, 0.1 - 2 µCi added). Uptake of radioactivity by fibroblasts in culture (Figure 16) was consistently 3 - 4 times lower than that observed for myotube cultures.

During the first 2 years of work for this project, D,L-[Me-^{3}H] carnitine hydrochloride of specific activity 2 Ci/mmol, 1 mCi/ml was utilised. This product was discontinued by Amersham International and later work involved use of the L-isomer, L-[Me-^{3}H] carnitine hydrochloride of high specific activity (87 Ci/mmol, 1 mCi/ml). Uptake of radioactivity by rat cultures during incubation with increasing concentrations of the L-isomer (1.5 - 15 nM, 0.1 - 1.0 µCi added) for 18 h at 37°C was similar to that obtained by using D,L-[Me-^{3}H] carnitine. Unless otherwise stated, all results given for rat cultures were obtained by labelling with D,L-[Me-^{3}H] carnitine.

FIGURE 15

ACETYLCHOLINESTERASE AND CREATINE PHOSPHOKINASE

ACTIVITY IN RAT MUSCLE CELLS IN CULTURE
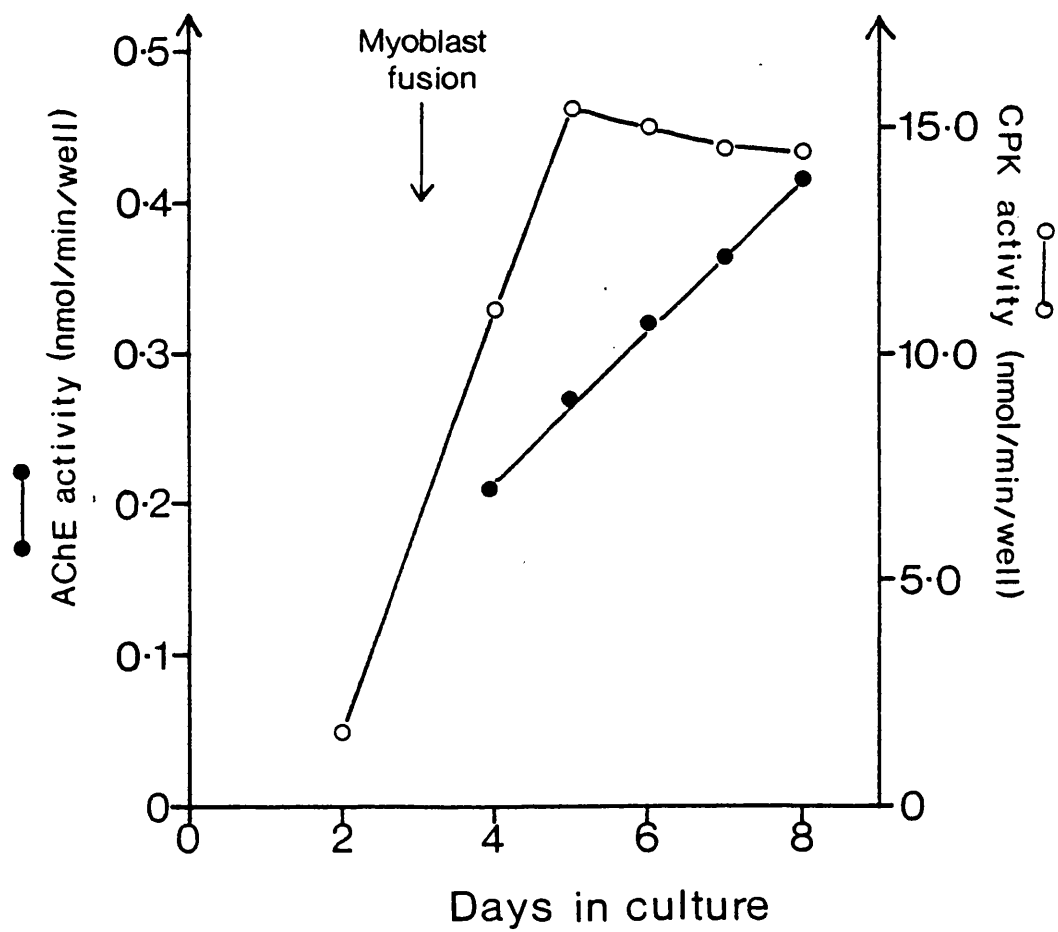
FIGURE 16

UPTAKE OF [Me-^{3}H] CARNITINE BY RAT CULTURES

The uptake of radioactivity by replicate cultures of
rat myotubes or rat skin fibroblasts (6 days in culture)
was determined after incubation with increasing
concentrations of [Me-^{3}H] carnitine for 18h at 37°C as
described in the "Methods" section 7.

Each point is the mean $\pm$ SD of 3 different experiments,
each of which included 4 culture wells.

● Myotube cultures :-

      [^{125}I]α-BGT binding (20nM) = 58.4 $\pm$ 3.5

                                  fmol/culture well

    Protein = 115 $\pm$ 7 ug/culture well

        (mean $\pm$ SD, 3 different cultures)

O Fibroblast cultures :-

      [^{125}I]α-BGT binding (20nM) not detected

    Protein = 54 $\pm$ 3 ug/culture well

        (mean $\pm$ SD, 3 different cultures)
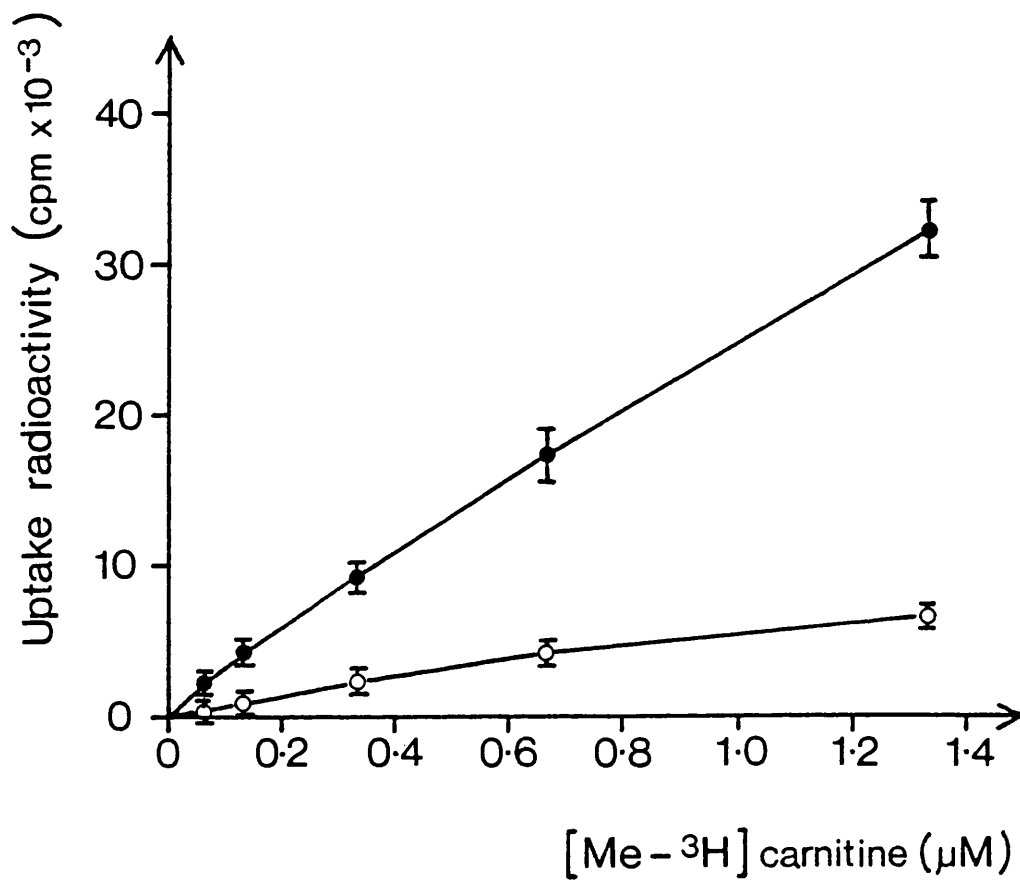
1.11 Spontaneous release of [Me-^{3}H] carnitine

After being labelled with [Me-^{3}H] carnitine for 18 h at 37°C

(o.67 µM, 1 µCi), myotube cultures were washed and fresh growth medium

(0.75 ml) was added. The cultures were again incubated (37°C) and, at

various time points, 4 replicate culture wells were washed and their

contents were solubilised in 0.1 M sodium hydroxide. After the final

time point, the solubilised cultures were counted for retained

radioactivity. Figure 17 shows the spontaneous loss of radioactivity

which was essentially linear over the first 8 hours. This represents

an average release of radioactivity of 6.4%/hour.

Fibroblast cultures labelled with DL- or L- [Me-^{3}H] carnitine

(0.67µM and 15nM respectively, 1µCi added) and myotube cultures labelled

with L-[Me-^{3}H] carnitine (15nM, 1µCi) showed a similar loss of

radioactivity when tested under similar conditions over a 3h time

period.

1.12 Effect of D,L-carnitine on [^{125}I]∝-BGT binding to myotube cultures

Rat myotube cultures (6 days in culture) were incubated with

unlabelled D,L-carnitine (0.15 - 1.5 µM, 18h at 37°C) before washing

and then determining the number of [^{125}I]∝-BGT binding sites (at 20nM

toxin concentration) as described in the "Methods" section 3. No

subsequent reduction in the number of specific [^{125}I]∝-BGT binding

sites after treatment with D,L-carnitine was observed (Figure 18).

FIGURE 17

SPONTANEOUS RELEASE OF $[Me-^3H]$ CARNITINE FROM RAT
MYOTUBE CULTURES

Replicate myotube cultures were labelled with $[Me-^3H]$
carnitine as described in Section 2.2.1. The cultures
were washed and fresh growth medium (0.75ml),
containing no radiolabel, was added. The cultures were
incubated at 37°C and the retention of radioactivity
by the cultures was determined at various time points
as described in Section 1.11. Each point is the
mean $\pm$ SD of 3 different experiments, each of which
included 4 culture wells.
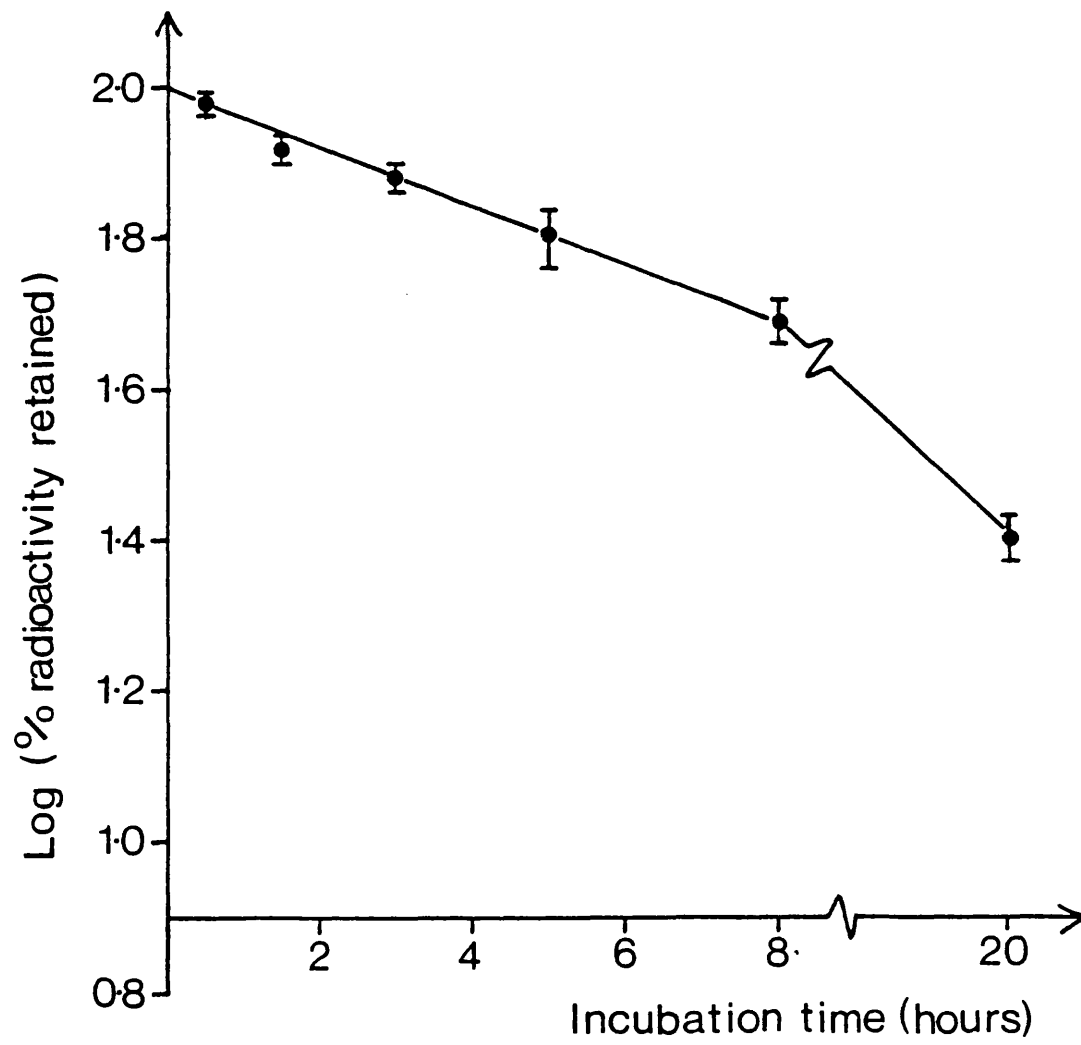
FIGURE 18

$[^{125}I]\alpha$-BGT BINDING TO RAT MYOTUBE CULTURES AFTER
PRE-INCUBATION WITH INCREASING CONCENTRATIONS OF
D,L-CARNITINE FOR 18h AT 37°C
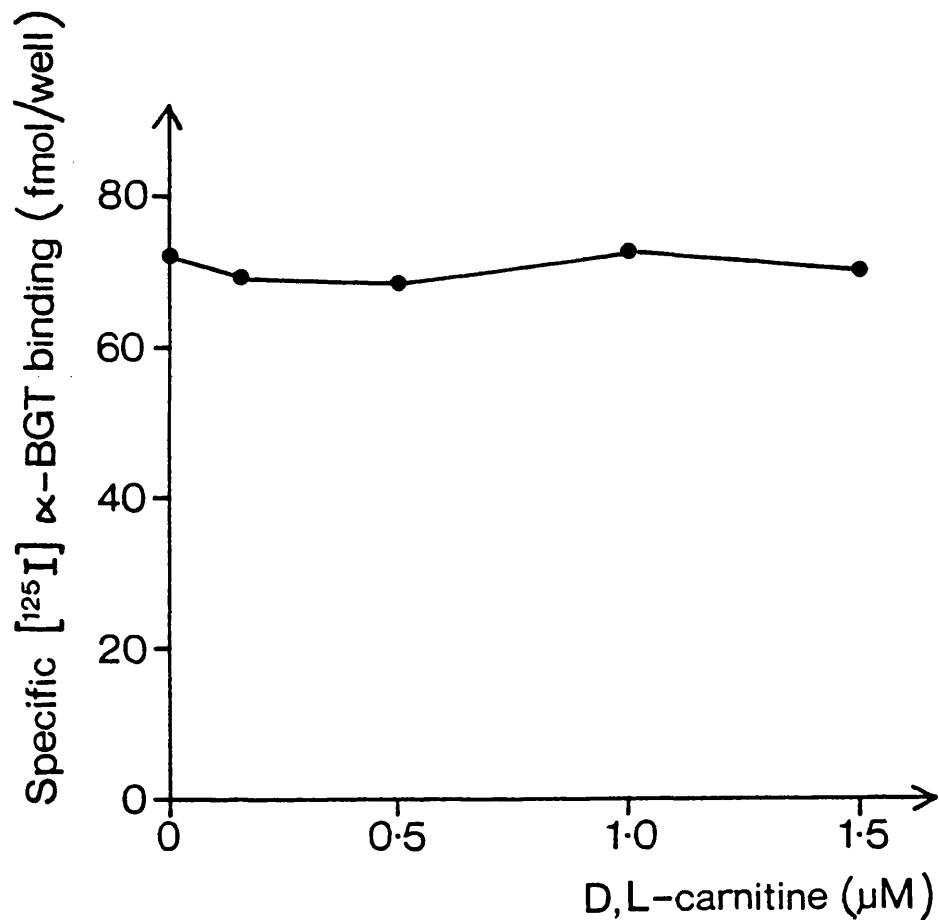
$[^{125}I]\alpha$-BGT binding was determined (20 nM)
as described in the "Methods" section 3.
Each concentration of D,L-carnitine was
added to 8 replicate culture wells.

2. Effects of myasthenic serum on cultures

2.1 Binding of serum components

2.1.1 Binding of serum immunoglobulins

Figure 19 illustrates the binding of heat-inactivated normal and myasthenic serum immunoglobulins detected by using [^{125}I]-labelled goat anti-human light chain antibodies ([^{125}I]-GAHL) (see "Methods" section 10.1). [^{125}I]-GAHL binding in the absence of test serum represented non-specific binding to the cultures (in the range 1750 - 3000 cpm). This value, obtained in each experiment, was arbitrarily taken as 100% and the binding in the presence of test serum was expressed as a relative value to allow comparison between different cultures. [^{125}I]-GAHL binding after incubation with normal sera gave a mean value of 205.3 ± 21.6% (3 different sera), mean ± SD (n). After incubation with myasthenic sera, 3 out of 4 samples gave values significantly higher than those obtained for the normal sera. The mean value was 338.0 ± 81.9% (4 different sera), mean ± SD (n). Binding of [^{125}I]-GAHL correlated with the anti-AChR antibody titre of the serum samples, the lowest titre serum tested, from patient MG 13, sample (iii), giving values similar to those obtained for one of the normal serum samples.

2.1.2 Loss of [^{125}I]α-BGT binding sites

Heat-inactivated normal and myasthenic serum samples were tested for their ability to reduce the number of [^{125}I]α-BGT binding sites in myotube cultures as described in the "Methods" section 10.2. Four out of seven myasthenic sera tested significantly reduced the number of available [^{125}I]α-BGT binding sites (Figure 20a). These sera

FIGURE 19

BINDING OF SERUM IMMUNOGLOBULINS TO RAT MYOTUBE

CULTURES

The binding of normal and myasthenic serum

immunoglobulins to myotube cultures (7 days in

culture) was determined as described in the "Methods"

section 10.1. The results are expressed as the

percentage of radioactivity bound to the cultures

in the absence of test serum (taken as 100%). The

heights of the columns represent the mean $\pm$ SD of 3

different experiments, each of which included 4

culture wells.
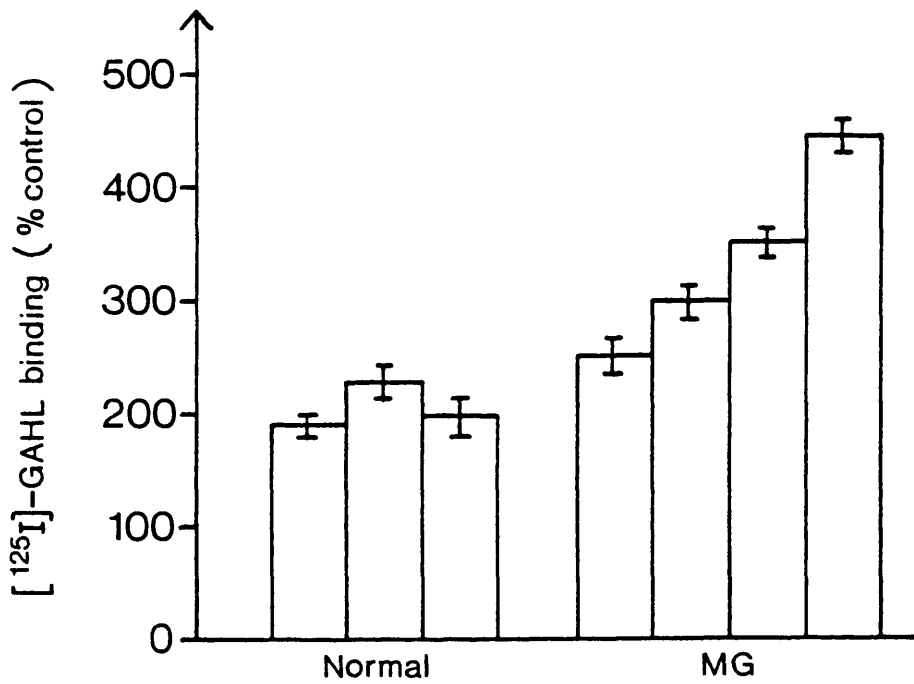
	Normal			MG			
Donor	1	2	3	13	13	4	14
Sample				(iii)	(vii)	(ii)	
Anti−AChR antibody titre (nM)	−	−	−	16·2	36·5	42·0	81·3

FIGURE 20

LOSS OF $[^{125}I]\alpha$-BGT BINDING SITES ON RAT MYOTUBE

CULTURES AFTER TREATMENT WITH MYASTHENIC SERUM

a) Replicate myotube cultures (7 days in culture) were
   incubated with heat-inactivated normal or myasthenic
   serum (100μl, 20% v/v) for 1h at 37°C. The cultures
   were washed and the remaining $[^{125}I]\alpha$-BGT binding
   sites were determined as described in the "Methods"
   section 3 (at 20nM toxin). The heights of the columns
   represent the mean $\pm$ range of 2 different experiments,
   each of which included 8 culture wells.

b) Replicate myotube cultures (7 days in culture) were
   incubated for 1h at 37°C with increasing concentrations
   (5-100 μl, 1-20% v/v) of heat-inactivated myasthenic
   serum (from patient MG 13, sample viii). The cultures
   were washed and the remaining $[^{125}I]\alpha$-BGT binding
   sites determined as described in the "Methods"
   section 3 (at 20nM toxin). The results were gained
   from one experiment in which 8 culture wells were
   tested for each serum concentration.

a)
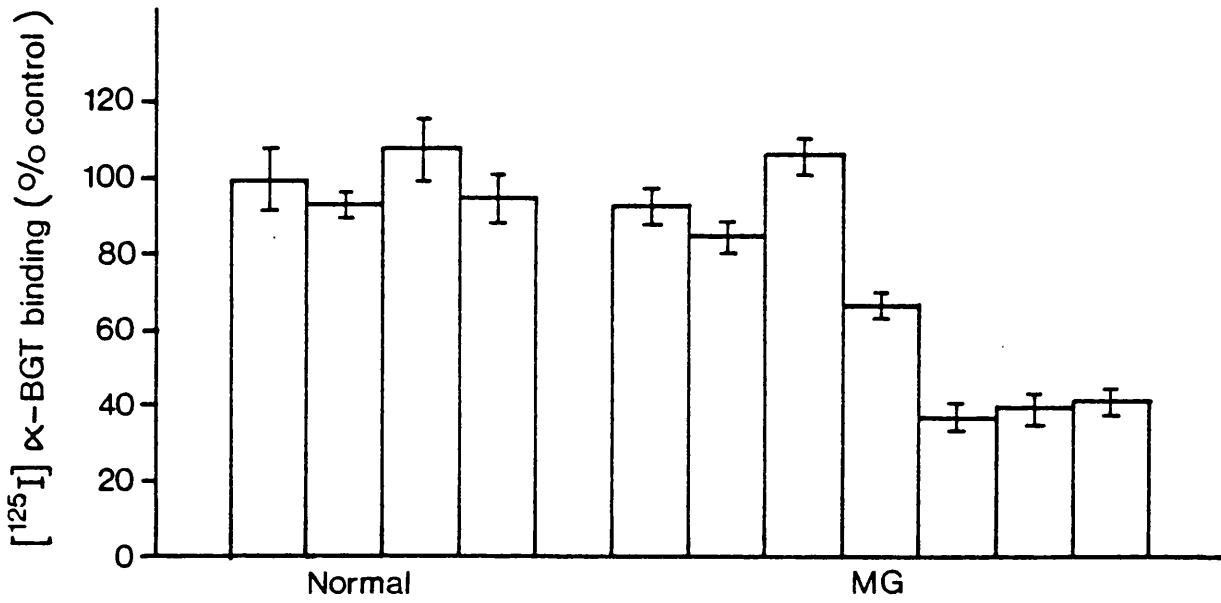
Donor	1	2	3	4	1	15	4	5	13	4	13
Sample							(i)		(vii)	(ii)	(viii)
Anti–AChR antibody titre nM	–	–	–	–	0·8	7·8	12·2	14·8	36·5	42·0	43·8

b)
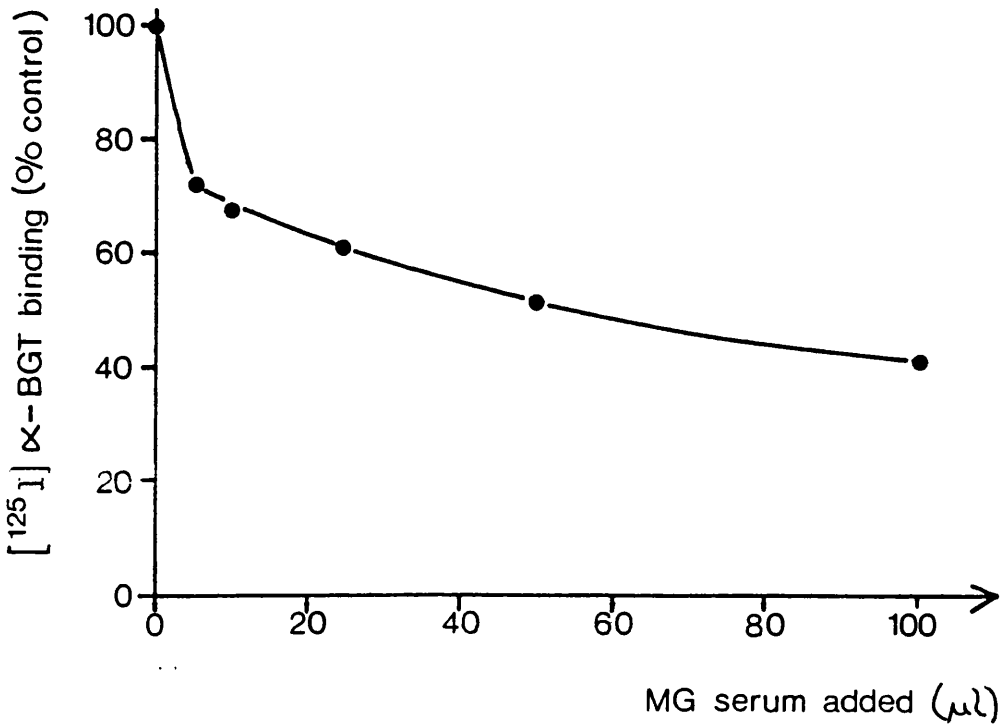
represented those with the highest anti-AChR antibody titres, although the reduction in available toxin sites was not proportional to the titre. Cultures incubated with test sera at $4^{O}C$ showed similar values for reduction of available toxin binding sites suggesting that the effect of myasthenic sera arose from blockade of binding rather than from energy dependent degradation of AChR.

One myasthenic serum sample (patient MG 13, sample viii) was shown to block $[^{125}I]\alpha$-BGT binding in a concentration-dependent manner (Figure 20b), the lowest concentration tested (5 µl, 1% v/v) reducing the number of available binding sites by 29%.

2.2 Myotoxicity studies

2.2.1 Labelling of cultures with [Me-^{3}H] carnitine - retention and variability

For myotoxicity studies, [Me-^{3}H] carnitine was added to rat myotube cultures on the 6th day in culture and serum myotoxicity was estimated on the 7th day in culture. Rat myotube cultures were routinely labelled with [Me-^{3}H] carnitine (0.67 µM, 1 µCi) for 18 h at $37^{O}C$. This concentration was chosen to give a clear difference between myotube and fibroblast uptake (Figure 16), with minimum radiolabel.

Under standard myotoxicity test conditions (3h further incubation at $37^{O}C$, see section 2.2.6) successful cultures without added test serum retained between 10,250 and 19800 cpm (13826 ± 2742, 28 different experiments, mean ± SD,n). Less dense myotube cultures, initially seeded at a lower plating density or demonstrating poorer growth, took up and retained less radioactivity after labelling with [Me-^{3}H] carnitine. Except where shown, these were not used for myotoxicity assays.

Within one preparation of myotube cultures, [Me-^{3}H] carnitine

retention was tested after incubation with radiolabel (0.67 µM, 1 µCi)

for 18 h at 37°C followed by further incubation (3 h at 37°C) with

fresh medium (0.66 ml) to which no additions of test serum were made

(control cultures). Within 24 replicate culture wells (1 culture

plate) retention of radioactivity was in the range 12092 - 16891 cpm,

with a mean value of 14324 ± 1415 (24), mean ± SD (n).

Serum myotoxicity assays were carried out in quadruplicate on

replicate culture wells. To minimise variation resulting from the

occasional poor cultures at the ends of the plate (resulting in lower

uptake of radioactivity), tests were spread over the plate as

illustrated in Figure 21. Economical use of cultures was made by

running one control and five tests per 24-well plate. Taking as an

example the 24-well plate tested for variation in retention of

radioactivity, comparison between 6 sets of quadruplicates gave a mean

value of 14324 ± 732 cpm, mean ± SD, from the range 13371 - 15566 cpm.

The standard error of the mean (SE) for each 'test', carried out in

quadruplicate, was usually less than 5%.

Later work involved the use of the L-[Me-^{3}H] carnitine (see

section 1.10). Myotube cultures were routinely labelled with this

isomer (15nM, 1µCi) for 18h at 37°C before myotoxicity assays were

carried out.

FIGURE 21

ARRANGEMENT OF MYOTOXICITY TEST SAMPLES FOR 6 TESTS ON A 24-WELL
CULTURE PLATE

1	3	5	6	2	4
1	3	5	6	2	4
2	4	6	1	3	5
2	4	6	1	3	5

2.2.2 Reference to experiments using human myotubes in culture

Preliminary myotoxicity studies carried out by using human myotube cultures indicated that there was no significant difference between the effects of non heat-treated myasthenic or normal sera when added to cultures (100 μl, 20% v/v) for 3 h at 37°C. The myotoxicity values obtained in these experiments (section B 2.2.2) were 13.1 ± 8.6% (9 sera) and 8.9 ± 5.0% (7 sera) for myasthenic and normal sera respectively. The sera utilised in these studies had been previously stored at -20°C for varying lengths of time, conditions under which endogenous complement activity might be expected to be decreased or destroyed (Whicher, 1978). Consequently, a complement-mediated myotoxicity assay on rat myotube cultures was assessed by using initially, fresh human serum from normal donors and finally,

commercial guinea-pig complement serum (GPC) as a source of complement activity. Serum from one myasthenic patient (MG 13) was continually assessed in the following studies as, using human myotube cultures, it gave the highest myotoxicity value (27.6%) when a non heat-treated sample (100 µl, 20% v/v) was incubated with cultures for 3 h at $37^{\circ}C$ (see section B 2.2.2).

2.2.3 Effect of non heat-treated and heat-treated serum

Two non heat-treated serum samples from MG 13 gave rise to myotoxicity values of 22.0% and 28.7% respectively when incubated with cultures for 3 h at $37^{\circ}C$ (100 µl serum added, 20% v/v) (Table 4). Serum samples from the 5 other myasthenic patients tested caused measured myotoxicity of between -3.1 - 17.7%. However, similar treatment of myotube cultures with fresh, non heat-treated serum from normal donors (subsequently used as a source of active complement) gave, in several cases, similar myotoxicity to that observed for patient MG 13 (donors 2,3,4,5 and 7, 22.6 - 29.9%, Table 4). As shown in Table 4, heat-inactivation of serum for 30 min at $56^{\circ}C$ reduced the measured myotoxicity of these normal serum samples and of the serum samples from MG 13 to less than 15%. Absorption of 'toxic' fresh normal serum on myotube cultures (1 h, $37^{\circ}C$) before use made no difference to the subsequent toxicity of the fresh serum when tested alone. Absorption with rat liver homogenate (see "Methods" section 11) reduced measured myotoxicity in a similar way to heat treatment. The mean values obtained for heat-treated normal and myasthenic sera were 7.8 ± 4.9% (13 samples) and 5.2 ± 7.2% (7 samples) respectively suggesting that measured myotoxicity values of up to 15% could be regarded as falling within normal values (see also section 2.2.13).

TABLE 4

MYOTOXICITY OF INDIVIDUAL MYASTHENIC AND NORMAL SERUM
SAMPLES BEFORE AND AFTER HEAT-TREATMENT

Rat myotube cultures were labelled with $[\text{Me-}^3\text{H}]$ carnitine
(section 2.2.1) and exposed to samples of test serum
($100\mu\text{l}$, 20% v/v) for 3h at 37°C. Control cultures to
which no additions were made were run simultaneously.
At the end of the incubation time, the cultures were
washed and solubilised for counting. Myotoxicity was
calculated as described in the "Methods" section 8 by
comparison with the control cultures. Each serum sample
was added to 4 replicate culture wells.

Test serum	Donor	Anti-AChR antibody titre (nM)	Myotoxicity	
			Not heat-treated (%)	Heat-treated[b] (%)
Normal	1	-	12.3	0.7
(freshly	2[a](i)	-	9.1	4.7
taken)	(ii)	-	11.6	10.9
	(iii)	-	25.4	5.0
	(iv)	-	23.6	11.2
	3[a](i)	-	15.2	14.2
	(ii)	-	25.7	8.4
	(iii)	-	15.2	4.7
	4[a](i)	-	7.1	11.8
	(ii)	-	22.6	8.8
	5	-	24.4	- 0.8
	6	-	2.2	6.8
	7	-	29.9	14.9
Myasthenia	4[a](i)	12.2	3.7	- 6.3
gravis	13[a](i)	5.8	28.7	8.2
	(vii)	36.5	22.0	9.9
	15	7.8	- 3.1	- 2.6
	16	11.0	12.1	14.2
	17	19.8	2.0	8.3
	18	25.0	17.7	4.5

[a] Serial samples taken at different times from the same donor

[b] Heat-treated at 56°C for 30 min

2.2.4 Effect of human complement

Fresh, non heat-treated serum from normal donors was used as a source of active complement. This serum (100 µl, 20% v/v) was incubated with myotube cultures for 3 h at $37^{o}C$, together with heat-inactivated normal or myasthenic serum (100 µl, 20% v/v). Cultures to which no additions were made (control) or to which human complement alone or heat-inactivated test serum alone were added, were incubated simultaneously. As described in the previous section, some samples of human complement tested alone gave rise to relatively high myotoxicity values (Table 4). The measured myotoxicity caused by addition of these samples to heat-inactivated test sera was similar to that of the 'toxic' samples tested alone (up to 30%).

The measured myotoxicity caused by addition of 'non- toxic' human complement (ie. less than 15% when tested alone) to heat-inactivated normal serum gave a mean value of 8.2 ± 6.4%, mean ± SD, 6 serum samples (Table 5). The previous measured myotoxicity by fresh samples from normal donors 2,3 and 7 (see Table 4) did not appear to be restored by addition of human complement. The measured myotoxicity caused by heat-inactivated myasthenic serum plus complement gave a mean value of 17.0 ± 7.4%, mean ± SD, 5 serum samples (Table 5). In one experiment (see Table 5) the measured myotoxicity by serum from MG 13 plus added human complement was 42.3%. However this was not substantiated in 2 other experiments using human complement from different donors.

Use of fresh human serum, previously absorbed with rat liver homogenate, as a complement source produced measured myotoxicity values of 8.2 ± 3.4%, (mean ± SD, 3 different experiments) when incubated with heat-inactivated serum from MG 13 in the myotoxicity

assay as described above.

2.2.5 Effects of complement from other species

The use of fresh human serum as a source of active complement was clearly unsatisfactory for two reasons. First, many samples were quantitatively toxic when tested alone. Secondly, it was possible that complement activity could vary from sample to sample thereby explaining the disparity in results obtained for the heat-inactivated serum from MG 13 upon addition of different samples of fresh normal serum (see Table 5). Thus, heat-inactivated myasthenic serum (from patient MG 13) was tested on myotube cultures in the presence of fresh serum (as a complement source) from various species (Table 6). Use of rat complement serum caused measured myotoxicity of 20%. Rabbit serum was found to be toxic to the cultures (myotoxicity = 65.2%) when tested by itself and complete disruption of the cell monolayer was clearly visible through the light microscope. This toxicity was reduced in the presence of normal or myasthenic serum. The most effective and non-toxic source of complement was that obtained from commercial guinea-pig serum (GPC). On addition to the heat-inactivated myasthenic serum, a myotoxicity value of 43.4% was obtained. A similarly treated heat-inactivated normal serum sample gave a value of 7.4% (Table 6).

Commercial GPC serum was subsequently used as a source of active complement. This allowed standardisation of the complement source in myotoxicity experiments. Test sera were routinely heat-inactivated (56°, 30 min) before use in these experiments.

TABLE 5

MYOTOXICITY OF INDIVIDUAL MYASTHENIC AND NORMAL SERUM

SAMPLES IN THE PRESENCE OF HUMAN COMPLEMENT

Rat myotube cultures were labelled with [Me-^{3}H] carnitine

(Section 2.2.1) and exposed to samples of heat-inactivated

test serum (100μl, 20% v/v) plus fresh human complement

serum (100μl, 20% v/v) for 3h at 37°C. Cultures to which

no additions were made (control) or to which human

complement alone (100μl, 20% v/v) were added were run

simultaneously. At the end of the incubation time, the

cultures were washed and solubilised for counting.

Myotoxicity was calculated as described in the "Methods"

section 8 by comparison with the control cultures. The

data shown are the measured myotoxicity of heat-inactivated

test sera plus complement where the results for complement

alone were less than 15%. Each serum sample was added to

4 replicate culture wells.

Test serum	Donor	Anti-AChR antibody titre (nM)	Myotoxicity (%)
Normal	1	–	4.7
	2[a](ii)	–	6.0
	(iii)	–	-0.9
	(iv)	–	13.0
	3[a](ii)	–	17.2
	7	–	9.1
Myasthenia	4[a](i)	12.2	16.4
gravis	13[a](vii)	36.5	20.8 [b]
			42.3
			19.7
	15	7.8	20.1
	17	19.8	8.1
	18	25.0	12.9
Complement alone			8.5 ± 4.1 (mean $\pm$ SD, 5 samples)

[a] Serial samples taken at different times from the same donor

[b] Results from 3 different experiments

TABLE 6

MYOTOXICITY OF MYASTHENIC AND NORMAL SERUM IN THE PRESENCE
OF COMPLEMENT FROM DIFFERENT SOURCES

Rat myotube cultures were labelled with $[Me-^3H]$ carnitine
(section 2.2.1) and exposed to samples of heat-inactivated
test serum (100μl, 20% v/v) with or without fresh serum
(100μl, 20% v/v) from various species, as a source of
active complement, for 3h at 37°C. Cultures to which no
additions were made (control) or to which complement
alone (100μl, 20% v/v) was added, were run simultaneously.
At the end of the incubation time, the cultures were
washed and solubilised for counting. Myotoxocity was
calculated as described in the "Methods" section 8 by
comparison with the control cultures. Each test was
carried out on 4 replicate culture wells.

Test serum	Myotoxicity (%)			Complement source
	HI serum alone	Plus added complement	Complement alone	
[a]Normal	12.3 ± 2.2	9.4 ± 3.5	9.3 ± 2.2	Human
[b]MG	6.2 ± 4.6	27.6 ± 12.7		
Normal	- 0.9	7.7	15.4	Rat
[b]MG	3.0	20.0		
Normal	- 0.9	7.4	- 0.3	Guinea-pig
[b]MG	3.0	43.4		(Miles Labs.)
Normal	- 0.9	37.1	65.2	Rabbit
[b]MG	3.0	48.0		

[a] data from 3 previous experiments (Tables 4 and 5) in which serum from 3 different normal donors were tested on the same cultures as serum from MG 13

[b] MG 13 sample (vii) anti-AChR antibody titre = 36.5nM

2.2.6. Length of incubation time

The loss of radioactivity from myotube cultures labelled with [Me-^{3}H] carnitine (section 2.2.1) was followed in replicate cultures incubated with heat-inactivated myasthenic or normal serum plus added GPC (Figure 22). Cultures treated with normal serum plus GPC lost radioactivity in a similar pattern to that shown by cultures to which no additions were made. Cultures incubated with myasthenic serum plus complement showed an accelerated loss of radioactivity typified by the pattern shown in Figure 22.

Figure 23 illustrates the measured myotoxicity by 2 normal and 2 myasthenic serum samples (heat-inactivated) plus GPC at various time points. These studies suggested an optimal incubation time of 3h for detecting maximum differences in myotoxicity.

2.2.7 Titration of GPC and serum

The effects of increasing concentrations of GPC (20 - 160 μl, 2.7 - 21.6% v/v) in the presence of a constant concentration of heat-inactivated test serum (80 μl, 10.8% v/v) were tested on myotube cultures labelled with [Me-^{3}H] carnitine (section 2.2.1). The measured myotoxicity was found to be dependent on the concentration of GPC serum added (Figure 24a). Similar experiments,in the presence of a constant amount of GPC (80 μl, 10.8% v/v), showed that the measured myotoxicity was dependent on the concentration of myasthenic serum added (Figure 24b).

FIGURE 22

RETENTION OF RADIOACTIVITY BY RAT MYOTUBE CULTURES

TREATED WITH HEAT-INACTIVATED SERUM AND GPC

Replicate myotube cultures were labelled with $[Me-^3H]$
carnitine (Section 2.2.1) and exposed to aliquots
(80µl, 12.1% v/v) of heat-inactivated normal (■) or
myasthenic (●) serum plus GPC (80µl, 12.1% v/v) at
37°C for varying lengths of time. Cultures to which
no additions were made were run simultaneously (O).
At the end of the incubation period, the cultures
were washed and solubilised for counting. Results are
expressed as the retention of radiolabel by cultures
as a percentage of time 0. Each point represents the
mean $\pm$ SD of 4 culture wells. The myasthenic serum
tested was from patient MG 13, sample (vi), anti-AChR
antibody titre = 28.5 nM.

