SOME EFFECTS OF ANTIBODY ON LYMPHOCYTIC FUNCTION

submitted by

Marion Hanbury Brown

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

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In this study effects of antibody *in vitro* on metabolic behaviour of neoplastic B lymphocytes from guinea pigs with L_2C leukaemia have been investigated. Two major effects have been found:
(1) When cultured with antibody to surface immunoglobulin (Ig), the spontaneous migration of L_2C cells along plastic surfaces was inhibited in a concentration-dependent manner. The relative inhibition decreased with time. Bivalent $(Fab'\gamma)_2$ fragments from antibody were able to inhibit migration whereas the monovalent $Fab'\gamma$ which is not able to cross-link surface Ig did not inhibit migration. The mechanism of inhibition did not involve the release of migration inhibition factor (MIF). In fact, no spontaneous or antibody-induced release by L_2C cells of MIF-like substances capable of inhibiting migration of L_2C cells themselves or of normal guinea pig macrophages could be demonstrated.
(2) Anti-Ig antibodies produced a temporary inhibition of synthesis of total cellular proteins in L_2C cells. Inhibition was achieved with very low concentrations of antibody (2 μg/ml) and increasing the concentration of antibody had no further effect. Removal of antibody from the culture medium after 30 minutes at $37^{o}C$ did not alter the response of the cells. Again, $(Fab'\gamma)_2$ from antibody was able to inhibit protein synthesis. The inhibition yielded by the monovalent $Fab'\gamma$ was less and is of doubtful significance. Antibodies to other molecules found on the cell surface, β_2 microglobulin and Ia antigens, did not inhibit protein synthesis. Cellular Ig synthesis was also inhibited by antibody to surface Ig. In lymphocytes from normal guinea pigs and from human patients with chronic lymphocytic leukaemia (CLL) there was no inhibition of protein synthesis by antibody.

Some preliminary studies on the cytotoxic effects of diphtheria toxin conjugated to antibody were done. Evidence was obtained for antibody specificity of the conjugates in killing L_2C cells and for increased cytotoxicity of antibody when coupled to diphtheria toxin.

CONTENTS

CHAPTER 1
GENERAL INTRODUCTION

1.1 B Lymphocytes

1.1.1 Normal

1.1.2 Neoplasms

1.1.3 A neoplasm in guinea pigs

1.2 Lymphocytic Surface Immunoglobulin

1.3 Capping and Endocytosis

1.3.1 Surface immunoglobulin

1.3.2 Molecular specificity

1.3.3 Mechanism

1.3.4 Molecular specificity of mechanism

1.3.5 Endocytosis

1.3.6 Regeneration of surface immunoglobulin

1.4 Lymphocytic Motility

1.4.1 Spontaneous movement

1.4.2 Stimulated movement

1.4.2.1 Antibody

1.4.2.2 Molecular specificity

1.4.3 Migration inhibition factor (MIF)

1.4.4 Tumour cell migration

1.5 Some Biological Effects of Antibody

1.5.1 DNA synthesis

1.5.2 Differentiation

1.6 Immediate Biochemical Effects of Antibody

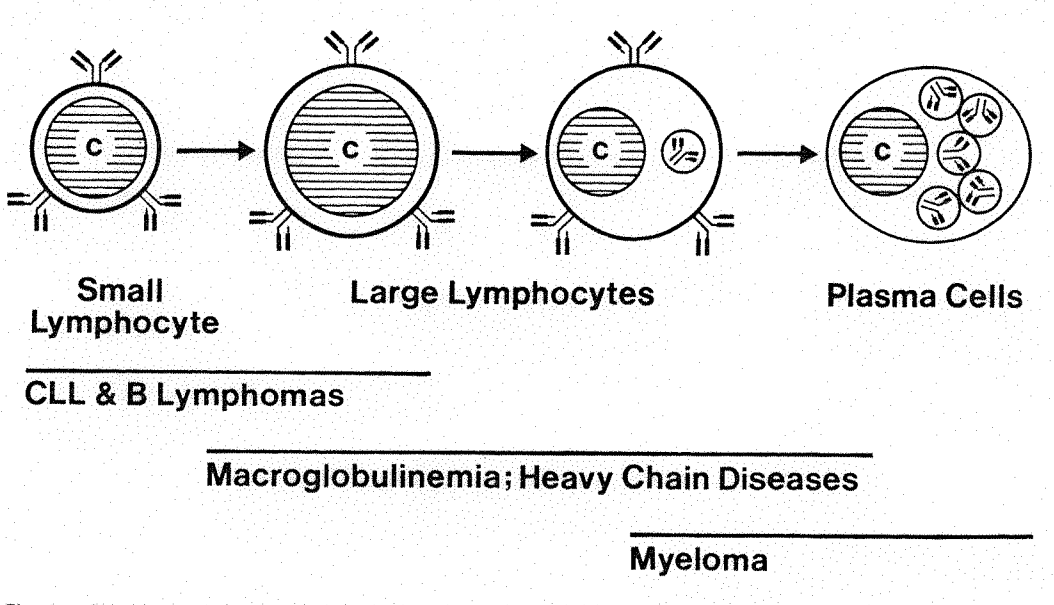


Fig. 1. Clonal expansions, physiological and neoplastic, of B lymphoid cells. The arrows depict antigen-driven physiological expansion. Y-shaped Ig molecules can be seen first as surface Ig, then as export Ig within secretory vesicles. As indicated by the horizontal lines, neoplasms can apparently arise from cells at various stages of maturation, to give clones with differentiation and mode of Ig production relatively static.