FIGURE 23

EFFECTS OF INCUBATION TIME ON MEASURED MYOTOXICITY

Replicate myotube cultures were labelled with $[Me-^3H]$ carnitine (section 2.2.1) and exposed to samples of heat-inactivated normal or myasthenic serum (80μl, 12.1% v/v) plus GPC (80μl, 12.1% v/v) for varying lengths of time at $37^{\circ}C$. Control cultures to which no additions were made were run simultaneously. At the end of the incubation time, the cultures were washed and solubilised for counting. Myotoxicity was calculated as described in the "Methods" section 8 by comparison with the control cultures at each time point. Each serum was tested for each time point on 4 replicate culture wells.

□ Patient MG 6   Anti-AChR antibody titre = 16.0 nM

                       (GPC source - Flow laboratories)

■ Patient MG 13 sample (vi)

                Anti-AChR antibody titre = 28.5 nM

                (GPC source - Miles laboratories)

o Normal donor 1   (GPC source - Flow laboratories)

● Normal donor 2   (GPC source - Miles laboratories)

FIGURE 24

MYOTOXICITY OF INCREASING CONCENTRATIONS OF HEAT-
INACTIVATED MYASTHENIC OR NORMAL SERUM AND GPC

Rat myotube cultures were labelled with $[Me-^3H]$
carnitine (Section 2.2.1) and exposed to:- a) samples
of heat-inactivated myasthenic or normal serum (80µl,
10.8% v/v) plus increasing concentrations of GPC
(20-160µl, 2.7-21.6% v/v) or b) samples of GPC (80µl,
10.8% v/v) plus increasing concentrations of heat-
inactivated myasthenic or normal serum (10-120µl,
1.4-16.2% v/v) for 3h at $37^{\circ}C$. Control cultures to
which no additions were made were run simultaneously.
At the end of the incubation time, the cultures were
washed and solubilised for counting. Myotoxicity was
calculated as described in the "Methods" section 8
by comparison with the control cultures. The tests
were as follows:-

		Anti-AChR antibody titre (nM)	GPC source
a)	▲ MG 6	16.0	Flow Labs.
	▢ MG 12	180.0	Flow Labs.
	▪ MG 13 sample (ii)	12.9	Miles Labs.
	● Normal serum	–	Flow Labs.
	○ GPC tested alone	–	Flow Labs.
b)	▲ MG 4 sample (ii)	42.0	Miles Labs.
	▪ MG 13 sample (ii)	12.9	Miles Labs.
	○ MG 13 sample (v)	20.6	Miles Labs.
	● Normal serum	–	Miles Labs.

Each point is the a) mean $\pm$ SD of 3 different experiments
or b) mean $\pm$ range of 2 different experiments, each of
which included 4 replicate culture wells.

a)
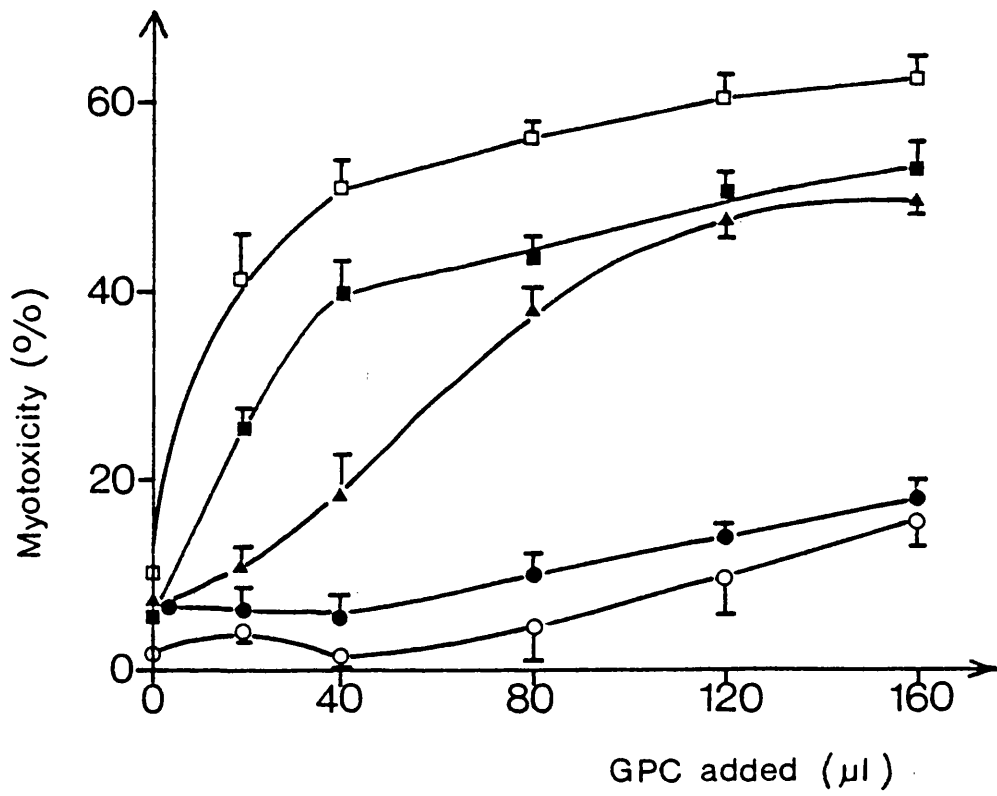
b)
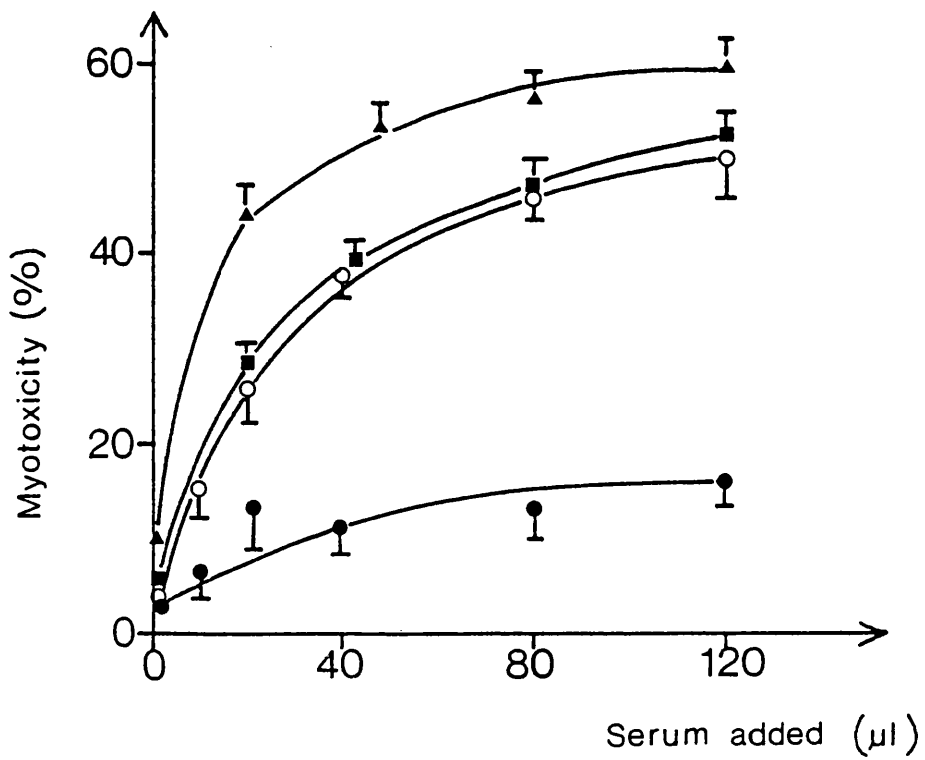
2.2.8 Activity of GPC

Two sources of GPC serum were used in myotoxicity experiments. During the course of this project, it became apparent that GPC supplied by Flow Laboratories (stated activity of $C'H_{50}$/ml = 250) was less active than GPC supplied by Miles Laboratories (stated activity of +ve haemolysis at 0.04 ml when used at a 1:10 dilution). This was particularly apparent with test serum samples where maximal levels of myotoxicity were not reached using GPC from Flow Laboratories, e.g. MG 4 (ii) and MG 6 - see Table 7. In both these cases, twice the concentration of Flow Laboratory GPC was necessary to effect approximately similar myotoxicity to that observed using Miles laboratory GPC tested under similar conditions. Subsequently, GPC supplied by Miles Laboratories was routinely used for determination of complement-mediated serum myotoxicity, and unless otherwise stated, all results were obtained using GPC from this source.

2.2.9 Morphological appearance of cultures after treatment with serum plus GPC

Myotubes treated with heat-inactivated myasthenic serum and GPC, causing measured myotoxicity of greater than approximately 50-55%, were clearly grossly damaged when examined by light microscopy (Figures 25 and 26). The normal appearance of myotubes was of a 'plump' morphology, phase-bright and agranular. After treatment with myotoxic myasthenic serum plus GPC, the myotubes appeared shrunken, phase-dark and granular showing, in parts, complete fragmentation. Where measured myotoxicity was in the range 60-65%, complete disruption of the myotube monolayer was seen, indicating that this

TABLE 7

COMPARISON OF MYOTOXICITY DETERMINED WITH TWO DIFFERENT

SOURCES OF GPC

Rat myotube cultures were labelled with $[$ Me-^{3}H$]$ carnitine
(section 2.2.1) and exposed to samples of heat-inactivated
serum (80μl) plus GPC (80μl or 160μl) for 3h at 37°C.
Control cultures to which no additions were made were run
simultaneously. At the end of the incubation time, the
cultures were washed and solubilised for counting.
Myotoxicity was calculated as described in the "Methods"
section 8 by comparison with the control cultures. Each
result is the mean $\pm$ SD of 3 different experiments, each
of which included quadruplicate culture wells.

Test serum	Anti-AChR antibody titre (nM)	GPC source	GPC added (ul)	Myotoxicity (%)
MG 4 sample (ii)	42.0	Flow Labs.	[a] 80	40.4 ± 2.2
			[b] 160	46.2 ± 5.5
		Miles Labs.	[c] 80	54.8 ± 2.7
MG 6	16.0	Flow Labs.	[a] 80	38.5 ± 2.0
			[b] 160	50.1 ± 2.0
		Miles Labs.	[c] 80	56.7 ± 2.7
MG 12	180.0	Flow Labs.	[a] 80	56.0 ± 0.8
			[b] 160	62.4 ± 1.8
		Miles Labs.	[c] 80	60.3 ± 2.9

[a] Final concentration of test serum and GPC was 10.8% (v/v)

[b] Final concentration of test serum and GPC was 10.8% (v/v) and 21.6% (v/v) respectively

[c] Final concentration of test serum and GPC was 12.1% (v/v)

FIGURE 25

PHASE CONTRAST PHOTOMICROGRAPHS OF 7-DAY OLD RAT

MYOTUBE CULTURES

A) Culture treated with heat-inactivated normal

serum (80 μl, 12.1% v/v) plus GPC (80 μl,

12.1% v/v) for 3h at 37°C.

(x 80 magnification)

Measured myotoxicity (see "Methods" section 8)

= 9.0%

B) Culture treated with heat-inactivated

myasthenic serum (80 μl, 12.1% v/v) plus

GPC (80 μl, 12.1% v/v) for 3h at 37°C.

(x 80 magnification)

Measured myotoxicity (see "Methods" section 8)

= 52.0%

A

B
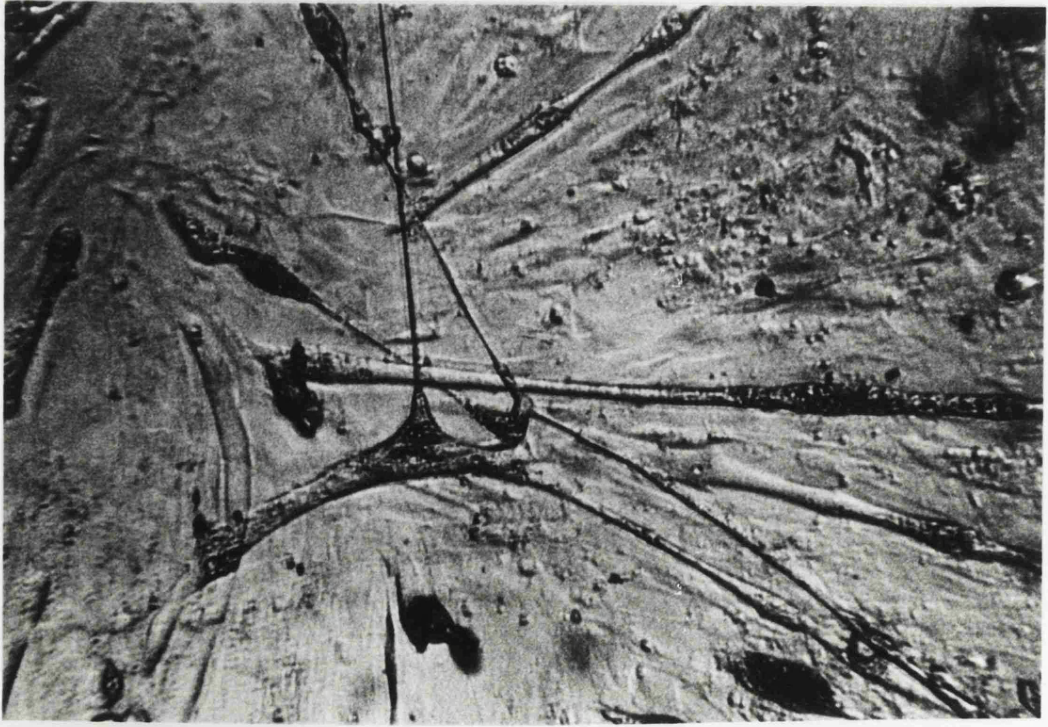
FIGURE 26

PHASE CONTRAST PHOTOMICROGRAPHS OF 7-DAY OLD RAT

MYOTUBE CULTURES

A) Culture treated with heat-inactivated normal

serum (80 μl, 12.1% v/v) plus GPC (80 μl,

12.1% v/v) for 3h at 37°C.

(x 200 magnification)

Measured myotoxicity (see "Methods" section 8)

= 9.0%

B) Culture treated with heat-inactivated

myasthenic serum (80 μl, 12.1% v/v) plus

GPC (80 μl, 12.1% v/v) for 3h at 37°C.

(x 200 magnification)

Measured myotoxicity (see "Methods" section 8)

= 52.0%
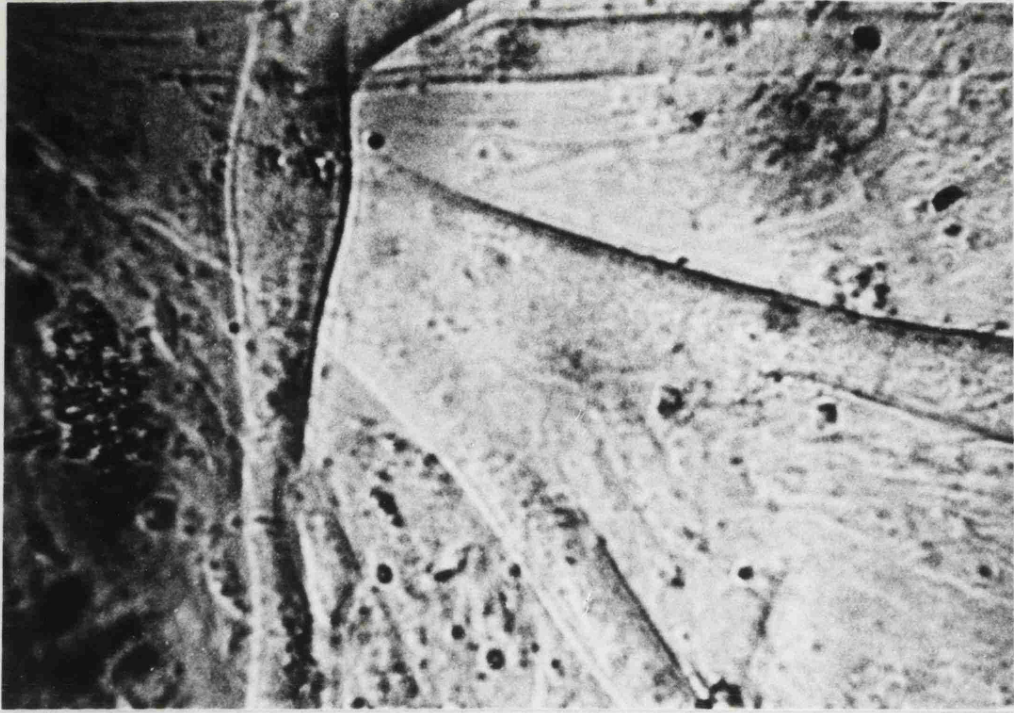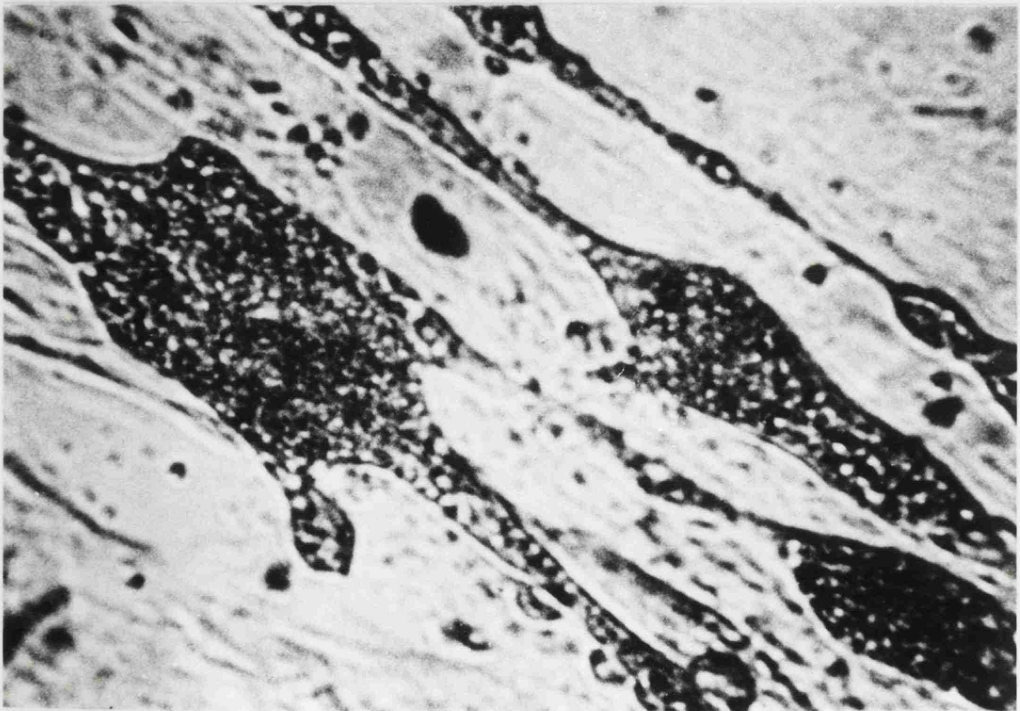
value represented maximum myotoxicity. Where lower levels of myotoxicity were demonstrated (in the range 40-50%) discernible patches of damage to individual myotubes could often be observed.

2.2.10 Toxicity of serum plus GPC to fibroblast cultures

Morphologically, the fibroblasts present in myotube cultures treated with myotoxic myasthenic serum plus GPC appeared unaffected when examined by light microscopy. Fibroblast cultures, prepared as described in the "Methods" section 1.5, were tested alone for the effects of heat-inactivated myasthenic and normal serum plus GPC under the same conditions as used for myotube cultures. As shown in Table 8, the complement-mediated toxicity by heat-inactivated myasthenic or normal serum was less than 17% in contrast to values of 60.5% and 9.1% respectively when the same sera were tested on myotube cultures.

2.2.11 Reduction of $[^{125}I]$∝-BGT binding sites by serum plus GPC

Myotube cultures were tested for their ability to bind $[^{125}I]$ ∝-BGT after treatment with heat-inactivated test serum (80 µl, 12.1% v/v) for 3h at $37^{\circ}C$, with or without the addition of GPC (80 µl, 12.1% v/v). Sets of the same cultures were labelled with [Me-^{3}H] carnitine (section 2.1.1) and tested with the same test serum under the same conditions for assessment of myotoxicity. Table 9 shows that the heat-inactivated myasthenic serum samples MG 4 (ii) and MG 12 tested alone reduced the number of available toxin binding sites by 55.0% and 64.0% respectively, indicating the blockade or loss of AChR on the myotubes in culture. No parallel myotoxicity was present. On addition of complement, relatively few $[^{125}I]$∝-BGT binding sites were available (76.4% and 89.2% reduction from control respectively) parallelled by

TABLE 8

TOXICITY OF HEAT-INACTIVATED SERUM PLUS COMPLEMENT TO

FIBROBLAST CULTURES

Replicate rat skin fibroblast cultures (tested over 6/7

days in culture) were labelled with $[Me-^3H]$ carnitine

(section 2.2.1) and exposed to aliquots of heat-

inactivated normal or myasthenic serum (80μl, 12.1% v/v)

plus GPC (80μl, 12.1% v/v) for 3h at 37°C. Control

cultures to which no additions were made were run

simultaneously. At the end of the incubation time, the

cultures were washed and solubilised for counting.

Toxicity was calculated as described in the "Methods"

section 8 by comparison with the control cultures. In

parallel experiments, replicate myotube cultures were used

to measure complement-mediated serum myotoxicity under

the same conditions using the same test sera. Each result

is the mean $\pm$ SD of 4 replicate culture wells.

Culture	Test	cpm retained (mean $\pm$ SD)	Myotoxicity (%)
Fibroblast	Control	3654 ± 216	0
	MG serum + GPC	3036 ± 147	16.9
	Normal serum + GPC	3081 ± 204	15.7
Myotube	Control	12116 ± 1110	0
	MG serum + GPC	4788 ± 284	60.5
	Normal serum + GPC	11018 ± 216	9.1

TABLE 9

EFFECT OF HEAT-INACTIVATED SERUM PLUS COMPLEMENT ON THE NUMBER OF $[^{125}I]\alpha$-BGT BINDING SITES

Rat myotube cultures (7 days in culture) were exposed to samples of heat-inactivated myasthenic or normal serum (80μl, 12.1% v/v) with or without added GPC (80μl, 12.1% v/v) for 3h at 37°C. At the end of the incubation time, the cultures were washed and the number of remaining $[^{125}I]\alpha$-BGT binding sites determined as described in the "Methods" section 3 (20nM toxin concentration). Results are expressed as a percentage of $[^{125}I]\alpha$-BGT binding to control cultures to which no additions were made. Parallel experiments were carried out on replicate cultures to determine complement-mediated serum myotoxicity under the same conditions using the same test sera. Each addition of serum $\pm$ GPC was tested on 4 or 8 replicate culture wells for myotoxicity or $[^{125}I]\alpha$-BGT binding experiments respectively.

Test serum	Anti-AChR antibody titre (nM)	GPC	$[^{125}I]\alpha$-BGT binding (% control)	Myotoxicity (%)
None	–	–	100	0
Normal	–	–	[a] 86.0 ± 3.2	9.3 ± 6.7
		+	[a] 93.9 ± 4.6	6.9 ± 4.4
MG 4 [b](i)	12.2	–	89.0	4.3
		+	87.0	19.5
(ii)	42.0	–	45.0	8.7
		+	23.6	57.5
MG 12	180.0	–	36.0	10.5
		+	10.8	64.3

[a] Results from 3 different experiments

[b] Serial samples taken at different times from the same patient

high myotoxicity (57.5% and 64.3% respectively). Treatment with normal

serum or MG 4 (i) serum (of a relatively lower anti-AChR antibody

titre - Table 9) resulted in similar small reductions of [^{125}I]α-BGT

binding (14.0% and 11.0% respectively) which was not enhanced on

addition of complement. The measured myotoxicity of MG 4 (i) in the

presence of complement showed a small increase to 19.5%.

2.2.12 Effects of culture variation on myotoxicity measurement

The variation in growth within replicate myotube cultures,

judged morphologically, by [^{125}I]α-BGT binding and by retention of

[Me-^{3}H] carnitine, was small but greater variation could occur between

myotube cultures established at different times (see sections 1.8 and

2.2.1). The effect of variation on measured myotoxicity was tested by

establishing replicate myotube cultures at varying initial cell

densities (see section 1.1). Table 10 illustrates the results obtained

when the effects of normal and myasthenic sera were tested in the

complement-mediated myotoxicity assay on these cultures. The

myotoxicity values determined from cultures of high or medium density

(61.2 and 39.0 fmol [^{125}I]α-BGT binding sites respectively) myotube

cultures were comparable. However, a low density culture, with sparse

non-interacting myotubes, gave no measurable evidence of myotoxicity.

The results confirmed that where a low density of myotubes was

present, specific release of radioactivity could not be detected,

being only a small proportion of the total radioactivity retained by

the cells in culture (contributed to by the fibroblasts present and

any non-specific retention of radioactivity).

The precision of measurement of [Me-^{3}H] carnitine retention,

after treatment with serum, was calculated from values gained for GPC

TABLE 10

COMPLEMENT-MEDIATED MYOTOXICITY BY HEAT-INACTIVATED

SERUM TO RAT MYOTUBE CULTURES INITIATED AT DIFFERENT

CELL DENSITIES

Rat myotube cultures were established after initiation

at different cell densities. The cultures were labelled

with L-[Me-^{3}H] carnitine (section 2.2.1) and the

complement-mediated serum myotoxicity assay carried out

as described in Table 9. The same heat-inactivated

myasthenic and normal serum samples were used on each

type of culture. Each result is the mean $\pm$ SD of 4

replicate culture wells. [^{125}I]α-BGT binding to the

cultures was determined as described in the "Methods"

section 3 (at 20nM toxin concentration).

Cell density ($\times 10^{-5}$/well)	$[^{125}I]$ α-BGT bound (fmol/well)	Test serum	GPC	$[Me-^3H]$ carnitine retained (cpm)	Myotoxicity (%)
2.50	61.2	None	-	11530 ± 940	0
		MG	-	10590 ± 579	8.2
			+	4555 ± 220	60.5
		Normal	-	9817 ± 453	14.9
			+	10739 ± 320	6.9
1.25	39.0	None	-	7881 ± 725	0
		MG	-	7535 ± 1109	4.4
			+	2755 ± 115	65.0
		Normal	-	6683 ± 550	15.2
			+	6801 ± 332	13.7
0.50	9.8	None	-	2818 ± 113	0
		MG	-	3001 ± 171	- 6.5
			+	2690 ± 235	4.5
		Normal	-	3143 ± 607	-11.5
			+	2733 ± 208	3.0

tested alone in the myotoxicity study (section 2.2.13). Retention of radioactivity was in the range 84.9% - 102.2% of replicate cultures to which no additions were made (control cultures). The mean value was 93.0 ± 4.6% (12 different experiments), mean ± SD (n).

2.2.13 Myotoxicity study

A standardised myotoxicity assay was used to study the effects of a range of normal and myasthenic sera on rat myotube cultures labelled with [Me-^{3}H] carnitine (section 2.1.1). For economical use of both test serum and GPC, myotube cultures were tested in quadruplicate with 80µl heat-inactivated test serum (12.1% v/v) with or without added GPC (80 µl, 12.1% v/v) for 3h at 37°C. Control cultures, to which no additions were made (0.66 ml growth medium only), were run simultaneously. Within each experiment, the effects of GPC (80µl, 12.1% v/v), added alone, were tested on cultures in quadruplicate. The tests were set out on each 24-well culture plate as illustrated in Figure 21 and myotoxicity was calculated as described in the "Methods" section 8.

Using the conditions described, the complement-mediated myotoxicity of serum samples from 12 normal and 13 myasthenic subjects was assessed. In addition, serum samples from 6 patients with polymyositis (chosen as an example of muscle degenerative disease) were tested for complement-mediated myotoxicity as described. GPC serum tested alone showed a variation between different muscle cultures that fell within the range -2.2 - 15.1% (mean ± SD, n, = 7.0 ± 4.6% (12 experiments). Heat-inactivated normal sera, with or without added GPC, gave similar myotoxicity values (Table 11 and Figure 27) indicating that values of up to 20% could be regarded as falling

within the normal range. Heat-inactivated myasthenic sera without

added GPC caused measured myotoxicity well within the normal range. In

the presence of added complement, heat-inactivated serum samples from

the 13 myasthenic patients in the study showed myotoxicity values

within the range 7.6 - 65.0% with a mean value of 41.9% (Table 11 and

Figure 27). The data for individual sera are shown in Table 12 where

it can be seen that 9 out of 13 different myasthenic patients have

sera with myotoxicity values that are clearly abnormal, as defined

above. Serum from each of the 6 patients with polymyositis caused

myotoxicity within the normal range (Tables 11 and 12, Figure 27).

Statistical comparison of complement-mediated myotoxicity caused by

different groups of subjects showed that the values given by normal or

polymyositis sera were significantly lower than the values obtained

for myasthenic sera (Mann-Whitney rank sum test, p = 0.01).

The measured complement-mediated myotoxicity by myasthenic

sera did not correlate with the anti-AChR antibody content of the

serum samples. However, it was notable that myasthenic serum samples

with myotoxicity values within the normal range tended to have

relatively low anti-AChR antibody titres (i.e. less than 20 nM).

Nevertheless some serum samples demonstrating high myotoxicity also

had lower antibody levels.

2.2.14 Myotoxicity by previously 'non-myotoxic' myasthenic serum

When the standardised complement-mediated myotoxicity assay

was carried out (see previous section), no significant myotoxicity

values were given by five myasthenic serum samples, notably those of

lower anti-AChR antibody titre (Table 12). Using myotoxic serum

(values outside normal range) measured myotoxicity was found to be

TABLE 11

MYOTOXICITY OF MYASTHENIC, POLYMYOSITIS AND NORMAL SERA

TESTED WITH RAT MYOTUBES IN CULTURE

The myotoxicity of test samples was determined as described in section 2.2.13. Unless otherwise stated, serum samples were tested with 3 different myotube cultures and the mean values taken.

Test serum	Number of donors	Complement	Myotoxicity (%)	
			Range	Mean $\pm$ SD
None	12	+	- 2.2 - 15.1	7.0 $\pm$ 4.6
Normal	12	-	- 0.3 - 16.4	9.2 $\pm$ 4.7
	12	+	3.5 - 14.5	10.3 $\pm$ 3.4
Myasthenia	13 [a]	-	- 7.7 - 11.4	3.8 $\pm$ 4.6
gravis	13 [a]	+	7.6 - 65.4	41.9 $\pm$ 22.4
Polymyositis	6 [b]	+	7.8 - 19.1	13.5 $\pm$ 4.6

[a] Multiple samples were taken at different times from 3 myasthenic patients (see Table 12). The mean values from the multiple samples were taken as the value for each patient.

[b] Serum samples from polymyositis patients were tested in one (3) or two (3) myotube cultures.

FIGURE 27

MYOTOXICITY OF NORMAL, MYASTHENIC AND POLYMYOSITIS
SERA TESTED WITH RAT MYOTUBES IN CULTURE
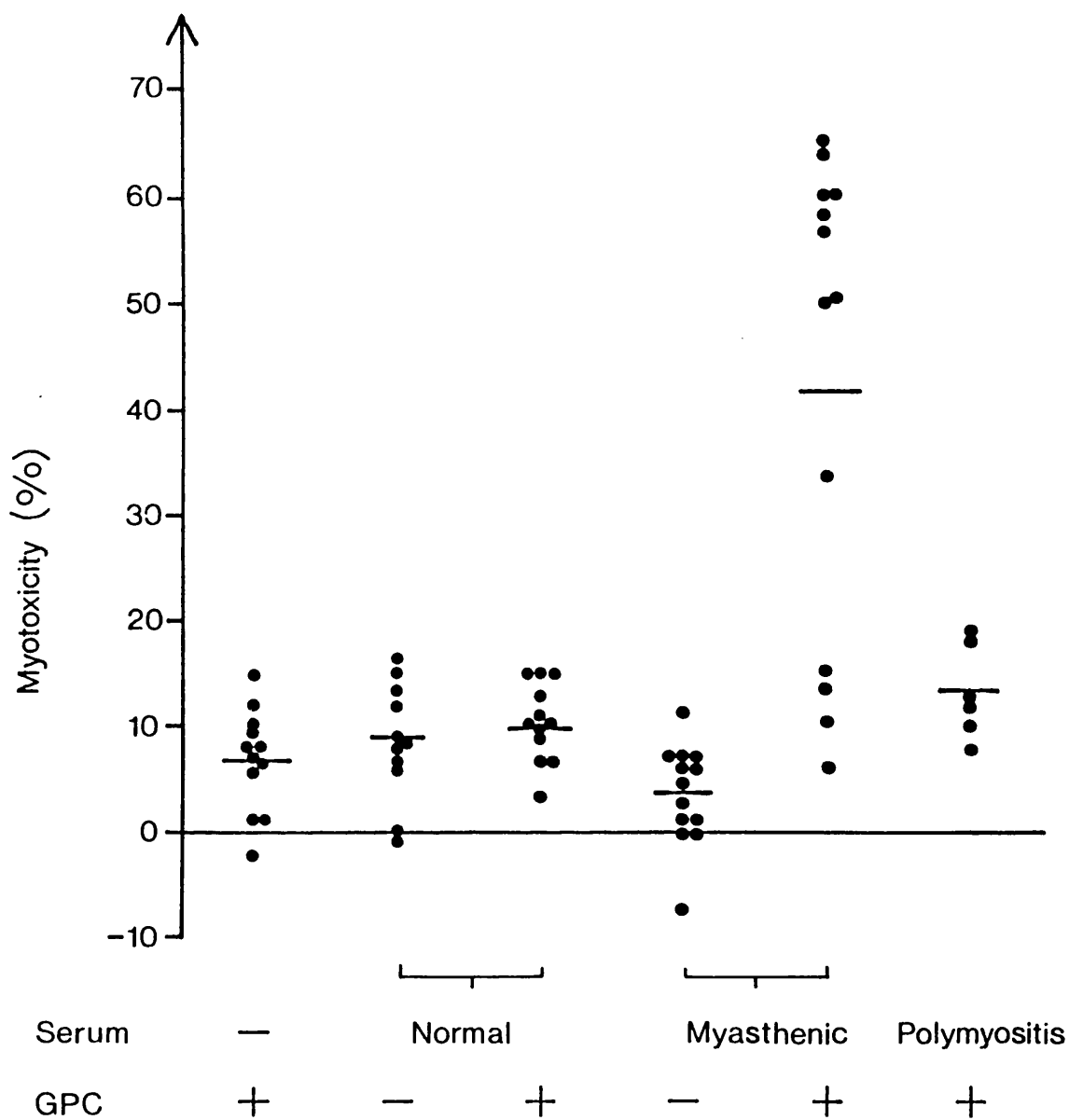
The data summarised in Table 11 are shown
graphically for each donor of serum.
The bar lines represent the mean value for
each group of serum samples tested.

TABLE 12

MYOTOXICITY OF INDIVIDUAL MYASTHENIC, POLYMYOSITIS AND
NORMAL SERUM SAMPLES IN THE PRESENCE OF COMPLEMENT

The myotoxicity of test samples was determined as
described in section 2.2.13 and the results summarised
in Table 11 and Figure 27.

Test serum	Donor		Myotoxicity (%) (mean $\pm$ SD)	Anti-AChR antibody titre (nM)
Myasthenia gravis	1		7.6 ± 6.2	0.8
	2		15.7 ± 4.2	4.2
	3		13.9 ± 1.6	9.2
	4 [a]	(i)	11.7 ± 7.5	12.2
		(ii)	54.8 ± 2.7	42.0
	5		63.9 ± 3.2	14.8
	6		56.7 ± 5.4	16.0
	7		10.8 ± 0.0	16.3
	8		60.3 ± 2.1	24.2
	9 [a]	(i)	59.8 ± 0.5	25.6
		(ii)	38.6 [b]	42.3
	10		65.0 ± 1.2	72.1
	11		58.4 ± 0.7	72.5
	12		60.3 ± 2.9	180.0
	13 [a]	(i)	62.3 ± 1.9	5.8
		(ii)	43.2 ± 1.6	12.9
		(iv)	65.4 ± 2.9	17.2
		(v)	47.0 ± 3.5	20.6
		(vi)	52.0 ± 5.4	28.5
		(viii)	30.7 ± 0.9	43.8

Test serum	Donor	Myotoxicity (%) (mean $\pm$ SD)	Anti-AChR antibody titre (nM)
Polymyositis	1	12.7 [b]	–
	2	7.8 $\pm$ 4.2 [c]	–
	3	12.3 $\pm$ 5.6 [c]	–
	4	19.1 $\pm$ 1.1 [c]	–
	5	18.7 [b]	–
	6	10.2 [b]	–
Normal	1	11.3 $\pm$ 6.9	–
	2	9.3 $\pm$ 5.9	–
	3	6.9 $\pm$ 4.4	–
	4	6.8 $\pm$ 6.9	–
	5	14.2 $\pm$ 8.7	–
	6	9.5 $\pm$ 6.0	–
	7	9.8 $\pm$ 1.8	–
	8	12.9 $\pm$ 3.1	–
	9	14.2 $\pm$ 3.2	–
	10	3.5 $\pm$ 7.9	–
	11	14.5 $\pm$ 3.4	–
	12	10.5 $\pm$ 6.7	–

[a] Repeat samples were taken over 17 months (patient 9), 27 months (patient 4) and 3 years (patient 13).

[b] One assay only

[c] Two assays only - value given is mean $\pm$ range

All other values represent the mean $\pm$ SD of 3 different experiments.

dependent on the amount of heat-inactivated serum and GPC added to the cultures (see Section 2.2.7). It was therefore apparent that each serum sample could require optimal concentrations of GPC and serum for maximum myotoxicity to be observed. Myasthenic sera shown to be non-myotoxic under the standard assay conditions (Table 13) were therefore tested for complement-mediated myotoxicity after increasing the amounts of serum and GPC added to the incubation medium (Figure 28). The total volume of growth medium and tests added to each culture was increased to 1.5 ml as it was noted that a high concentration of added serum (i.e. greater than 40%) caused myotubes to become detached from the culture plate. At higher added volumes of GPC and serum (additions 2 and 3, Figure 28), the measured myotoxicity increased in 3 of the 4 myasthenic samples tested. At the highest volume used, myotoxicity was evident when viewed through the light microscope. One of the myasthenic serum samples tested, notably of the lowest anti-AChR antibody titre (from patient MG 19), showed no increase in measured myotoxicity, remaining within normal values.

Manipulation of myotoxicity assay conditions as described did not alter the measured myotoxicity by normal sera plus GPC (values below 18%, see Figure 30) or by normal, myasthenic or GPC serum tested alone (all values below 20% - data not shown). The measured myotoxicity by the control myasthenic serum (MG 13 v) was also not altered at the two lowest additions used (see Table 13 and Figure 28).

TABLE 13

COMPLEMENT-MEDIATED MYOTOXICITY BY MYASTHENIC SERA

OF RELATIVELY LOW ANTI-AChR ANTIBODY TITRE

The complement-mediated myotoxicity of heat-inactivated myasthenic serum samples of relatively low anti-AChR antibody titre was determined by using the standard conditions of assay (section 2.2.13). The same serum samples were subsequently used in myotoxicity experiments in which the conditions of assay were altered (see Figure 28).

Test serum	Donor	Myotoxicity (%)	Anti-AChR antibody titre (nM)
Myasthenia	4 (i)	11.7 ± 7.5 [a]	12.2
gravis	19	9.8 [b]	3.6
	20	8.4 [b]	5.1
	21	14.1 [b]	8.4
[c]	13 (v)	47.0 ± 3.5 [a]	20.6

[a] mean $\pm$ SD of 3 different experiments

[b] one assay only

[c] serum sample used as a control (see Figure 28)

FIGURE 28

COMPLEMENT-MEDIATED MYOTOXICITY BY PREVIOUSLY

'NON-MYOTOXIC' MYASTHENIC SERA

Replicate rat myotube cultures were labelled with

$[Me-^3H]$ carnitine as described in Section 2.2.1.

Increasing volumes of heat-inactivated myasthenic

serum (shown to be 'non-myotoxic' when tested under

standard myotoxicity conditions - see Table 13) plus

complement were added to the cultures as follows:-

Addition	Tests			
	Serum added		GPC added	
	(µl)	(% v/v)	(µl)	(% v/v)
1	80	5.3	80	5.3
2	160	10.7	160	10.7
3	240	16.0	240	16.0

The cultures were incubated for 3h at $37^{\circ}C$. Control

cultures to which no additions of test serum or GPC

were made (1.5 ml of growth medium only) were run

simultaneously. At the end of the incubation time,

the cultures were washed and solubilised for counting.

Myotoxicity was calculated as described in the "Methods"

section 8 by comparison with the control cultures.

Each addition of test serum and GPC was tested on 4

culture wells.

148
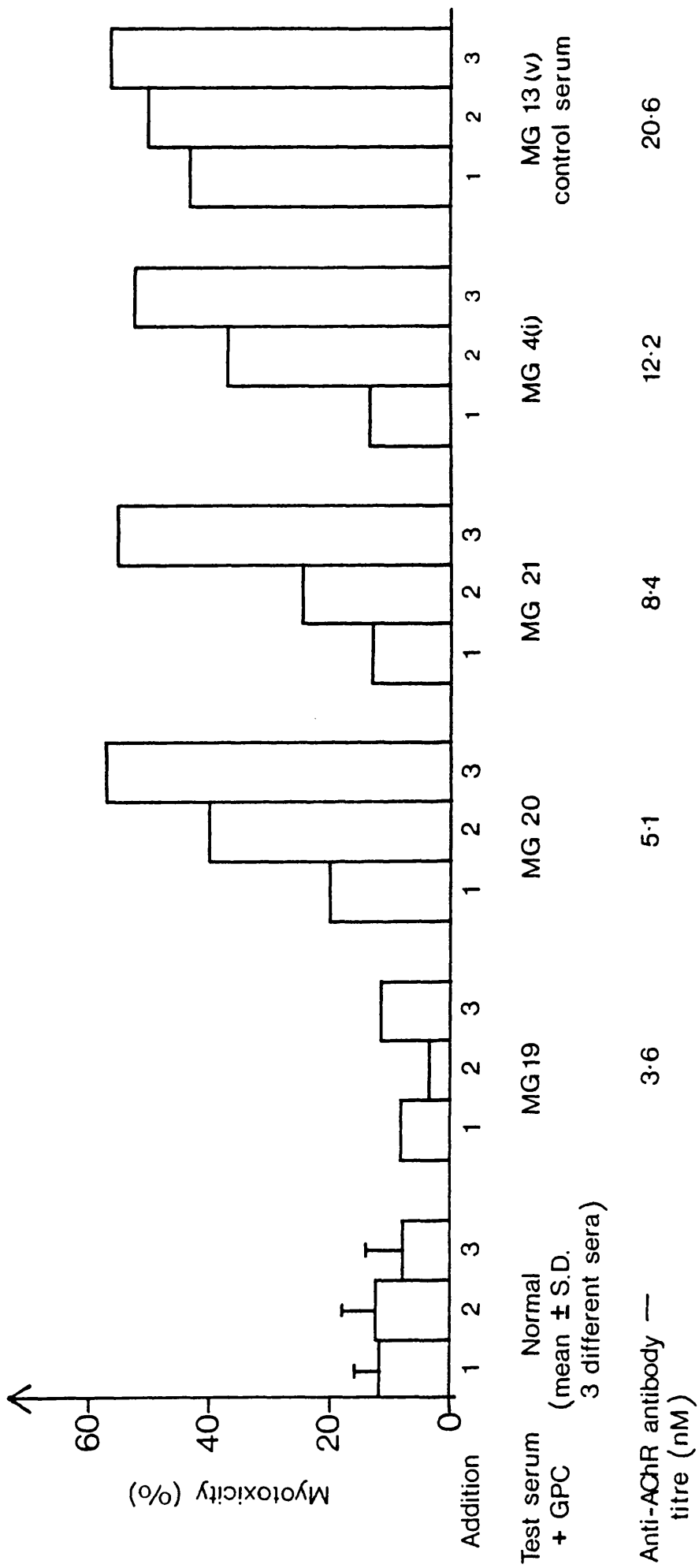
2.2.15 Effects of anti-AChR antiserum raised against purified AChR in

    animals

        The complement-mediated myotoxicity of antiserum raised

against purified AChR in animals was tested on rat myotubes in culture

by using the experimental conditions described in Section 2.2.13

(Table 14). Heat-inactivated rabbit anti-foetal calf AChR antiserum

and rabbit anti-rat junctional AChR antiserum caused a measured

myotoxicity of 48.2% and 37.5% respectively in the presence of

complement. The values obtained for the heat-inactivated antisera

tested alone were 11.8% and 4.5% respectively. Normal rabbit serum

tested alone, before and after heat-inactivation, produced myotoxicity

of 6.2% and 5.4% respectively. This serum was stored at -20°C for over

a year before use. The myotoxicity value obtained before

heat-inactivation, was in sharp contrast to the same serum used fresh

(as a source of complement) in previous myotoxicity studies when the

serum was clearly toxic (see section 2.2.5). The addition of

complement to the heat-inactivated serum did not restore the

previously observed toxicity (myotoxicity value of 16.4% - Table 6).

2.3 Depletion of anti-AChR antibodies from myasthenic serum

2.3.1 Preparation of affinity columns

    Detergent extracts of human muscle (49.5 - 210 pmol [^{125}I]α-BGT

binding sites at a concentration of 0.45 - 1.3 pmol/ml extract) were

applied as a batch to α-BGT-Sepharose 4B as described in the "Methods"

section 14.2. The amount of AChR coupled to the α-BGT-Sepharose 4B was

determined by assay of the supernatant and washings for AChR content

TABLE 14

COMPLEMENT-MEDIATED MYOTOXICITY BY ANTISERUM RAISED

AGAINST PURIFIED AChR IN ANIMALS

Rat myotube cultures were labelled with L-$[$Me-^{3}H$]$ carnitine

(section 2.2.1) and exposed to samples of serum (80µl,

12.1% v/v) with or without added GPC (80µl, 12.1% v/v)

for 3h at 37°C. Control cultures to which no additions

were made were run simultaneously. Myotoxicity was

calculated as described in the "Methods" section 8 by

comparison with the control cultures. Each result is the

mean $\pm$ range of 2 different experiments, each of which

included quadruplicate culture wells.

Test serum	Anti-AChR antibody titre (nM)	Treatment	GPC	Myotoxicity (%)
Normal rabbit	–	None	–	6.2 $\pm$ 3.4
		HI	–	5.4 $\pm$ 4.6
		HI	+	16.4 $\pm$ 3.2
Rabbit anti-foetal	700 [a]	HI	–	11.8 $\pm$ 7.7
calf AChR		HI	+	48.2 $\pm$ 4.9
Rabbit anti-rat	105 [b]	HI	–	4.5 $\pm$ 5.7
junctional AChR		HI	+	37.5 $\pm$ 1.8
Sheep anti-	1713 [c]	HI	–	9.9 $\pm$ 3.2
Torpedo AChR		HI	+	5.3 $\pm$ 7.1
None	–	None	+	6.8 $\pm$ 7.8

[a] Determined using crude detergent extract of foetal calf AChR
[b] Determined using crude detergent extract of rat junctional AChR
[c] Determined using purified Torpedo AChR

by ammonium sulphate precipitation (see "Methods" section 12.2). The

highest specific binding obtained was 8.2 pmol AChR/ml affinity beads

(Table 15).

2.3.2 Depletion of anti-AChR antibodies

The affinity columns were equilibrated with phosphate

buffered saline (PBS), pH 7.2, as described in the "Methods" section

14.2. Use of PBS, pH 7.2, containing 1% (v/v) Triton X-100, to

equilibrate and wash the affinity column, resulted in the presence of

Triton X-100 in serum fractions passed through the column. Even after

extensive dialysis of the resulting serum fractions, the Triton X-100

present was observed to solubilise the myotube cultures when

subsequent myotoxicity assays were carried out.

Heat-inactivated myasthenic serum (MG 4 (ii), 2 ml, containing

84 pmol of anti-AChR antibody) was applied to the AChR-∝-BGT-Sepharose

4B column as described in the "Methods" section 14.3. Maximum

depletion of anti-AChR antibody was achieved by use of the highest

density AChR-∝-BGT-Sepharose 4B column (column 3, 8.2 pmol AChR/ml

beads). Table 16 gives the results obtained, indicating a maximum

depletion of 67.9%. The myasthenic serum was recirculated through this

affinity column for 2h at 23°C (see "Methods" section 14.3). After

collection, a further sample of the same serum was applied to the

column and recirculated overnight at 23°C. Further anti-AChR antibody

depletion was achieved (Table 16) indicating that the AChR on the

affinity column was not saturated and that a longer application time

could be beneficial. Passage of the same myasthenic serum (2 ml)

through a control ∝-BGT-sepharose 4 B column with no coupled AChR

resulted in little loss of anti-AChR antibody (Table 16). The recovery

TABLE 15

PREPARATION OF AChR -α BGT - SEPHAROSE 4B AFFINITY COLUMN

Prep.	AChR applied (pmol)	[a]αBGT-Sepharose 4B (ml)	AChR bound (%)	Specific AChR binding (pmol/ml beads)
1	49.5	25	100	2.0
2	110.0	25	100	4.1
3	210.0	25	97.1	8.2

[a] 0.04 mg α-BGT / ml Sepharose 4B

TABLE 16

DEPLETION OF ANTI-AChR ANTIBODIES FROM MYASTHENIC SERUM ON THE AFFINITY COLUMN

Column	a AChR bound (pmol)	b Anti-AChR applied (pmol)	a Anti-AChR antibody content of effluent (pmol)	Anti-AChR antibodies removed (%)	Anti-AChR antibody titre of concentrated effluent (nM)
1	49.5	84.0	54.0	35.7	27.0
2	110.0	84.0	37.6	55.2	18.8
3	204.0	84.0	27.0	67.9	13.5
re-applied -		84.0	57.3	31.8	28.6
Control	-	84.0	81.6	3.0	40.8

Each value is the mean of 2 (a) or 3 (b) separate determinations

of protein and serum IgG after passage of serum through the affinity column 3 and the control column is given in Table 17. The levels of IgG were apparently unchanged.

2.3.3 Purification of anti-AChR antibody

The adsorbed anti-AChR antibody from application of heat-inactivated myasthenic serum (4 ml) to the AChR-α-BGT-Sepharose 4B column was eluted with 2M KI as described in the "Methods" section 14.4 (Figure 29). Measurement of anti-AChR antibody content of the pooled and concentrated fractions indicated a recovery of 18.7% (Table 18).

2.3.4 Myotoxicity by anti-AChR antibody depleted serum

The complement-mediated myotoxicity of the anti-AChR depleted, heat-inactivated serum samples (affinity column 3, Table 16) was assessed on rat myotube cultures, labelled with [Me-^{3}H] carnitine (section 2.2.1). The effects of increasing concentrations of serum (20 - 80 µl, 2.7% - 10.8% v/v) in the presence of a constant concentration of GPC (Flow Laboratories, 160 µl, 21.6% v/v) were tested (Figure 30). Serum depleted of anti-AChR antibody by 67.9% (final anti-AChR antibody titre of 13.5 nM) gave myotoxicity values, for each serum concentration, within the normal range expected (i.e. less than 20%, see section 2.2.13). The same serum before treatment (anti-AChR antibody titre of 42.0 nM) or after treatment with the α-BGT-Sepharose 4B column to which no AChR was coupled (anti-AChR antibody titre of 40.8 nM) gave, at each concentration, myotoxicity values greater than 40%. The second serum sample applied overnight to the affinity column (see Table 16), showing anti-AChR antibody depletion of 31.8% (final

TABLE 17

RECOVERY OF PROTEIN AND IgG AFTER PASSAGE OF MYASTHENIC
SERUM THROUGH AFFINITY COLUMN 3

Serum treatment	Protein (mg)	Recovery (%)	IgG (mg)	Recovery (%)
Before column	92.0	-	6.16	-
Application 1	77.4	84.1	6.16	100
Application 2	87.4	95.0	6.16	100
Control column	81.4	88.5	6.16	100

TABLE 18

PURIFICATION OF ANTI-AChR ANTIBODIES FROM MYASTHENIC
SERUM

[a] Anti-AChR applied (pmol)	[a] Anti-AChR bound (pmol)	Anti-AChR eluted		[c] IgG (mg)
		[b] (pmol)	recovery (%)	
168.0	83.7	15.7	18.7	0.57

[a] see Table 16   [b] mean of 2 separate determinations
[c] estimated from $A_{1cm}^{1\%} = 14.0$ for IgG at 280 nM

FIGURE 29

ELUTION PROFILE FROM THE AChR – αBGT – SEPHAROSE 4B AFFINITY COLUMN 3
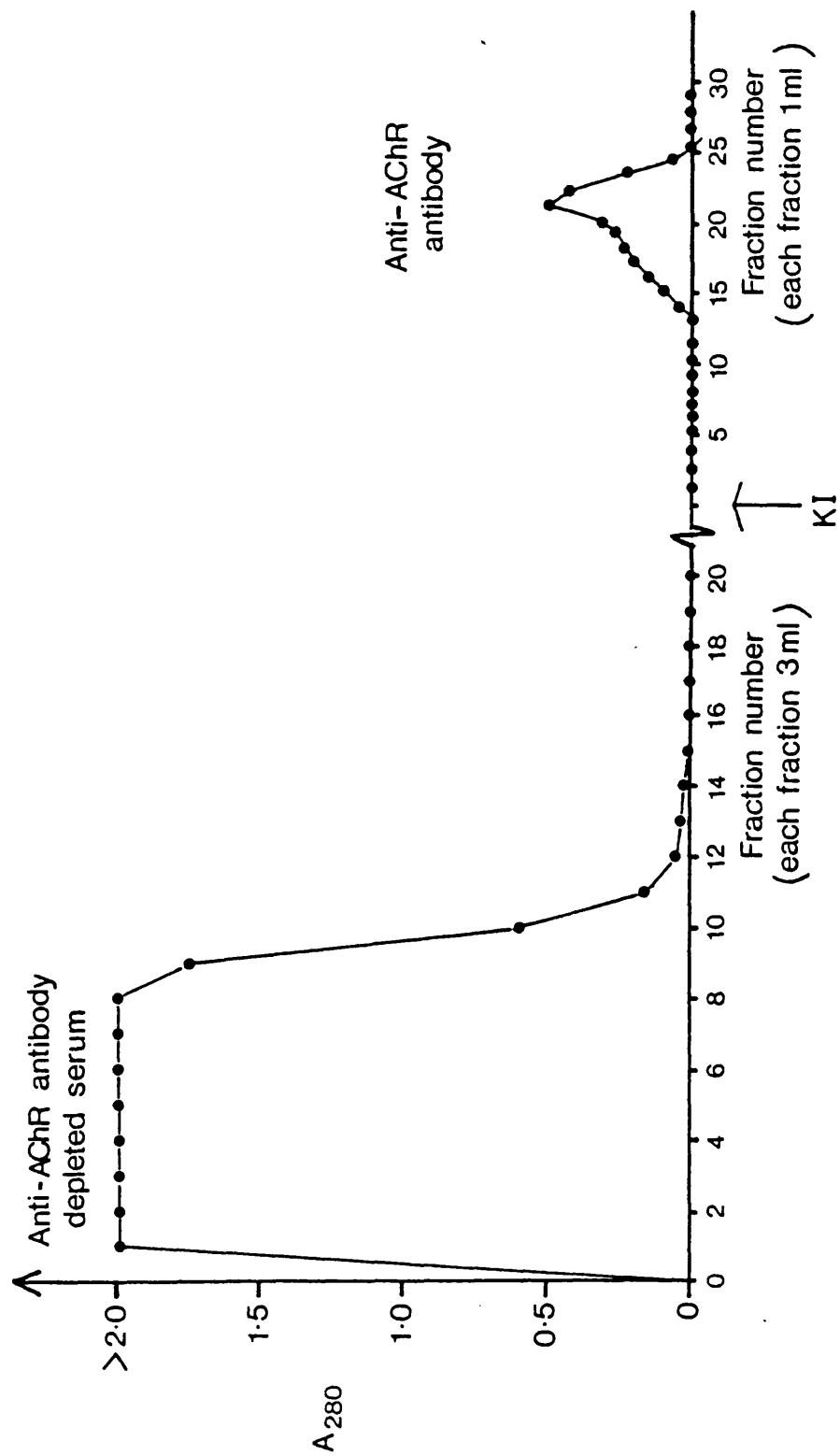
FIGURE 30

COMPLEMENT-MEDIATED MYOTOXICITY BY ANTI-AChR ANTIBODY

DEPLETED SERUM

Replicate rat myotube cultures were labelled with

$[$ Me-^{3}H$]$ carnitine as described in Section 2.2.1. The

complement-mediated myotoxicity of heat-inactivated

myasthenic serum before (●) and after (O) anti-AChR

antibody depletion on an AChR-αBGT-Sepharose 4B

affinity column (column 3, Table 16) was tested by

exposing cultures to aliquots of increasing

concentrations of serum (20-80μl, 2.7-10.8% v/v) in the

presence of GPC (Flow Labs. 160μl, 21.6% v/v) for 3h

at 37°C. Myasthenic serum treated with a control

αBGT-Sepharose 4B column with no coupled AChR (□) or

applied to the previously used column 3   (■)

(see Table 16) were similarly tested. Myotoxicity was

calculated as described in the "Methods" section 8 by

comparison to control cultures with no additions run

simultaneously. Each point is the mean $^+_-$ range of 2

separate experiments, each of which included 4 replicate

culture wells.
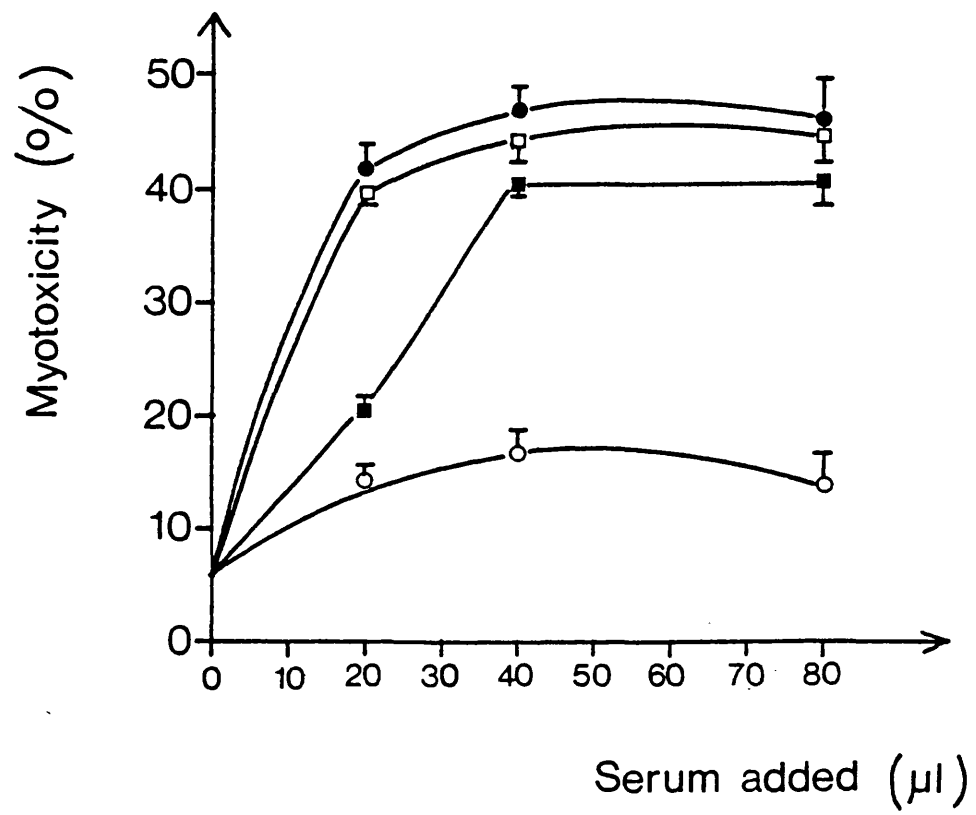
anti-AChR antibody titre of 28.6 nM), demonstrated reduced myotoxicity

only at the lowest concentration of serum tested (Figure 30).

2.3.5 Myotoxicity by purified anti-AChR antibody

The complement-mediated myotoxicity of the purified anti-AChR

antibody, prepared as described in Section 2.3.3, was tested on rat

myotube cultures. The final anti-AChR antibody titre of the purified

antibody was 15.7 nM. Addition of purified antibody (80 µl, 10.8% v/v)

and GPC (Flow Laboratories, 160 µl, 21.6% v/v) to cultures for 3h at

37°C resulted in a measured myotoxicity of 16.3% (mean of 2 separate

experiments) which fell within the normal range expected (i.e. less

than 20%).

2.3.6 Reduction of [^{125}I]α-BGT binding sites by anti-AChR antibody
depleted serum and purified anti-AChR antibody

Heat-inactivated myasthenic serum, before and after treatment

with the AChR-α-BGT-Sepharose 4B column 3 (Table 16) and the eluted

anti-AChR antibody were tested for their ability to reduce the number

of [^{125}I]α-BGT binding sites on rat myotube cultures. The cultures

were pre-incubated with serum or purified antibody (80 µl, 10.6% v/v)

for 90 min at 37°C before estimation of remaining toxin-binding sites

as described in the "Methods" section 3. Figure 31 shows that

anti-AChR antibody depleted serum samples and purified anti-AChR

antibody reduced the number of available [^{125}I]α-BGT binding sites to

a similar extent as did the non-depleted myasthenic serum.

FIGURE 31

LOSS OF $[^{125}I]\alpha$-BGT BINDING SITES ON RAT MYOTUBE

CULTURES AFTER TREATMENT WITH MYASTHENIC SERUM

DEPLETED OF ANTI-AChR ANTIBODY AND PURIFIED ANTI-AChR

ANTIBODY

Replicate myotube cultures (7 days in culture) were

incubated for 90 min at 37°C with heat-inactivated

myasthenic serum (80μl, 10.6% v/v) before (a) or

after (b,c) treatment with an AChR-αBGT-Sepharose 4B

affinity column (column 3, Table 16). The cultures

were then washed and the remaining $[^{125}I]\alpha$-BGT binding

sites determined as described in the "Methods" section

3 (at 20 nM toxin). Cultures preincubated with purified

anti-AChR antibody eluted from the affinity column

(Table 18) 80μl, 10.6% v/v) were similarly tested. The

heights of the columns represent $[^{125}I]\alpha$-BGT binding as

a percentage of that of control cultures to which no

additions of test serum or purified antibody were made.

The results were gained from one experiment in which

6 replicate culture wells were tested for each serum or

the antibody sample.

Anti–AChR
antibody titre
(nM)
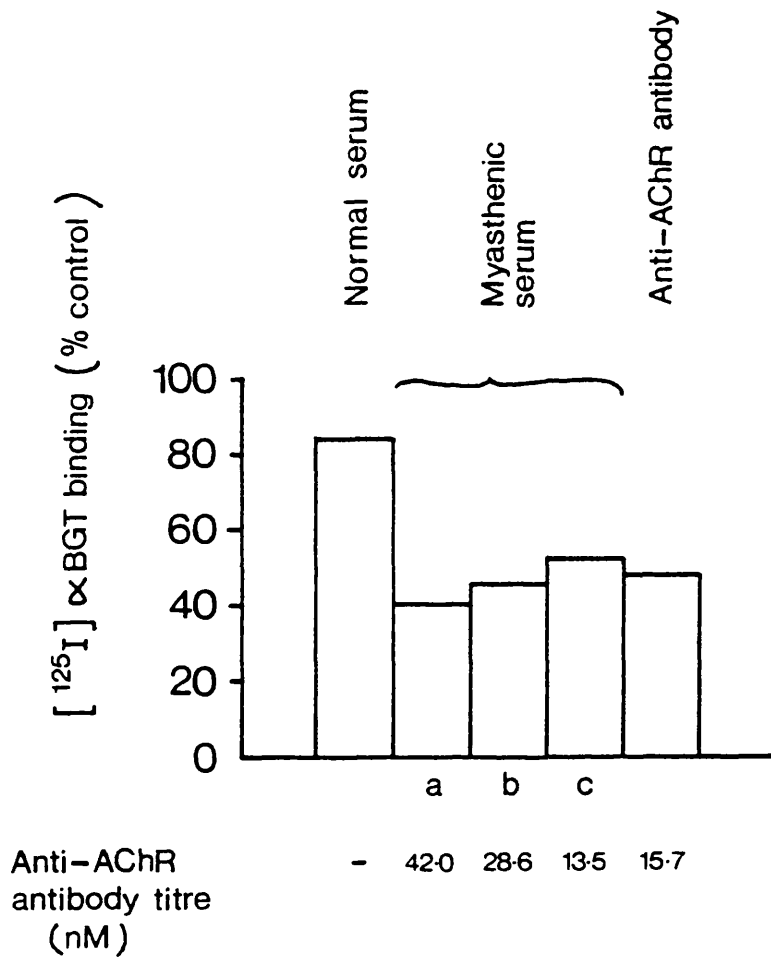
2.4 Effects of myasthenic IgG

2.4.1 Purification of IgG

The IgG fraction was purified from 2 myasthenic and 1 normal

heat-inactivated serum samples (10 ml) as described in the "Methods"

section 15.1. Anti-AChR antibodies were determined in sera before

purification and in the IgG fraction after purification (see "Methods"

section 13). The recoveries of specific antibody and IgG are given in

Table 19.

2.4.2 Depletion of IgG subclass 3 from IgG

Purified IgG (5 ml containing 8.75 - 20 mg) was applied to a

protein-A sepharose column (see "Methods" section 15.2), and the

non-bound fractions containing IgG subclass 3 collected. The bound

IgG, consisting of subclasses 1,2 and 4, was then eluted with 0.1 M

citrate, 0.1 M phosphate buffer, pH 3 (Figure 32). Anti-AChR

antibodies were determined in the IgG fractions, after passage through

the column. The recoveries of specific antibody and IgG are given in

Table 20.

2.4.3 Myotoxicity of IgG and IgG depleted of subclass 3

The complement-mediated myotoxicity of purified IgG and IgG

depleted of subclass 3, was assessed on rat myotube cultures labelled

with L-[Me-^{3}H] carnitine (section 2.2.1). The volumes of IgG

preparations were adjusted so that equivalent concentrations of

anti-AChR antibody to that in each respective serum sample tested,

were added to the cultures. From Figure 33 it can be seen that the IgG

depleted of subclass 3 gave approximately comparable myotoxicity

TABLE 19

RECOVERIES OF ANTI-AChR ANTIBODY AND IgG FROM SERA

Serum [a]	Anti-AChR antibody (pmol) in		IgG (mg) in	
	serum	IgG (% recovery)	serum	· IgG (% recovery)
Normal	N.D.	N.D. (-)	115	100 (87.0)
Myasthenic 1 (patient 4)	420 ± 12	348 ± 6 (82.9)	30.8	17.5 (56.8)
Myasthenic 2 (patient 13)	200 ± 8	93 ± 5 (46.5)	57.5	30.0 (52.2)

N.D. - None detected

[a] Each value is the mean $\pm$ SD of 3 separate determinations

FIGURE 32

ELUTION PROFILE OF IgG AFTER PASSAGE THROUGH A PROTEIN A-SEPHAROSE 4B COLUMN

165
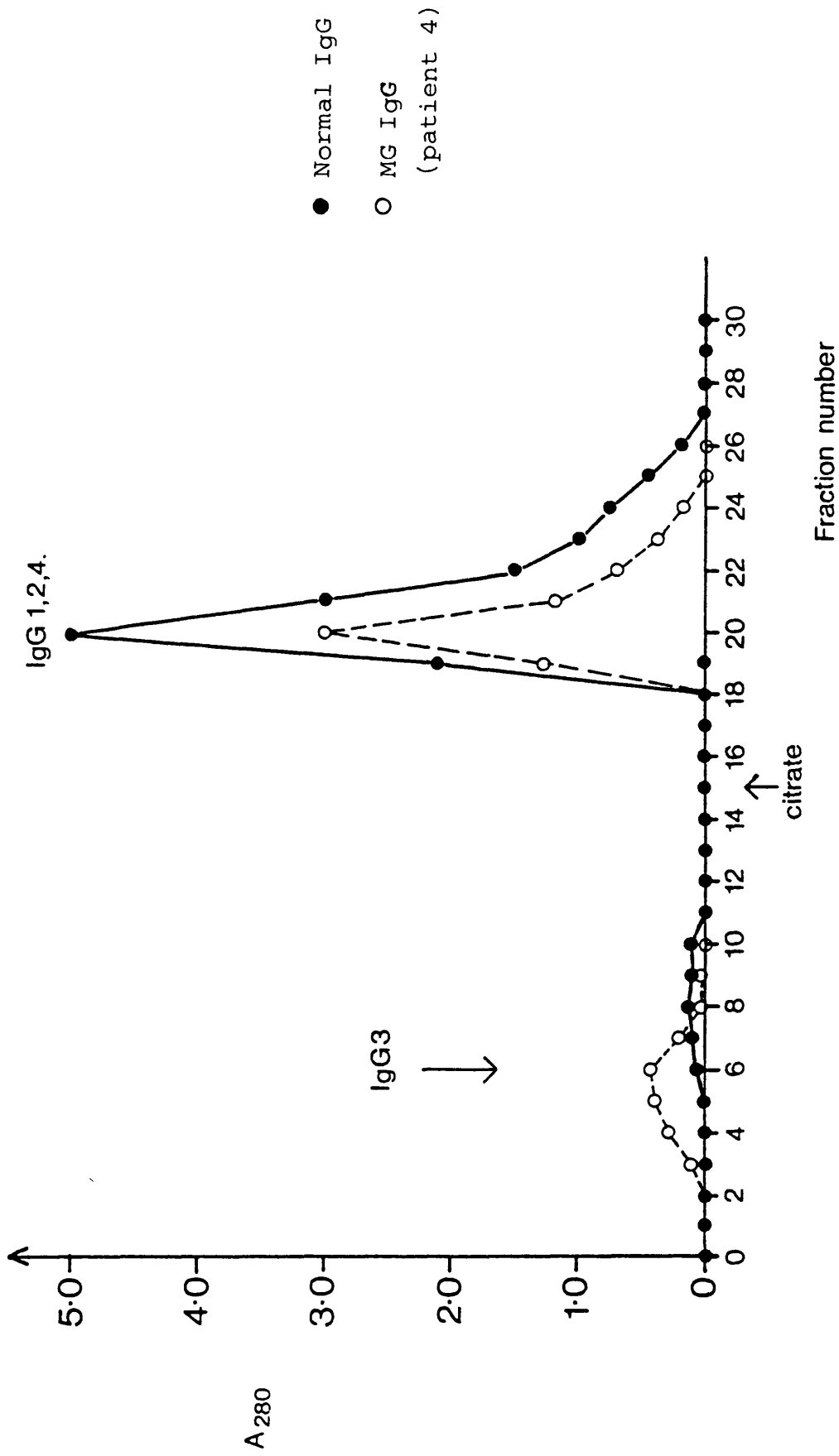
TABLE 20

RECOVERIES OF ANTI-AChR ANTIBODY AND IgG AFTER PASSAGE OF PURIFIED IgG THROUGH A PROTEIN A-SEPHAROSE 4B COLUMN

IgG	Anti-AChR antibody (pmol) in			IgG (mg) in		
	IgG	[a] IgG 1,2,4	[a] IgG 3	IgG	IgG 1,2,4	[b] IgG 3
		(% recovery)			(% recovery)	
Normal	N.D.	N.D. (-)	N.D. (-)	20.0	10.5 (52.5)	0.2 (1.0)
Myasthenic 1 (patient 4)	174.0	118.0 (67.8)	1.1 (0.6)	8.75	5.0 (57.1)	1.5 (17.1)
Myasthenic 2 (patient 13)	46.5	39.0 (83.9)	1.5 (3.2)	15.0	13.75 (91.7)	0.15 (1.0)

N.D. - None detected

[a] Each value is the mean of 2 separate determinations

[b] Estimated from $A_{1cm}^{1\%} = 14.0$ for IgG at 280 nM

values to those obtained from the respective IgG preparations.
However, the myotoxicity demonstrated by one of the preparations of
IgG was considerably reduced when compared to that shown by its
respective serum sample (Figure 33a). In the above experiments, the
complement-mediated myotoxicity values for heat-inactivated normal
serum, IgG and IgG depleted of subclass 3, added at equivalent volumes
to the highest of those for the myasthenic 2 fractions (Table 20) were
16.1%, 4.4% and 16.2% respectively. The purified IgG and IgG depleted
of subclass 3, were stored at $-20^{o}C$ for 1-2 weeks prior to use in the
myotoxicity assays. A sample of myasthenic 1 IgG (Figure 33a) was
tested for complement-mediated myotoxicity after a further 6 weeks of
storage at $-20^{o}C$. Measured myotoxicity was found to be reduced from
45.7% to 14.9% indicating deterioration of the stored IgG.

For these experiments, myotube cultures were labelled with
L-[Me-^{3}H] carnitine. It was noted that the measured myotoxicity by the
myasthenic serum sample 1 (from patient MG 4, sample ii), in the
presence of complement, was higher (67.2%) than had previously been
noted when the same volume of serum (80μl) was added to cultures (see
Table 12).

FIGURE 33

COMPLEMENT-MEDIATED MYOTOXICITY OF MYASTHENIC SERUM,

IgG AND IgG DEPLETED OF SUBCLASS 3

Replicate rat myotube cultures were labelled with L-
$[Me-^3H]$ carnitine as described in Section 2.2.1. The

complement-mediated myotoxicity of heat-inactivated

myasthenic serum (●), IgG (O) or IgG depleted of

subclass 3 (■) was tested by exposing cultures to

increasing concentrations of anti-AChR antibody in

the serum or serum fractions in the presence of GPC

(80µl, 8.0% v/v) for 3h at $37^{\circ}C$. Myotoxicity was

calculated as described in the "Methods" section 8

by comparison with control cultures to which no

additions were made. The value for no addition of

anti-AChR antibody represents that for GPC tested alone.

Each point represents the mean $\pm$ range of 2 separate

experiments, each of which included 4 culture wells.

    a)   Myasthenic serum 1 (from patient MG 4)

    b)   Myasthenic serum 2 (from patient MG 13)

a)
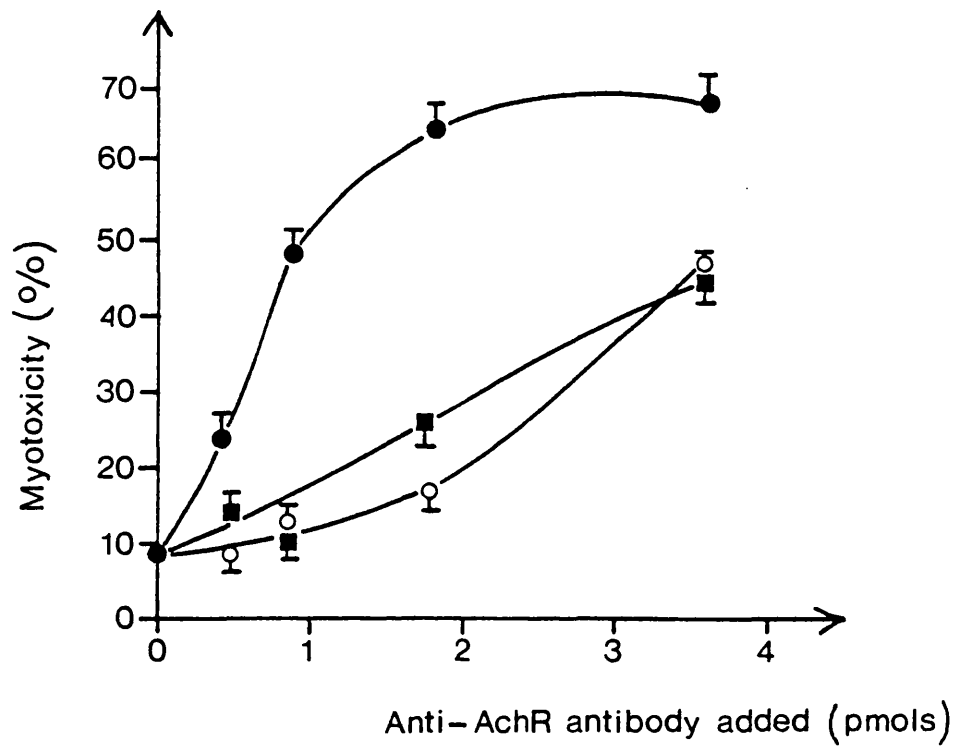
b)
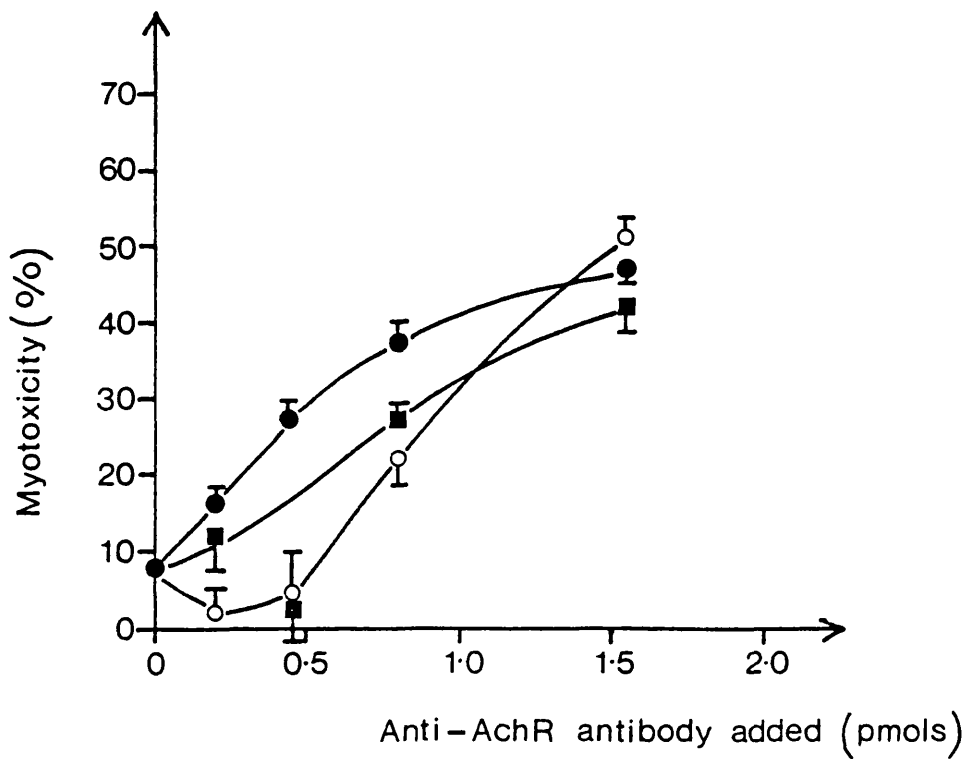
B. HUMAN SKELETAL MUSCLE CELLS IN CULTURE

1. Growth and characteristics

1.1 Preparation of cultures

Single cell suspensions were prepared from human foetal limbs as described for newborn rat limbs (see "Methods" section 1.2). Cell yields ranged from 1.8 - 6.0 x 10^6 cells per limb. Dissociation of tissue with 0.2% (w/v) trypsin plus 0.1% (w/v) collagenase for 1h at 37°C resulted in similar cell yields to portions of the same tissue dissociated in 0.25% (w/v) trypsin alone. The latter procedure was therefore routinely used. Cell viability, as judged by exclusion of trypan blue dye, was greater than 95% and was not affected by previous storage of limbs in growth medium for up to 72h.

Human muscle cultures were more susceptible to bacterial and fungal infection than the rat muscle cultures. This may arise from the non-sterile conditions of the foetuses when collected, as infection occurred within 2-3 days of culture. Foetal limbs were accordingly collected and stored before dissection in growth medium containing 1000 U/ml penicillin and streptomycin, and fungizone (2.5 μg/ml). Of 52 human muscle cultures prepared, 39 produced myotube cultures of reasonable density with no apparent infection. In one case of suspected infection, a 2-day old culture was treated overnight with kanamycin (500 μg/ml), and kanamycin (50 μg/ml) was subsequently added to the growth medium. This treatment greatly reduced, but did not eliminate, the resulting infection and was even less successful with a later infected culture.

1.2 Growth media

The nature and concentration of serum components were varied to test the effects on subsequent myotube growth (see "Materials" for the resulting media - GM1, GM2 and GM3). Other media additions tested were insulin (0.4 U/ml, 1.5 µM), dibutyryl cAMP (dbcAMP - 1mM) and dexamethasone (1 µM). The use of serum-free chemically defined growth media was also assessed (SFM - see "Materials"). The criteria used to judge growth were the morphological appearance of myotubes and the extent of fibroblast growth. The level of $[^{125}I]\alpha$-BGT binding sites, considered to reflect the expression of AChR on the surface of the myotubes (Vogel et al.,1972) was also measured. The results are summarised in Tables 21,26 and 27, and are discussed in greater detail in the following sections.

1.3 Optimal plating density of cells

The optimal plating density of cells was dependent on the serum content of the growth medium. Initiation of cultures in GM3 (containing 20% FCS) supported proliferation of cells but myoblast fusion did not occur. Consequently, cells could be initially plated at a lower density (0.5-1 x 10^5 cells per 15.5mm culture well) and allowed to grow to confluence (5-7 days) before changing to fusion medium with a lower serum content (GM1, GM2 or SFM). Such a procedure yielded myotubes of density and $[^{125}I]\alpha$-BGT binding that were similar to those of sister cultures initially plated at higher densities.

The optimal plating density of cells initiated in GM1 (containing 10% HS) was 2.5 x 10^5 cells per 15.5mm culture well. Higher or lower plating densities gave similar results to those

observed with rat myotube cultures (see Section A 1.1). Initiation of cultures in GM2 (containing 2% HS) or in SFM did not support cell growth.

1.4 Morphology of myotube cultures

The attachment of cells to the collagen substratum and the subsequent morphology of myoblasts and fibroblasts in the cultures was as described for rat cultures (Section A 1.2, Figure 34 A). The time and apparent synchrony of myoblast fusion to form multinucleated myotubes was dependent on the serum content of the growth media utilised. In cultures initiated in GM3 (containing 20% FCS), fusion of myoblasts was not observed. After changing to fusion medium (GM1 or SFM) myoblast fusion commenced and was complete within 2-3 days. Use of SFM improved the time of onset and synchrony of myoblast fusion. Cultures initiated and grown in GM1 (containing 10% HS) showed a similar period of myoblast fusion beginning spontaneously after 3-4 days in culture. Again, the time of onset and the synchrony of myoblast fusion was improved by reducing the serum content of the growth medium (GM2 or SFM) after 3 days growth.

Myotube formation and growth were promoted by all the media tested. Morphologically, the myotubes appeared similar, being mainly thin and refractile (an indication of their roundness) with flattened, broader, non-refractile areas occurring principally at branching points but also occasionally at points along one myotube (see Figures 34; 35 A,B and 36C). Occasionally, insulin-containing growth medium (insulin added throughout the culture period to GM1/GM2 or GM1/SFM combinations) gave rise to a different type of morphology in which all the myotubes present appeared broad, flattened and

FIGURE 34

LIGHT PHOTOMICROGRAPHS OF HUMAN MUSCLE CELLS IN CULTURE

A) 1 day after plating the single cell suspension

at 2.5 x 10^5 cells/culture well in GM1

containing 10% HS.

(x 80 magnification)

B) The appearance of a 5 day culture grown in

GM1 containing 10% HS.