Fig. 1.1 Clonal expansions, physiological and neoplastic, of B lymphoid cells
(taken from Stevenson, 1978)

1.1 B Lymphocytes

1.1.1 Normal

Lymphocytes of the B cell series can be identified by their ability to synthesize immunoglobulin (Ig) and express it on their surfaces at some stage of their differentiation. They are believed to develop from a haematopoietic stem cell, an ancestor common to B and T lymphocytes. The differentiation of stem cells into B lymphocytes first occurs in embryonic life. B cells derive their name from the site of B cell differentiation and antigen-independent multiplication shown to be the bursa of Fabricius in birds (Glick, Chang and Jaap, 1956). There is no one bursa-equivalent in the mammal and the sites of differentiation have been established in the foetal mouse as liver and spleen (Owen, Cooper and Raff, 1974; Melchers, von Boehmer and Phillips, 1975) with the bone marrow apparently being the major site in the adult (Osmond and Nossal, 1974).

In their ontogeny, B lymphocytes reach a stage where antigenic stimulus is required for further differentiation (Fig. 1.1). These small resting B cells synthesize Ig primarily for display on the cell surface and it is the Ig molecules which recognize antigen (reviewed Warner, 1974). To respond to antigen, the B cell requires additional help from collaborating T cells (Miller and Mitchell, 1969) and macrophages (Unanue, 1972). These collaborating cells or their products may act through a separate mitogen receptor on the B cell. The antigen-induced activation of B cells can lead to stimulation of growth and final differentiation into a plasma cell with an increased cytoplasm to nucleus ratio, the cytoplasm being heavily laden with rough endoplasmic reticulum. The plasma cell produces Ig for secretion, inserting little or none into the surface and is the antibody-producing cell in the humoral immune response.

Throughout the changes in Ig synthesis all the Ig from a single clone of cells has the same antigenic specificity (Burnet,

1959). All the light chains from a single clone of cells are of one type, κ or λ but the class of Ig synthesized by a B cell may change in association with maturation. IgM is first detected on the surface of B cells (Vitetta, Melcher, McWilliams, Lamm, Phillips-Quagliata and Uhr, 1975) and mature antigen-sensitive B lymphocytes can express both IgM and IgD on the same cell (Vitetta and Uhr, 1976). Antigenic stimulation can cause a further switch in the class of Ig synthesized by a cell.

1.1.2 Neoplasms

Recognized neoplasms of B lymphocytes represent autonomous proliferation of a particular clone of cells and give rise to diseases such as chronic lymphocytic leukaemia (CLL), many lymphomas, heavy chain diseases, macroglobulinaemia and multiple myeloma. It is widely believed that neoplastic B cells represent normal lymphoid cells arrested at a specific point in maturation (Salmon and Seligmann, 1974) (Fig. 1.1). In this case, bearing in mind possible perturbations due to the neoplastic process, tumours formed from these cells provide pure populations of cells for the study of different stages of differentiation as well as the characterization of the cancer itself. CLL in man results from multiplication of a small cell displaying surface Ig (Aisenberg and Bloch, 1972) whereas multiple myeloma arises from an excess production of plasma cells usually secreting Ig. The neoplastic cells synthesize a restricted type of Ig as expected from cells of a single clone. Thus in a particular neoplasm all the Ig will have the same idiotypic specificity and only one type of light chain will be recognized. There may be some variation in the class of Ig expressed. Both IgM and IgD have been identified on the surface of CLL cells (Fu, Winchester and Kunkel, 1974) with the same idiotypic specificity (Fu, Winchester, Feizi, Walzer and Kunkel, 1974).

1.1.3 A neoplasm in guinea pigs

Studies on B cell neoplasms have been facilitated by an animal

model, the L_2C leukaemia which is a lymphocytic leukaemia of strain 2 guinea pigs. L_2C leukaemia was the only experimental leukaemia indubitably derived from B lymphocytes available for study in any animal species until recent descriptions of murine B cell lymphomas (Slavin and Strober, 1978; Lanier, Lynes and Haughton, 1978; Mckeever, Kim, Nero, Laskov, Merwin, Logan and Asofsky, 1979).

The L_2C leukaemia arose spontaneously in 1953 in a female guinea pig of the inbred strain 2 and has since been passaged continuously in vivo within this strain (Congdon and Lorenz, 1954). The female diploid karyotype has been retained in the leukaemic cells with some variation among the five sublines (Whang-Peng, 1977). The disease is manifested by lymphoblasts with little cytoplasm and no significant rough endoplasmic reticulum and its distribution in the animal is widespread with large accumulations of tumour cells in the blood, spleen, liver and lymph nodes. L_2C leukaemic cells display surface markers characteristic of B cells: Ig and receptor for the third component of complement (Shevach, Ellman, Davie and Green, 1972). Four of the sublines also have the antigens of the major histocompatibility complex including the Ia antigens (Shevach and Schwartz, 1977) which are involved in cell collaboration in the immune response.