(x 40 magnification)

C) 8 days in culture showing a network of

branched myotubes but also a heavy growth

of fibroblasts (culture grown in GM1

containing 10% HS). (x 80 magnification)
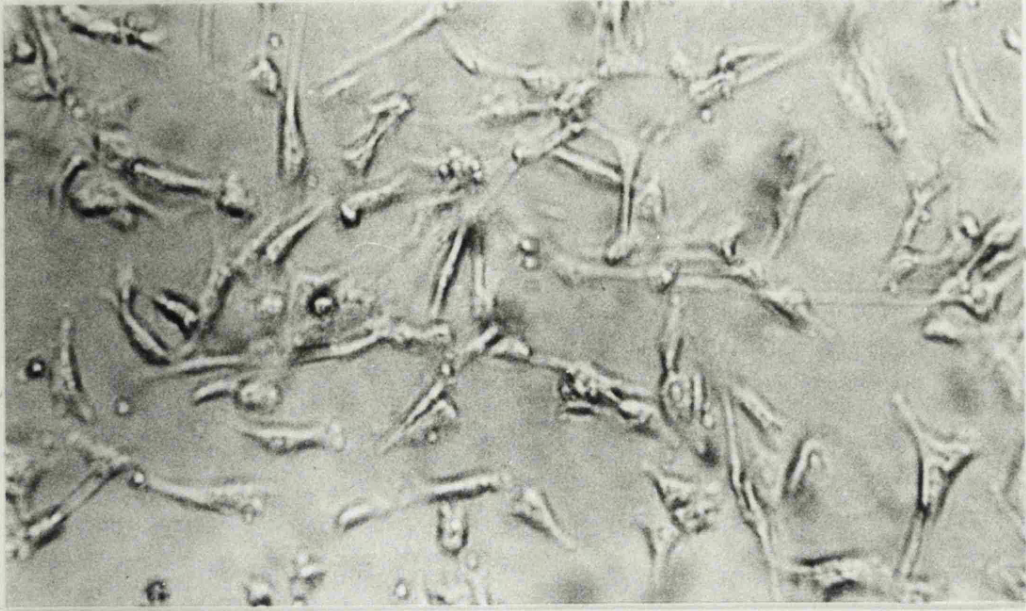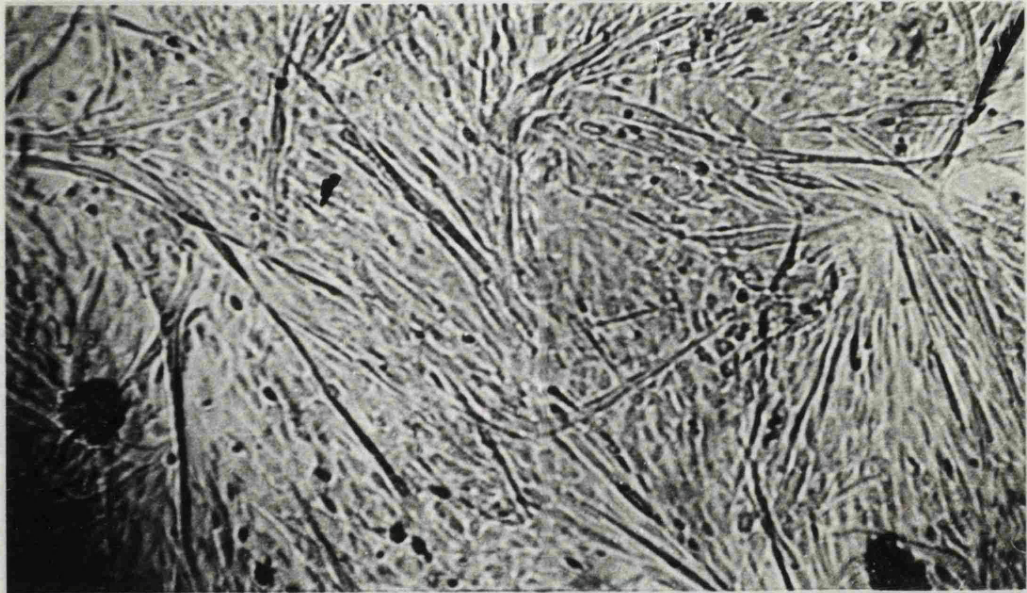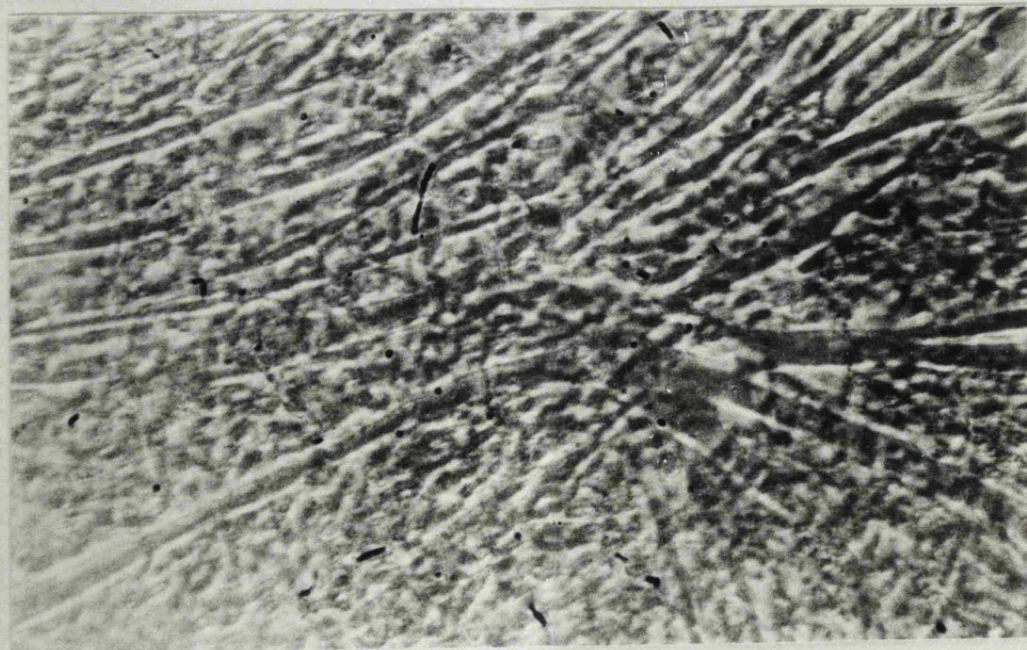
FIGURE 35

PHOTOMICROGRAPHS OF HUMAN MUSCLE CELLS IN CULTURE

A) Light photomicrograph of an 8 day culture
   initiated in GM3, containing 20% FCS, and
   switched to GM1, containing 10% HS, after
   3 days growth. The myotubes present are
   almost overgrown by fibroblasts.
   (x 40 magnification)

B) Phase contrast photomicrograph of a 7 day
   culture initiated in GM1, containing 10% HS,
   and switched to GM2, containing 2% HS, after
   3 days growth.
   (x 40 magnification)

C) Light photomicrograph of a 7 day culture
   initiated in GM1, containing 10% HS, and
   switched to SFM after 3 days growth. Insulin
   (0.4 U/ml, 1.5 µM) was added throughout the
   culture period. The myotubes adopted a broad,
   flattened morphology.
   (x 40 magnification)

A
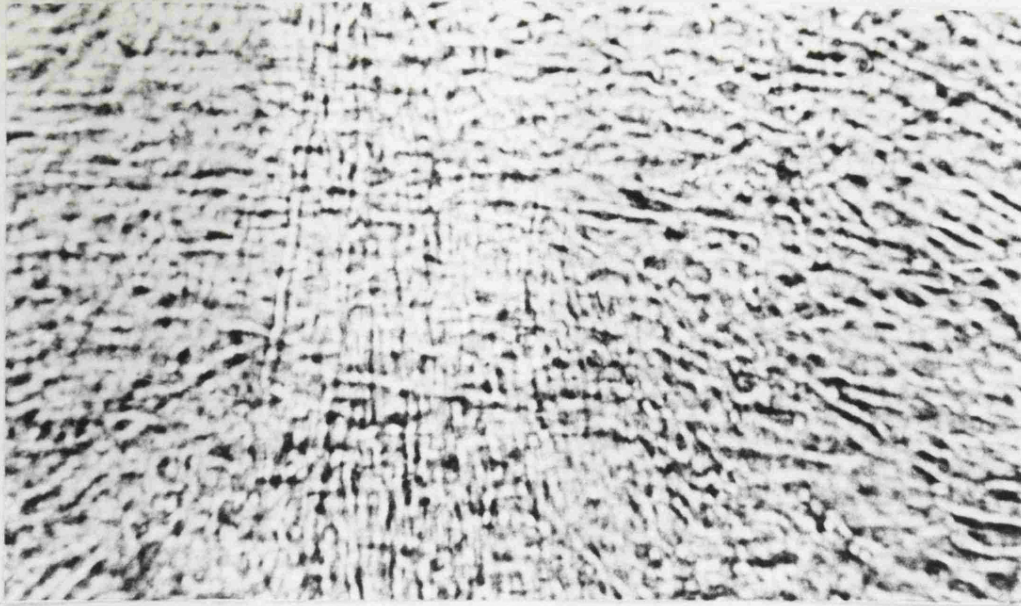
B
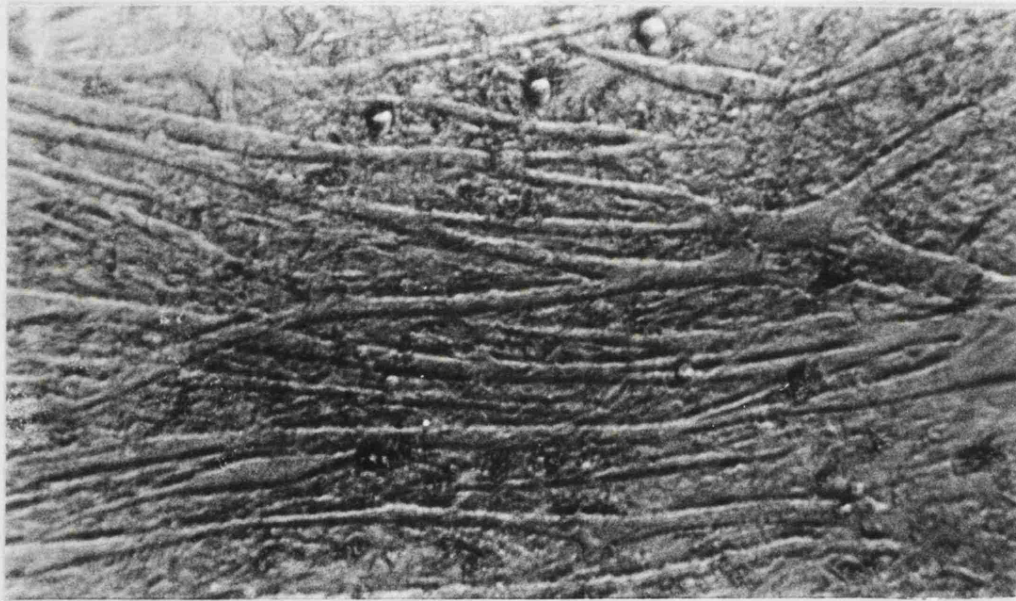
C
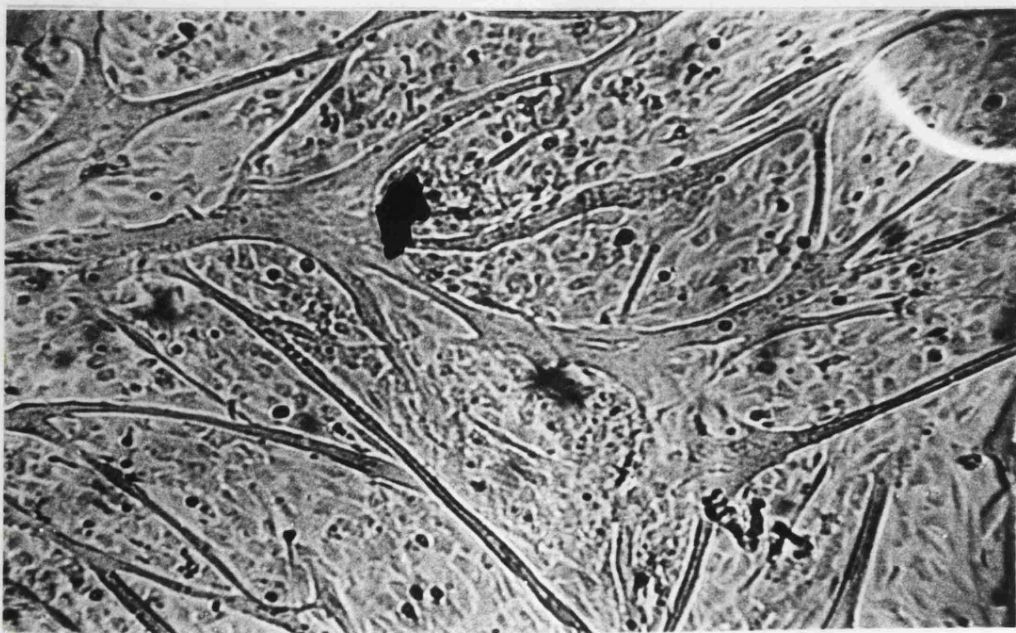
FIGURE 36

LIGHT PHOTOMICROGRAPHS OF HUMAN MUSCLE CELLS IN CULTURE

A) The appearance of a 7 day culture initiated in
   GM1, containing 10% HS, and switched to GM2,
   containing 2% HS and dbcAMP (1 mM) after 3
   days growth. The myotubes adopted a broad,
   flattened morphology, the myotube membranes
   acquiring a 'wavy' appearance.
   (x 80 magnification)

B) A sister culture to that shown in A) which
   was initiated in GM1, containing 10% HS, and
   switched to GM2, containing 2% HS, after 3
   days growth. Insulin was added throughout the
   culture period (0.4 U/ml, 1.5 μM).
   (x 80 magnification)

C) A sister culture to those shown above which
   was initiated in GM1, containing 10% HS, and
   switched to GM2, containing 2% HS, after 3
   days growth.
   (x 80 magnification)

A
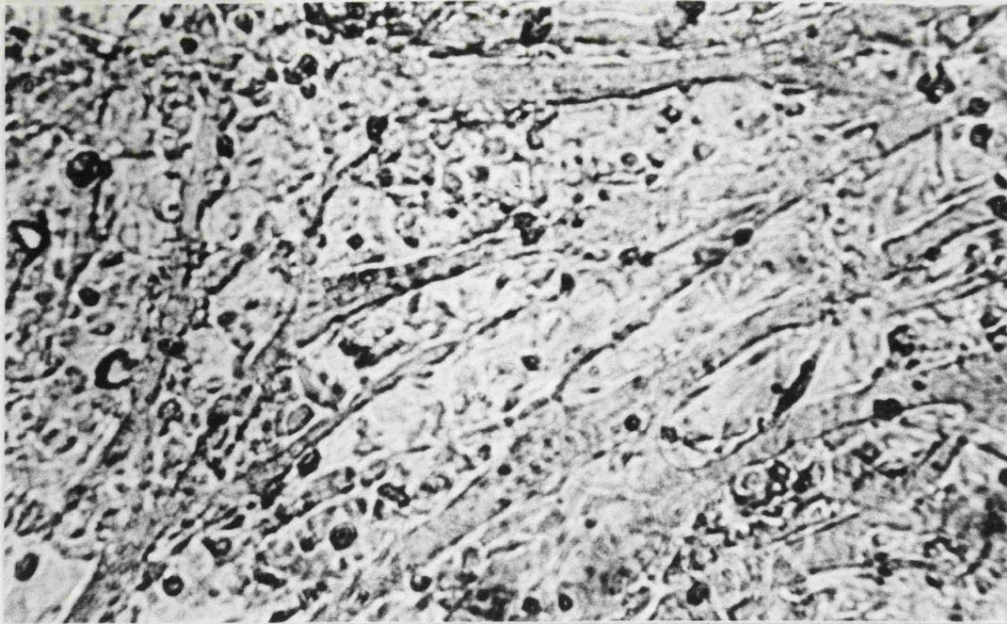
B

C
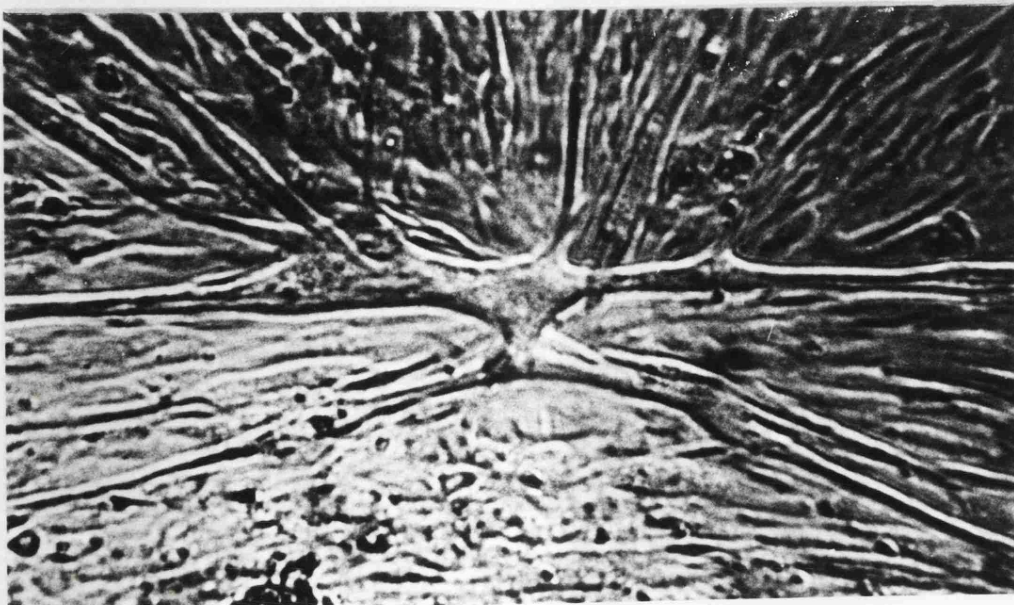
TABLE 21

SUMMARY OF GROWTH MEDIA USED TO SUPPORT GROWTH AND DIFFERENTIATION OF HUMAN MUSCLE CELLS IN CULTURE

Serum composition		Other additions		Myotube growth	Fibroblast growth	Number of preps. observed
initiation	fusion	initiation	fusion			
FCS 20%	HS 10%	-	-	+	+++	12
FCS 20%	SFM	-	-	+	+ or ++	3
HS 10%	HS 10%	-	-	+	++	6
HS 10%	HS 2%	-	-	++	+ or ++	8
HS 10%	SFM	-	-	+ or ++	+	4
HS 10%	HS 2%	Insulin	Insulin	++	++	8
HS 10%	HS 2%	-	Insulin	++	++	2
HS 10%	SFM	Insulin	Insulin	+ or ++	++	2
HS 10%	HS 2%	Insulin	Insulin + dbcAMP	++	++	2
HS 10%	HS 2%	-	dbcAMP	++	++	2
HS 10%	HS 2%	-	Dexamethasone	++	++	1

Myotube growth was judged morphologically by appearance and final density, and by the subsequent [^{125}I]α-BGT binding when compared to replicate cultures grown sequentially in GM3 (containing 20% FCS)/GM1(containing 10% HS), GM1/GM2 (containing 2% HS) or grown wholly in GM1 (see Tables 26 and 27).

non-refractile (Figure 35C and 36B). This morphology was not seen in cultures to which no insulin (or in the case of SFM, no additional insulin) was added. A similar morphology was also obtained from cultures to which dbcAMP (1 mM) was added on the onset of myoblast fusion. In addition, the myotube membranes acquired a 'wavy' appearance (Figure 36A).

In a series of studies in which replicate cultures were grown and maintained in various media combinations, denser and more branched populations of myotubes were obtained from the sequential use of GM1 (containing 10% HS) /GM2 (containing 2% HS) or GM1/GM2 with additional insulin (0.4 U/ml, 1.5 µM) added throughout the culture period (see also section 1.9). The addition of dexamethasone (1µM) to one culture grown sequentially in GM1/GM2 made no apparent difference to the subsequent morphology of the myotubes.

No spontaneous contractility of myotubes was ever observed in human muscle cultures. The lifetime of the cultures was approximately 14-17 days, by which time the myotubes had become granulated, phase-dark and shrunken, indicative of cell death.

1.5 Inhibition of fibroblast growth

Cultures initiated in GM3 (containing 20% FCS) and maintained in GM1 (containing 10% HS) showed a heavy overgrowth of fibroblasts. This overgrowth was greatly reduced by maintaining the cultures in SFM or by initiating and maintaining the cultures in GM1 alone. The mitotic inhibitors Ara C and FDU were tested for their ability to reduce numbers of fibroblasts and for their effect on myotube growth (Table 22). When, one day after the onset of myoblast fusion, either inhibitor was included for a period of 72h in cultures

initiated in GM3 or GM1 and maintained in GM1, poor myotube growth

resulted and was paralleled by low levels of [^{125}I]α-BGT binding. As

noted for the rat myotube cultures (see Section A 1.3), it was

probable that the mitotic inhibitors interfered with myoblast fusions.

The poorest growth of human myotubes occurred after treatment with

FDU. Addition of inhibitor two days after the onset of myoblast

fusion, made little difference to the fibroblast growth, as this was

already pronounced by this stage. These mitotic inhibitors were not

routinely added to human myotube cultures.

Reduction in fibroblast growth was greatest with cultures

initiated in GM3 or GM1 and switched to SFM to promote myoblast

fusion, or in cultures initiated in GM1 (containing 10% HS) and

maintained in GM2 (containing 2% HS). The clearest background was

obtained when GM1 or GM3 were changed to fusion medium after 3 days

growth. In the case of GM3, this necessitated using a higher initial

plating density of 2.5×10^5 cells/culture well, as lower densities

did not produce cell confluence within this time, resulting in a less

dense population of myotubes. Initiating cultures in GM3 at a low cell

density and changing to SFM after confluence was reached, did not

greatly reduce the extent of fibroblast growth.

The addition of insulin to growth media throughout the

culture period (0.4 U/ml, 1.5 µM or in the case of SFM - additional

insulin to give a final concentration of 0.4 U/ml, 1.5 µM), promoted

the growth of fibroblasts in comparison to replicate cultures grown

in the absence of insulin (or additional insulin in the case of SFM).

Additional procedures tested, to improve the purity of

myoblasts in single cell suspensions, were the preplating of

suspensions for 20 min at 37°C (see Section A 1.3) or the repassaging

TABLE 22

EFFECTS OF MITOTIC INHIBITORS ON SUBSEQUENT HUMAN

MYOTUBE GROWTH

Replicate myotube cultures, initiated in GM3, were

treated after one day's growth in GM1 with

fluorodeoxyuridine (15μg/ml) and uridine (35μg/ml) (FDU)

or cytosine arabinoside (Ara C, 10μM) for 72h. After

5 days growth in GM1, the cultures were tested for

$[^{125}I]$ α-BGT binding as described in the "Methods" section

3 (at 30nM toxin concentration). Each result is the

mean $\pm$ SD of 4 culture wells.

Test	$[^{125}I]$ α-BGT binding			Protein
	Total (cpm/well)	Non-specific (cpm/well)	Specific (fmol/well)	(μg/well)
No addition	12228 $\pm$ 1204	4832 $\pm$ 367	13.7	148 $\pm$ 6
+ FDU	5423 $\pm$ 504	3411 $\pm$ 420	3.7	98 $\pm$ 4
+ Ara C	8145 $\pm$ 442	3497 $\pm$ 260	8.6	125 $\pm$ 6

of cultures initiated for 3 days in GM3 to promote cell proliferation (see "Methods" section 1.4). The preplating of suspensions did not reduce the subsequent growth of fibroblasts in myotube cultures; therefore this procedure was not routinely used. The repassaging of cultures was observed to reduce the overgrowth by fibroblasts of cultures initiated in GM3 and changed to GM1 after 3 days in culture, and of cultures grown wholly in GM1. In the latter case, myoblast fusion was observed to commence after 2-3 days in culture as apposed to 3-4 days in cultures established from the original cell suspension and plated at the same initial cell density. Specific [^{125}I]α-BGT binding to repassaged cultures was increased when compared to primary cultures established from the original cell suspension (see Table 23). The repassaging of cultures made no difference to the fibroblast growth in cultures switched, after 3 days growth, to GM2 (containing 2% HS) or SFM for fusion. Switching the medium at this stage of growth was a procedure which in itself reduced fibroblast growth. The principal disadvantage in using the repassaging procedure as described, was the decreased cell yield obtained when compared to the original cell suspension i.e. a reduction of 34.5 $\pm$ 6.1%, mean $\pm$ SD (3 experiments), resulting in a reduced number of replicate culture wells available for one experiment. As this procedure did not appear to increase the level of [^{125}I]α-BGT binding sites above that obtained for cultures grown sequentially in GM1/GM2 (see Section 1.9) it was not subsequently used.

TABLE 23

$[^{125}I]\alpha$-BGT BINDING BY PRIMARY AND REPASSAGED HUMAN MUSCLE CELL CULTURES

Single cell suspensions were prepared from human muscle tissue as described in the "Methods" section 1.3. Half of the cells obtained were grown as a primary culture while the other half were repassaged after 3 days growth in GM3 (see "Methods" section 1.4). Each resulting culture, grown sequentially in GM3/GM1 (2 cultures) or wholly in GM1 (1 culture) was tested for specific $[^{125}I]\alpha$-BGT binding (30nM) after 8 days growth and results were compared.

Preparation	Initial cell density (x10^-5/well)	Specific $[^{125}I]\alpha$-BGT binding		Protein range (µg/well)	Number of cultures tested
		% primary culture	range (fmol/well)		
Primary culture	2.5	100	11.2 – 16.1	143 – 172	3
Repassaged culture	2.5	136.4 $\pm$ 22.6	14.3 – 26.1	112 – 133	3

1.6 Foetal age

In most culture preparations, tissues from several foetuses were pooled in order to increase the number of replicate cultures for various studies. In one experiment, tissue samples from small foetal limbs (approximate age 9 weeks) and from larger foetal limbs (approximate age 14-15 weeks) were dissociated and cultured separately under the same conditions (Initiation in GM3 for 3 days before switching to SFM). On the seventh day in culture, myotubes originating from the older foetal tissue appeared morphologically denser and more branched than those originating from the younger foetal tissue. $[^{125}I]$ ∝-BGT binding was 65% higher in the former (Table 24). Further correlation of morphology and $[^{125}I]$∝-BGT binding with foetal age of tissue source was not made, because of the difficulty in supply of the foetal tissue.

1.7 Protein content of cultures

The variation in total protein within one myotube culture was small i.e. in one representative culture, grown sequentially in GM3/GM1, protein equalled 161.6 ± 15.2 µg/culture well, mean ± SD (24 culture wells), determined after 5 days in GM1. The variation in total protein content between cultures established at different times and in different growth media, determined 4-5 days after onset of myoblast fusion, fell in the range 84.6 - 172.4 µg/culture well. As noted for the rat myotube cultures (Section A 1.6), the cultures originate from a mixture of cell types giving rise to both myoblast and fibroblast growth, each of which contributes to total protein levels. All experimental results are therefore expressed as per culture well as opposed to per mg protein.

TABLE 24

EFFECTS OF FOETAL AGE ON SUBSEQUENT HUMAN MYOTUBE

GROWTH

Myotube cultures were established at the same time
under the same conditions from foetal tissue of
different gestational age. Cultures were initiated in
GM3 and subsequently maintained in SFM for 4 days
before determining $[^{125}I]\alpha$-BGT binding (20nM) as
described in the "Methods" section 3. Each result is
the mean $\pm$ SD of 4 culture wells.

Approximate age (weeks)	$[^{125}I]\alpha$-BGT binding			Protein (ug/well)
	Total (cpm/well)	Non-specific (cpm/well)	Specific (fmol/well)	
14 - 15	8468 $\pm$ 660	2711 $\pm$ 281	10.6	104 $\pm$ 2
9	5141 $\pm$ 324	1666 $\pm$ 172	6.4	97 $\pm$ 4

1.8 Time course of the appearance of [^{125}I]α-BGT binding sites in cultures

One representative myotube culture, initiated in GM3 and maintained in GM1, was tested over 11 days growth for [^{125}I]α-BGT binding and total protein content (Figure 37). When fusion of myoblasts was complete (2 days in fusion medium, GM1), specific toxin binding increased 4-fold to a maximum level of 24.9 fmol/culture well within 3 days (determined at 30 nM toxin). After this time, toxin binding decreased. The protein content of cultures increased consistently over the time period studied, paralleled by the observed continued growth of fibroblasts in the myotube cultures.

When cultures were grown in alternative growth media (Section 1.2), maximal [^{125}I]α-BGT binding always occurred within 4-5 days of initiation of myoblast fusion (data not shown). Human myotube cultures were therefore routinely used for assays at this time.

1.9 Equilibrium binding of [^{125}I]α-BGT to cultures

After incubation of cultures with [^{125}I]α-BGT, 3 washes with growth medium were sufficient to remove free radiolabel. Counting errors (SE) of 4 replicate culture wells were usually less than 5%. Pre-incubation of myotube cultures (30 min, 23°C) with decamethonium bromide inhibited [^{125}I]α-BGT binding in a concentration dependent manner (Figure 38). Maximum inhibition (50.5%) was achieved by using 1 mM decamethonium. The inhibition of toxin binding by decamethonium, in different experiments using cultures established in different media, represented 46% - 82% of the total toxin binding determined in the absence of inhibitor. Experiments in which the use of excess unlabelled α-BGT (1μM) or decamethonium (1mM) were compared as

FIGURE 37

INCREASE OF $[^{125}I]\alpha$-BGT BINDING SITES IN HUMAN MUSCLE

CELL CULTURES

Replicate muscle cell cultures, grown sequentially in

GM3/GM1, were tested daily for specific $[^{125}I]\alpha$-BGT

binding (30nM) as described in the "Methods" section 3.

The total protein content of the cultures was also

determined.

(O——O) Specific $[^{125}I]\alpha$-BGT binding/culture well.

Each point is the difference between

total and non-specific binding, each

determined from 4 culture wells.

(●----●) Protein content/culture well. Each

point is the mean $\pm$ SD of 8 culture wells.
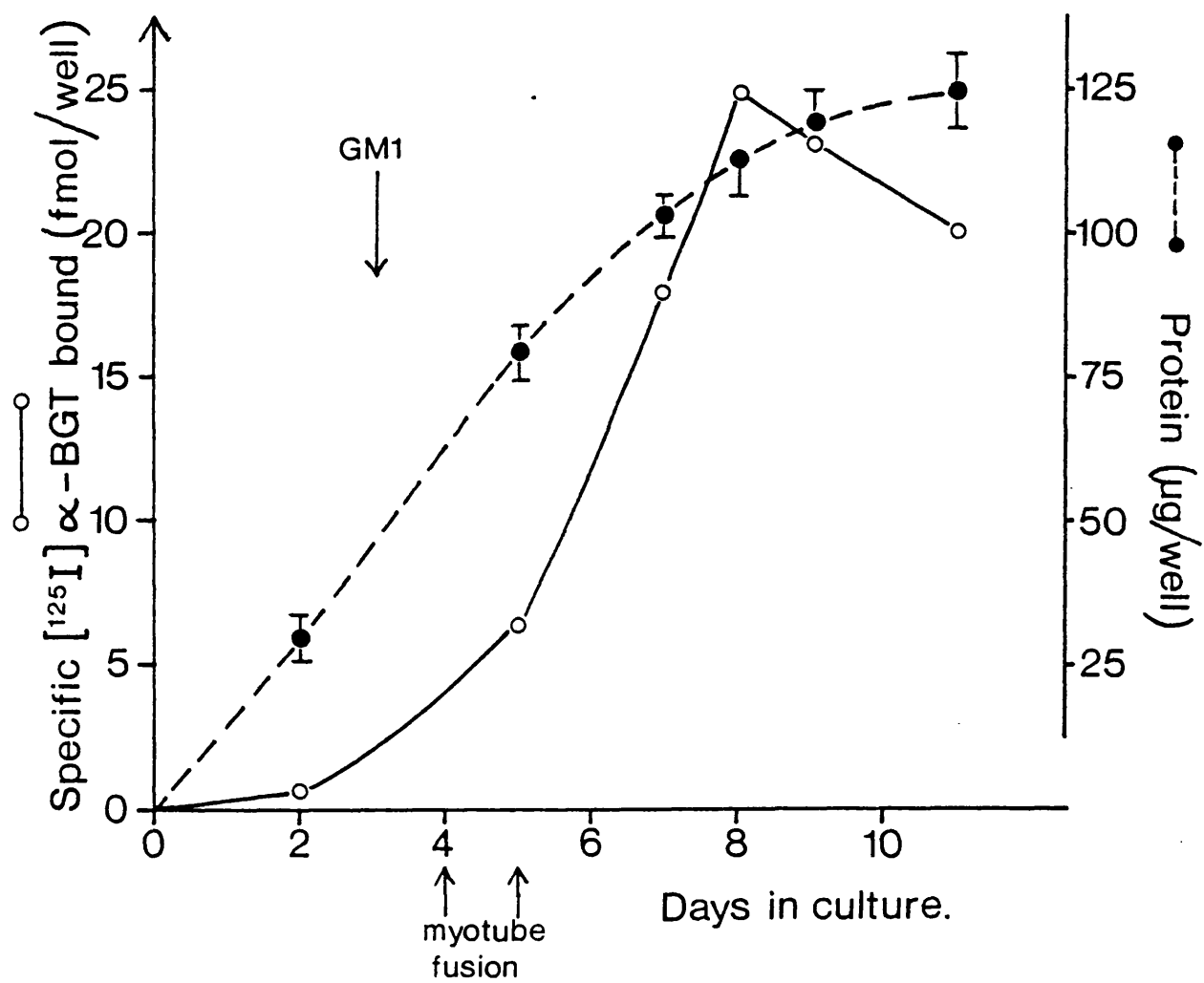
FIGURE 38

INHIBITION OF $[^{125}I]\alpha$-BGT BINDING TO HUMAN MYOTUBE

CULTURES BY DECAMETHONIUM BROMIDE

The binding of $[^{125}I]\alpha$-BGT (30nM) to replicate myotube

cultures (8 days in culture, grown sequentially in

GM3/GM1) was determined as described in the "Methods"

section 3 after pre-incubation of the cultures (30 min,

23°C) with increasing concentrations of decamethonium

bromide. Each point is the mean $\pm$ SD of 4 culture wells.
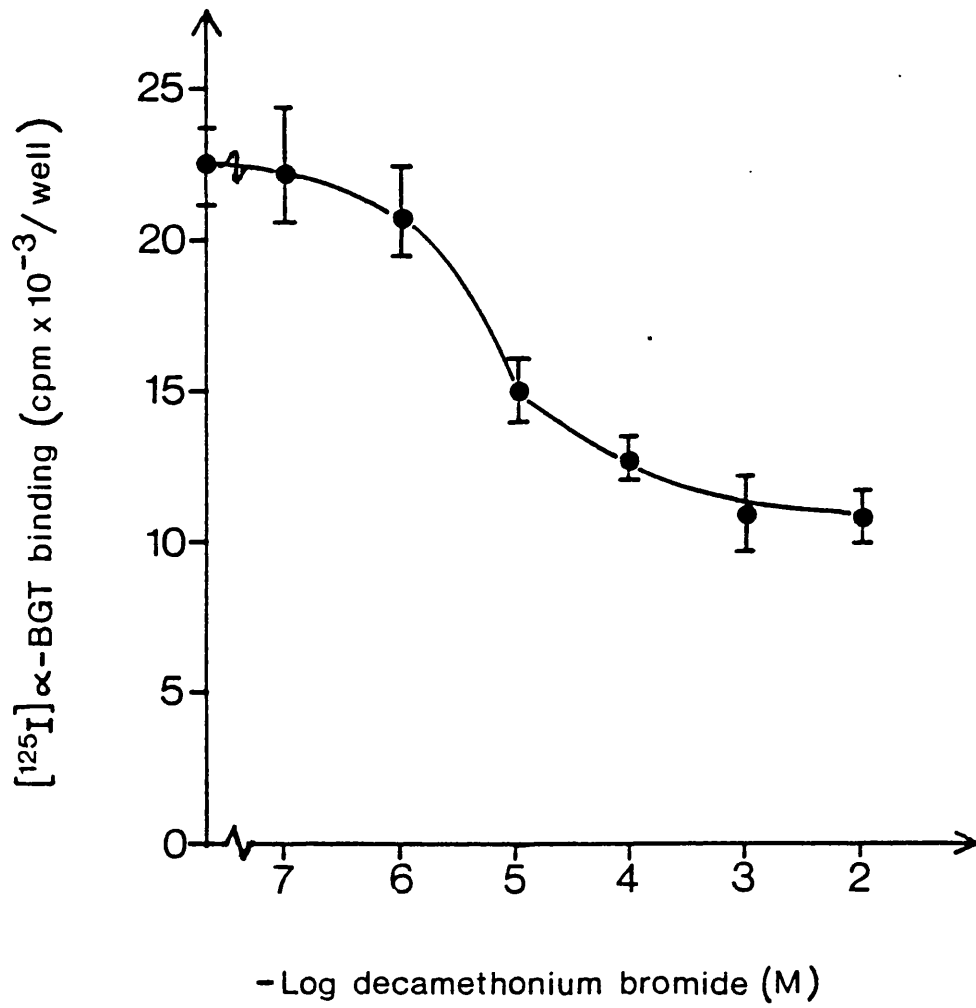
TABLE 25

INHIBITION OF $[^{125}I]$ $\propto$-BGT BINDING BY DECAMETHONIUM

BROMIDE AND BY EXCESS UNLABELLED $\propto$-BGT

$[^{125}I]$ $\propto$-BGT binding (30nM) was determined on replicate

human myotube cultures in the presence and absence of

decamethonium bromide (1mM) or $\propto$-BGT (1µM) as described

in the "Methods" section 3. Each result is the mean $\pm$ SD

of 4 culture wells treated similarly.

Culture number	$[^{125}I]$ -BGT binding			Inhibition	
	Total	+ $\propto$-BGT	+ DBr	(% total)	
	(cpm/well)	(cpm/well)	(cpm/well)	+ $\propto$-BGT	+ DBr
[a] 47	13628 $\pm$ 1250	7235 $\pm$ 252	6831 $\pm$ 635	46.9	49.9
[a] 48	17706 $\pm$ 1789	7754 $\pm$ 755	9371 $\pm$ 1255	56.2	47.0
[b] 49	23288 $\pm$ 2148	6661 $\pm$ 604	11955 $\pm$ 1048	71.4	48.7

[a] Cultures initiated in GM1 + insulin (0.4 U/ml, 1.5uM)

and changed after 3 days growth to SFM + additional

insulin (final concentration 0.4 U/ml, 1.5 uM)

[b] Cultures initiated in GM1 + insulin (0.4 U/ml, 1.5uM)

and changed after 3 days growth to GM2 + insulin

(0.4 U/ml, 1.5uM)

inhibitors of toxin binding suggested that the former was occasionally more effective (Table 25). However, non-specific binding of $[^{125}I]$ α-BGT was routinely determined in the presence of decamethonium (1mM).

The binding of $[^{125}I]\alpha$-BGT to myotube cultures was determined over a range of toxin concentrations. Figure 39 illustrates binding by a representative culture grown sequentially in GM3/GM1. Non-specific binding, measured in the presence of decamethonium (1mM) accounted for 40-50% of the total binding. Figure 40 illustrates $[^{125}I]\alpha$-BGT binding by cultures of which half were grown and maintained wholly in GM1 and half changed to GM2 after 3 days growth. In the latter case, non-specific binding accounted for only 18-20% of the total radioactivity, as opposed to 25-35% in the former case. This may well reflect the reduced fibroblast growth and subsequent lower total protein content of cultures grown sequentially in GM1/GM2. As discussed in Section 1.4, initiating myotube cultures in GM1 and changing to GM2 after 3 days growth improved the density and morphology of myotubes in these cultures. This was reflected in an increase in specific $[^{125}I]\alpha$-BGT binding (Figure 40).

In each binding experiment, specific $[^{125}I]\alpha$-BGT binding approached saturation (Figures 39 and 40). Scatchard analysis (Figure 41) of the binding data gave B_{max} values in the range 29.6 - 42.0 fmol/culture well and K_D values in the range 4.42 - 5.71 pmol/culture well equivalent to 16.6 $\pm$ 2.2 nM in the assay conditions used (see "Methods" section 3).

In a series of studies in which replicate cultures were grown and maintained in various media combinations, cultures grown sequentially in GM1/GM2 or in GM1/GM2 with additional insulin (0.4 U/ml, 1.5 µM) added throughout the culture period, gave the highest

FIGURE 39

EQUILIBRIUM BINDING OF $[^{125}I]\alpha$-BGT TO HUMAN MYOTUBE

CULTURES GROWN SEQUENTIALLY IN GM3/GM1

The binding of increasing concentrations of $[^{125}I]\alpha$-BGT

to replicate myotube cultures (8 days in culture) was

determined as described in the "Methods" section 3.

The binding data from one representative culture

shows:-

- ● Binding in the absence of decamethonium bromide
  (total binding)

- O Binding in the presence of 1mM decamethonium
  bromide (non-specific binding)

- □ Specific binding

Each point is the mean $\pm$ SD of 4 culture wells.

Protein content = 162 $\pm$ 15 µg/culture well (mean $\pm$ SD,

8 culture wells)

Inset - Specific binding expressed as
         fmol $[^{125}I]\alpha$-BGT binding/culture well.
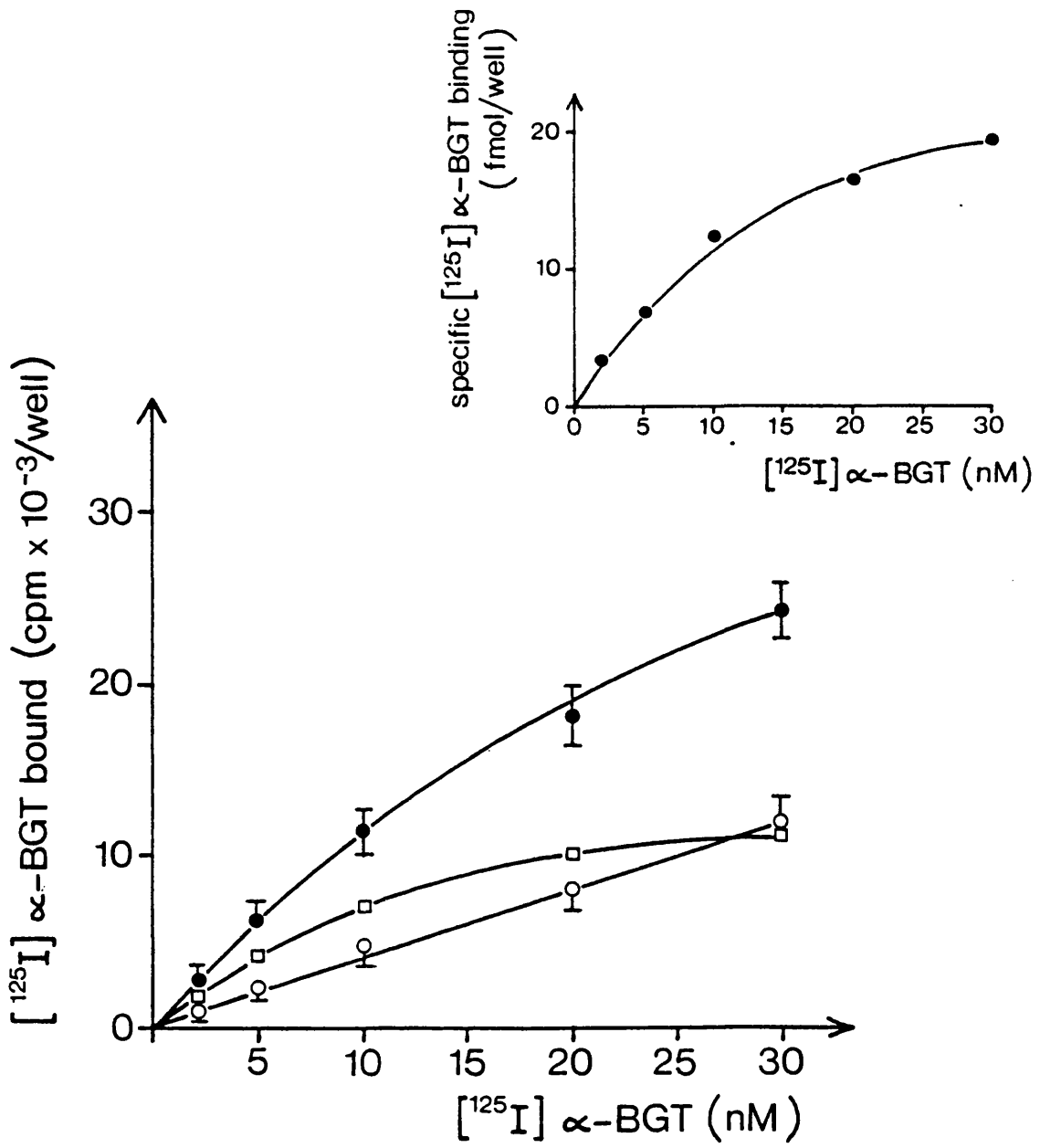
FIGURE 40

EQUILIBRIUM BINDING OF $[^{125}I]\alpha$-BGT TO HUMAN MYOTUBE

CULTURES GROWN IN GM1 OR SEQUENTIALLY IN GM1/GM2

The binding of increasing concentrations of $[^{125}I]\alpha$-BGT

to replicate myotube cultures grown and maintained

wholly in GM1 (a) or switched to GM2 after 3 days growth

in GM1 (b) was determined after 8 days in culture as

described in the "Methods" section 3.

● Binding in the absence of decamethonium

bromide (total binding)

O Binding in the presence of 1mM decamethonium

bromide (non-specific binding)

□ Specific binding

Each point is the mean $\pm$ SD of 4 culture wells.

Protein content = a) 149 $\pm$ 10 µg/culture well

b) 94 $\pm$ 3 µg/culture well

(mean $\pm$ SD, 8 wells)

Insets - specific binding expressed as

fmol $[^{125}I]\alpha$-BGT bound/culture well

a)

b)
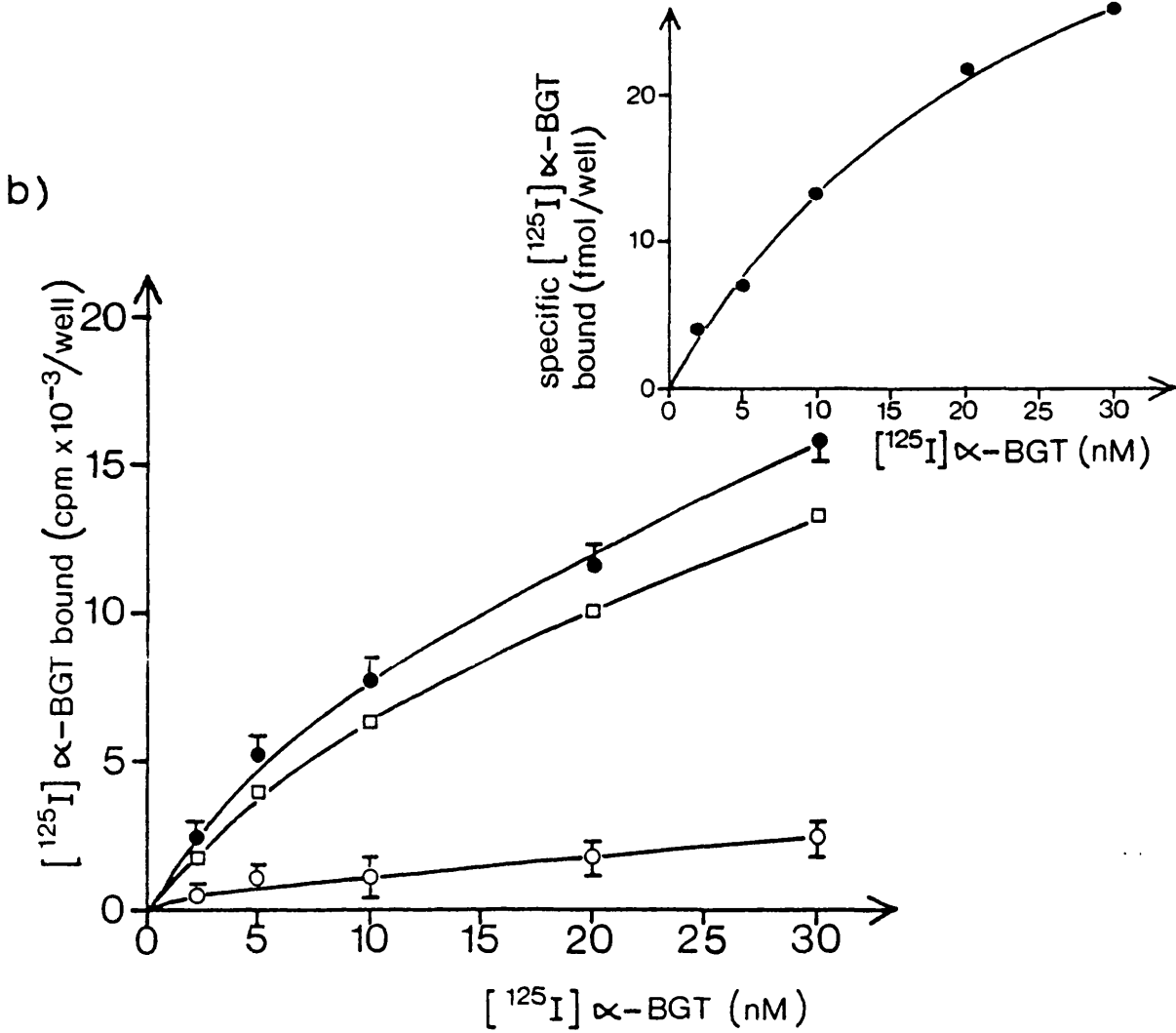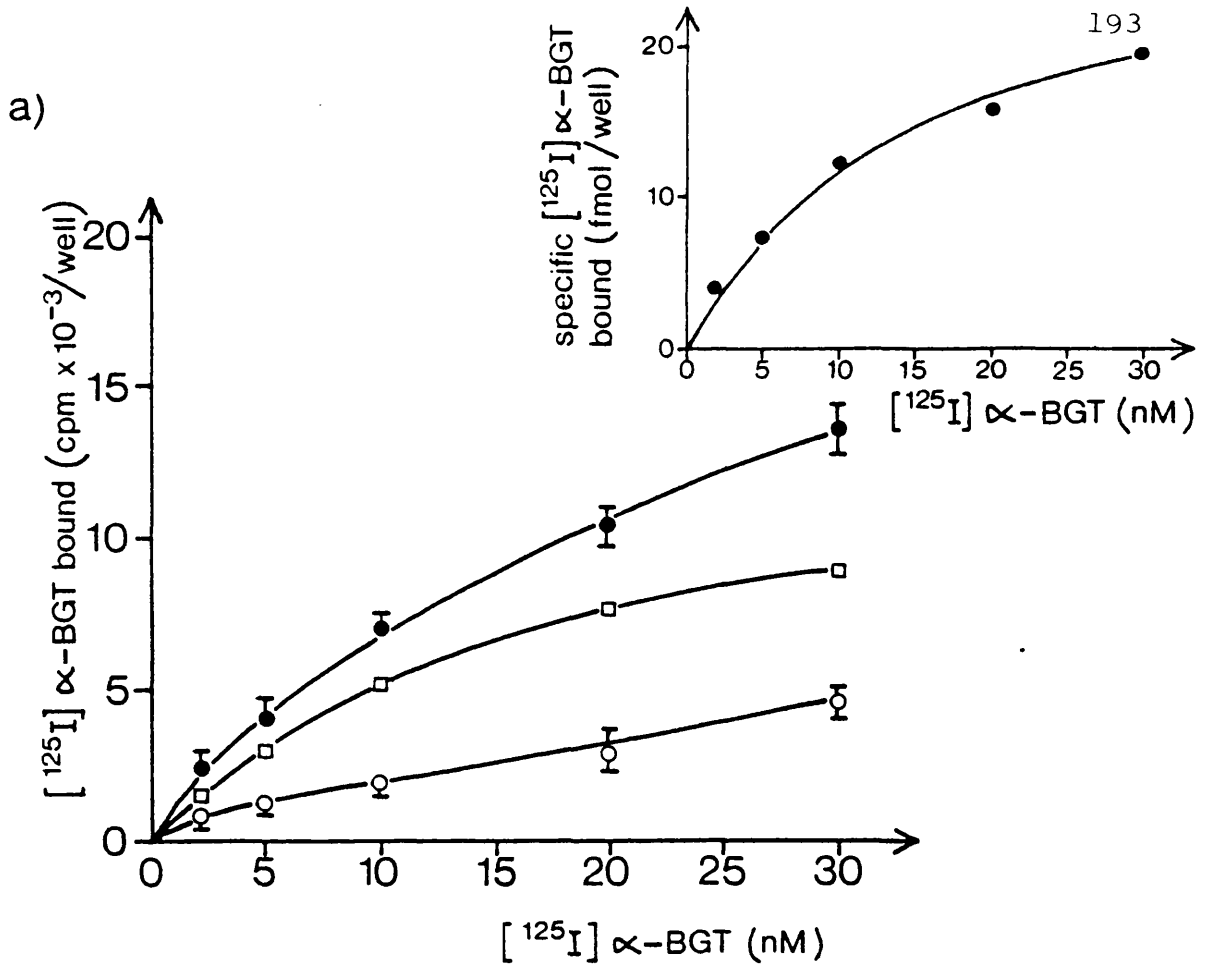
FIGURE 41

SCATCHARD ANALYSIS OF THE BINDING DATA IN FIGURES 39 & 40

Lines were constructed by linear regression by using the data from Figures 39 and 40.

	Culture medium	K_D	B_{max}	r^2
●	GM3/GM1	14.8 nM	29.6 fmol/well	0.99
○	GM1	16.0 nM	30.9 fmol/well	0.99
□	GM1/GM2	19.1 nM	42.0 fmol/well	1.00

Mean K_D = 16.6 nM
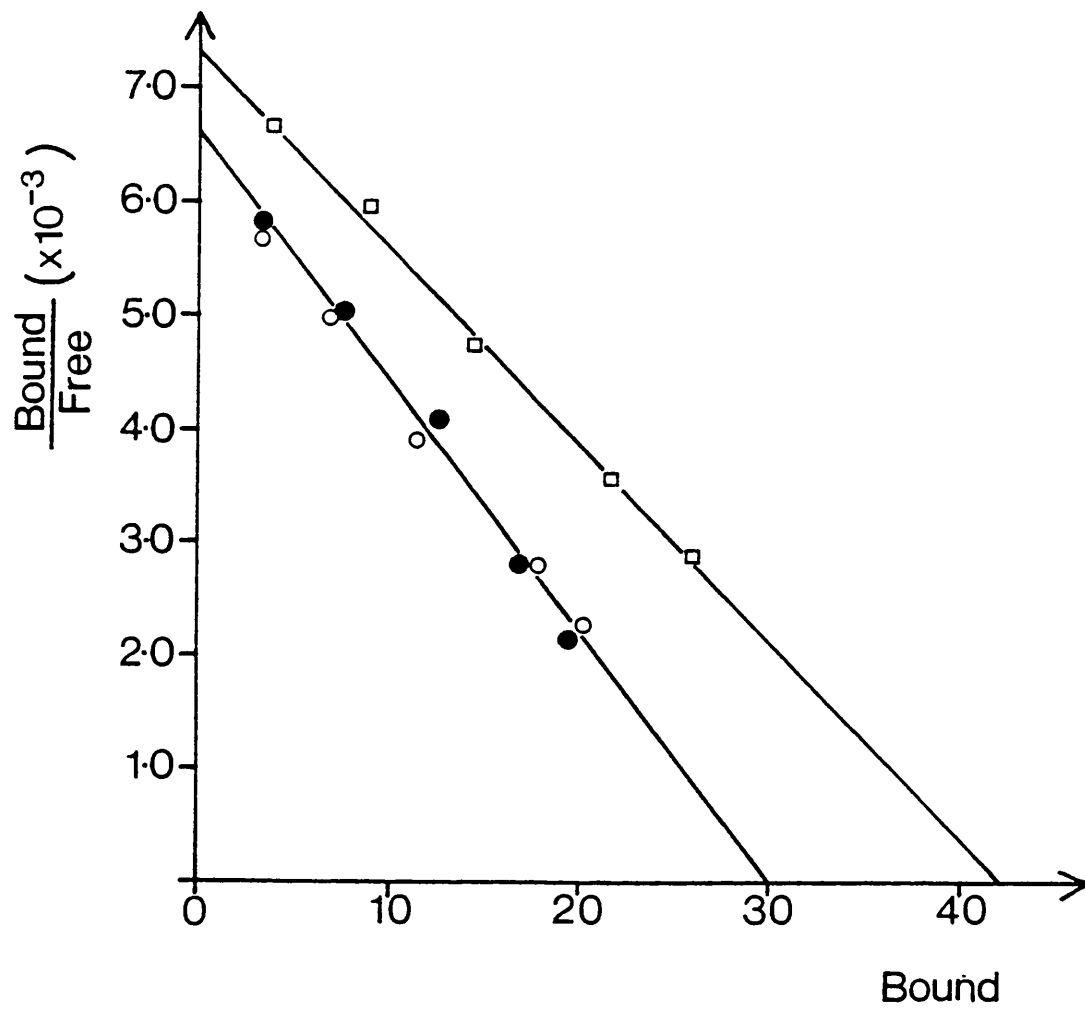
relative specific [^{125}I]α-BGT binding, determined at 30 nM

concentration after 7-8 days in culture (Tables 26 and 27). However

the growth of human myotubes in culture, judged morphologically and by

[^{125}I]α-BGT binding, was very variable in all serum compositions of

growth media. Table 28 summarises the results obtained for toxin

binding in these experiments.

1.10 Acetylcholinesterase and creatine phosphokinase activity

AChE activity in human myotube cultures, grown sequentially in

GM1/GM2, was 0.15 ± 0.03 nmol substrate converted/min/culture well

(mean ± SD, 3 experiments) determined on the 8th day in culture. CPK

activity, determined on the same day, was 7.7 ± 2.5 nmol substrate

converted/min/culture well (mean ± SD, 3 experiments).It was not

possible to study the time course of the appearance of AChE and CPK

activity in human cultures because of the large number of replicate

cultures that would be needed for each experiment. These could not be

obtained with the limited amount of foetal tissue available.

1.11 Uptake of [Me-^{3}H] carnitine

Human myotube cultures (grown sequentially in GM3/GM1) and skin

fibroblast cultures were tested, over the 7th and 8th day in culture,

for uptake of radioactivity after incubation with [Me-^{3}H] carnitine as

described in the "Methods" section 7. Figure 42 illustrates, as noted

with the rat cultures (Section A 1.10), that uptake of radiolabel was

not saturating over the concentration range tested (0.067 - 1.33 μM,

0.1 - 2 μCi). The myotube cultures took up approximately 6 times more

radioactivity than the confluent fibroblast cultures. The 2 myotube

cultures tested were of similar morphology and expressed similar

TABLE 26

[125I]α-BGT BINDING BY HUMAN MYOTUBE CULTURES GROWN IN MEDIUM WITH DIFFERENT SERUM COMPOSITIONS

Replicate muscle cell cultures were initiated and maintained in different serum-supplemented media for comparison of growth (see Table 21) and [125I]α-BGT binding (30nM) measured after a 5 days growth or b 4 days growth in fusion medium (total time in culture 8 or 7 days respectively).

Culture	Serum composition		Specific [125I]α-BGT binding		Protein	Number
	initiation	fusion	% control	range (fmol/well)	range (µg/well)	of preps.
Control	FCS 20%	HS 10%	100	a 12.4 - 15.2	138 - 156	3
Test	FCS 20%	SFM	98.0 ± 4.1	b 11.7 - 15.0	84 - 110	
Control	FCS 20%	HS 10%	100	a 8.4 - 17.0	138 - 162	3
Test	HS 10%	HS 10%	104.6 ± 6.1	a 9.1 - 15.5	116 - 147	
Control	HS 10%	HS 10%	100	b 7.4 - 20.4	115 - 134	4
Test	HS 10%	HS 2%	134.1 ± 10.6	b 9.8 - 30.3	99 - 122	
Control	HS 10%	HS 2%	100	b 9.8 - 20.3	99 - 120	3
Test	HS 10%	SFM	68.4 ± 18.4	b 8.1 - 15.2	74 - 98	

TABLE 27

[125I]α-BGT BINDING BY HUMAN MYOTUBE CULTURES GROWN IN MEDIUM WITH DIFFERENT ADDITIONAL COMPONENTS

Replicate muscle cell cultures were initiated and maintained in growth media with or without the addition of insulin (0.4 U/ml, 1.5uM), dexamethasone (1uM) or dbcAMP (1mM). After 4 days growth in fusion medium (total time in culture of 7 days), [125I]α-BGT binding (30nM) was determined.

| Serum composition | | Addition | | Specific [125I]α-BGT binding | | Protein | Number |
initiation	fusion	initiation	fusion	% control	range (fmol/well)	range (µg/well)	of preps.
HS 10%	HS 2%	-	-	100	8.4 - 17.1	104 - 131	4
HS 10%	HS 2%	Insulin	Insulin	118.6 ± 6.0	10.5 - 20.6	127 - 156	
HS 10%	HS 2%	-	-	100	17.4, 20.4	124, 131	2
HS 10%	HS 2%	-	Insulin	100.5	17.9, 20.0	128, 142	
HS 10%	HS 2%	-	-	100	8.4	118	1
HS 10%	HS 2%	-	Dexamethasone	125.0	10.5	124	
HS 10%	HS 2%	Insulin	Insulin	100	15.2, 15.5	132, 156	2
HS 10%	SFM	Insulin	Insulin	82.3	11.5, 13.8	128, 134	
HS 10%	HS 2%	-	-	100	11.2, 17.1	118, 124	2
HS 10%	HS 2%	-	dbcAMP	108.6	12.4, 18.2	120, 130	
HS 10%	HS 2%	Insulin	Insulin + dbcAMP	123.0	14.2, 20.4	131, 159	

TABLE 28

SUMMARY OF $[^{125}I]$α-BGT BINDING BY HUMAN MYOTUBE CULTURES

Human myotube cultures were initiated and maintained

in growth media with different serum compositions.

The binding of $[^{125}I]$α-BGT (30nM) was determined after

4 or 5 days in fusion medium as described in the

"Methods" section 3. The results given do not include

cultures grown in medium with hormonal or dbcAMP additions.

Serum composition of growth media		Specific $[^{125}I]$α-BGT binding		No. of cultures
		(fmol/well)	range	
Initiation	Fusion	mean $\pm$ SD	(fmol/well)	
FCS 20%	HS 10%	[a] 16.5 $\pm$ 6.3	8.4 - 26.1	12
HS 10%	HS 10%	[b] 14.5 $\pm$ 5.4	7.4 - 20.4	7
HS 10%	HS 2%	18.5 $\pm$ 7.4	8.4 - 30.3	8
FCS 20% or HS 10%	SFM	12.0 $\pm$ 2.8	8.1 - 15.2	6

[a] Results include two or [b] one repassaged culture

FIGURE 42

UPTAKE OF [Me-^{3}H] CARNITINE BY HUMAN CULTURES

The uptake of radioactivity by human myotube cultures
(grown sequentially in GM3/GM1) or human skin
fibroblasts (7 days in culture) was determined after
incubation with increasing concentrations of [Me-^{3}H]
carnitine for 18h at 37°C as described in the "Methods"
section 7. Each point is the mean of 2 different
experiments, each of which included 4 culture wells.

● Myotube cultures :-

    [^{125}I]∝-BGT binding (30nM) = 16.8 and 18.2

    fmol/culture well respectively

    Protein = 156 and 138 µg/culture well

        respectively

○ Fibroblast cultures :-

    [^{125}I]∝-BGT binding (30nM) not detected

    Protein = 48 and 50 µg/culture well

        respectively
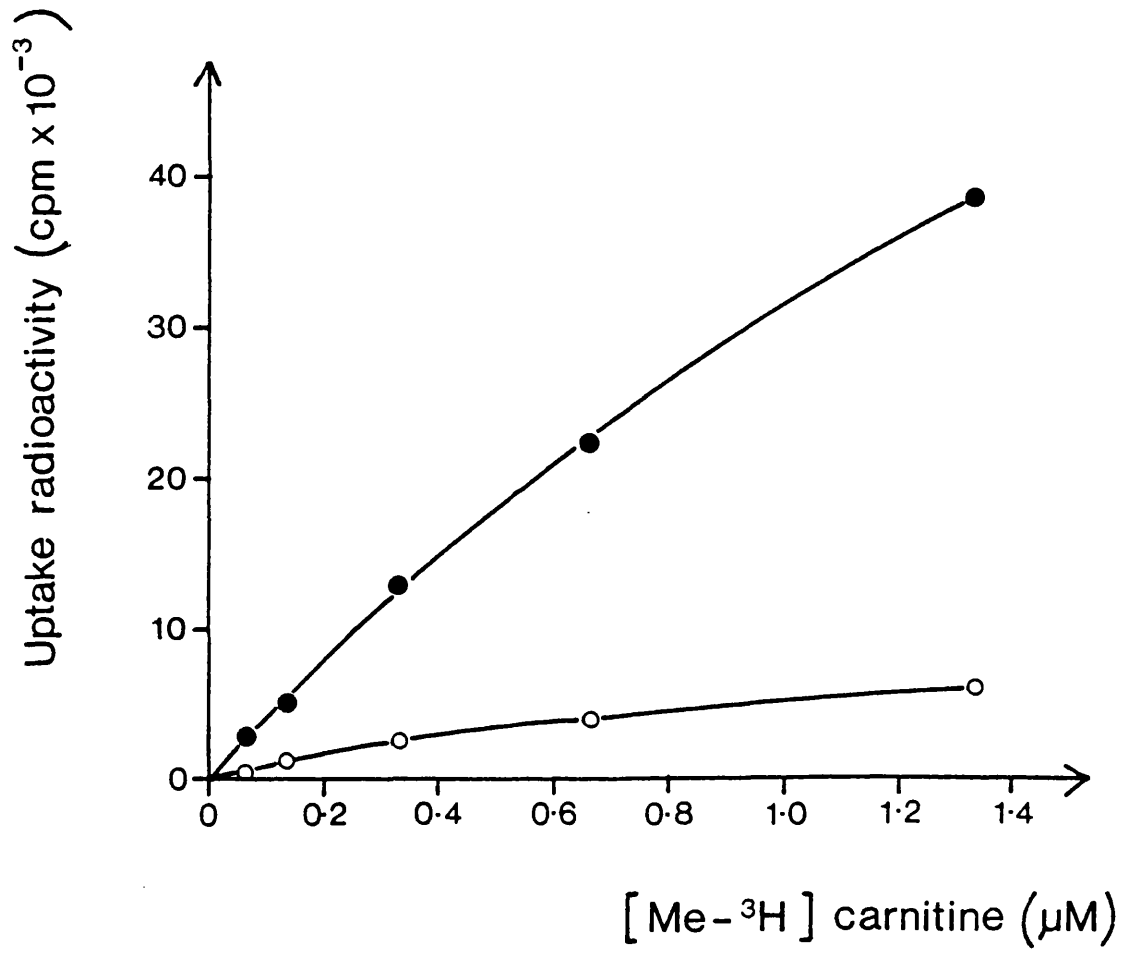
[^{125}I]∝-BGT binding (16.8 and 18.2 fmol/culture well respectively).

As explained in Section A 1.10, use was made of L-[Me-^{3}H] carnitine in later studies for this project. Uptake of radioactivity by human myotubes in culture during incubation with L-[Me-^{3}H] carnitine for 18 h at 37°C, was observed to be higher than that seen after incubation with D,L-[Me-^{3}H] carnitine (Table 29). Similar results were obtained after incubation of fibroblast cultures with L-[Me-^{3}H] carnitine under the same conditions, but uptake by these cultures remained approximately 5-6 times lower than that for the myotube cultures tested in parallel. Unless otherwise stated, results given for human myotubes in culture were obtained after labelling with D,L-[Me-^{3}] carnitine.

1.12 Spontaneous release of [Me-^{3}H] carnitine

Myotube cultures (7 days in culture) were labelled with [Me-^{3}H] carnitine (0.67 µM, 1 µCi) for 18h at 37° C, washed and fresh growth medium was added (0.75 ml). The retention of radioactivity by the cultures after further incubation at 37°C was calculated as described for rat myotube cultures in Section A 1.11. Figure 43 illustrates the spontaneous release of radioactivity with increasing time of incubation. This was essentially linear over the time followed (5 h), indicating an average release of 4.8%/h. Equivalent experiments with cultures preincubated with L-[Me-^{3}H] carnitine (7.5 nM, 0.5 µCi) and further incubated for 3 h at 37°C in fresh growth medium, gave rise to similar values for the spontaneous release of radiolabel (4.2%/h). Fibroblast cultures labelled with D,L (0.67µM, 1µCi) or L-[Me-^{3}H] carnitine (7.5 nM, 0.5µCi) and tested for the spontaneous release of radiolabel as described above gave similar results.

TABLE 29

UPTAKE OF DIFFERENT ISOMERS OF $[Me-^3H]$ CARNITINE

BY HUMAN MYOTUBES IN CULTURE

Myotube cultures, grown sequentially in GM3/GM1, were

incubated with $[Me-^3H]$ carnitine for 18h at 37°C and

the uptake of radioactivity determined as described in

the "Methods" section 7. The cultures were tested over

the 7th and 8th day of growth. Each result is the

mean of 2 separate experiments. Each culture tested

demonstrated $[^{125}I]\alpha$-BGT binding of 13.4 and 18.2

fmol/culture well respectively.

Isomer	$[Me-^3H]$ carnitine added		Uptake of radioactivity
	nM	μCi	(cpm)
[a] L	15.0	1.0	43134
	7.5	0.5	23025
[b] DL	670	1.0	22474
	335	0.5	13167

[a] Specific activity 87 Ci/mmol

[b] Specific activity 2 Ci/mmol

FIGURE 43

SPONTANEOUS RELEASE OF $[$ Me-^{3}H$]$ CARNITINE FROM HUMAN

MYOTUBE CULTURES
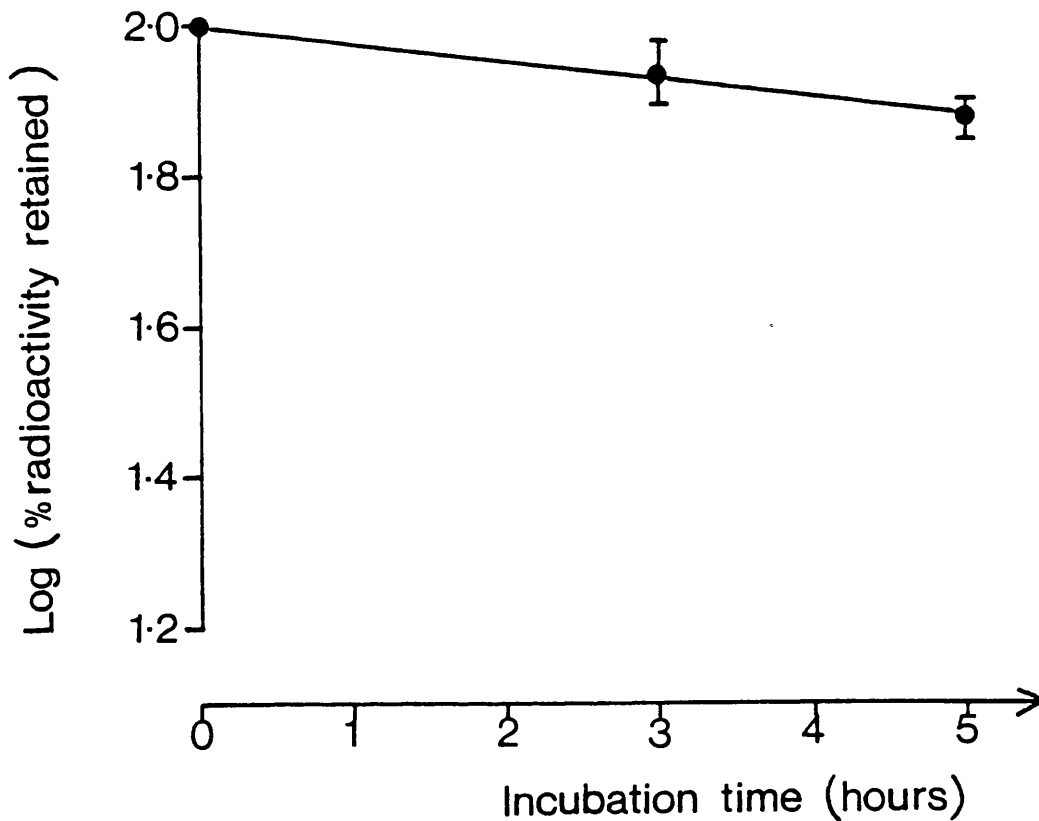
Replicate myotube cultures, grown sequentially in
GM3/GM1, were labelled with $[$ Me-^{3}H$]$ carnitine as
described in Section 2.2.1. The cultures were washed
and fresh growth medium (0.75ml) containing no
radiolabel was added. The retention of radioactivity
by the cultures was determined after 3h and 5h
further incubation at 37°C. Each point is the
mean $^+_-$ SD of 3 different experiments, each of which
included 4 culture wells.

1.13 Effect of D,L-carnitine on [^{125}I]α-BGT binding to crude detergent extracts of human muscle

Crude detergent extract of adult human muscle (0.9 nM [^{125}I] α-BGT binding sites) was incubated in the presence and absence of benzoquinonium chloride (2mM) or in the presence of increasing concentrations of unlabelled D,L-carnitine for 30 min at 23°C. The mixtures were then further incubated with [^{125}I]α-BGT (1 nM, 60 min at 23°C) before determination of toxin binding to the crude extract by ammonium sulphate precipitation on GFC glass filters as described in the "Methods" section 12.2. No inhibition of [^{125}I]α-BGT binding by D,L-carnitine in the concentration range $10^{-2} - 10^{-6}$M was observed (Figure 44). Inhibition of toxin binding to the crude extract by benzoquinonium chloride was 62.6%.

2. Effects of myasthenic serum on cultures

2.1 Binding of serum immunoglobulins

[^{125}I] -labelled goat anti-human light chain antibodies ([^{125}I]-GAHL) were used to detect the binding of serum immunoglobulins to myotube cultures (8 days in culture) as described in the "Methods" section 10.1. Non-specific binding of [^{125}I]-GAHL to the cultures in the absence of test serum was in the range 1850 - 2500 cpm. This value, obtained in each experiment, was arbitrarily taken as 100% and the binding in the presence of test serum expressed as a relative value to allow comparison between different cultures. 3 out of 4 myasthenic serum samples tested gave binding values significantly higher than those obtained for normal sera (Figure 45). The values obtained were 131.0 ± 11.0% (3 serum samples) and 318.9 ± 124.5% (4

FIGURE 44

$\left[\,^{125}I\,\right]\alpha$-BGT BINDING TO DETERGENT EXTRACTS OF ADULT

HUMAN MUSCLE AChR AFTER PRE-INCUBATION WITH

D,L-CARNITINE FOR 30 MIN AT $37^{\circ}C$
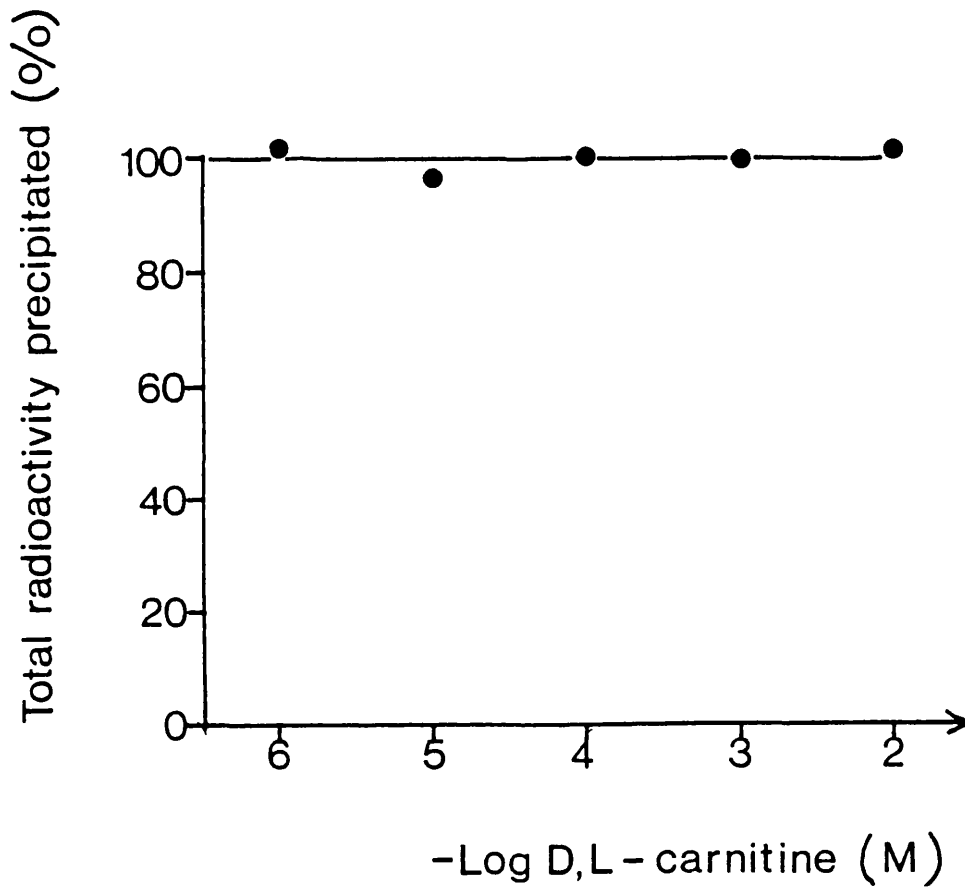
Each point is the mean of 2 different

experiments each carried out in quadruplicate.

serum samples), mean ± SD,n, for normal and myasthenic sera

respectively. As noted with rat myotube cultures (Section A 2.1.1),

the reactive sera were of relatively higher anti-AChR antibody titre,

the binding of these myasthenic immunoglobulins appearing proportional

to the anti-AChR antibody content of the respective sera (Figure 45).

Myotoxicity studies

2.2.1 Labelling of cultures with [Me-^{3}H] carnitine - retention and variability

For myotoxicity studies, [Me-^{3}H] carnitine was added to

human myotube cultures on the 7th day in culture and serum myotoxicity

was estimated on the 8th day in culture. Human myotube cultures were

routinely labelled with D,L-[Me-^{3}H] carnitine (0.67 µM, 1µCi) for 18h at

37°C. This concentration was chosen so as to give a clear difference

between myotube and fibroblast uptake (Figure 42). Under standard

myotoxicity assay conditions (3h further incubation at 37°C, see

Section A 2.2.6), successful cultures without added test serum

retained between 15299 - 23064 cpm (19023 ± 2458, 8 different

experiments, mean ± SD,n). For later myotoxicity studies, myotube

cultures were routinely labelled with L-[Me-^{3}H] carnitine (7.5 nM, 0.5

µCi) for 18h at 37°C. Under the standard myotoxicity conditions

described above, these cultures retained between 21660 - 26427 cpm

(24037 ± 1814, 5 different experiments, mean ± SD,n).

Within one preparation of myotube cultures, the retention of

radiolabel was tested after incubation with D,L-[Me-^{3}H] carnitine

(0.67µM, 1µCi) for 18h at 37°C, followed by further incubation (3h at

37°C) with fresh medium (0.66 ml) to which no additions of test serum

FIGURE 45

BINDING OF SERUM IMMUNOGLOBULINS TO HUMAN MYOTUBE

CULTURES

The binding of normal and myasthenic serum

immunoglobulins to myotube cultures grown sequentially

in GM3/GM1 (8 days in culture) was determined as

described in the "Methods" section 10.1. The results

are expressed as the percentage of radioactivity

bound to the cultures in the absence of test serum

(taken as 100%). The heights of the columns represent

the mean $\pm$ SD of 3 different experiments, each of

which included 4 culture wells.
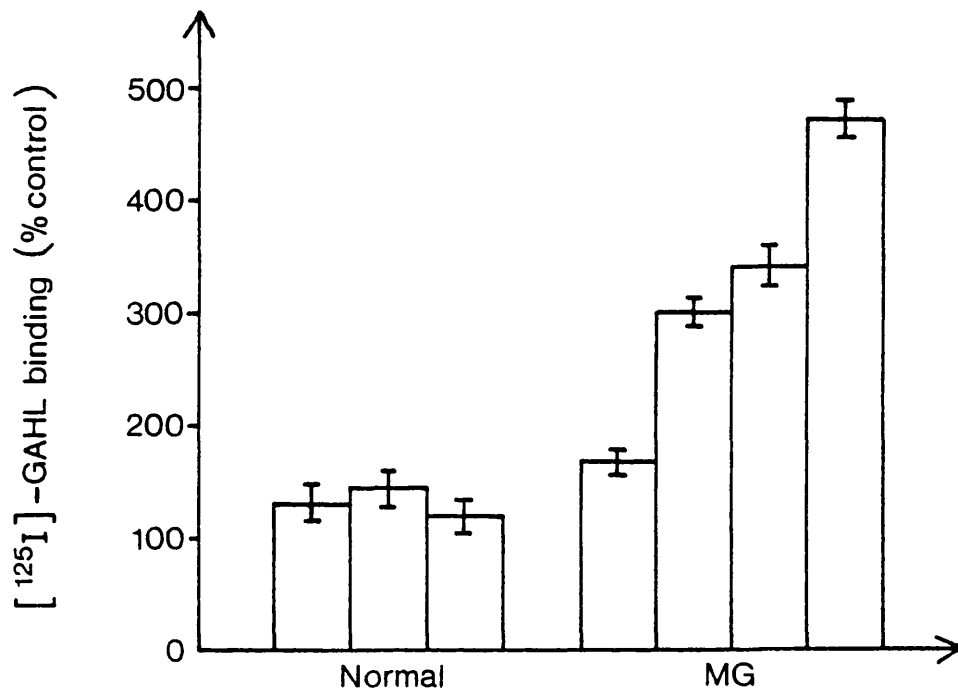
were made (control cultures). Within 24 replicate culture wells (1

culture plate), retention of radioactivity was in the range 14886 -

18157 cpm per culture well, with a mean value of 17109 ± 1188 cpm (24),

mean ± SD (n). Serum myotoxicity assays were carried out in

quadruplicate as described for rat myotube cultures in Section A

2.2.1. Dividing one 24-well culture plate into 6 groups of 'tests'

gave values in the range 16205 - 18073 cpm per test (17109 ± 705 cpm, 6

tests, mean ± SD, n) in the example cited above. The standard error of

the mean (SE) for each 'test', carried out in quadruplicate, was

usually less than 5%.

2.2.2 Effect of non heat-treated serum

Preliminary myotoxicity studies observed the effects of non

heat-treated normal and myasthenic serum samples (both stored before

use at $-20^{\circ}C$ for varying lengths of time) on human myotubes in

culture. All myotube cultures were labelled with [Me-^{3}H] carnitine as

described in Section 2.2.1 before use in the myotoxicity assays. The

myotube cultures were incubated with normal or myasthenic serum (100

μl, 20% v/v) for 3h at $37^{\circ}C$ and myotoxicity was measured as described

in the "Methods" section 8. The measured myotoxicities given by normal

serum samples were in the range 1.0 - 14.4% (8.9 ± 5.0%, mean ± SD, 7

sera). Corresponding values given by myasthenic serum samples were in

the range -1.0 - 27.6% (13.1 ± 8.6%, mean ± SD, 9 sera). The results for

each individual serum sample are shown in Table 30. Only one

myasthenic serum (from patient MG 13) gave higher myotoxicity values,

in each of 2 experiments, than the highest value noted for a normal

serum (from donor 2, 17.1% in one experiment). Statistical treatment

of the data obtained, showed that there was no significant differenc⌐

TABLE 30

MYOTOXICITY OF NON HEAT-TREATED MYASTHENIC AND NORMAL

SERUM SAMPLES

Human myotube cultures, grown sequentially in GM3/GM1,

were labelled with $[\text{Me-}^3\text{H}]$ carnitine (section 2.2.1) and

exposed to samples of test serum (100μl, 20% v/v) for

3h at 37°C. Control cultures to which no additions were

made were run simultaneously. At the end of the incubation

time, the cultures were washed and solubilised for counting.