The surface Ig has been identified as IgM (Stevenson, Eady, Hough, Jurd and Stevenson, 1975a) of light chain class λ (Stevenson, Mole, Raymont and Stevenson, 1975b). The L_2C cell synthesizes its IgM mainly for insertion into the surface with an excess production of light chain which is released from the cell (Hough, Chapple, Stevenson and Stevenson, 1978). Leukaemia-bearing animals produce a urinary λ light chain apparently identical to that on the surface (Stevenson et al, 1975b). Stevenson and Stevenson (1975) raised a tumour specific antiserum against fragments of IgM cleaved proteolytically from the surface of L_2C cells. Using this antiserum idiotypic determinants demonstrated

on the surface Ig are the same for all sublines of the tumour in which they have been investigated (Forni, Shevach and Green, 1976). In neoplasms such as the L_2C leukaemia and CLL in man which do not export significant amounts of Ig,antibodies to tumour specific antigens on the cell surface have therapeutic potential because there will be little tumour Ig to form an extracellular antigenic barrier (Stevenson, Elliott and Stevenson, 1977a).

1.2 Lymphocytic Surface Immunoglobulin

Surface Ig, excluding Ig adsorbed from the serum is synthesized by the B lymphocyte and inserted into the membrane (Raff, Sternberg and Taylor, 1970). The pathways of Ig destined for secretion and for insertion into the membrane diverge at some point in biosynthesis, possibly in the Golgi complex (Vitetta and Uhr, 1974). Once in the membrane, the surface Ig which is monomeric (Vitetta, Grundke-Iqbal, Holmes and Uhr, 1974) can be removed intact only by dispersing the membrane in detergent (Melchersand Andersson, 1973). The way in which Ig is attached to the membrane has not been definitively established but it is generally accepted that the Fab portion is exposed to the outside environment. Part of the Fc portion will not react with antibodies (Fu and Kunkel, 1974) suggesting that a short sequence is buried in the membrane. There is speculation that Ig is destined to be anchored in the lipid bilayer of the membrane by virtue of a hydrophobic extra piece cleaved from secretory Ig (Burstein and Schechter, 1977; Schechter, Wolf, Zemell and Burstein, 1979). Melcher and Uhr (1973) suggested that incomplete glycosylation is the feature which retains Ig on the membrane rather than release it.

1.3 Capping and Endocytosis

1.3.1 Surface immunoglobulin

The distribution of Ig on the membrane which is initially diffuse can be changed by binding of antibody. The redistribution

of surface receptors has implications in the delivery to the cell of signals involved in the activation of lymphocytes, antigenic modulation and tolerance induction. Taylor, Duffus, Raff and de Petris (1971) described the phenomenon of capping, a process in which antibody redistributes the receptors to one pole of the cell. The events after interaction of receptors with ligand leading up to cap formation can be separated into different stages. Binding is followed by formation of patches of antibody-Ig complexes and will occur at $4^{o}C$. Patching does not require metabolic energy whereas capping does. At $37^{o}C$, the patches move rapidly to one pole and condense into caps leaving the surface of the cell free of Ig receptors. Both patching and capping depend on cross-linking of receptors which requires a bivalent ligand (Unanue, Perkins and Karnovsky, 1972; de Petris and Raff, 1973). Antibody at a concentration below that required to saturate all the surface Ig can induce patching but not capping (Unanue et al, 1972).

1.3.2 Molecular specificity

The redistribution of Ig and other receptors shows molecular specificity although individual receptors do not always behave independently. Selective redistribution, which is the capping of one receptor by its specific antibody without causing capping of any others, was first described by Taylor et al (1971). It was found that capping of Ig by its specific antibody on murine B cells did not change the distribution of the histocompatibility antigen (H2). Similarly, Preud'homme, Neauport-Sautes, Piat, Silvestre and Kourilsky (1972) demonstrated independent redistribution of Ig and histocompatibility antigens in human cells. However, independent redistribution of receptors is not always observed. Bourguignon, Hyman, Trowbridge and Singer (1978) found that when a major surface glycoprotein T200 on a mouse cell line was capped with its specific antibody, H2, thymus leukaemia antigen (TL) and Thy-1 would co-cap.

Krammer and Pernis (1976) reviewed conflicting data on co-capping of Fc^{γ} receptors with Ig. They found Fc^{γ} receptors

co-capped with surface Ig when the latter was capped by anti-Ig. Abbas and Unanue (1975) showed that Fc^{γ} receptors co-capped with Ig but the converse did not occur: capping of Fc^{γ} receptors did not change the distribution of Ig. Thus Fc^{γ} receptors did not seem to be associated with surface Ig but an association was formed when Ig was capped with anti-Ig. Other workers (Ramasamy and Lawson, 1975) did not observe co-capping of Fc^{γ} receptors. Explanations for these differences remain speculative. However it has been found that loss of Fc^{γ} receptor interaction with surface Ig was associated with cell activation (Scribner, Weiner and Moorhead, 1977).

Schreiner and Unanue (1977) discussed two distinct forms of capping. Capping of Ig (see below) fell into one category and the other included Con A receptors, H2 antigens, β_2 microglobulin and antigens binding the antibodies in anti-lymphocyte globulin. Capping in the latter cases is relatively slow (Unanue et al, 1972) and with most of these receptors a single ligand alone is not sufficient to induce capping. An antibody directed against the first ligand provides the necessary cross-linking.