Myotoxicity was calculated as described in the "Methods"

section 8 by comparison with the control cultures.

Each result is the mean $\pm$ range of 2 different experiments

in each of which were included 4 culture wells.

Test serum	Donor	Anti-AChR antibody titre (nM)	Myotoxicity (%)
Normal	1	-	6.7 ± 5.0
	2	-	14.4 ± 2.7
	3	-	9.7 ± 3.5
	4	-	1.0 ± 0.9
	5	-	4.7 ± 0.9
	6	-	12.5 ± 0.9
	7	-	13.6 ± 1.5
Myasthenia gravis	1	0.8	15.3 ± 2.4
	6	16.0	$- 3.7 \pm 4.1$
	8	24.2	[b] 12.8
	13[a](vii)	36.5	27.6 ± 2.6
	14	81.3	20.4 ± 4.2
	22	76.2	[b] $- 1.0$
	23	100.0	13.8 ± 4.5
	24	24.1	8.6 ± 10.5
	25	32.6	17.1 ± 1.8

[a] Serial samples taken from one patient at different times

[b] One assay only

between the two groups of sera (Mann-Whitney rank sum test, p = 0.025). No culture treated with serum appeared morphologically damaged when viewed by light microscopy.

2.2.3 Effect of heat-treated serum

Heat-inactivation ($56^{\circ}C$, 30 min) of the sera used in the above experiments, resulted in myotoxicity values in the range 1 - 18% for both normal and myasthenic sera. This indicated that values within this range are probably not significant. The only marked difference in myotoxicity after heat-inactivation, was observed with serum from patient MG13 (Table 30), heat-inactivation of which reduced measured myotoxicity from 27.6% to 12.8% (mean of 2 experiments).

2.2.4 Effect of guinea-pig complement

The standardised conditions used to test the complement-mediated myotoxicity of heat-inactivated serum to rat myotubes in culture (Section A 2.2.13) were used to carry out equivalent experiments on human myotubes in culture (Table 31). Of the 7 myasthenic sera tested, 6 had previously been shown to be myotoxic to rat myotubes in culture when tested under the same conditions (resulting myotoxicity values between 38.6% and 60.3% - see Table 12). However, this myotoxicity was not repeated with human cultures, all values falling under 20%.

Using rat myotubes in culture, previously 'non-myotoxic' myasthenic serum was shown to become myotoxic when volumes of test serum and GPC added to the cultures were increased (see Section A 2.2.14). Similar experiments were carried out with human myotubes in culture by increasing the volumes of test serum and GPC added in the

TABLE 31

COMPLEMENT-MEDIATED MYOTOXICITY BY HEAT-INACTIVATED

MYASTHENIC AND NORMAL SERUM SAMPLES TO HUMAN MYOTUBE

CULTURES

Myotube cultures, grown sequentially in GM3/GM1 or

wholly in GM1, were labelled with L-$[$Me-^{3}H$]$ carnitine

(section 2.2.1). The cultures were exposed to samples

of heat-inactivated test serum (80μl, 12.1% v/v) in

the presence or absence of GPC (Miles Labs., 80μl,

12.1% v/v) for 3h at 37°C. Control cultures to which

no additions were made and cultures to which GPC alone

was added were run simultaneously. Myotoxicity was

calculated as described in the "Methods" section 8 by

comparison with the control cultures. Each serum was

tested on 4 replicate culture wells.

The final column gives the

myotoxicity values obtained when

the same myasthenic serum samples

were tested on rat myotube cultures

in the presence of GPC using the same

conditions as above (see Table 12).

Test serum	Donor	Anti-AChR antibody titre (nM)	Myotoxicity (%) heat-inactivated	+GPC	Values obtained with rat cultures (%)
Normal	1	–	14.1	0.4	
	2	–	4.0	16.9	
	3	–	5.3	8.0	
	4	–	4.0	18.9	
Myasthenia gravis	4 [a] (i)	12.2	3.3	16.4	11.7
	(ii)	42.0	6.3	9.8	54.8
	6	16.0	7.0	10.5	56.7
	9 [a] (i)	25.6	5.0	14.4	59.8
	(ii)	42.3	12.5	17.2	38.6
	12	180.0	4.7	1.8	60.3
	13 [a] (v)	20.6	16.1	18.0	47.0
GPC tested alone	–	–	11.2 ± 7.5 (mean $\pm$ SD, 3 experiments)		

[a] Serial samples taken from the same patient at different times

myotoxicity assay. Figure 46 illustrates the myotoxicity results obtained after replicate human myotube cultures were incubated with each addition for 3h at 37°C. The myasthenic serum (anti-AChR antibody titre of 42.0 nM) gave a myotoxicity value of 34.9% with the highest addition tested. The same serum tested on rat myotube cultures under standard myotoxicity test conditions gave a value of 54.8% (see Table 12).

Human myotube cultures were subsequently incubated with increasing concentrations of heat-inactivated serum (50 - 300 µl, 3.3 - 20% v/v) in the presence of a constant concentration of GPC (300 µl, 20% v/v), or alternatively with increasing concentrations of GPC (50 - 300 µl, 3.3 - 20% v/v) in the presence of a constant concentration of test serum (300 µl, 20% v/v) to determine if there was an optimal level of either addition for the routine measurement of myotoxicity. From Figure 47 it can be seen that large additions of both test serum and GPC (200 - 300 µl for the 2 myasthenic sera tested) were necessary to cause myotoxicity values of greater than 20.0%. In these experiments, no damage to the myotubes in culture was observed when they were examined by light microscopy.

FIGURE 46

COMPLEMENT-MEDIATED MYOTOXICITY BY MYASTHENIC SERUM
PREVIOUSLY SHOWN TO BE 'NON-MYOTOXIC' UNDER STANDARD
MYOTOXICITY ASSAY CONDITIONS

Replicate human myotube cultures (grown in GM1) were
labelled with L-[Me-^{3}H] carnitine as described in
Section 2.2.1. Increasing volumes of heat-inactivated
myasthenic serum (shown to be 'non-myotoxic' when
tested under standard myotoxicity conditions - see
Table 31) plus complement were added to the cultures
as follows:-

Addition	Tests			
	Serum added		GPC added	
	(µl)	(% v/v)	(µl)	(% v/v)
1	80	5.3	80	5.3
2	120	8.0	120	8.0
3	160	10.7	160	10.7
4	240	16.0	240	16.0
5	300	20.0	300	20.0

The cultures were incubated for 3h at 37°C. Control
cultures to which no additions of test serum or GPC were
made (1.5 ml of growth medium only) were run
simultaneously. At the end of the incubation time, the
cultures were washed and solubilised for counting.
Myotoxicity was calculated as described in the "Methods"
section 8 by comparison with the control cultures.
Each addition of test serum was tested on 4 culture wells.
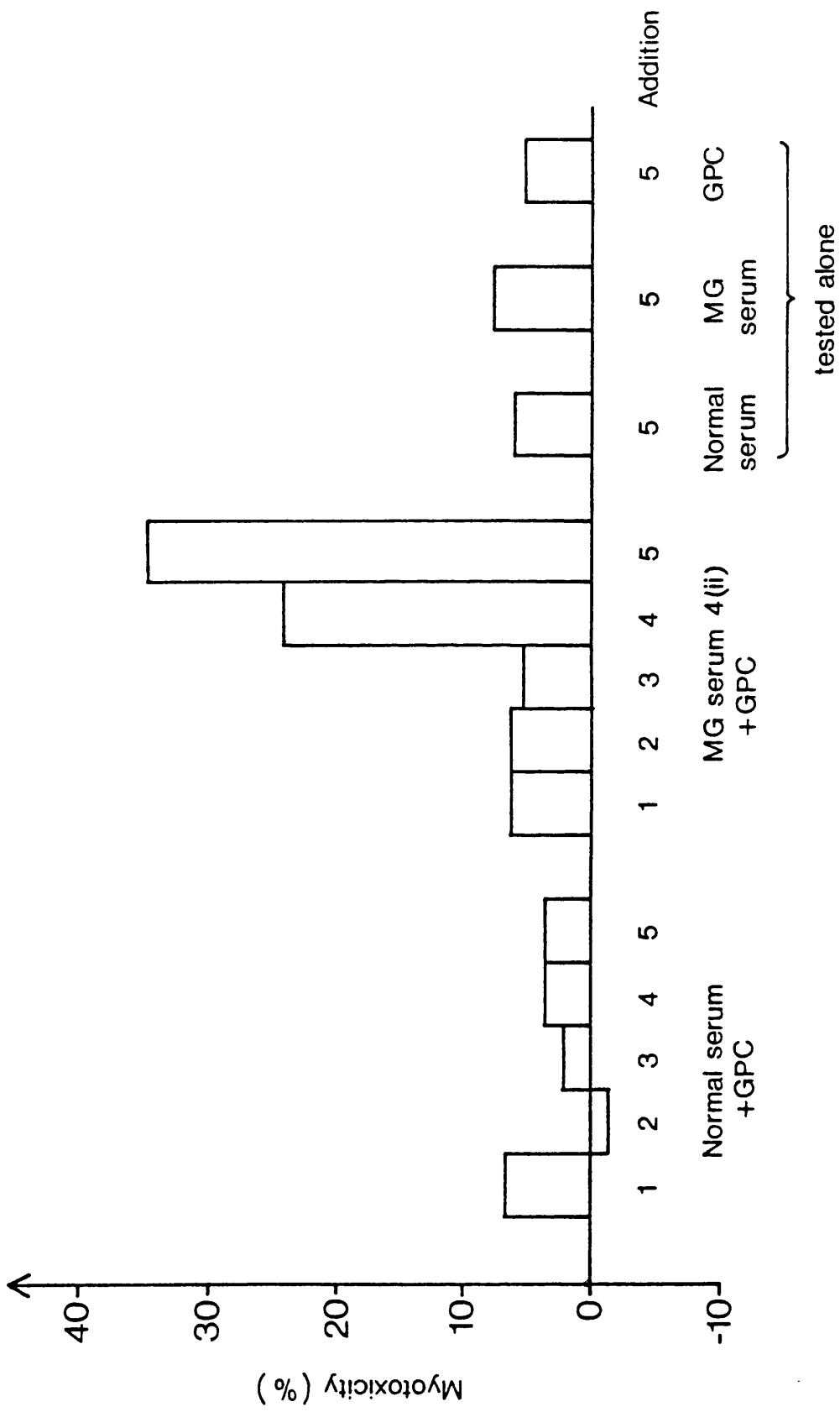
FIGURE 47

MYOTOXICITY OF INCREASING CONCENTRATIONS OF HEAT-
INACTIVATED MYASTHENIC OR NORMAL SERUM AND GPC

Human myotube cultures (grown sequentially in GM1/GM2)
were labelled with L-[Me-^{3}H] carnitine as described
in Section 2.2.1. The cultures were exposed to:-
a) aliquots of heat-inactivated myasthenic or normal
serum (300μl, 20% v/v) plus increasing concentrations
of GPC (50-300μl, 3.3-20.0% v/v) or b) aliquots of GPC
(300μl, 20% v/v) plus increasing concentrations of heat-
inactivated myasthenic or normal serum (50-300μl,
3.3-20.0% v/v) for 3h at 37°C. Control cultures to which
no additions were made were run simultaneously. At
the end of the incubation time, the cultures were washed
and solubilised for counting. Myotoxicity was calculated
as described in the "Methods" section 8 by comparison with
the control cultures. The tests were as follows:-

● Normal serum 1

○ Normal serum 2

□ MG 13 sample (v) Anti-AChR antibody titre = 20.6nM

■ MG 4 sample (ii) Anti-AChR antibody titre = 42.0nM

Each concentration was tested on 4 replicate culture wells.
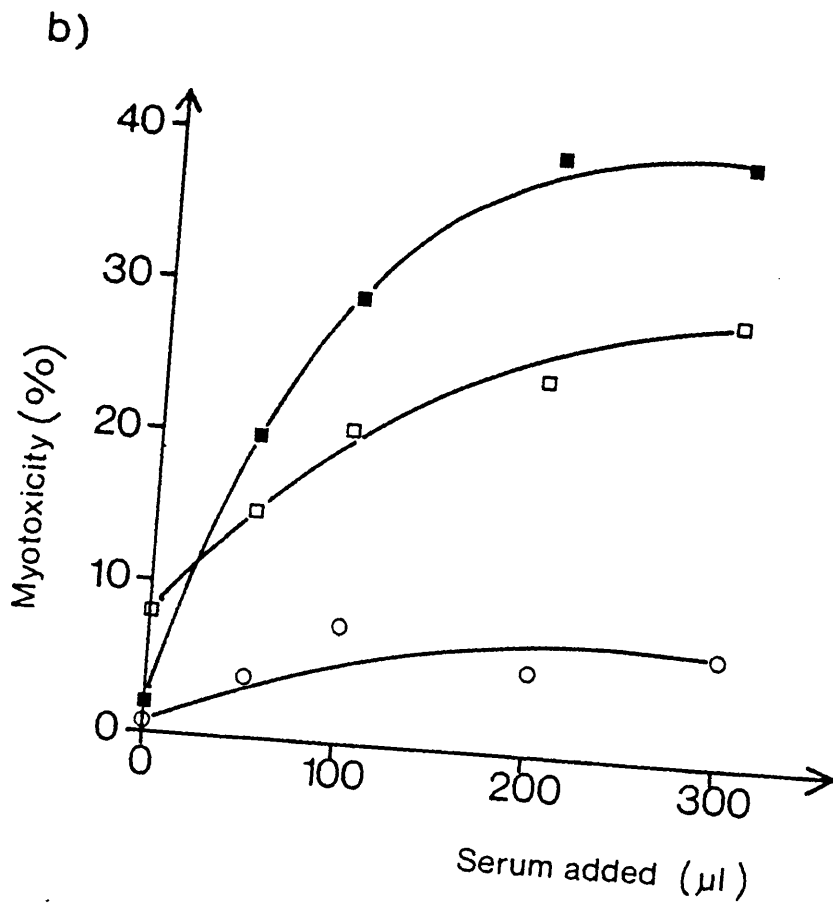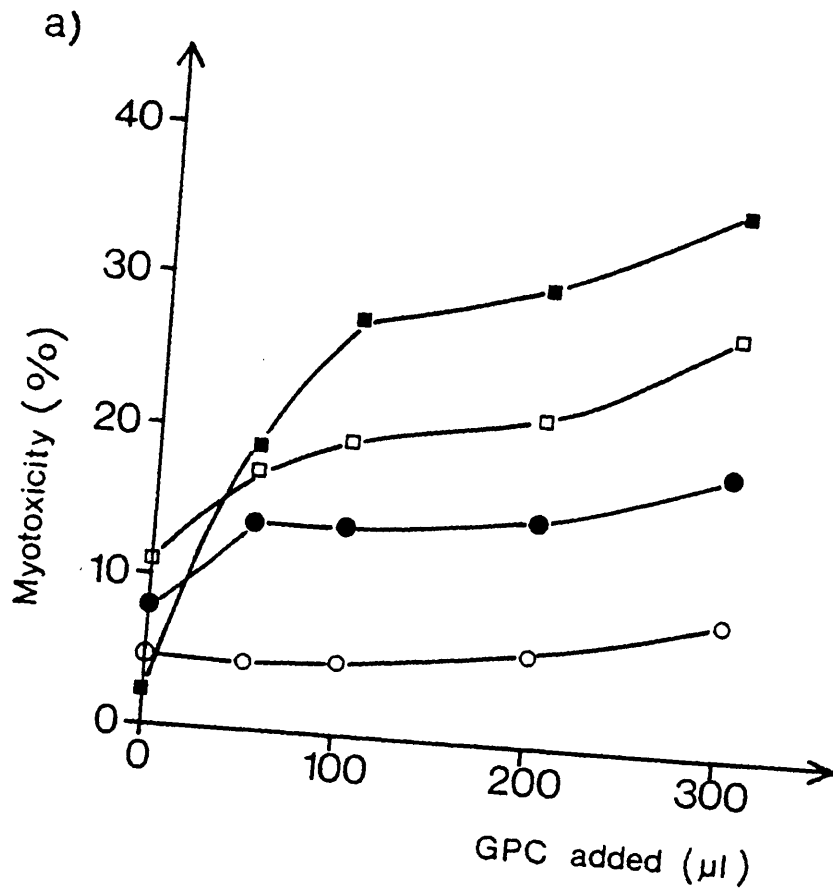
DISCUSSION

The basic defect in MG is a reduction in the number of AChRs at neuromuscular junctions which accounts for the impairment of neuromuscular transmission. Although the mechanisms initiating this disease remain unknown, it is well established that the humoral immune system, via anti-AChR antibodies, plays a central role in the pathogenesis of MG (for reviews see Lindstrom, 1979; Vincent, 1980; Newsom-Davis and Vincent, 1982; Harrison and Behan, 1986). One suggested mechanism for the pathogenicity of anti-AChR antibodies is complement-mediated destruction of the post-synaptic membrane. The possibility that these antibodies could initiate such a mechanism was studied by using a mammalian tissue-culture system. Rat muscle cells in culture were initially used because of their ready availability and known differentiation characteristics. These studies were extended to foetal human muscle cells in culture.

1. Growth of rat muscle cells in culture

1.1 Growth and differentiation

Cultures of rat skeletal muscle cells were successfully established; their growth and maturation in vitro following the expected pattern. Mononucleated cells settled on the collagen substratum and proliferated for 2-3 days. After this time, the spontaneous fusion of myoblasts occurred; the resulting myotubes continuing to grow to form a dense network of branched, spontaneously contracting myotubes. Well differentiated myotube cultures were thus obtained within a culture time of 7 days. With the onset of fusion, a marked increase in CPK and AChE activity occurred. The number of

AChRs, as judged by the binding of [^{125}I]∝-BGT, also increased rapidly

after the onset of fusion. This increase in muscle specific proteins

following the fusion of myoblasts, is well documented for both rat and

chick muscle cultures and is consequently used as a marker of

differentiation (Shainberg et al.,1971; Patrick et al.,1972; Morris

and Cole, 1972; Sytkowski et al.,1973; Prives and Paterson, 1974;

Devreotes and Fambrough, 1975; Prives et al.,1976; Spector and Prives,

1977; Oh and Markelonis, 1978; Shainberg and Brik, 1978).

The specific binding of [^{125}I]∝-BGT to the cultured rat muscle

AChR was demonstrated by the use of decamethonium bromide, an ACh

agonist that shows a high specificity for the AChR at the

neuromuscular junction. This agonist inhibited [^{125}I]∝-BGT binding in

a concentration-dependent manner; maximum inhibition of greater than

90% being achieved at a 1mM concentration of agonist. Equilibrium

binding studies of [^{125}I]∝-BGT indicated a K_D of 13.4 nM under the

conditions of this assay system. Saturating conditions were not used

in routine determinations of [^{125}I]∝-BGT binding because of the large

amounts of iodinated toxin that would be necessary.

During the experiments designed to determine optimal

plating densities, it was noticed that high initial cell densities

resulted in earlier myoblast fusion. The biochemical events involved

in the fusion process are not well understood. The timing of the onset

of fusion is known to be dependent upon growth medium composition and

to be influenced to some extent also by the initial cell density.

Myoblast fusion is Ca^{2+} dependent; low concentrations in growth media

allowing cell proliferation but inhibiting any subsequent fusion. The

onset of fusion is accelerated by restoring optimal Ca^{2+}

concentrations in the culture medium (Shainberg et al.,1969; Fambrough

and Rash, 1971). This procedure has proved useful in experiments designed to examine fusion as a synchronous process. Media supplemented with high concentrations of serum also inhibit the fusion process, which is accelerated by subsequent serum depletion (Yaffe, 1971; Hauschka, 1974a). This procedure has proved useful in cloning techniques for expansion of muscle cell populations without fusion taking place. As already mentioned, the onset of spontaneous myoblast fusion is also dependent on cell density, a high cell density accelerating the fusion process (Konigsberg, 1971; Morris and Cole, 1972; Hauschka, 1974a). These observations would suggest that the onset of spontaneous fusion results from the production of fusion stimulating factors or from the depletion of growth-promoting factors and requires optimal levels of calcium (approximately 1.4mM). It is currently thought that the fusion process is stimulated by activation of a putative receptor that results in the breakdown of membrane inositol phospholipids, the formation of prostaglandins E_2 and a subsequent increase in cAMP and protein synthesis (see Wakelam, 1985 for review).

The fusion of myoblasts is thought to trigger the production of many muscle-specific proteins, including the cholinergic receptors. Electrophysiological measurements of ACh sensitivity (Dryden, 1970; Fambrough and Rash, 1971) and determinations of [^{125}I] ∝-BGT binding (Patrick et al.,1972; Sytowski et al.,1973; Prives and Paterson, 1974) have revealed few, if any, AChRs on the myoblast surface before fusion with a subsequent rapid increase in levels thereafter. Myoblasts appear to be, however, capable of differentiation in the absence of fusion. If fusion is inhibited by lowering the Ca^{2+} concentration in the growth medium of chick muscle

cultures, the myoblasts differentiate in the absence of fusion with a subsequent increase in surface AChR and AChE (Paterson and Prives, 1973; Shainberg and Brik, 1978) and cytoplasmic CPK (Turner et al.,1976; Shainberg and Brik, 1978). It has been suggested, however, that these observations are indicative of a species specific phenomenon; in rat muscle cultures, the appearance of AChR, AChE and CPK depends on cell fusion. The increase in surface AChR on rat myotubes has been shown (Shainberg and Brik, 1978) to result from synthesis de novo in response to fusion, as apposed to the presence of a pool of pre-existing AChRs inside the myoblasts. The latter proposal has been suggested by the observation that chick myoblasts contain relatively high levels of intracellular, as opposed to surface, AChR (Teng and Fiszman, 1976).

1.2 Reduction of fibroblast growth

Primary cell cultures prepared from muscle are mixtures of both myoblasts and fibroblasts although other cell types, such as smooth muscle cells from vascular walls, could also be present. The contaminating fibroblasts usually overgrow the myotubes in culture unless their growth is minimised. The fibroblasts present can obscure the visual assessment of myotube growth and may affect this growth by competition for nutrients. Their presence can also make the design and interpretation of many experiments difficult. In the context of the work carried out for this project, minimal fibroblast contamination is also desirable because of the contribution by fibroblasts to total [Me-^{3}H] carnitine uptake by the cultures.

Several methods have been reported whereby the number of fibroblasts present in cultures can be reduced. These include the

pre-plating of the primary cell suspension (Yaffe, 1968), the exposure of cells in primary suspensions to X-irradiation (Friedlander et al.,1978) and the use of cytotoxic drugs to inhibit fibroblast cell division (Fischbach, 1972; Wood, 1976). In the present studies, pre-plating was found not to reduce fibroblast growth effectively and was not routinely used. It was also found moreover, that some of the supposed fibroblasts affixed to the pre-plates, when maintained in growth medium, developed into spontaneously contracting myotubes suggesting the additional removal of myogenic cells. These myotubes developed in the absence of a collagen-coated surface, probably as a result of fibronectins being produced by the fibroblasts present (Chiquet et al.,1979).

Fibroblast growth in the rat muscle cultures prepared for this project, was routinely minimised by the addition of fluorodeoxyuridine (FDU) or cytosine arabinoside (Ara C). FDU and Ara C are mitotic inhibitors which exert their effects by inhibiting DNA synthesis and the replicative DNA polymerases respectively (Cozzarelli, 1977). It has been reported that FDU can also prevent the fusion of myoblasts in culture (Fambrough and Rash, 1971) which could explain the reduction in the number of $[^{125}I]\alpha$-BGT binding sites seen in the cultures treated with these drugs (see Table 2). It is also possible that the drugs were cytotoxic to some of the myogenic cells (Cozzarelli, 1977). Despite these possible disadvantages, the effectiveness of FDU and Ara C in minimising the fibroblast contamination was felt to warrant their use.

A factor to be considered when interpreting experimental results obtained from muscle cultures, is the contribution of fibroblasts to the parameters being determined. It is common practice

to present comparative data with respect to the total protein content of the culture, the percentage of total nuclei present in myotubes or to the total DNA present. These factors could only be relevant if the contribution by fibroblasts to each count was negligible as these cells contribute protein, nuclei and continued DNA synthesis in the cultures under examination. For this reason, it can be difficult to compare results as culture conditions for the preparation and growth of muscle cells are not standardised between laboratories. An example of using such criteria could be demonstrated by using the experimental results obtained after treatment of the rat muscle cultures with cytotoxic drugs. The number of $[^{125}I]\alpha$-BGT binding sites in the control, FDU and Ara C treated cultures would be 295.6, 400.0 and 426.1 fmol per mg protein respectively (see Table 2) as apposed to the presented result of 47.3, 36.8 and 39.2 fmol per culture well respectively. The former result would clearly indicate a stimulatory effect of the drugs. Therefore, as many experiments were of a comparative nature, all results for this project are presented as per culture well.

1.3 Serum composition of growth medium

There was little variation in growth, as judged by $[^{125}I]$ α-BGT binding, carnitine uptake and total protein content, between cultures established from the same primary cell suspension. The greater variability which was observed between cultures prepared at different times could result from a number of factors including mixed cell types in the original cell suspension, differing plating efficiencies and the composition of the particular serum batch used to supplement growth media. It is not easy to identify fibroblasts and

myoblasts in cell suspensions as they are morphologically similar. The development of monoclonal antibodies specific for myoblasts (Walsh and Ritter, 1981) will help in this respect. The plating efficiencies of the cultured muscle cells were not determined as the high cell densities used made these determinations impracticable.

The composition of serum added to growth medium can vary from batch to batch. The components of serum include hormones, vitamins, lipids and trace metals, variations in all of which may significantly affect cultures (see Hauschka, 1972; Paul, 1975; Gospodarowicz and Moran, 1976; Sato and Reid, 1978; for reviews). Each batch of serum was routinely pre-tested for its ability to promote muscle cell growth. One particular serum batch apparently deteriorated, giving rise to relatively poor cultures with lower myotube densities and reduced levels of [^{125}I]α-BGT binding (for an example see Figure 13b, culture C).

The serum factors promoting muscle cell growth have still not been defined but are thought to be mainly hormonal (see Sato and Reid, 1978 for review). Attempts have been made to clarify these factors with the use of serum-free chemically defined media which allow more detailed analyses of the influence of environmental factors on muscle differentiation, changes in membrane components and identification of components secreted by cells during different stages of development. A further aim is to produce reproducibly differentiated muscle cells. Several investigators have described defined serum-free media which support both growth and differentiation of primary rat and the L6 cell line (Florini and Roberts, 1979) and chick muscle cells (Kumegawa et al.,1980; Dollenmeier et al.,1981). The medium described by Florini and Roberts (1979) was based on the addition of fetuin, insulin and

dexamethasone. Kumegawa et al. (1980) defined a medium with added insulin and thyroxine while Dollenmeier et al. (1981) used a complex composition of hormones, vitamins and growth factors which again included insulin. A serum-free chemically defined medium is routinely used in this department for the growth of neuronal cells in culture after initial cell attachment has taken place in serum containing medium (Digby et al.,1985 - see "Materials" p.50). The use of this medium to promote rat muscle growth in culture was not successful; only poor growth, as judged by light microscopy and levels of [^{125}I] ∝-BGT binding, being demonstrated. The cultures grown in SFM were, however, remarkably free of fibroblast contamination, a consequence also noted by Florini and Roberts (1979) and Dollenmeier et al. (1981).

2. Growth of human muscle cells in culture

There have been relatively few reports of human foetal muscle cells in culture; a fact reflecting the difficulty in supply. Table 32 lists studies in which the successful growth of human foetal muscle cells was reported and which gave details of the conditions used for growth. As can be seen, a wide variety of growth medium compositions has been used. Although many of these studies based their techniques on those described by Yasin et al. (1977) for the growth of dissociated adult human tissue, or on those described by Askanas and Engel (1975) and Witkowski et al. (1976) for the growth of adult human explant cultures, inevitably each group has added modifications. Table 33 lists similar details for adult human muscle cultures prepared by dissociation of tissue. In many cases, these studies do not give details describing the morphology or differentiation of the muscle

TABLE 32   CONDITIONS FOR THE GROWTH OF HUMAN FOETAL MUSCLE CELLS IN CULTURE

Culture technique	Growth medium	Serum supplement (v/v)	[a] Other additions (v/v)	Reference
Explant	33% Tyrode's BSS	33% Human serum	[b] 33% CEE	Kakulas et al.,1968
	199	10% HS	25% CEE	Currie, 1970
	DMEM	10% FCS	-	Bevan et al.,1977,1978
	199	1) 10% FCS	-	Harvey et al.,1979,1930
		2) 2.5% FCS after fusion		
Dissociation of explant outgrowths	Ham's F 10	10% FCS	Calcium to 0.7mM	Emery & McGregor,1977
	Eagle's minimal EM	1) 15% FCS	Enriched Earles salts	Meola et al.,1983
		2) 10% FCS on fusion	-	
Dissociation of tissue	Ham's F 10	15% HS	5% CEE	Hauschka, 1974a
	DMEM	10% FCS	-	Bevan et al.,1978
	199	1) 10% FCS	-	Cambridge & Stern,1981
		2) FCS reduced for fusion		
	Minimal essential	10% HS	2% CEE	Strohman et al.,1983
	DMEM	1) 16% FCS	1.8% CEE	Yasin & Van Beers,1983
		2) 8% HS for fusion	1.8% CEE	
	DMEM	10% FCS	50ng/ml cholera toxin for fusion	Adams & Bevan,1985

a All media contained antibiotics   b Chicken embryo extract

TABLE 33   CONDITIONS FOR THE GROWTH OF HUMAN ADULT MUSCLE CELLS IN CULTURE PREPARED BY ENZYMIC DISSOCIATION OF TISSUE

Growth medium	Serum supplement (v/v)	[a] Other additions (v/v)	Reference
DMEM	9% HS	[b] 1.8% CEE	Yasin et al.,1977 Franklin et al.,1980
DMEM	1) 16% FCS 2) 8% HS for fusion	1.8% CEE 1.8% CEE	Yasin et al.,1981,1982
1) Ham's F 10 2) DMEM	1) 20% FCS 2) 2% HS for fusion	0.5% CEE –	Blau & Webster,1981 Blau et al.,1983
Minimal essential	10% Human serum	3% CEE	Cossu et al.,1980
DMEM/199 4:1	1) 20% FCS 2) 5% HS for fusion	– –	Neville et al.,1983 Graham et al.,1984
DMEM	1) 20% FCS 2) 10% HS for fusion	2% CEE	Bolhuis et al.,1985

[a] All media contained antibiotics   [b] Chicken embryo extract

cultures obtained.

2.1 Conditions for growth

The human foetal muscle cultures established in this study

showed a similar growth pattern to that observed for rat muscle cells

in culture. Mononucleated cells settled on the collagen substratum and

proliferated; the onset of fusion occurring spontaneously after 3-4

days in culture, approximately one day later than noted in the rat

cultures. The resulting myotubes continued to grow, as judged by light

microscopy, but not to the same extent as rat myotubes in culture,

being generally thinner, not as long, less dense and less branched. No

spontaneous contractions were ever seen in the human cultures.

Use of media supplemented with 20% FCS stimulated the

proliferation of cells and inhibited myoblast fusion as described by

Hauschka (1974a) and by Yaffe (1971). This technique allowed lower

initial cell densities to be used, fusion being initiated by serum

depletion at cell confluence. The resulting myotubes were, however,

heavily contaminated with fibroblasts and it is probable that this

procedure would only prove useful where the percentage of fibroblasts

in the initial cell suspension is low. This technique is used to

promote human myoblast proliferation in cloning techniques (Blau and

Webster, 1981; Yasin et al.,1981, 1982) or where human muscle cells

are obtained from one or more repassaging procedures (Neville et

al.,1983; Yasin and van Beers, 1983; Graham et al.,1984; Bolhuis et

al.,1985). Repassaging procedures were recommended by Konigsberg

(1979) as yielding purer populations of cells, the fibroblasts tending

to adhere to a plastic surface following the trypsinisation of primary

muscle cultures. The repassaging procedure carried out in this work

resulted in less fibroblast contamination in the secondary cultures and an increase in the number of $[^{125}I]\alpha$-BGT binding sites per culture well when compared to the primary culture (see Table 23). This indicated that a purer population of muscle cells was present in the secondary culture. Although the final cell yield was reduced on repassaging, this procedure may be worth pursuing. The growth of cells to confluence from a smaller initial cell density than used in this study, and subsequent repassaging of cultures, perhaps 2-3 times, could result in a purer population of muscle cells and an increased cell number. The problems encountered with fibroblast contamination in this study were probably also contributed to by the difficulty in dissecting away all the foetal skin from the muscle pieces.

The addition of FDU or Ara C to restrict fibroblast growth in human muscle cultures resulted in a sparse myotube population which was reflected in the reduced number of $[^{125}I]\alpha$-BGT binding sites (Table 22). The deleterious effects of FDU and Ara C probably arose from inhibition of myoblast fusion (Fambrough and Rash, 1971) and possibly from the cytotoxic effects of the drugs (Cozzarelli, 1977). The time over which human myoblasts fused to form myotubes was longer than that observed for rat myoblasts when fusion took place in GM1 (containing 10% HS). This could explain the more severe effects on growth when human, as opposed to rat, muscle cultures were treated with these drugs.

The use of serum-free, chemically defined media (see "Materials" p.50) supported the fusion of myoblasts in a similar manner to that of control cultures maintained in media supplemented with 10% HS, although not as well as cultures maintained in media supplemented with 2% HS. These judgements were made on the basis of

morphology and final numbers of [^{125}I]α-BGT binding sites (Tables 21 &
26). SFM did not, however, support initial cell proliferation and
growth although some cell attachment to the collagen substratum took
place. This is in agreement with the observation of Yasin and van
Beers (1983) who tested the effects of SFM on adult and foetal human
muscle cell growth in culture. These workers used defined growth media
with added fetuin, insulin and dexamethasone or alternatively with
added insulin and thyroxine. Their SFM's also failed to support
initial cell attachment and growth. When used at a later stage to
promote myoblast fusion, both types of SFM supported myotube growth
but to a lesser extent than did serum supplemented medium. Yasin and
van Beers (1983), however, judged the differentiation of the myotubes
present, based on morphological appearance and CPK levels, to be
improved, particularly in the case of adult human muscle. The same
workers also noted that fibroblast contamination was considerably
reduced. The lack of cell adhesion and growth in the presence of SFM,
both in this study and in that of Yasin and van Beers (1983), probably
reflects the lack of fibronectin (Chiquet et al.,1979). The small
amount of cell attachment seen in the presence of SFM probably arose
from the presence of serum in the initial cell suspension, which was
subsequently diluted in SFM. The experiments carried out with rat
muscle cultures grown in SFM (see "Discussion" section 1.3) indicated
that a deficiency of serum components resulted in poor growth and
development. This was not the case with the human muscle cells
suggesting that a better medium for the growth of human muscle in
culture may exist.

The use of nutrient deprivation to stimulate myoblast fusion in
human muscle cultures has been reported by several groups of workers

(Cambridge and Stern, 1981; Blau and Webster, 1981; Neville et al.,1983; Graham et al.,1984; Bolhuis et al.,1985). This procedure was subsequently used in the present investigations and it was observed that the use of GM2 (containing 2% HS) resulted in an improved synchrony of fusion and an apparent increase in the extent of the fusion process. The latter observation was suggested by the increased myotube densities achieved and by the increased levels of $[^{125}I]\alpha$-BGT binding when compared to control cultures maintained in GM1 (containing 10% HS). Nutrient deprivation also reduced the level of fibroblast contamination. Minimal proliferation of fibroblasts after nutrient deprivation of human muscle cultures has also been noted by other investigators (Harvey et al.,1979; Neville et al.,1983; Bolhuis et al.,1985). In this study, the use of GM1 followed by GM2, to stimulate myoblast fusion, was considered to be the most successful growth medium combination to be used for the growth of human foetal muscle cell cultures.

2.2 Addition of hormones or growth activators

The addition of cholera toxin or dbcAMP to growth media has been reported to stimulate fusion in cultured rat muscle (Stygall and Mirsky, 1980), and cholera toxin was included in the growth medium by one set of workers to increase the degree of fusion in foetal human muscle cells in culture (Adams and Bevan, 1985). Reports on the effects of adding dbcAMP to myoblasts in culture are variable. Stygall and Mirsky (1980) suggested that stimulation of myoblast fusion only occurs if the intracellular levels of cAMP are 'sub-optimal'. The addition of dbcAMP to cultured foetal human muscle cells in this study did not appear to increase greatly the extent of myoblast fusion, as

judged by final levels of [^{125}I]α-BGT binding sites (Table 27).

Dexamethasone, a steroid hormone, has been shown to stimulate cell proliferation in rat muscle cultures (Florini and Roberts, 1979) and has been included in the growth medium, together with insulin, to promote myoblast fusion in mass cultures of human muscle clonal cells (Kedes et al., 1984). In the present study, the addition of dexamethasone to human muscle cells resulted in a 25% increase in final levels of [^{125}I]α-BGT binding sites (Table 27). This result was obtained from only one experiment in relatively poor cultures. It could, however, be profitable to consider the addition of dexamethasone to growth medium in future studies.

Insulin is a known growth promoter (see Froesch et al.,1985; Kahn, 1985 for reviews) and has been shown to stimulate muscle cell growth (Mandel and Pearson, 1974; Florini and Roberts, 1979; Kumegawa et al.,1980) and fusion in culture (de la Haba et al.,1966; Mandel and Pearson, 1974; Kumegawa et al.,1980). In this study, insulin did not increase the extent of fusion of human myoblasts but did appear to increase cell proliferation. Addition of insulin to the growth medium throughout the culture period resulted in an average 19% increase in the final numbers of [^{125}I]α-BGT binding sites (Table 27). No increase in toxin binding sites was observed if insulin was added at a later stage to promote myoblast fusion. Unfortunately, the early addition of insulin resulted in increased fibroblast contamination, which was reflected in increased total protein levels.

The addition of dbcAMP and on occasion, also of insulin to the growth medium resulted in an altered morphology of the resulting myotubes. These appeared broad and flat, and in the case of added dbcAMP acquired a 'wavy' membrane appearance. Clonal studies carried

out by Hauschka (1974b) demonstrated that the morphology of human
foetal myotubes altered with increasing age of the foetal tissue.
Young foetal tissue gave narrow myotubes in culture whereas older
tissue (~17 weeks) resulted in broad, flat myotubes. A later study by
Harvey et al. (1979) showed that the morphology of myotubes arising
from human foetal explant cultures, changed with time in culture. The
first myotubes to appear were broad and flat, subsequently giving rise
to long, narrow refractile myotubes. With increasing time in culture,
broad flat myotubes were again apparent, and after 11 weeks, the
narrow refractile form had become flattened and adopted a 'wavy'
appearance. Both types of myotube were observed to contract but the
narrow refractile form exhibited greater responsiveness to
iontophoretically applied ACh. It would appear from the studies of
Harvey et al. (1979) that the narrow refractile form of myotube is the
more developed but the significance of human myotube morphology in
culture is unclear. The broad, flat myotubes in cultures treated with
dbcAMP or insulin could suggest that these cultures had 'aged'.

A recent report by Askanas et al. (1984) described the
effects of adding insulin, epidermal growth factor and fibroblast
growth factor to adult human muscle in explant culture. These workers
reported stimulated and extended myoblast fusion in these cultures and
increased development, as judged by CPK activity and the number of
AChRs, at an earlier age. The effects of these additives on
dissociated human foetal muscle in culture would therefore be worth
investigating.

2.3 Variation between cultures

The variation between human foetal muscle cultures prepared from the same primary cell suspension was small, as judged by light microscopy, carnitine uptake and the number of $[^{125}I]\alpha$-BGT binding sites. The greater variability between cultures established at different times was possibly attributable to the reasons outlined for rat muscle cultures (see "Discussion" section 1.3) but could also arise from the age of the foetal tissue used. In the experiment carried out comparing the growth of 9 week and 14 week foetal muscle under identical conditions, the older tissue gave denser, more branched myotube cultures but otherwise the morphology of the myotubes appeared similar. The latter observation is in contrast to those of Hauschka (1974b) (discussed in the previous section). Hauschka (1974b) also demonstrated that the proportion of cells in the human foetal limb which could form muscle colonies rose from 15% at 40 days to over 90% at 14 weeks.

2.4 Differentiation of human muscle cultures

The extent of differentiation in the human foetal muscle cultures was investigated by determining the number of $[^{125}I]\alpha$-BGT binding sites present in culture. Decamethonium bromide, an ACh agonist, inhibited $[^{125}I]\alpha$-BGT binding in a concentration-dependent manner, maximum inhibition being achieved at 1mM decamethonium. The degree of inhibition achieved by using this agonist was dependent on the extent of fibroblast growth in the cultures. High levels of fibroblast contamination led to relatively high levels of non-specific binding of $[^{125}I]\alpha$-BGT to the cultures. The use of unlabelled α-BGT to detect non-specific binding was observed in two experiments to

increase the apparent specific binding of [^{125}I]α-BGT (Table 25).
However, this procedure was considered to be less reliable and was not
routinely used (Lunt, 1985).

The specific binding of [^{125}I]α-BGT to the foetal human muscle
cultures was observed to increase rapidly after the onset of myoblast
fusion, reaching a peak within 4-5 days. Similar observations were
made by Franklin et al. (1980) and Blau and co-workers (1981, 1983)
for adult human muscle cultures prepared from dissociated tissue or
from pooled clonal cells respectively. In accordance with the
observations of Blau and co-workers (1981, 1983) it was observed that
the number of [^{125}I]α-BGT binding sites decreased after a peak value
was obtained. These results are also in agreement with the findings of
Prives et al. (1976) for cultured chick muscle cells. In the latter
case, however, there is a suggestion that loss of surface AChRs
results from muscle contractions (Shainberg et al.,1976; Birnbaum et
al.,1980). The cultured human muscle in this study never showed
spontaneous contractility. It is possible that a neural factor is
necessary to stabilise or increase the AChRs on the surface of the
myotubes (Christian et al.,1978; Podleski et al.,1978; Schaffner and
Daniels, 1982; Salpeter et al.,1982; Buc-Caron et al.,1983).