There is a relationship between Con A capping in lymphocytes and cell movement. Con A capping is seen after a lymphocyte has initiated a cycle of cell movement. The cap forms at the trailing pole and disperses when the cell rounds up. These caps can be reversed by suppressing motility with low doses of cytochalasin B (de Petris, 1975). Schreiner and Unanue (1977) categorized this Con A type of capping as movement-dependent capping. More recently, Braun, Fujiwara, Pollard and Unanue (1978a) have relegated Con A capping to a position intermediate between the two types, probably due to Con A binding to various surface molecules.

Schreiner and Unanue pointed out that capping of Ig molecules appeared to be fundamentally different. Included in this type of capping are TL and Fc^{γ} receptors (Braun et al, 1978a). It is very rapid, more than 90% of normal mouse B cells capping their surface

Ig within 2 - 3 minutes of exposure to the ligand. However, neoplastic cells have been observed to cap surface Ig much less readily (Liebes, Quagliata and Silber, 1977). Capping of Ig can occur in non-motile cells (Unanue, Ault and Karnovsky, 1974). Ig caps are not spontaneously reversible and are not disrupted by sodium azide nor other metabolic inhibitors (Schreiner and Unanue, 1976a).

1.3.3 Mechanism

Taylor et al (1971) first suggested that Ig capping was associated with contractile elements of a cell because of its partial inhibition by cytochalasin B which is thought to interfere with microfilament function. Subsequent evidence has suggested attachment of antibody to surface Ig involves a linkage with the microfilaments of the cytoskeleton.

Fluorescent techniques in which ligand (bound to surface receptors) and intracellular contractile proteins are differentially stained have enabled associations between them to be observed. Working with human fibroblasts, Ash, Louvard and Singer (1977) observed that initially, three independent integral membrane proteins were distributed between rows of organized microfilaments. If clustering of these surface receptors by antibody was induced at 37^{o}C before fixation and staining, the surface spots of fluorescence appeared larger and were now superimposed on those of microfilaments. $(Fab'\gamma)_2$ from antibody was also effective in forming linkages with microfilaments, and microtubules did not appear to be involved. Bourguignon and Singer (1977) found that capping with lectins or antibody produced a concentration of intracellular myosin and actin under caps in mouse splenic B and and T lymphocytes. The redistribution of cytoplasmic myosin together with surface Ig after antibody-induced capping was also observed by Schreiner, Fujiwara, Pollard and Unanue (1977). Monovalent $Fab'\gamma$ from antibody was not able to form the connection with microfilament clusters (Schreiner et al, 1977).

The underlying similarities of capping in different cells were discussed by Ash et al (1977). The receptors on fibroblasts which are flattened cells become immobilized over organized microfilaments but in the rounded lymphocyte and cells in suspension, the actin and myosin are not as organized or restrictive, and receptors are able to cap. However, the early events are the same in both cases, an energy-independent clustering of integral membrane proteins leading to a transmembrane linkage. Bourguignon and Singer (1977) suggested that there is an integral protein or class of proteins, X, in the plasma membrane of all eukaryotic cells to which membrane-bound actin is attached directly or indirectly. α-actinin is thought to be involved in linkage of actin to the membrane (Geiger and Singer, 1979). To enable specific redistribution to occur individual receptor molecules are not linked to actin or myosin until it is specifically cross-linked.

Flanagan and Koch (1978) showed that surface components such as Ig which are normally not attached to actin form stable associations with it when cross-linked by antibody. They used a technique (Koch and Smith, 1978) in which isolated myosin filaments were added to cell lysates; myosin filaments bind specifically to actin. Radioactively-labelled antibody was bound to surface Ig on lymphocytes and after cell lysis was detected bound to isolated myosin filaments. Lymphocytes prevented from capping with sodium azide, or P3 myeloma cells which do not cap still showed attachment of surface Ig to actin following treatment with bivalent antibody. Flanagan and Koch (1978) speculated that there is an equilibrium between Ig and microfilaments which can be altered by cross-linking surface components. This is similar to Edelman's theory of anchorage modulation (1976) in which receptors exist in two states, anchored and free. Cross-linking of receptors alters the equilibrium towards anchorage.

Once the transmembrane linkage with actin has been formed it is thought that the patches are collected into a cap by a sliding filament mechanism involving activation by Ca^{2+} similar to muscle

contraction (Bourguignon and Singer; Schreiner and Unanue, 1976b). Schreiner and Unanue (1976b) proposed a mechanism for capping in which Ca^{2+} activates the contractile system immediately under the antibody-receptor complex whereas the neighbouring areas would be relaxed. Net displacement would be possible with alternating contraction and relaxation.

There is no well-defined role for microtubules in capping. Con A capping is enhanced by colchicine which interferes with polymerization of microtubules. This effect can be explained by the increase in cell movement produced by treatment with the drug (Unanue and Karnovsky, 1974). A more fundamental role for microtubules is inferred from the results of Unanue and Karnovsky in which cytochalasin B and colchicine had a synergistic effect in inhibiting both Con A capping and Ig capping. Edelman's theory of anchorage modulation proposed a role for microtubules as anchors and a means of propagation of signals to and from the cell surface.

1.3.4 Specificity of mechanism

Singer's group (Singer, Ash, Bourguignon, Heggeness and Louvard, 1978) concluded that all surface receptors including Con A receptors (Ash and Singer, 1976; Toh and Hard, 1977) they had investigated are able to form transmembrane linkages after cross-linking by a ligand and although variations exist in different capping processes, all operate by a basically similar mechanism. Variations in capping could be accounted for by differences in stoichiometry of receptor binding to X, or the capacity of an aggregate to trigger the Ca^{2+} activation of the actin-myosin collection mechanism (Bourguignon and Singer, 1977).

Schreiner and Unanue (1977) considered it unlikely that all membrane proteins can interact with the cell's contractile system. They suggested that each cell type contains a particular set of membrane proteins that can functionally link to the contractile apparatus. That the association with the cytoplasm is a property

unique to Ig was suggested by the observation of mitogen-activated B lymphocytes which cap their surface Ig spontaneously without affecting the distribution of other membrane proteins (Schreiner, Braun and Unanue, 1976; Braun et al, 1978a). Further evidence for two distinct forms of capping was provided from experiments with tertiary amino anaesthetics and a Ca^{2+} ionophore (Braun, Fujiwara, Pollard and Unanue, 1978b). Increasing the intracellular concentrations of Ca^{2+} by use of ionophores stopped capping of Ig, FcγR and TL receptors and disrupted already formed caps of the same receptors in an energy-requiring process. These effects may have resulted from a systemic contractility of the microfilaments. Tertiary amino anaesthetics passively disrupted these caps displacing Ca^{2+} from the membrane, suggesting that a link between microfilaments and receptors had been broken. Caps of receptors which appeared not to be associated with cytoplasmic myosin were disrupted by neither anaesthetic nor Ca^{2+} ionophore. Schreiner and Unanue (1977) proposed that redistribution of Con A and the other inefficiently-capping receptors does not require membrane-cytoplasmic associations. It could occur through some organized lipid flow such as in a model proposed by Bretscher (1976) in which there is a continuous membrane lipid flow dragging patches into a cap.

1.3.5 Endocytosis

Ligand-receptor complexes on the cell surface may be endocytosed, remain on the cell surface, be released into the extracellular environment, or dissociate. Most of the complexes involving surface Ig with antibody are removed by endocytosis. The linkage of actin and myosin with patches and caps provides the local contractile machinery required to invaginate and pinch off regions of the capped membrane thereby providing a connection between capping and endocytosis (Bourguignon and Singer, 1977). Even if receptors besides Ig can associate with microfilaments in the same cell there must be a peculiarity of the Ig association which enables it to cap and be endocytosed more efficiently.

Interiorization has been described as a very fast energy-dependent process (Taylor et al, 1971).

Endocytosis of antibody-Ig complexes can follow capping or take place from multiple sites on the cell surface. For example, antibody at doses too low to induce significant capping was still internalized (Unanue et al, 1972). As in human or rabbit B cells, endocytosis could occur without receptors capping (Ault, Karnovsky and Unanue, 1973; Linthicum and Sell, 1974). Monovalent Fab'γ from antibody has been reported to interiorize to a limited extent (de Petris and Raff, 1973), but the bivalent antibody was much more efficient.

Morphological studies of endocytosis of antibody-Ig complexes (reviewed Schreiner and Unanue, 1976a) showed that, once inside the cell, complexes moved toward the Golgi region and fused with lysosomes where they were degraded. The lymphocyte is capable of hydrolyzing and digesting the interiorized complexes (Engers and Unanue, 1973; Ault, Unanue, Katz and Benacerraf, 1974). These workers showed that when B cells were incubated with ^{125}I-labelled antibody, ^{125}I was identified bound to amino acids and small protein fragments released from the cell into the culture medium.

As discussed by Singer et al (1978) the mechanism of endocytosis of surface Ig-antibody complexes may be related to receptor mediated endocytosis of coated pits (Goldstein, Anderson and Brown, 1979). Coated pits are regions of the cell surface containing clusters of surface receptors. The region of the plasma membrane containing the coated pit is pinched off and internalized. Once inside the cell the vesicle has various fates (Doyle and Baumann, 1979) such as fusion with lysosomes and consequently degradation, or the vesicle can be recirculated back to the cell surface.

1.3.6 Regeneration of surface immunoglobulin

After capping and endocytosis of surface Ig, the Ig molecules are replaced on the membrane of B cells (Unanue et al, 1972). For this re-expression to occur, cells cleared of surface Ig had to be incubated for 12 - 24 hours without antibody present in the medium. The presence of antibody presumably continuously eliminates new surface Ig. The ability to re-express surface Ig varies with the stage of maturation of the cells. B cells from mice younger than 14 days could not re-express surface Ig within the period of observation (Raff, Owen, Cooper, Lawton, Megson and Gathings, 1975; Sidman and Unanue, 1975). The immature cell could however re-express surface Ig after its removal with pronase, so it appears that the clearing of surface Ig by antibody produced a unique suppressive signal.