The presence of occasional spontaneous contractions of adult
human myotubes in dissociated cultures has been observed by Yasin et
al. (1977), Blau and co-workers (1981, 1983) and Bolhuis et al.
(1985). They have also been observed in foetal muscle explant culture
(Harvey et al.,1979) but not in foetal muscle cultures prepared from
dissociation of explant outgrowths (Meola et al.,1983). Other reports
have given no details concerning the observation of spontaneous
contractions.

It was not possible to correlate the CPK and AChE activities
of foetal human muscle cultures with time in this study because of the
number of replicate cultures that would be necessary. These activities
were determined on 7-day cultures, and found to be low compared to
those of rat muscle cultures. The human myotube cultures established
in the investigations carried out for this project generally appeared
immature when compared to the rat myotube cultures. Although the two
types of muscle AChR were shown to have similar affinities for [^{125}I]
x-BGT, the number of binding sites expressed and the enzyme activities
measured per culture were far lower in the human cultures (Table 34).
One contribution to these factors is the lower myotube densities
achieved in the human cultures. However, the rat myotube cultures had
similar or much higher levels of [^{125}I]x-BGT binding per culture when
initiated at low cell densities (giving rise to sparse or low density
myotubes) and assayed at lower concentrations of iodinated toxin.
Therfore it is safe to assume that the human myotubes were each
expressing lower numbers of AChRs.

2.5 Maturity of cultures

A similar lack of maturation in human muscle cultures, to that
described above, has been indicated in other studies. Using the
measurement of muscle specific proteins and other biochemical
parameters of cell metabolism as criteria for the differentiation of
human adult muscle in culture, relative lack of maturity has been
noted by Iannaccone et al. (1982) and Bolhuis et al. (1985). Both
groups of workers used techniques based on the trypsinisation of
explant outgrowths. Iannaccone et al. (1982) noted that early
myogenesis, as judged by CPK and pyruvate kinase activity and myosin

synthesis, was similar to that observed with chick muscle culture. However, the degree of differentiation observed in chick cultures after 7-10 days growth was not equalled by the human cultures after 36 days growth. Using similar parameters, Bolhuis et al. (1985) concluded that only an early differentiation phase has been reached by the human muscle cultures although spontaneous contractions were occasionally observed.

Franklin et al. (1980) found that the levels of AChRs expressed in adult human dissociated muscle cultures were 5-fold lower than those expressed by neonatal mouse muscle cultures established under similar conditions. Determinations of ACh activated channel currents and autoradiographic analysis of $[^{125}I]\alpha$-BGT binding to human foetal dissociated muscle cultures have shown a very low density of AChRs to be present on the myotubes (Adams and Bevan, 1983, 1985) when compared to other species (Table 35). Askanas et al. (1977), using an indirect immunoperoxidase method for bound AChR, noted only faint staining of adult human myotubes in culture in contrast to the heavy staining obtained with rat and chick cultured myotubes. No basal laminar membrane was observed in the human cultures (Askanas et al.,1977) and there was negligible histochemical staining for AChE (Kobayashi and Askanas, 1985). Later studies involving co-cultures of human muscle and foetal rat spinal cord (Kobayashi and Askanas, 1985) found increased levels of AChR and AChE forming at the synaptic junctions, and the partial appearance of a basement membrane. Recently, in this department, successful co-cultures of human foetal muscle and human neuronal cells has been achieved. The myotubes present appeared morphologically to attain a higher degree of maturity than noted when cultured aneurally. Many of the neurones appeared to be in functional

TABLE 34

COMPARISON OF RAT AND HUMAN MUSCLE CELLS IN CULTURE

(THIS STUDY)

1) Specific $[^{125}I]\alpha$-BGT binding

K_D	(fmol/culture well)	Protein (μg/culture well)	Source
13.4 nM	55.5 ± 9.9 (19)	103.3 ± 14.4	Rat
16.6 nM	15.4 ± 5.3 (33)	124.3 ± 26.0	Human

2) CPK activity nmol substrate converted/min/culture well

18.1 ± 2.1 (5) Rat

7.7 ± 2.5 (4) Human

3) AChE activity nmol substrate hydrolysed/min/culture well

0.23 ± 0.06 (5) Rat

0.15 ± 0.03 (3) Human

All results are the mean $\pm$ SD (number of preparations)

TABLE 35

DENSITY OF ACETYLCHOLINE RECEPTORS ON CULTURED MUSCLE CELLS

Muscle source		Receptor density (sites/μm^2)	Reference
Chick embryo	(cluster)	9000	Sytowski et al.,1973
	(diffuse)	900	
Rat embryo	(cluster)	8000	Axelrod et al.,1976
	(diffuse)	2000	
	(cluster)	3000-4000	Land et al.,1977
	(diffuse)	54-900	
Rat L-6 cell line		5-400	Land et al.,1977
Human foetal		1 - 4	Adams & Bevan, 1983, 1985

contact with the myotubes and after two weeks of growth, many of the myotubes were observed to contract spontaneously. These studies indicate that neural factors could be necessary for further development of cultured human muscle to take place.

Investigations by Harvey and co-workers (1979, 1980) have indicated that a long maturation time was necessary for aneurally cultured human muscle, prepared from explants, to attain fully differentiated membrane characteristics. These studies (Harvey et al.,1979, 1980) are the only reports for foetal human muscle correlating growth with any but morphological criteria. After the first appearance of myotubes, sensitivity to applied ACh and resting membrane potentials increased with time in culture. Although the myotubes appeared morphologically well differentiated after 4-6 weeks growth, having cross-striations and some spontaneous contractions, ACh sensitivity did not reach maximal levels until 8-10 weeks growth. The long development time of these cultures contrasts sharply with those observed for rat or chick muscle cultures (Harvey, 1984). The need for a long maturation period for fully differentiated aneurally cultured human muscle could reflect the long development period of muscle in vivo (Minguetti and Mair, 1981). Maintenance of dissociated cell cultures for such long periods would appear to be difficult and the human muscle cultures tested in this work deteriorated after approximately two weeks in culture. This could reflect the high cell densities used, the lack of suitable growth medium or the nature of the dissociation techniques used to establish muscle cells. These techniques would appear to result in the more rapid growth of cultured muscle when compared to the time taken for outgrowths of explant tissue to mature (eg. Askanas et al.,1977; Bevan et al.,1978; Harvey

et al.,1979;   Blau and Webster, 1981; Iannaccone et al.,1982).

3. Uptake of [Me-^{3}H] carnitine by muscle cultures

Carnitine (ɣ-amino-ꞵhydroxy-butyric acid. 3-methyl-betaine) is found in a number of cell types; its concentration in muscle cells is particularly high (Greville and Tubbs, 1968). 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 role of carnitine is to promote the oxidation of fatty acids by facilitating their transfer across the inner mitochondrial membrane (Fritz and Marquis, 1965).

Carnitine is a structural analogue of choline and its O-ester, acetylcarnitine, is an analogue of acetylcholine. From the structural similarity of carnitine to choline, and acetylcarnitine to acetylcholine (see Figure 48); it has accordingly been assumed that acetylcarnitine could play a role in cholinergic neurotransmission (Sass and Werness, 1973). L-Acetylcarnitine, the naturally occurring form in the CNS, is effective in increasing the spontaneous activity and potentiating cholinergic and serotonergic responses of brainstem neurones in the rat (Tempesta et al.,1985). Acetylcarnitine has been shown to have a specific nicotinic action on the neuromuscular junction, blocking transmission in the cat anterior tibialis muscle by depolarisation, and augmenting the effects of other depolarising blockers (Blum et al.,1971). The same study demonstrated the depolarising effects of carnitine and acetylcarnitine on isolated frog muscle. These effects were less potent than those of acetylcholine, more closely resembling those of choline. Acetylcarnitine was nevertheless shown to be acting on the same receptor sites as

acetylcholine. Both the L- and D- isomers showed nicotinic effects.

The suggestion that there is a competitive action between carnitine and acetylcholine on neuromuscular junctional receptors is interesting in the context of MG. The administration of D,L-carnitine to uraemic patients on haemodialysis (in order to lower levels of endogenous triglycerides) was shown to produce a myasthenic like syndrome in 3 out of 30 patients, which resulted in altered electromyograms and decreased muscle action potentials (Bazzato et al.,1981). The syndrome was prevented by use of L-carnitine at similar dosages. The myasthenic effects of treatment with D,L-carnitine were also noted in a later study (Clair et al.,1984), one patient developing muscle fatiguability, diplopia and difficulty in chewing and swallowing. In view of these reports, the effects of D,L-carnitine on the binding of [^{125}I]α-BGT to human crude muscle extracts or to rat myotube cultures (see "Results" sections B 1.13 and A 1.12 respectively) were studied. In the former case, incubation of detergent-solubilised AChR with D,L-carnitine ($10^{-2} - 10^{-6}$M) for 30 min at 23°C did not inhibit the subsequent binding of [^{125}I]α-BGT. In the latter case, incubation of myotube cultures with D,L-carnitine (0.15 - 1.5μM) for 18h at 37°C, followed by washing (conditions under which myotoxicity assays were carried out) had no effect on the number of [^{125}I]α-BGT binding sites expressed. It was concluded that, under the conditions used, D,L-carnitine did not affect the function of AChRs.

In order to study the myolytic effects of myasthenic serum, use was made of a quantitative assay for muscle lysis originally developed by Cambridge and Stern (1981) to study cell-mediated myotoxicity in polymyositis. The method depends upon the preferential

FIGURE 48 245
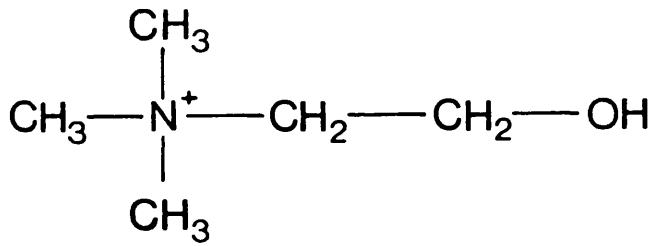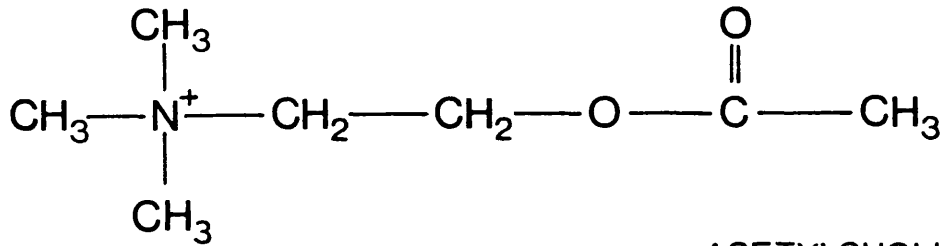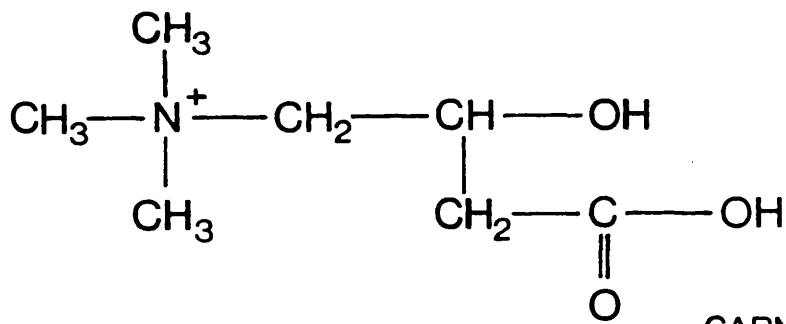
Structures of choline, acetylcholine, carnitine and acetylcarnitine

uptake by cultured myotubes of [Me-^{3}H] carnitine, loss of which can be monitored following cytolytic damage. A major advantage of this procedure was reported to be the very much slower uptake of carnitine by fibroblasts, which, as extensively discussed earlier, contaminate myotube cultures (Cambridge and Stern, 1981). Carnitine uptake by cultured human foetal myotubes was shown by these authors to be five-fold higher than by cultured human skin fibroblasts. A study of carnitine uptake and loss by human myocardial cells and mouse fibroblast cells from established cell lines (Bohmer et al.,1977) demonstrated a 3-10 fold higher uptake of [Me-^{3}H] carnitine by the muscle cells than by the fibroblasts. In the present study carnitine uptake by human muscle cells was approximately six times higher than that by fibroblasts; the corresponding figure for rat myotubes was 3-4 fold. Loss of radiolabelled carnitine from the muscle cells in the study of Bohmer et al. (1977) was 7% per hour; a figure similar to those obtained in the present work for rat myotube cultures (6.4%/h) and human myotube cultures (4-5%/h). The human myotube cultures retained similar or greater amounts of radioactivity than the rat myotube cultures, when labelled with D,L-[Me-^{3}H] carnitine under the same conditions. This was unexpected in view of the greater density and differentiation of the rat cultures. Differences between carnitine uptake of human and rat cultures was greatest with the L-isomer of carnitine; the human cultures appearing to take up approximately twice that of the rat cultures. It is not clear whether these results reflect species differences or different states of differentiation. It has been reported that cultured human foetal muscle cells, as is the case with adult human muscle, have a higher oxidative than glycolytic metabolism (Meola et al.,1983) which the authors suggested was a

species-specific phenomena.

4. Effects of myasthenic serum on muscle cells in culture

4.1 Binding of serum immunoglobulins to muscle cultures

The cross-reactivity of myasthenic immunoglobulins with rat

muscle cells in culture was determined for 4 serum samples covering a

range of anti-AChR antibody titres. Binding was detected by using

$[^{125}I]$ goat anti-human light chain antibodies. Under the conditions

used, normal serum gave a mean binding value of 205% relative to

cultures to which no additions of serum were made. The binding by

myasthenic serum gave a mean value of 338%, indicating the specific

binding of myasthenic immunoglobulins to cultured rat muscle antigens.

Equivalent experiments, using the same normal and myasthenic serum

samples, were carried out with human muscle cells in culture. The

binding demonstrated by normal and myasthenic serum samples gave mean

values of 131% and 319% respectively. The higher binding of normal

human immunoglobulins to rat muscle cultures as compared with human

cultures could indicate specific binding to antigens on rat muscle

cultures or alternatively, simply reflect a generally higher

non-specific level of binding to these cultures. Overall, it would

appear that, as expected, myasthenic serum immunoglobulins showed a

higher degree of reactivity with human muscle cultures (2.4 x mean

normal levels) than with rat muscle cultures (1.7 x mean normal

levels). Although the number of myasthenic sera tested was small, it

appeared that myasthenic serum binding, as detected by $[^{125}I]$ goat

anti-human light chain antibodies, was correlated with anti-AChR

antibody titre (Figures 19 and 45). The results also suggested that

the binding of myasthenic serum of relatively low anti-AChR antibody

titres (ie. less than 16.2nM) would not be detected by this method.

4.2 Reduction of [^{125}I]α-BGT binding sites by myasthenic serum

The reduction in the number of [^{125}I]α-BGT binding sites on rat myotube cultures by myasthenic serum samples was measured by determining the number of sites remaining after pre-incubation of the muscle cultures with myasthenic serum. Significant reductions, relative to normal serum controls, were shown by 4 out of 7 myasthenic serum samples tested. These effects were independent of complement as the sera were heat-inactivated before use. A reduction in the number of toxin binding sites was not observed after incubation of the cultures with 3 myasthenic serum samples of relatively low anti-AChR antibody titre (ie. less than 14.8nM). Overall, however, the correlation between anti-AChR antibody titre and site reduction was poor. The loss of [^{125}I]α-BGT binding sites was dependent on serum concentration for one particular sample. Pre-incubation of rat muscle cultures with serum samples at 4°C, gave similar results, suggesting that the effect arose from direct blockade of the α-BGT binding site rather than from increased energy-dependent receptor degradation. It should be noted, however, that the serum samples tested were added at room temperature before transferral to a 4°C environment and subsequent [^{125}I]α-BGT binding assays were carried out at room temperature. It is therefore possible that degradation of the AChR played a part in the effects of myasthenic sera.

Drachman et al. (1982) recommended a procedure by which unequivocal blockade of cultured rat muscle AChR was demonstrated. This involved the addition and incubation of myasthenic immunoglobulins to rat muscle cultures for 18h at 4°C, before

subsequent saturation of the cultures with α-toxin for 4h at 4°C. In this way, degradation was eliminated and possible dissociation of antibody was minimised. Using this procedure, Drachman et al. (1981, 1982) reported that despite potential cross-reactivity problems, 88% of the 48 myasthenic serum samples tested produced significant blockade of AChRs and furthermore, that the extent of blockade correlated with the clinical status of the patient. The studies described in the present work suggest that, under the conditions used, significant differences in binding of myasthenic immunoglobulins and in blockade of toxin binding were only observed at higher levels of anti-AChR antibody content. It could be that the use of purified immunoglobulin or IgG would increase the sensitivity of the assay methods used which could be complicated by the non-specific binding of serum proteins. However, cross-reactivity between cultured rat muscle AChR and myasthenic serum anti-AChR antibodies was clearly indicated.

Theoretically, it is possible that antibodies could produce blockade of AChRs by binding directly at, or near to the active sites of the receptors, thereby sterically hindering access to the sites, or by allosterically interfering with α-BGT binding (see Figure 49). It has been reported that only a relatively small fraction of antibodies to the AChR in myasthenic patients bind directly to the α-BGT binding site itself (Dwyer et al.,1979; Mittag et al.,1981; Vincent and Newsom-Davis 1979, 1980, 1982; Whiting et al.,1983) although in other studies using chick muscle cells in culture (Fulpius et al.,1981) nearly half the patients had some antibody capable of binding to the site. However, as pointed out in the "Introduction" (p. 33), the ability of antibodies to block binding of α-BGT may not necessarily parallel their ability to interfere with ACh binding at the

FIGURE 49
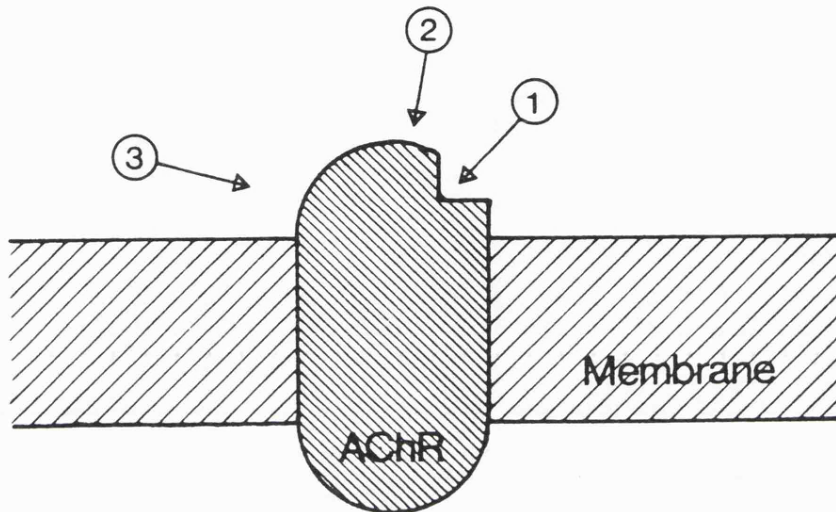
Schematic representation of possible sites of
attachment of anti-AChR antibody causing 'blockade'
of receptor. In 1) the antibody attaches to the ACh
combining site. In 2) antibody attaches near the ACh
binding site and sterically hinders the binding of ACh
and α-BGT. In 3) the antibody does not directly
interfere with the ACh binding site, but alters the
AChR molecule thus inhibiting the ACh or α-BGT binding.

neuromuscular junction.

4.3 The myotoxicity of myasthenic serum to rat muscle cells in culture

The myolytic effects of myasthenic serum were tested using the
$[Me-^3H]$ carnitine release method of Cambridge and Stern (1981).
Initial experiments tested the effects of normal and myasthenic serum
samples which had not been subjected to heat treatment. Some of the
fresh normal serum samples caused an increased release of
radioactivity from the myotube cultures, comparable to that observed
by some samples of myasthenic serum (up to 30% myotoxicity). The
myolytic effects of these sera were reduced by heat-treatment of the
sera for 30 min at 56^oC and restored in the case of myasthenic sera
but not of normal sera, by the subsequent addition of guinea-pig
complement. The measured myotoxicity of the fresh normal sera was also
reduced by absorption with rat liver homogenate which would suggest
that these sera contain non-tissue specific components which were
toxic to the rat muscle cultures. The observation, however, that
toxicity was not restored, after heat-treatment, by subsequent
addition of GPC would suggest that the factor responsible was not an
antibody directed against rat muscle antigens. In this context, the
high toxicity to rat muscle cultures demonstrated by a fresh rabbit
serum sample was interesting (see "Results" section A 2.2.5). This
toxicity was reduced to normal levels (less than 20% under the assay
conditions used) after storage at -20^oC for over a year. As noted for
the normal human samples, this toxicity was not restored by the
addition of complement under the conditions used to demonstrate the
complement-mediated myotoxicity of myasthenic sera. It is known that
some serum batches used to supplement growth media in tissue culture

studies can be toxic to cultured cells (see Paul, 1975) and for this reason all such sera are routinely heat-treated before use. Other workers have also noted the heat-labile toxicity of some normal human serum samples when added to rat muscle cells in culture (Obata et al.,1976). The mechanism of this toxicity remains unclear but appears to differ from that shown by normal human sera to chick muscle cultures; this is associated with the widespread occurence of anti-chick tissue antibodies (Harvey et al.,1978b). In order to avoid complications arising from this labile, toxic factor, and also to obviate variations arising from the lability of endogenous complement, all serum samples subsequently tested for myotoxicity were routinely heat-inactivated before use. Heat-inactivated serum samples tested alone, in the absence of complememt, caused a measured myotoxicity of less than 20%. Myotoxicity measurements falling below this value were therefore taken as lying within normal limits.

The myotoxic effects of myasthenic sera in the presence of complement are consistent with antibody-initiated lysis of the rat myotube membranes. Specificity of the lytic action was shown by the observation that a highly myolytic myasthenic serum was not toxic to rat fibroblast cultures, in the presence of GPC, when these cultures were tested in parallel with muscle cultures under the same conditions (Table 8). The myotoxicity demonstrated by myasthenic serum samples in the presence of GPC was dependent on the amount of GPC added (Figure 24a) and on the activity of the GPC source tested. Complement is commonly assayed in terms of its activity expressed by the degree of lysis of a standard suspension of antibody-coated sheep red blood cells. The volume of complement added correlates with the degree of lysis attained (Lachmann and Hobart, 1978). Fresh human or rat sera

were generally ineffective as sources of complement. These results are consistent with the lack of complement-mediated myotoxicity shown by myasthenic sera in other studies where fresh rat serum (Lennon, 1978b) or low amounts of GPC (5% v/v, total volume unstated, Liveson et al.,1976) were added as a source of complement.

Drachman et al. (1980) tested the effects of fresh human serum, as a source of complement, on myasthenic immunoglobulin-induced loss of AChRs on cultured rat muscle. This study failed to show enhancement by complement in vitro even though the passive transfer of these immunoglobulins to mice resulted in complement-dependent EAMG. In the present work, a similar in vitro study using rat muscle cultures, showed that myasthenic serum samples reduced the numbers of $[^{125}I]\alpha$-BGT binding sites independently of GPC and in the absence of myotoxic effects. However, the addition of complement greatly enhanced the loss of $[^{125}I]\alpha$-BGT binding sites which was paralleled by the increased measured myotoxicity (Table 9). One myasthenic serum (from patient MG 4, sample i) failed to reduce the number of available toxin binding sites relative to controls or to cause myotoxicity either with or without added complement. This serum had a relatively low anti-AChR antibody titre (12.2nM). Another serum sample from the same patient (anti-AChR antibody titre = 42.0nM) however, both blocked toxin binding and showed myotoxicity. These results would suggest that under the conditions used, the toxicity depends on the titre of cross-reacting antibodies.

The degree of complement-mediated myotoxicity produced by myasthenic sera did not appear to be affected by variations in the density of myotubes, as judged morphologically and by the number of $[^{125}I]\alpha$-BGT binding sites (see Table 10). It is possible that, in the

experiments carried out, optimal conditions for maximum lysis were achieved at the highest myotube density tested which would therefore also be optimal where a lower number of myotubes were present. For this reason, cultures demonstrating similar myotube densities were used for all myotoxicity studies, and studies of a comparative nature were carried out on replicate muscle cultures. High density muscle cultures would appear to be required for the myotoxicity assay, to optimise the differences in [Me-^{3}H] carnitine uptake by myotubes and fibroblasts present in the cultures. Throughout the course of myotoxicity studies in this project, it was observed that where myasthenic serum samples demonstrated high myotoxicity values, damage to myotubes was clearly visible in the light microscope thus supporting the implications of the quantitative assay procedure used.

A myotoxicity study, using optimised conditions for assay, was carried out to determine the complement-mediated effects of a range of myasthenic and normal serum samples. Under the conditions used, serum samples from 9 out of 13 myasthenic donors caused accelerated loss, relative to normal serum controls, of labelled carnitine from rat myotubes. In every case, the observed myotoxicity was dependent on the addition of complement. The myotoxicity values given by the normal, myasthenic or complement sera tested alone, and by the normal sera in the presence of complement, were all below 20% (Table 11 and Figure 27).

Serum samples from patients with polymyositis were also tested for complement-mediated myotoxicity. These patients were chosen as an example of muscle degenerative disease also possibly of autoimmune origin (Currie, 1970; Currie et al.,1971). It has recently been shown that the neuromuscular junctions of these patients have

reduced numbers of AChRs and that polymyositic sera or IgG could
increase the degradation of AChR in vitro in a similar way, but to a
lesser extent, to that of myasthenic sera (Pestronk and Drachman,
1985). The same study, however, demonstrated only a very low (< 1.2nM)
or non-detectable level of anti-AChR antibodies in these sera.
Patients with polymyositis are known to have serum antibodies directed
against cytoplasmic elements of skeletal muscle, such as myosin (Wada
et al.,1983) but the observed damage to muscle which occurs in this
disease is principally thought to be mediated by lymphocytotoxic
mechanisms (Currie, 1970; Currie et al.,1971; Kakulas et al.,1971;
Dawkins and Mastaglia, 1973; Cambridge and Stern, 1981). In the study
by Dawkins and Mastaglia (1973), addition of heat-inactivated
polymyositic serum (100μl, 10% v/v) in the presence of GPC (50μl, 5%
v/v) was not found to be toxic to chick muscle cells in culture, as
judged by release of ^{51}Cr from the muscle cells. In the myotoxicity
study carried out for this project, serum from polymyositis patients
did not cause complement-mediated myotoxicity of rat myotubes in
culture, relative to the normal controls, when tested under the same
conditions as sera from myasthenic patients (Table 11 and Figure 27).
It would thus appear that, under the conditions used for the study,
the observed myotoxicity is specific for MG.

In the myotoxicity study, all the myasthenic serum samples
tested contained anti-AChR antibodies, the involvement of which in the
lytic process accordingly seems likely. Although correlation of
anti-AChR antibody titre with myotoxicity values is not good, it does
seem that myasthenic serum samples with myotoxicity values within the
normal range tend to have relatively low anti-AChR antibody titres
(ie. less than 20nM). Nevertheless, some samples with high myotoxicity

also had lower antibody titres (Table 12) and it may be that, in such
cases, the percentage of the anti-AChR antibodies present which
cross-react with rat muscle AChR and/or which can effectively activate
complement is higher. It was obvious from the experiments carried out
to determine optimal levels of added myasthenic serum and GPC to
detect myotoxicity (Figure 24) that each serum demonstrated different
characteristics which probably reflects a difference in ability of the
antibodies present to both bind to muscle components and to activate
complement.

That most myasthenic serum samples can cause
complement-mediated myotoxicity in rat muscle cultures was further
indicated by experiments where the conditions used for assay were
adapted in order to detect myotoxicity by sera of relatively low
anti-AChR antibody titre (see "Results" section A 2.2.14). Under these
conditions, in which larger amounts of serum and GPC were added to the
myotube cultures, an accelerated loss, when compared to normal serum
controls, of radiolabelled carnitine was detected in 3 of the 4 sera
tested. The myasthenic serum demonstrating no relative myotoxicity was
of the lowest anti-AChR antibody titre tested (3.6nM) and it is
probable that in this case, optimal requirements for myotoxicity were
still not reached.

The ability of antibodies to muscle membrane surface antigens
to cause damage to muscle cells in culture in the presence of
complement, has been demonstrated in studies unrelated to MG. Chick
muscle cells in culture treated with antisera to chick-specific
antigens demonstrate characteristic staining of the muscle membrane
after detection of bound antibodies by an indirect immunofluorescence
technique (Dawkins and Holborow, 1972). No staining was present on

chick muscle cultures when these were treated with antisera directed against intracellular muscle antigens. In the same study, it was demonstrated that the antibodies to surface antigens of chick muscle, but not antibodies directed at intracellular components, caused cytotoxicity in the presence of GPC when detected by a ^{51}Cr release method. The degree of cytotoxicity attained also correlated with the intensity of fluorescent staining achieved (Dawkins and Holborow, 1972). The cytotoxicity conditions used by these workers involved the incubation of the muscle cultures for 18 hours with antiserum and GPC added at equal concentrations (50µl, 5% v/v).

A later study by Stephens and Henkart (1979) showed lysis of cultured rat muscle by GPC after trinitrophenol (TNP) modification of the muscle membrane with 2,4,6-trinitrobenzenesulphonate and addition of rabbit anti-TNP serum (10% v/v) and complement (final dilution 1:2 to 1:4). Lysis was judged morphologically by failure to exclude trypan blue dye from the muscle cells. These authors showed that while low concentrations of added GPC affected the electrical properties of the muscle membranes, lysis was only achieved by addition of high concentrations of GPC and occurred within one hour of incubation. The effects on the electrical membrane properties, in response to antibody and complement, was reversible by washing the cells shortly after depolarisation or if low concentrations of GPC were used. These studies suggested that while muscle cells coated with antibody could bind complement with the subsequent formation of the membrane attack complex (C5 - C9) and the influx of extracellular ions, the muscle cells possessed an efficient repair mechanism that could reverse the effects of membrane damage. The effectiveness of these mechanisms was overcome, however, in the sustained presence of large amounts of added

complement. The studies by Stephens and Henkart (1979) could readily explain the results of Lennon (1978b) who incubated EAMG serum with rat muscle cells in the presence of rat serum as a source of complement. No lysis was detected despite the presence of IgG and C3 on the rat cells.

In the myotoxicity experiments carried out for this project, it seemed probable that the antibodies responsible for the observed lysis were directed at the AChR on rat muscle membranes. However, in addition to antibodies to the AChR, patients with MG have circulating antibodies to various other skeletal muscle antigens and these should be considered. The occurrence of anti-striational antibodies, directed against cytoplasmic elements of the contractile apparatus, is well documented in MG (see Newsom-Davis and Vincent, 1982). The presence of these antibodies was demonstrated before that of specific anti-AChR antibodies was known (Strauss et al.,1960; Van der Geld et al.,1963; Beutner et al.,1966). It has been shown that while approximately only 45% of all myasthenic patients have circulating anti-striational antibodies, almost all patients with a thymoma have these antibodies (Oosterhuis et al.,1976). However, the presence of these antibodies is not specific for MG as 25% of patients with a thymoma not associated with MG also have circulating anti-striational antibodies (Oosterhuis et al.,1976; Limburg et al.,1983). These antibodies interact with intracellular antigens hence it is unlikely in the tissue culture system used for myotoxicity studies, that the antibodies giving rise to complement-mediated lysis are directed at any but surface antigens.

Other studies have demonstrated the presence of antibodies to a citric acid extract of skeletal muscle which again can be detected in nearly all myasthenic sera from patients with a thymoma, but not at

all or in a low incidence, in serum from myasthenic patients with no associated thymoma (Aarli et al.,1981; Gilhus et al.,1983a,b). The antibodies to citric acid extract have been shown by immunofluorescence techniques, to bind near the surface of striated muscle cells and to be directed at antigens not associated with the AChR (Gilhus et al.,1983a,b). The presence of these antibodies in the myasthenic serum samples used for the myotoxicity studies cannot be discounted although it is known that where clinical details were available for a few of the myolytic samples, the donors had no associated thymoma (personal communication Drs. Wallington, Campbell and Wakefield). However, as described in the next section, attempts were made to define the specificity of the myotoxic components of myasthenic sera.

4.4 Anti-AChR antibody-dependent myotoxicity

Experiments designed to determine the specificity of the antibody-dependent complement-mediated myotoxicity to rat muscle AChR were carried out by using two different approaches. These experiments utilised antisera raised in animals to purified AChR or human myasthenic serum from which anti-AChR antibodies were specifically removed.

The use of rabbit anti-foetal calf AChR and rabbit anti-junctional rat AChR antisera both produced accelerated loss, relative to the normal rabbit serum control, of radiolabelled carnitine from rat muscle cells when used in the complement-mediated myotoxicity assay. The conditions for assay were the same as used in the myotoxicity study to detect the lytic activity of myasthenic sera. The antiserum to rat junctional AChR only gave a myotoxicity value of

37.5% in the presence of complement even though it had a high

anti-AChR antibody titre (105 nM) when tested against crude muscle

extracts of rat junctional AChR. It is possible that this antisera

contained a low proportion of complement-fixing anti-AChR antibodies.

Sheep anti-Torpedo AChR antiserum did not cause myotoxicity in the

presence of complement. It is known that anti-AChR antibodies can

differ in their cross-reactivity with AChR from different species

(Lindstrom et al.,1978b; McAdams and Roses, 1980; Savage-Marengo et

al.,1979, 1980; Harrison et al.,1981; Vincent and Newsom-Davis, 1982)

and that anti-Torpedo AChR antisera in particular have limited

cross-reactivity with mammalian AChR (Lindstrom et al.,1978b).

Nevertheless, the demonstration of complement-mediated myotoxicity by

rabbit anti-foetal calf and anti-rat junctional AChR antisera would

indicate that antibodies specific for the AChR can cause

complement-mediated myotoxicity in a similar manner to that observed

for human myasthenic sera.

Evidence that anti-AChR antibodies are the major factor

in mediating myotoxicity in the assay system used, was provided by the

consequence of their specific immunoabsorption from human myasthenic

serum. AChR bound to toxin-agarose affinity columns has been used in

several studies to purify anti-AChR antibodies directed at Torpedo

(Schwartz et al.,1979; Hinman et al.,1985) and human AChR (Lang et

al.,1982; Whiting et al.,1983).

Lang et al. (1982) coupled human AChR to a covalently

linked α-BGT-Sepharose 4B matrix through the α-BGT binding site. They

showed that the column was capable of adsorbing over 90% of the

anti-AChR antibodies when limiting concentrations were applied to the

column. However, when excess antibody was applied, different amounts

of anti-AChR antibody were adsorbed, the molar ratios of IgG to AChR varying from 1:1 to 1:20 for the 3 myasthenic sera tested. In the present project, maximum removal of antibody was 67.9% (Table 16) when the molar ratio of antibody to AChR on the column was approximately 1:4. Application of a second sample of the myasthenic serum to the same affinity column resulted in further removal of antibody indicating that the AChR on the column was not saturated. This could indicate that a longer period than 2 h was necessary to effectively remove a maximum amount of antibody. Alternatively, the affinity column could have separated sub-populations of antibody based upon their specificity for different antigenic determinants on the AChR.

The unbound fractions from the affinity column were concentrated to the original volume of serum and subsequently used in the myotoxicity assay. The complement-mediated myotoxicity of the myasthenic serum fell from over 40% to values within the normal range (less than 20%) when the maximally depleted serum was tested. It is of interest that immunoabsorption lowered the anti-AChR antibody titre from 42.0 nM to a value (13.5 nM) comparable with that (12.2 nM) of a sample obtained from the same patient at a different time which showed a myotoxicity value (11.7%) also well within the normal range (patient MG 4, Table 12). Reduction of the anti-AChR antibody titre to 28.6 nM from 42.0 nM only reduced myotoxicity at lower levels of added serum (Figure 30). These results indicated that in the myasthenic serum sample tested, the extent of anti-AChR antibody removal could be correlated with its ability to cause complement-mediated myotoxicity.

The reduction in the number of [^{125}I]α-BGT binding sites on rat myotube cultures by myasthenic serum, before and after anti-AChR antibody depletion, was measured by determining the number of sites

remaining after pre-incubation of the muscle cultures with the myasthenic serum samples. Anti-AChR antibody depleted serum reduced the number of toxin binding sites in a similar manner to that of the non-depleted sample. This was in contrast to the effects of another sample of serum from the same patient which showed a similar anti-AChR antibody titre to the depleted sample (12.2 and 13.5 nM respectively - see Figure 20a). It is not clear, because of the assay conditions used, whether the reduction in [^{125}I]α-BGT binding sites is a result of direct blockade or accelerated degradation of the AChRs. However, it would appear that both the anti-AChR antibody depleted sample and the sample taken from the same patient at a different time but showing a similar antibody titre, contain antibodies with different characteristics.

The bound anti-AChR antibody on the affinity column was eluted with 2M KI with a final recovery of 18.7%. Yields of greater than 50% were obtained by Lang et al. (1982) and Whiting et al. (1983) using 3M potassium thiocyanate which was reported by these workers to be the most effective elution reagent. These workers also covalently cross-linked the AChR to the affinity column to prevent any subsequent elution of AChR with the purified antibody. It is therefore possible that the eluted antibody fraction contained AChR. The IgG content of this fraction was high (0.57 mg) suggesting that antibodies other than anti-AChR antibody were bound by the affinity column. This observation was also made by Lang et al. (1982).

The purified antibody fraction was concentrated to give a similar anti-AChR antibody content (15.7 nM) to the serum sample maximally depleted of specific antibody. The purified antibody was able to reduce the number of available [^{125}I]α-BGT binding sites after

incubation with rat muscle cultures (Figure 31) but also did not show any complement-mediated myotoxicity under the conditions used, the value obtained (16.3%) being within the normal range. These results would again suggest, as discussed for the anti-AChR antibody depleted serum, that although antibody could bind to the rat muscle cultures, complement was not being activated in sufficient amounts to effect lysis. It is possible that this is related to specific sub-populations of antibody or alternatively, that the Fc portion of the antibody which binds complement, was damaged during the elution procedure or during the subsequent dialysis, concentration and storage.

4.5 Myotoxic effects of myasthenic IgG and IgG depleted of subclass 3

The demonstration of a direct myolytic role for myasthenic sera raises the question as to whether particular anti-AChR antibody sub-populations are especially active in this respect and whether the titres of such antibody sub-populations could correlate with clinical state. Studies of IgG subclasses in myasthenic sera have produced conflicting results. Lefvert and co-workers (Lefvert and Bergstrom, 1978; Lefvert et al.,1981) have stressed the predominance of IgG3 in anti-AChR antibodies whereas other groups of workers have reported only low proportions of IgG3 (Tindall, 1981; Vincent and Newsom-Davis, 1982; Whiting et al.,1983) with predominance of IgG1 and IgG2 (Vincent and Bilkhu, 1982; Vincent and Newsom-Davis, 1982). The possible predominance of IgG1 and IgG3 is of especial interest in that these subclasses bind complement with high affinity (Burton, 1985). Use was made of a protein-A Sepharose column to separate purified IgG into subclasses IgG1,2 and 4 and subclass IgG3. The interaction between Staphylococcal protein-A and IgG subclasses represents a selective

biological activity. IgG subclasses 1,2 and 4 bind to protein-A via

their Fc regions whereas IgG3 does not (Kronvall and Williams, 1969).

IgG was prepared by ammonium sulphate precipitation and ion-exchange

chromatography. This procedure is reported as leaving only traces of

IgG4 in the purified IgG samples (Skvaril and Morell, 1970). As a

consequence, the IgG eluted from the protein-A Sepharose column

probably consisted of only IgG subclasses 1 and 2. The recovery of

IgG3 in the unbound fractions of the normal and one of the myasthenic

IgG samples was low (1.0% of initial IgG - Table 20). The normal level

of IgG3 in human serum is approximately 4-7% of total IgG (Stanworth

and Turner, 1978). The reasons for this relatively low recovery are

probably related to the known susceptibility of IgG3 to proteolytic

digestion (Michaelson and Natvig, 1974), a point to be noted in the

context of storage and assay of myasthenic serum samples (Fulpius et

al.,1981; Lefvert et al.,1981). Subsequent myotoxicity experiments

demonstrated that IgG depleted of subclass 3 was as effective in

producing complement-mediated lysis in cultured muscle cells as the

IgG fraction (Figure 33). However, both of these fractions appeared

less effective, when added at similar anti-AChR antibody

concentrations, to the original serum samples. A subsequent IgG

sample, stored for a longer period at $-20^{\circ}C$, proved ineffective in

producing complement-mediated lysis. These results would suggest that

purified IgG or IgG subclasses are susceptible to degradation on

storage and should be stored in a lyophilised form to avoid this

complication. In complement studies, it is particularly important that

the Fc region of the antibody, which binds complement, remains

functionally intact. An alternative explanation for the reduced

effects of purified IgG in promoting myotoxicity, which was

particularly apparent in one preparation (Figure 33a) could be that some lytic anti-AChR antibodies were of the IgM class.

In these latter experiments, use was made of L-[Me-^{3}H] carnitine to label rat muscle cultures. It was noted that serum from patient MG 4, sample (ii), gave a higher myotoxicity value than previously noted when muscle cultures were labelled with D,L-[Me-^{3}H] carnitine. Subsequent experiments (not shown) showed that the non-specific uptake of radiolabelled carnitine in the presence of a high concentration of unlabelled carnitine (1mM) was higher in the case of D,L- as opposed to L-carnitine (approximately 35% and 12% of total radioactivity respectively). Thus, it would be expected that the maximum myotoxicity obtainable would be higher in cultures labelled with L- as opposed to D,L-[Me-^{3}H] carnitine. In further experiments, designed to assess the contribution of non-specific radioactivity to spontaneous release of radiolabel or measured myotoxicity, the percentage of total radioactivity contributed to by non-specific radioactivity was found to remain constant.

4.6 The myotoxicity of myasthenic serum to human muscle cells in culture

Preliminary experiments tested the effects of non heat-treated myasthenic and normal serum samples when added to human muscle cells in culture. With the exception of one myasthenic serum sample, which gave a mean myotoxicity value of 27.6% (Table 30), the myasthenic sera tested caused myotoxicity of similar values to those obtained for normal sera. In subsequent experiments, all sera were heat-inactivated before use and a known amount of GPC serum was added as a standardised source of complement. However, using the same conditions under which

high myotoxicity was shown towards rat muscle cells in culture, no

equivalent myotoxicity was evident towards human muscle cells in

culture (Table 31). Manipulation of assay conditions, in a similar

manner to that by which myotoxicity towards rat muscle cells was

demonstrated by previously 'non-toxic' sera, resulted in a myotoxicity

value for the myasthenic serum tested of 34.9% (Figure 46). Subsequent

experiments demonstrated that the myotoxic effects of myasthenic serum

were dependent on the amount of complement added and dependent on the

amount of myasthenic serum added (Figure 47). No damage to the human

myotubes was observed when examined by light microscopy, as high

myotoxicity levels (ie.> 50%) were not reached. These myotoxicity

results indicated, as described for rat myotubes, that the effects

could be explained in terms of antibody-mediated lysis of the human

myotube membranes.

The results gained from studies in which the binding of

myasthenic immunoglobulin to human muscle cultures was estimated,

suggested that these immunoglobulins bound as well, if not better, to

the human cultures than to the rat cultures (see "Discussion" section

4.1). One of the myasthenic serum samples used in the latter study

(from patient MG4, sample ii) was subsequently used in the myotoxicity

studies on human cultures. However, under the conditions used to

detect myotoxicity in rat cultures, no myotoxicity was observed in

human cultures. Myotoxicity was only apparent when the volume of sera

and GPC added to cultures were each increased 3-4 fold. The reasons

for the differences observed with rat and human muscle cultures are

not immediately clear. The factors responsible could include:-

1) Different sub-populations of antibody are binding to the human

cultures which are not so effective at activating GPC

2) The different nature of the antigenic sites on the human myotube membranes

3) The stability or recovery aptitude of the human myotube membranes

4) The immaturity of the human myotubes in culture

or a combination of these factors.

Assuming that the classical pathway of complement activation is involved in the myolytic process, then the initial component, the Clq molecule, has 6 binding sites for attachment to antibody-antigen complexes. An essential requirement for stable binding is that at least 2 of the sites are involved. When IgG is bound to antigen, Clq binds to 2 separate IgG molecules through their Fc regions. These IgG molecules must lie within 30-40 nm of each other, this being the maximum distance that Clq can span (Hughes-Jones et al.,1984; Burton,1985). In studies by Dower and Segal (1981), it was shown that in the case of Clq binding to antibody coated cells where the antibody is bound to a membrane mobile antigen, the multivalent Clq will bind preferentially to the region of highest antibody concentration. However, for low cell-surface densities, free IgG would serve as a potent inhibitor of Clq binding and may therefore modulate complement activation on cells bearing low levels of antibody. In effect, these studies showed that aggregated IgG has a greater affinity for Clq than monomeric IgG. Hence, a possible explanation for the difference in myotoxic effects on rat and human muscle cultures could lie in the number and distribution of surface antigens. In the case of rat muscle cultures, it has been shown that the density of AChRs on the surface membrane is high and that clusters or aggregates of receptors occur on the membrane (see Table 35). However, it has been demonstrated that

only a sparse distribution of AChRs is present on human foetal myotube

membranes at a density of 1-4 receptors per μm^2 and no clusters of

receptors have been observed (Adams and Bevan, 1983, 1985). It is

therefore possible that at lower added concentrations of myasthenic

serum to human myotube cultures, although antibody effectively binds

to receptor, the antibody-receptor complexes are too far apart to

activate Clq. High levels of added antibody could perhaps effectively

bind enough receptor so that Clq can bind, but in addition, high

levels of unbound IgG could inhibit maximum binding of Clq.

An alternative or additional factor to be considered

concerning the human myotube cultures is the apparent immaturity of

these cultures with respect to cultured muscle of other species (see

"Discussion" section 2.5). It is possible that because of the immature

physiological properties of the human foetal myotube membranes (Harvey

et al.,1979) that these membranes would be less susceptible to

complement-mediated attack.

Conclusions and Prospects

Use was made in this study of a quantitative assay for muscle

lysis in vitro based on the selective uptake of tritium-labelled

carnitine by myotubes in a tissue culture system (Cambridge and Stern,

1981). The studies carried out demonstrated that treatment of rat

muscle cultures with myasthenic serum caused specific myotube damage

in a manner that was dependent upon the concentration and activity of

added complement. All the myasthenic sera tested had elevated levels

of anti-AChR antibodies. The involvement of such antibodies in the

observed muscle lysis was suggested by their demonstrated

cross-reactivity with rat muscle AChR, by the loss of myolytic

activity following their depletion in serum and by the demonstrated complement-dependent myolytic effects of antisera to AChR raised in rabbits.

The demonstration of a direct myolytic role for myasthenic sera in vitro, raises the question of the significance of this effect in vivo. The ability of myasthenic sera or IgG to promote the lysis of post-synaptic membrane bearing junctional AChRs would be of more relevance to the disease state in vivo. In this context, the addition of neuronal factors to muscle cells in vitro to promote aggregation of AChRs or the use of nerve-muscle co-cultures in the myotoxicity assay would be interesting.

Complement undoubtedly plays a role in disturbing neuromuscular transmission in animals with EAMG (see "Introduction" p.37) and severe damage to muscle endplates, associated with the presence of IgG and the lytic component of complement (Sahashi et al.,1980), in the human disease has been observed. Destruction of the synaptic folds or of those segments of the folds that bear AChR represents a very obvious mechanism of AChR loss. Activation of the lytic phase of the complement reaction sequence by bound anti-AChR antibody, could result in the formation of trans-membrane ion channels made up of the C5-C9 membrane attack complex. The insertion of such channels into the post-synaptic membrane would be likely to cause an uncontrolled flux of extracellular ions, focal calcium excess (Campbell et al.,1979), protease activation and disruption of cytoskeletal elements within the folds. The subsequent shedding of damaged segments of the folds bearing AChR, IgG and the membrane attack complex could be the outcome of disturbed membrane dynamics and impaired cytoskeletal support (Engel and Fumagalli, 1982). The post-synaptic membrane would then

reseal itself over the junctional folds which would be shorter and bear fewer AChRs than the pre-existing folds.

It would be interesting to speculate about the role of complement in patients with less severe disease. It is possible that the complement sequence does not always go to completion because of efficient regulatory mechanisms such as circulating serum complement inactivators. Alternatively, a low concentration of active complement could lead to impaired muscle function, but not in sufficient quantity to overcome the reversible repair mechanisms of the muscle membrane (Stephens and Henkart, 1979). It is also possible that the activation of Clq by bound anti-AChR antibodies, could lead to enhanced receptor degradation by cross-linking previously non-interacting IgG-AChR complexes on the post-synaptic membrane. It could thus be hypothesised that the severity of MG is related to both the levels of active circulating complement and the levels of specific populations of anti-AChR antibodies which are capable of fixing and thus activating complement. In this context, it is interesting that the disease is often exacerbated as a result of viral or bacterial infections (Grob, 1981), conditions in which the levels of the acute phase proteins of inflammation, including complement, are known to increase (Whicher, 1978).

A myotoxicity assay that is able to quantitate the damaging effects of antibody to muscle cells could have an important role in the investigations of the pathogenesis of MG. Thus, this assay could be used to examine the anti-AChR antibody subclass patterns of highly lytic myasthenic serum samples. It could well be that low amounts of particular subclasses, such as IgG1 or IgG3 or low amounts of IgM with high complement-fixing ability play a major role in muscle lysis;

therefore correlations could be sought between subclass profile and
lytic activity. Such studies, together with the determination of
complement levels in freshly obtained myasthenic serum samples could
then be correlated with clinical state. Hopefully, this could lead to
serum assays of greater diagnostic significance than is the case for
determination of total anti-AChR antibody levels and also help to
clarify the role of complement-mediated lysis in the pathogenesis of
MG.

The human muscle cells in culture, established from foetal
tissue, demonstrated a lack of differentiation when compared to the
cultured rat muscle cells. Myotoxicity by the myasthenic sera tested,
was only demonstrated at high levels of added serum and GPC. These
observations could be directly related to the immaturity of these
cells in vitro. Human muscle in culture is increasingly being used as
an experimental model for the study of human muscle differentiation
and metabolism and for the further investigation of human disease. It
would therefore be of great benefit to establish optimal, standardised
conditions for growth in vitro which would result in a muscle system
of more relevance to that in vivo and enable comparable investigations
to be carried out in different laboratories.

APPENDIX

In the myotoxicity study (Section A 2.2.13), heat-inactivated serum samples from 9 out of 13 myasthenic patients showed clear complement-mediated myotoxicity towards rat myotubes in culture, in contrast to 0 out of 12 normal controls. The myotoxicity values obtained did not show a correlation with the clinical severity of disease in each patient (Table 36) and were not associated with the occurrence of a thymoma (Table 36) or on the presence of anti-striated muscle anti- bodies (Table 37). Anti-AChR antibodies were present in all the myasthenic serum samples tested, including a patient severely afflicted with myasthenic symptoms following D-penicillamine therapy (MG 6, Table 36). It is possible that the myotoxic nature of the myasthenic sera, or where tested, the myasthenic IgG is due to the presence of these antibodies but further work is needed to confirm this. Experiments utilising purified anti-AChR antibodies or monoclonal anti- bodies directed against AChRs would prove useful in this respect. As outlined in the Discussion, differences in the observed myotoxicity values could reflect varying antigenic cross- reactivities between rat and human AChRs. However, the myotoxicity values obtained from multiple serum samples from two patients did not correlate with the anti-rat AChR antibody titre (Table 37). Alternatively, the differences could be due to the importance of subpopulations of anti-human AChR antibodies, such as IgG subclasses which differ in their ability to activate complement (Burton, 1985).

TABLE 36 DETAILS OF MYASTHENIC PATIENTS USED IN THE MYOTOXICITY STUDY (see Section A 2.2.13)

Patient No.	Age (yrs)	Sex	Severity [a] of symptoms	Presence of thymoma	Thymectomy	Therapy [b]	Associated diseases	Myotoxicity (%)
1	71	M	MG	No	No	AC, IS	None	7.6
2	80	F	In remission	No	Yes	None	None	15.7
3	36	F	MG	No	Yes	AC	Possible lupus erythematosus	13.9
4	–	F	SG	–	Yes	AC, IS	None	11.7 – 54.8
5	–	F	MG	–	–	–	–	63.9
6	60	M	SG	No	No	None	D-penicillamine induced myasthenia	56.7
7	38	F	MG	No	Yes	AC	None	10.8
8	52	M	SG	No	Yes	AC	Hypertension	60.3
9	67	F	MG	No	No	AC	–	38.6, 59.8
10	–	–	MG	–	–	–	–	65.0
11	–	–	–	–	–	–	–	58.4
12	26	F	SG	No	Yes	AC, IS	–	60.3
13	33	F	SG	No	Yes	AC, IS	None	30.7 – 65.4

a MG Mild generalised   SG Severe generalised

b AC Anticholinesterases   IS Immunosuppressives

–   No information available

TABLE 37   SERUM ANTI-AChR AND ANTI-STRIATED MUSCLE

ANTIBODIES IN MYASTHENIC PATIENTS USED FOR

THE MYOTOXICITY STUDY (see Section A 2.2.13)

Patient Number	Anti-AChR antibody titre (nM) [a]Human	[b]Rat Junctional	Extra-junctional	[c]Anti-striated muscle antibody	Myotoxicity (%)
1	0.8	nt	nt	ND	7.6
2	4.2	nt	nt	nt	15.7
3	9.2	nt	nt	nt	13.9
4 [d](i)	12.2	0.24	1.48	nt	11.7
(ii)	42.0	0.96	4.22	nt	54.8
5	14.8	nt	nt	nt	63.9
6	16.0	nt	nt	ND	56.7
7	16.3	nt	nt	nt	10.8
8	24.2	nt	nt	ND	60.3
9 [d](i)	25.6	nt	nt	ND	59.8
(ii)	42.3	nt	nt	ND	38.6
10	72.1	nt	nt	nt	65.0
11	72.5	nt	nt	nt	58.4
12	180.0	nt	nt	nt	60.3
13 [d](i)	5.8	0.10	0.34	nt	62.3
(ii)	12.9	0.09	0.81	nt	43.2
(iv)	17.2	0.13	0.77	ND	65.4
(v)	20.6	0.34	1.22	Weak positive	47.0
(vi)	28.5	0.47	1.68	Weak positive	52.0
(viii)	43.8	0.73	2.59	ND	30.7

Anti-AChR antibody titres determined using crude extracts of [a]human or [b]rat leg muscle. Rat titres courtesy of Dr. Helen Lotwick. [c]Anti-striated muscle antibodies determined courtesy of the Bristol Royal Infirmary and Bath Royal United Hospital. [d]Serial samples taken at different times from the same patient.

nt - not tested   ND - none detected

TABLE 38   DETAILS OF NORMAL CONTROLS USED IN THE

MYOTOXICITY STUDY (see Section A 2.2.13)

Donor Number	Age (years)	Sex	Associated diseases
1	32	F	None
2	24	M	None
3	44	M	None
4	42	M	None
5	33	F	None
6	30	M	None
7	25	F	None
8	23	M	None
9	28	M	None
10	22	M	None
11	25	F	None
12	26	F	None

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Complement-Dependent Toxicity of Serum from Myasthenic Patients to Muscle Cells in Culture

Lisbeth A. Childs, Roger Harrison and George G. Lunt

Department of Biochemistry, University of Bath, Bath BA2 7AY (U.K.)

Summary

The toxicity of myasthenic sera to rat myotubes in monolayer culture was examined by measuring the release of [Me-^{3}H]carnitine from pre-loaded cells. In the presence of guinea pig complement, heat-inactivated serum samples from 9 out of 13 myasthenic patients showed clear myotoxicity, in contrast to 0 out of 11 normal controls and 0 out of 6 polymyositis patients. Neither heat-inactivated sera alone nor guinea pig complement sera alone showed myotoxicity. Removal of anti-acetylcholine receptor (anti-AChR) antibodies from a myasthenic serum sample by affinity absorption led to loss of myotoxicity. Myotoxicity of myasthenic sera could, in most cases, be confirmed by light microscopy.

These results support the idea that complement-mediated cell damage, initiated by anti-AChR antibodies, contributes to post-synaptic membrane degeneration in myasthenia gravis.

Introduction

It is now generally accepted that myasthenia gravis is an autoimmune disorder in which the autoantigen is the nicotinic acetylcholine receptor (AChR) of the post-syn-

aptic muscle membrane (for reviews see Vincent 1980; Harrison and Behan, in press). Neuromuscular transmission is accordingly impaired largely as a loss of functional AChR's. Elevated levels of circulating anti-AChR antibodies are present in approximately 90% of clinically diagnosed myasthenic patients and there is considerable evidence that these antibodies constitute the primary agents causing loss of AChR activity. The means by which such loss occurs are still unclear and three antibody-mediated mechanisms have been suggested (Drachman et al. 1981). These are:

(1) Direct blockade of access of the neurotransmitter to its binding site;
(2) Accelerated degradation of the membrane-bound receptor;
(3) Complement-mediated lysis of the post-synaptic membrane.

Although considerable experimental evidence has been produced supporting intermediacy of the first two of these mechanisms in the disease process, the extent of their involvement remains in doubt (Harrison and Behan, in press). Less data have been reported in support of the third proposed mechanism of receptor loss, complement-mediated lysis. Probably the most convincing evidence for this mechanism is indirect, involving ultramicroscopic demonstration of IgG, C-3 and C-9 on disintegrating junctional folds and on debris in the synaptic clefts of myasthenic patients (Engel et al. 1977; Sahashi et al. 1980). Little direct evidence for lytic action of myasthenic sera on muscle cells has been reported, however, and the publication of a procedure (Cambridge and Stern 1981) for quantifying myotube-specific cytotoxicity prompted us to apply this to such sera. The method, developed for measuring cell-mediated myotoxicity in polymyositis, depends upon the selective uptake by cultured myotubes of [Me-^{3}H]carnitine, loss of which can be monitored following cytolytic damage. A major advantage of the procedure is the very much slower uptake of carnitine by fibroblasts, which commonly contaminate myotube cultures.

Materials and Methods

Tissue culture

Myotube cultures were prepared from the thigh muscles of 1-2-day-old neonate white CFHB rats, essentially according to the method of Yaffe (1973). A single cell suspension was obtained from minced tissue by trypsinisation (0.2% trypsin) for 1 h at 37°C in Ca^{2+}- and Mg^{2+}-free balanced salt solution. After centrifugation at 400 $\times$ g for 10 min, the tissue pellet was resuspended in growth medium (Dulbecco's Modified Eagles Medium supplemented with 10% Donor Horse serum, 0.15% glucose, 100 U/ml penicillin and 100 μg/ml streptomycin). The cell suspension was filtered through two layers of nylon bolting cloth (53 μm aperture) and plated (2.5 $\times$ 10^5 cells/culture well) in 24-well culture plates (Linbro, Flow Laboratories, Irvine, Ayrshire, Scotland) precoated with rat tail collagen (Masurovsky and Peterson 1973). The cultures were grown at 37°C in an atmosphere of 10% carbon dioxide in air, the growth medium being replaced every 3 days. Myoblasts began to fuse, forming myotubes, after 3 days of growth. The number of fibroblasts present was minimised by treating 3-day-old cultures with fluorodeoxyuridine (15 μg/ml) for 72

h. For all experiments, myotube cultures were used after 7 days growth, at which time maximum expression of AChR's, as judged by binding of $[^{125}I]\alpha$-bungarotoxin (Vogel et al. 1972) was achieved.

Fibroblast cultures were prepared from neonatal rat endothelial tissue as described above for myotube cultures except that Donor Horse serum was replaced by foetal calf serum. A confluent layer of fibroblasts was formed within 7 days and used to assay uptake of tritiated carnitine at this stage.

Labelling with [Me-³H]carnitine

[Me-³H]Carnitine hydrochloride, specific activity 2 Ci/mmol (Amersham International plc, Amersham, Bucks., U.K.) was diluted in growth medium to a final concentration of 10 μmol/l. This solution (50 μl, 1 μCi) was added to growth medium (0.7 ml) in each culture well and incubated at 37°C, under an atmosphere of 10% carbon dioxide in air, for 18 h. Uptake of labelled carnitine at this stage was determined by washing the cultures 3 times with growth medium, harvesting and counting for radioactivity as described below.

Serum

Blood samples were obtained from 13 patients with clinically confirmed myasthenia gravis. A further 6 blood samples were obtained from patients with polymyositis, chosen as an example of muscle degenerative disease. Eleven control samples were obtained from members of the laboratory staff. Serum was removed from clotted blood and stored at −20°C until use. Radioimmunoassays for anti-AChR antibodies were carried out as described by Carter et al. (1981). Before measuring complement-mediated myotoxicity, all sera were incubated at 56°C for 30 min prior to use. Lyophilised guinea pig complement serum (Miles Laboratories, Stoke Poges, Bucks., U.K., stated activity of +ve haemolysis at 0.04 ml when used at 1/10 dilution) was reconstituted with commercial diluent (1 ml) at the time of use.

Myotoxicity assays

Monolayer cultures of rat myotubes were incubated with [Me-³H]carnitine as described above. The cultures were washed 3 times with growth medium and fresh medium (0.5 ml) was added to each culture well. Sera were tested by addition of a sample (0.08 ml) to each of 8 replicate culture wells. Guinea pig complement serum (0.08 ml) was added to 4 of these wells and the same volume of growth medium to the remaining 4 wells. Growth medium alone (0.16 ml) was added to each of a further 4 control wells. For each myotube culture an additional 4 wells containing complement (0.08 ml) but no serum (0.08 ml growth medium instead) were included. The cultures were incubated at 37°C for 3 h (except in time studies) in an atmosphere of 10% carbon dioxide in air. At the end of the incubation time, the cultures were washed 3 times with growth medium, harvested and counted for radioactivity as described below. The value of radioactive counts retained was, in each case, taken as the mean of those from each of the 4 replicate wells.

Myotoxicity was expressed as the percentage loss of radioactivity compared with

controls according to the formula

$$\frac{CRC - CRT}{CRC} \times 100\%$$

where CRC = counts retained in the control containing neither serum nor complement and CRT = counts retained in the test sample. Each test sample was assayed in 3 different myotube cultures to give the quoted mean value for myotoxicity.

Harvesting of carnitine-labelled cultures

Monolayer cultures were harvested for counting by incubation with 0.1 M sodium hydroxide (0.3 ml) for 30 min at room temperature. The contents of each culture well were transferred to scintillation vials. Each well was then washed with 0.1 M sodium hydroxide (0.2 ml) for 15 min and the washings transferred to the appropriate scintillation vials. Scintillation fluid (30% Triton X-100 in toluene, 5 g/l PPO, 5 ml) was added to each vial, the contents mixed on a vortex mixer and the radioactivity of each vial counted in a Packard Liquid Scintillation counter.

Depletion of anti-AChR antibodies from myasthenic serum

Anti-AChR antibodies were removed from a myasthenic serum sample [Patient 4 (ii)] by affinity absorption.

Human AChR was coupled to α-bungarotoxin-Sepharose 4B by using an adaptation of the method of Lang et al. (1982). α-Bungarotoxin (2 mg) was covalently linked to cyanogen bromide-activated Sepharose 4B (March et al. 1974) (50 ml packed volume) according to the method of Lindstrom et al. (1981). The final density of α-bungarotoxin was 0.04 mg/ml beads. AChR was prepared as a crude detergent extract of human leg muscle and its α-bungarotoxin binding activity was determined by ammonium sulphate precipitation assay (Stephenson et al. 1981). The detergent extract of AChR (200 ml, 210 pmol α-toxin binding sites) was applied as a batch to α-bungarotoxin-Sepharose 4B (25 ml) and stirred gently overnight at 4°C. The beads were then washed extensively with 0.5 M NaCl in phosphate-buffered saline (PBS) followed by PBS alone. Assay of the supernatant and washings showed that 97% of the applied AChR was bound to the beads giving a final density of 8.2 pmol AChR/ml beads. The beads were then packed in a column (1.7 cm × 30 cm) and equilibrated with PBS at a flow rate of 30 ml/h. A control column containing α-bungarotoxin-Sepharose 4B, to which no AChR had been attached, was similarly prepared.

Myasthenic serum (2 ml, containing 84 pmol of anti-AChR antibodies) was applied to each column and circulated at 30 ml/h for 2 h at 23°C. The columns were then washed with PBS (75 ml) and fractions (3 ml) collected. Fractions showing absorption at 280 nm were pooled and concentrated on an Amicon B15 concentrator to the original volume (2 ml) of applied serum. Serum IgG was measured by a radial immunodiffusion assay using antiserum to human IgG (Immunostics RID plate, Seward laboratories, London). Anti-AChR antibody content and complement-mediated myotoxicity were assayed as described above.

Results

Seven-day-old rat myotube cultures incubated with [Me-^{3}H]carnitine routinely took up 10–20 × 10^3 cpm, representing 1–2% of the total carnitine of the culture medium. Variation between replicate cultures was less than 10%. The corresponding uptake of radioactivity by fibroblast cultures was 3–4 times lower than that of the myotubes.

Loss of radioactivity from labelled myotubes incubated with growth medium in the absence of serum and complement followed a time course typified by that shown in Fig. 1. Myotubes incubated with heat-inactivated normal human serum plus complement showed a very similar pattern. Cells incubated with heat-inactivated myasthenic sera together with complement, generally showed an accelerated loss of radioactivity. Initial studies indicated that this loss, relative to controls, was greatest after 3 h incubation (Fig. 1) and all subsequent experiments accordingly used these conditions.

The measured myotoxicity caused by the addition of guinea pig complement serum alone showed a variation between different myotube cultures that fell within the range −2.2 to 15.1% (mean ± SD 7.0 ± 4.6) (Table 1). Heat-inactivated normal human sera, with or without guinea pig complement, gave similar myotoxicities (Table 1) and values of up to 20% in our system can be regarded as falling within the normal range. Cultures showing such myotoxicity values never showed any evidence of cell damage when examined microscopically.
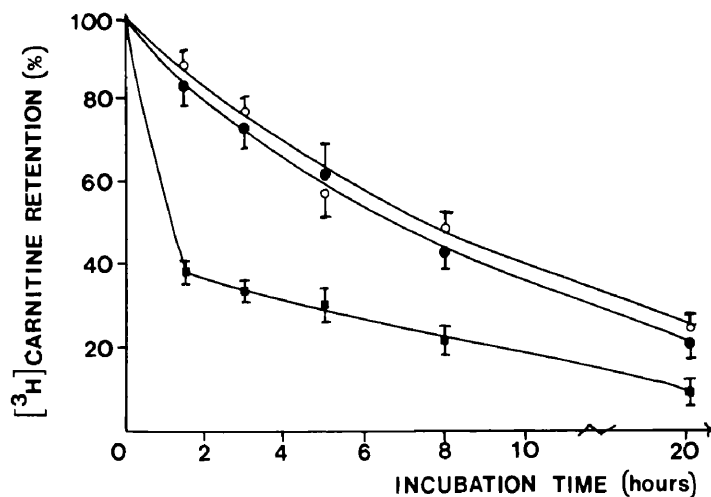

Fig. 1. Retention of [^{3}H]carnitine by myotube cultures after incubation with heat-inactivated serum and complement. Replicate myotube cultures were labelled with [^{3}H]carnitine (Materials and Methods section) and exposed to aliquots (0.08 ml) of heat-inactivated normal (●) or myasthenic (■) sera plus guinea pig complement (0.08 ml) at 37°C for varying lengths of time. Cultures to which no additions were made were run simultaneously (○). At the end of each incubation period, the cultures were washed and harvested for counting. Results are expressed as the retention of radiolabel by cultures as a percentage of time 0. Each point represents the mean ± SD of 4 replicate cultures.

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TABLE 1

MYOTOXICITY OF MYASTHENIC, POLYMYOSITIS AND NORMAL HUMAN SERA TESTED WITH RAT MYOTUBES IN CULTURE

Myotoxicity of test samples was determined as described in the Materials and Methods section. Unless otherwise stated, serum samples were tested with 3 different myotube cultures and the mean values taken.

Test serum	Number of donors	Complement	Myotoxicity (%)	
			Range	Mean ± SD
None	12	+	−2.2–15.1	7.0 ± 4.6
Normal	11	−	−0.3–16.4	9.3 ± 4.8
Normal	11	+	3.5–14.5	10.3 ± 3.4
Myasthenia gravis	13 [a]	−	−7.7–11.4	3.8 ± 4.6
Myasthenia gravis	13 [a]	+	7.6–65.0	41.5 ± 21.7
Polymyositis	6 [b]	+	7.8–19.1	13.5 ± 4.2

[a] Multiple samples were taken at different times from 3 myasthenic patients (see Table 2); the mean values from the multiple samples were taken as the value for each patient.

[b] Serum samples from polymyositis patients were tested in one (3) or two (3) myotube cultures.

Heat-inactivated myasthenic sera in the absence of added guinea-pig complement gave myotoxicity values well within the normal range. In the presence of added complement, on the other hand, heat-inactivated serum samples from 13 myasthenic patients showed myotoxicity values ranging from 7.6% to 65%, with a mean value of

TABLE 2

MYOTOXICITY OF INDIVIDUAL MYASTHENIC SERUM SAMPLES IN THE PRESENCE OF COMPLEMENT

Patient	Myotoxicity (%) (Mean ± SD)	Anti-AChR antibody titre ($\times 10^{-10}$ M)
1	7.6 ± 6.2	8.2
2	15.7 ± 4.2	41.8
3	13.9 ± 1.6	91.6
4 [a] (i)	11.7 ± 7.5	121.6
(ii)	43.2 ± 0.8	420.5
5	63.9 ± 3.2	147.7
6	56.7 ± 5.4	159.7
7	10.8 ± 0.0	162.6
8	60.3 ± 2.1	242.0
9 [a] (i)	59.8 ± 0.5	255.7
(ii)	38.6 [b]	422.7
10	65.0 ± 1.2	721.3
11	58.4 ± 0.7	725.4
12	60.3 ± 2.9	1800.0
13 [a] (i)	62.3 ± 1.9	58.2
(ii)	43.2 ± 1.6	129.0
(iii)	65.4 ± 2.9	172.4
(iv)	52.0 ± 5.4	285.1
(v)	30.7 ± 0.9	438.2

[a] Repeat samples were taken over 17 months (patient 9), 27 months (patient 4) and 3 years (patient 13).
[b] One assay only; all other values represent means of 3 separate experiments.
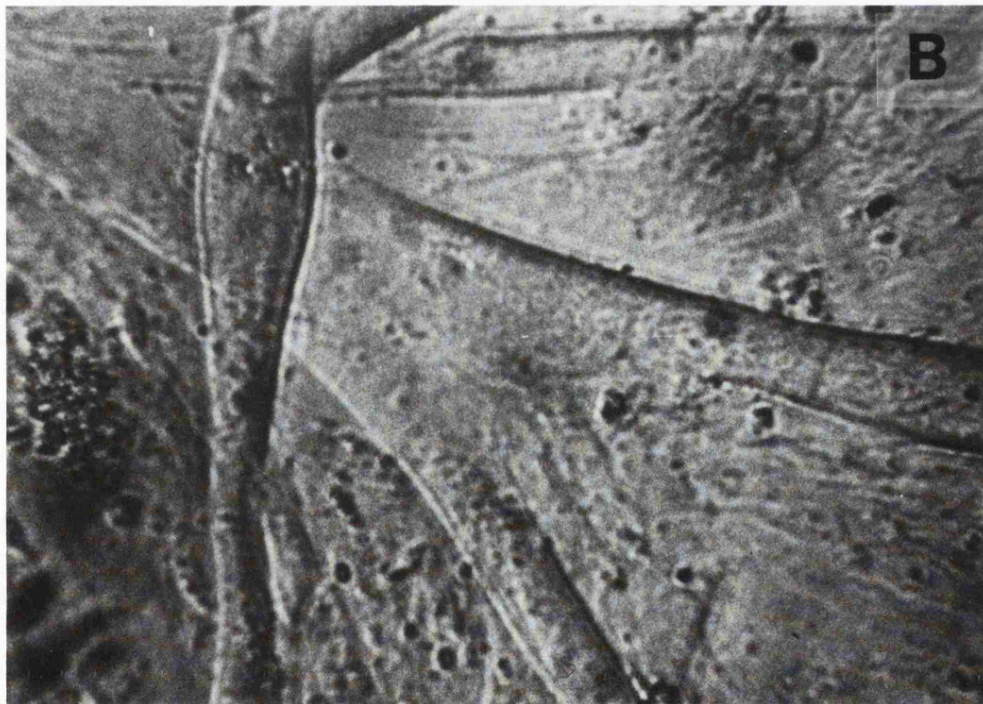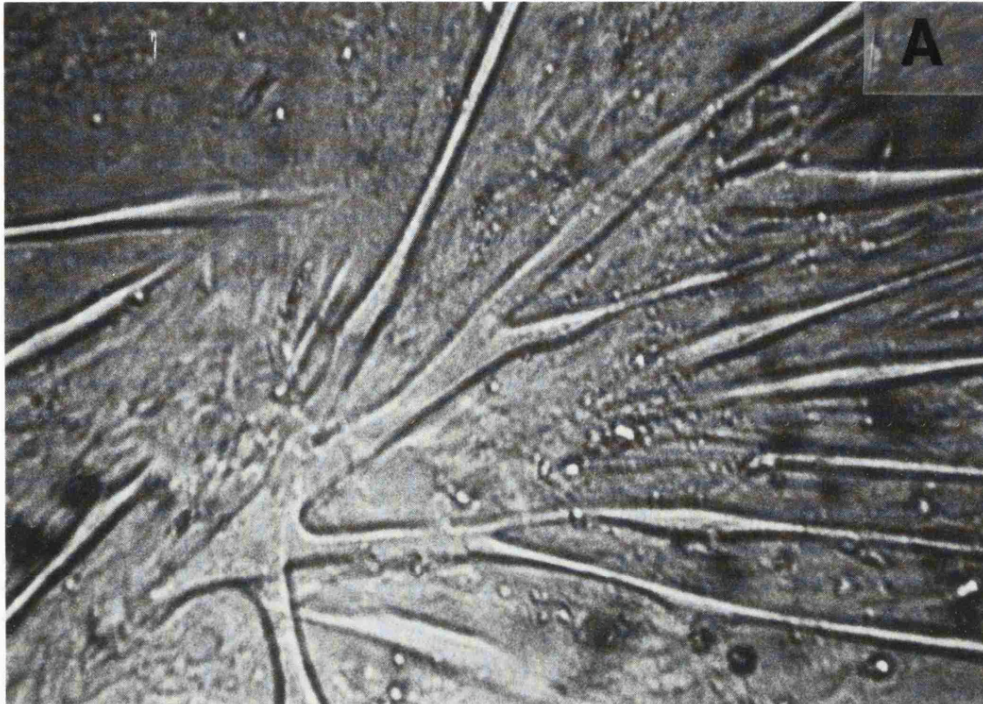
Fig. 2. Phase contrast photomicrographs of 7-day-old rat myotube culture. *A*, *B*: Cultures treated with heat-inactivated normal serum plus complement; *C*, *D*: Cultures treated with heat-inactivated myasthenic serum plus complement. Magnifications: ×80 (*A*, *C*); ×200 (*B*, *D*).
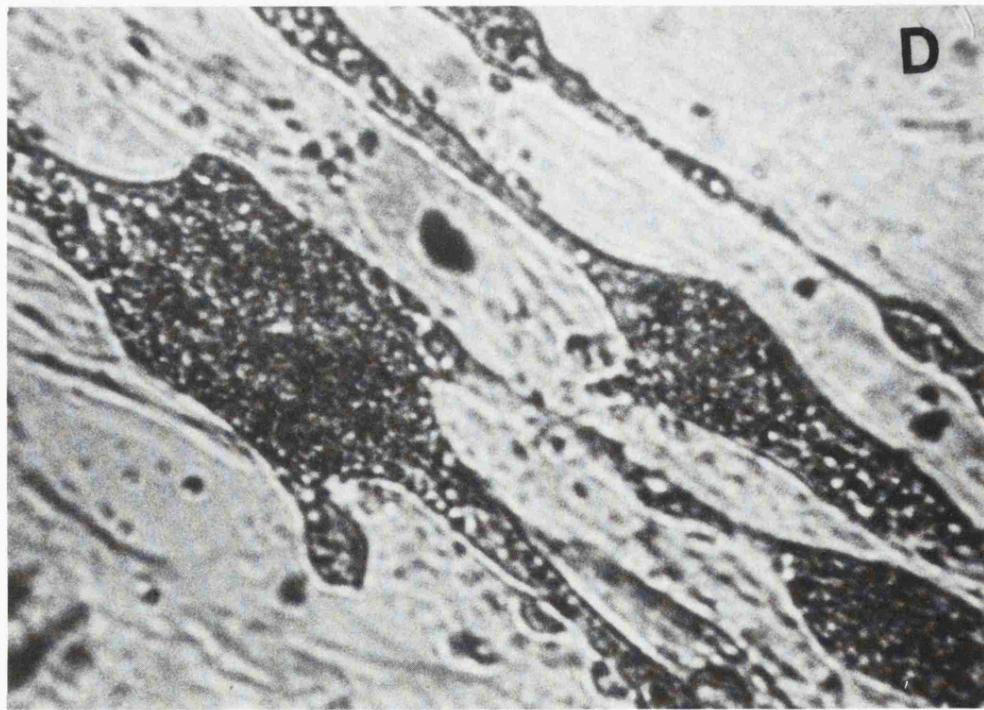
76

Fig. 2C and D.

TABLE 3

EFFECT ON MYOTOXICITY OF MYASTHENIC SERUM SAMPLE 4 (ii) OF DEPLETION OF ANTI-AChR ANTIBODIES

Treatment	Anti-AChR antibody titre ($\times 10^{-10}$ M)	IgG (mg/ml)	Myotoxicity (%)
None	420.5	3.08	43.2
AChR-affinity column	134.8	3.08	14.6
Control column	408.2	3.08	40.0

41.5% (Table 1). The data for myasthenic sera are shown in detail in Table 2, where it can be seen that 9 out of the 13 different patients have sera with myotoxicity values that are clearly abnormal, as defined above. Sera from each of 6 patients with polymyositis gave myotoxicity values within the normal range (Table 1).

In the cases of sera having myotoxicities greater than 50%, damage to the myotubes was clearly visible under the light microscope. Myotubes incubated with serum samples from normal (myotoxicity 9.0%) and myasthenic (myotoxicity 52.0%) donors are compared in the phase-contrast micrographs of Figs. 2A, B and 2C, D, respectively. Whereas myotubes treated with normal serum plus complement appear plump, phase-bright and agranular, those treated with myasthenic serum plus complement are shrunken, phase-dark and granular, showing, in parts, complete fragmentation. The background layer of fibroblast cells always appeared to be unaffected by either control of myasthenic serum.

A sample of myasthenic serum [patient 4 (ii)] having myotoxicity 43.2% was depleted of anti-AChR antibodies by affinity absorption on an AChR-α-bungarotoxin-Sepharose 4B column (Materials and Methods section). Table 3 shows that depletion of the anti-AChR antibody titre from 420.5×10^{-10} M to 134.8×10^{-10} M was paralleled by a fall in myotoxicity from 43.2 to 14.6%. Total IgG levels were apparently unchanged.

Discussion

A role for complement in the pathogenesis of myasthenia gravis was first suggested by Nastuk et al. (1960) following their observations that complement activity fell during exacerbation of the disease and rose to, or above, normal levels during periods of remission. Similar fluctuations were not, however, subsequently found by the same workers (Niemi et al. 1981) in experimental myasthenia in rabbits. In fact, as already mentioned, the strongest experimental evidence for complement-mediated lysis in myasthenia gravis is the presence of IgG and complement components on post-synaptic membrane fragments (Engel et al. 1977; Sahashi et al. 1980) and direct demonstrations of muscle cell lysis by myasthenic sera have hitherto been few. Nastuk et al. (1959), in studying the effects of myasthenic serum samples on frog sciatic nerve–sartorius muscle preparations in vitro, observed that two out of 22 such sera caused a rapid and irreversible reduction in twitch tension; a phenomenon which coincided with low resting potentials and apparent disintegra-

tion of many surface muscle fibres. However, similar effects were also shown by one of 9 normal sera tested, albeit after a much longer delay time. Liveson et al. (1976) described lysis, detected by light microscopy, of mouse somite cultures incubated with 3 out of 17 myasthenic serum samples. In one serum sample so tested, myolytic activity was shown to be destroyed by heating and not to be restored by subsequent addition of guinea pig complement.

Initial experiments (data not shown) indicated that both myasthenic and normal human control sera could sometimes cause release of carnitine from cultured myotubes (up to 30%); an effect that was destroyed by treatment of the sera at 56°C for 30 min and restored, in the case of myasthenic but not of normal sera, by the subsequent addition of guinea pig complement. Restoration of these properties in myasthenic sera was found to be dependent on the amount of complement added, which could explain the difference between our findings and that of Liveson et al. (1976). In order to obviate variations arising from the lability of endogenous complement and to standardise, as far as possible, the conditions of our assay system, all serum samples subsequently tested were heat-inactivated and a fixed amount of guinea pig complement was added at the time of testing. The procedure also avoids complications resulting from the heat-labile factor in some normal human sera, the nature of which is not clear.

Under the above conditions, serum samples from 9 out of 13 myasthenic donors caused accelerated loss, relative to normal controls, of labelled carnitine from rat myotubes. This effect was dependent on complement and can be readily explained in terms of antibody-initiated lysis of the myotube membranes. As is most commonly the case, all myasthenic serum samples tested contained anti-AChR antibodies, the involvement of which in the lytic process accordingly seems likely. Other autoantibodies directed against skeletal muscle membrane (Gilhus et al. 1983a,b; Mehl and Lang 1984) also occur in myasthenic serum, however, and must be considered. Evidence that anti-AChR antibodies are the major factor in mediating myotoxicity in our system, however, is provided by the consequence of their specific immunoabsorption from myasthenic serum. Following affinity chromatography on bound AChR, the myotoxicity of a myasthenic serum sample fell from 43% to a value (15%) well within the range characteristic of normal control sera. It is of interest that immunoabsorption lowered the anti-AChR antibody titre from 420×10^{-10} M to a value (135×10^{-10} M) comparable with that (122×10^{-10} M) of a sample [4 (i), Table 2] obtained 27 months later from the same patient; the later sample also showed a myotoxicity value (12%) well within the normal range. Although detailed correlation of anti-AChR antibody titre with myotoxicity values is not good, it does seem that serum samples with myotoxicity values within the normal range tend to have relatively low anti-AChR antibody titres (e.g. less than 200×10^{-10} M). Nevertheless, some samples with high myotoxicity also had lower antibody titres and it may be that, in such cases, complement fixation is affected by relatively high proportions of particular antibody subclasses, which are known to differ in their ability to activate complement.

In the case of serum samples with higher myotoxicity values, damage to myotubes was clearly visible in the light microscope, supporting the implications of our

quantitative assay procedure. Overall, it does appear that higher myotoxicity values are confined to myasthenic patients and are not given either by normal controls or by patients with polymyositis; chosen as an example of muscle degenerative disease.

We have preliminary evidence from experiments not reported in detail here that myasthenic sera have very similar effects on human myotubes in culture. Our findings provide further evidence for the possibility that complement-mediated lysis is an important factor in the pathogenesis of myasthenia gravis. Our observations that myotoxicity may depend upon factors other than anti-AChR antibody titre, as commonly assayed, suggest that levels of such factors, e.g. complement and possibly particular antibody subclasses, could profitably be considered in future studies on the correlations of biochemical parameters with disease state in myasthenia gravis.

Acknowledgement

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