1.4 Lymphocytic Motility

1.4.1 Spontaneous movement

Lewis (1931) described the "handmirror appearance" of rat lymphocytes in amoeboid movement. He observed the formation of a pseudopod, and a cytoplasmic tail which McFarland and his associates named the "uropod". The uropod is a specialized surface area for endocytosis or attachment of the cell to its surroundings, including other cells (McFarland, 1969), and appears to be associated with activation and motility of lymphocytes. There is a concentration of organelles and cytofibrils in the uropod in contrast to the advancing pseudopod. The uropod can be formed and retracted in minutes. Rosenstreich, Shevach, Green and Rosenthal (1972) found that all the guinea pig lymphocytes that formed uropods in tissue culture were T cells. Schreiner and Unanue (1975a) observed uropod formation and spontaneous movement in the occasional murine B cell. The actual mechanism of motility in eukaryotic cells is not well understood but could possibly be based on contractile events involving actin and myosin similar to those occurring in muscle cells (Korn, 1978).

1.4.2 Stimulated Movement

1.4.2.1 Antibody

Antibody has been shown to stimulate normal Ig-bearing lymphocytes to move (Unanue et al, 1974). Stimulation was transient under the conditions *in vitro*, lasting 15 - 25 minutes and it required bivalent antibodies and elevated temperature ($37^{o}C$). The cap was seen first, then the uropod formed under it (Schreiner and Unanue, 1976b). Stimulation of motility requires cell attachment to a solid substrate and this made it possible for Unanue et al (1974) to dissociate capping and stimulation of movement one from the other. When the cells were in suspension and unable to attach to a solid substrate antibody induced capping even though there was no subsequent motility. Drugs were also used to demonstrate the independence of capping from motility. Colchicine enhanced antibody-induced motility but did not affect capping whereas low doses of cytochalasin B inhibited motility without having a significant effect on capping (Unanue et al, 1974).

Both Singer's group (Bourguignon and Singer, 1977) and Schreiner et al (1977) pointed out that if the asymmetrical myosin distribution found in motile cells is essential to motility, the effect of Ig capping could be to produce a similar pattern in B cells.

Evidence for or against stimulation by antibody of directed movement comes from work with modified Boyden-type chambers. Schreiner and Unanue (1975b) reported that antibody stimulated murine lymphocytes to move through filters in a random fashion. In later work, Ward, Unanue, Goralnick and Schreiner (1977) claimed that there was true directional motility of rat B cells in a chemotactic response to very low concentrations of antibody diffusing towards them from a distal chamber.

1.4.2.2 Molecular specificity

Antibodies to some cell surface molecules including Ig, FcγR receptors and TL antigen induced a motile response (Schreiner and Unanue, 1975b; Braun et al, 1978a). The same workers found that anti-H2, anti-Thy 1 and rabbit anti-mouse lymphocyte antibody (ALS) would not produce stimulation of motility even after capping with a second antibody. In fact ALS has been reported to inhibit motility of lymphocytes (Biberfield, Holm and Perlmann, 1969; Schreiner and Unanue, 1975b). The distinction between these two groups of surface determinants corresponds to the receptors which interact with microfilaments and those that do not in Schreiner and Unanue's proposed models for capping.

There is some evidence that antigen-antibody complexes will stimulate human lymphocytes to move (Alexander and Henkart, 1976). Increasing cyclic GMP stimulated motility for both B and T cells, whereas agents which increase cyclic AMP inhibited antibody - stimulated movement of B cells and spontaneous movement of T lymphocytes (Schreiner and Unanue, 1975a; Unanue and Schreiner, 1975). Colchicine abrogated the effect of cyclic AMP suggesting that microtubule stabilization may have impeded lymphocyte movement (Unanue and Schreiner, 1975).

1.4.3 Migration inhibition factor (MIF)

A soluble cell-free substance which is capable of inhibiting normal macrophage migration is produced by sensitized T lymphocytes in response to antigen. This phenomenon was first reported by David and by Bloom and Bennett independently in 1966. It was later shown that B cells as well as T cells could produce migration inhibition factor (MIF), although there is some disagreement about the conditions required (Yoshida, Sonozaki and Cohen, 1973; Bloom, Stoner, Gaffney, Shevach and Green, 1975).

Yoshida et al (1973) found that non-immune B cells identified on the basis of complement receptors would produce MIF in response

to PPD, which can act as either mitogen or antigen, as efficiently as immune B cells. A soluble factor from normal T cells stimulated with PPD was capable of suppressing this B cell activity (Cohen and Yoshida, 1977). In contrast, Bloom et al (1975) found that T cell-depleted lymphocyte populations from non-immune guinea pigs did not produce MIF in response to PPD. From experiments with different strains of guinea pigs, Bloom and Shevach (1975) concluded that primed B lymphocytes were incapable of producing MIF in the absence of competent T cells, suggesting a helper rather than a suppressor role for T cells.

Rocklin, MacDermott, Chess, Schlossman and David (1974) found that immune human B cells stimulated with specific antigen produced MIF. They reported that the B cell preparation was highly purified after elution from a rabbit anti-human Fab affinity column. However, there is no means yet of separating B and T cells which ensures a completely pure population of each.

Human lymphoid cell lines of B cell origin and non-lymphoid cell lines were able to produce a factor which inhibited the migration of guinea pig macrophages and cultured lymphoid cells (Papageorgiou, Henley and Glade, 1972). Tubergen, Feldman, Pollock and Lerner (1972) correlated the release of MIF by lymphoid and fibroblast cell lines with the activation of cells to enter the mitotic cycle. The ability to produce MIF was found to be independent of the B or T cell origin of cell lines by Yoshida et al (Yoshida, Kuratsuji, Takada, Takada, Minowada and Cohen, 1976).

The actual nature of MIF has only been crudely defined. It is non-dialysable and stable to heating 56^{o}C for 30 minutes (Rocklin, Remold and David, 1972). The molecular weight has been estimated at 45,000 for guinea pig MIF (Yoshida et al, 1973) and 23,000 for human MIF (Rocklin et al, 1974). Differences between antigen-induced MIF and mitogen-induced MIF have been reported

by Remold, David and David (1972). Rocklin (1974) makes a distinction between a macrophage MIF and a leukocyte MIF. Supernatants from human sensitized lymphocytes and antigen could be separated into fractions which inhibit human polymorphonuclear leukocytes but not human monocytes or guinea pig macrophages, and fractions with the converse inhibitory properties. Other factors with various biological activities are also released under the conditions which produce MIF. None of these factors have been molecularly identified and have been collectively labelled "lymphokines" (Dumonde, Wolstencroft, Panayi, Matthew, Morley and Howson, 1969). The action of guinea pig lymphokines could be inhibited with some degree of specificity by antibodies raised against purified lymphokine fractions (Geczy, Friedrich and de Weck, 1975; Geczy, Geczy and de Weck, 1976; Yoshida, Bigazzi and Cohen, 1975).

1.4.4 Tumour cell migration

Neoplastic lymphoid cells, both animal and human, are actively motile in tissue culture. Cochran (1971) used the capillary tube technique (George and Vaughan, 1964; David, Al-Askari, Lawrence and Thomas, 1964) to describe the migration of tumour cells from a Moloney virus-induced lymphoma (YAC). He included results for the migration of other murine and human tumours including CLL. Migration was shown to be an active process requiring viable cells and metabolic energy.

The migration of tumour cells can be inhibited by antisera (Cochran, Klein and Keissling, 1972; Currie and Sime, 1973). Cochran et al (1972) showed that migration of YAC cells *in vitro* was inhibited by antisera to H2 and Moloney virus-related antigens. The degree of inhibition correlated with activity of the antisera in complement-dependent cytotoxic tests. The effect did not require complement to be added to the system and the active component could be specifically absorbed from the sera. Inhibition could be achieved with a pulse

of anti-H2 at concentrations which produced no detectable agglutination of cells. Currie and Sime (1973) found that syngeneic immune serum specifically impeded migration of tumour cells. They raised the antiserum by injecting irradiated neoplastic SL2 cells into DBA2 mice. The immune serum inhibited migration of SL2 cells but not that of normal spleen cells nor of a non-cross-reacting lymphoma. The serum inhibitor could bind to the target cells since a pulse exposure followed by washing still produced inhibition. Further evidence for the serum inhibitor being antibody was that the effective component had a molecular weight between 100,000 and 300,000. Stevenson and Stevenson (1975) demonstrated that the IgG fraction from sheep anti-idiotype serum inhibited the migration of the L_2C leukaemic cells from strain 2 guinea pigs.

The migration of tumour cells is sensitive not only to antibody. Friberg, Cochran and Golub (1971) reported that Con A in sub-agglutinating amounts inhibited the migration of murine ascitic tumour cells. The inhibition could be abolished by the addition of α-methyl-D-mannoside which inhibits attachment of Con A to the cell surface.

1.5 Some Biological Effects of Antibody

1.5.1 DNA synthesis

The ability of antibody to deliver a mitogenic signal has been widely studied. After an initial observation by Sell and Gell (1965) that antibody stimulated 3H-thymidine uptake in rabbit peripheral blood lymphocytes, variable results have been obtained by workers looking for the same phenomenon in different systems. Induction of DNA synthesis by antibody has been reported in other species (reviewed Warner, 1974) including man (Greaves, 1970; Frøland and Natvig, 1970; Gausset, Delespesse, Hubert, Kennes and Govaerts, 1976) and more recently mice (Weiner, Moorhead and Claman, 1976a; Sieckmann, Asofsky, Mosier, Zitron and Paul, 1978a;

Sidman and Unanue, 1978a). Frøland and Natvig (1970) found an increase in DNA synthesis in human peripheral blood lymphocytes in response to rabbit antisera to human $(Fab'\gamma)_2$ which would be polyspecific for the different Ig classes. Antibodies specific for μ, γ or α chains stimulated DNA synthesis in human lymphocytes from blood, tonsils, spleen and lymph nodes (Gausset et al, 1976). There has also been a report of stimulation of DNA synthesis in human peripheral blood lymphocytes by anti-δ (Kermani-Arab, Leslie and Burger, 1977). It has been claimed that specific activation of the μ chain of surface IgM is required to produce stimulation of DNA synthesis in mice (Weiner, Moorhead, Yamaga and Kubo, 1976b; Sidman and Unanue, 1978a). However, Sieckmann et al (1978a) found that antibody acting via the light chain was effective albeit to a lesser extent than anti-μ. Both groups (Sidman and Unanue, 1978a; Sieckmann et al, 1978a) only observed this proliferation in the presence of 2-mercaptoethanol which according to Sidman and Unanue activated a cofactor in serum.

The behaviour of immature cells differed from mature cell populations in that antibody did not stimulate DNA synthesis from young mice (Weiner et al, 1976a; Sidman and Unanue, 1978a,b). Sieckmann et al (1978b) who did observe stimulation by antibody in young mice correlated the effect with the development of a mature subset of B cells. Scribner, Weiner and Moorhead (1978) found that cells from young mice would respond to $(Fab'\gamma)_2$ from antibody which led them to suggest a regulatory role for the Fc piece which diminished with age. In experiments with various subpopulations of murine B cells Sidman and Unanue (1978b) found that adult B cells, with IgD-bearing cells removed, proliferated in response to anti-IgM whereas neonatal cells with IgM as the only surface Ig were inhibited. Simply the interaction of antibody with one particular receptor did not determine the response of the B cell. This is in contrast with claims that IgM and IgD convey different signals to the same cell, a postulate advocated by Kettman, Cambier, Uhr, Ligler and Vitetta (1979). Their

hypothesis is supported by experimental evidence in which removal of IgD from adult splenocytes resulted in increased susceptibility to tolerance induction. They suggest that it is the presence of IgD on the cell surface which prevents IgM in interacting with antibody from conveying an inhibitory signal to the cell. IgD appears later than IgM on splenocytes of neonatal mice at a time when mice develop a markedly increased immune responsiveness.

The homogeneity of the target cell population is one of the many variable influential factors in the systems mentioned. Gausset et al (1976) found that their human purified B cell populations obtained by rosetting with neuraminidase-treated sheep red blood cells did not respond to antibody. The addition of T cells enabled antibody to stimulate the cells. In contrast, Sieckmann, Scher, Asofsky, Mosier and Paul (1978b) in studies with murine B cells depleted of T cells by various means claim that T cell help is not necessary for stimulation of DNA synthesis by antibody. This latter group also found that depletion of macrophages did not diminish the antibody responses. However, a requirement for macrophage was demonstrated by Mongini, Friedman and Wortis (1978) for stimulation of murine cells by antibody.

No reports are to hand on the effect of antibody to Ig on the proliferative behaviour of leukaemic B lymphocytes bearing surface Ig. Low doses of antibodies directed to other cell surface antigens have been reported to stimulate nucleic acid turnover in a variety of tumour cell lines, including some of lymphoid origin (Shearer, Philpott and Parker, 1973; Shearer and Parker, 1978).

1.5.2 Differentiation

A requirement for cell collaboration is seen in the effects of antibody on differentiation. From a body of confusing, contradictory data to which he alluded in his editorial Möller (1978) drew one positive conclusion: anti-Ig sera by themselves do not induce Ig synthesis in pure populations of B cells. He

drew particularly on the work of Kishimoto and Ishizaka (1975) who showed that when spleen cells from rabbits immunized against DNP proteins were treated with antibody, washed and cultured with supernatants from cultures of antigen and presumably activated T cells, they would produce anti-DNP antibodies. Only with both treatments would they differentiate. Antibody can have a suppressive effect on mitogen-induced differentiation of B cells to antibody-secreting cells (Finkelman and Lipsky, 1978; Sidman and Unanue, 1978a). The latter group along with others (Andersson, Bullock and Melchers, 1974) also reported a suppression of mitogen-induced mitosis by antibody.

1.6 Immediate Biochemical Effects of Antibody

The immediate biochemical effects of antibody on lymphoid cells have received less attention. Nishizawa, Kishimoto, Kikutani and Yamamura (1977) demonstrated *in vitro*, increased phosphorylation of non-histone nuclear proteins in nuclei from rabbit lymphocytes stimulated with antibody to Ig. A cytoplasmic factor was maximally induced two hours after antibody stimulation and appeared to activate a non-histone protein protein kinase in quiescent nuclei. A transient rise in cyclic AMP which can affect enzyme activity was caused by antibody binding to surface Ig on L_2C leukaemic cells (Virji and Stevenson, 1979).

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Buffers

Buffers and solutions were prepared using glass distilled de-ionized water and "Analar" grade biochemicals. Chemicals unless stated otherwise were obtained from British Drug Houses, Chemicals Ltd., Dorset, U.K.

0.1M NaCl-0.02M Tris-HCl-0.001M EDTA-0.002% NaN_3 (Tris-NaCl-azide), pH8.0

	g/l
NaCl	5.84
Tris-HCl	2.42
Disodium EDTA. $2H_2O$ (EDTA)	0.372
NaN_3	0.02
1M HCl	10 ml

0.5M NaCl-0.1M Tris-HCl-0.05M EDTA-0.01% NaN_3 (high molarity Tris-NaCl-azide), pH8.0

As for Tris-NaCl-azide x5.

0.2M Tris-HCl-0.01M EDTA (TE8), pH8.0

	g/l
Tris-HCl	24.20
EDTA	3.72
5M HCl	20 ml

0.1M Tris-HCl 0.2M NaCl, pH8.0

	g/l
Tris-HCl	12.11
NaCl	11.69
5M HCl	10 ml

Phosphate buffered saline (PBS), pH7.3

	g/l
NaCl	7.01
KH_2PO_4	0.79
Na_2HPO_4	3.44

0.03M Phosphate-0.001M EDTA, pH7.3

	g/l
Na_2HPO_4	3.20
KH_2PO_4	1.02
EDTA	0.37

0.01M Phosphate buffer, pH8.0

	g/l
KH_2PO_4	0.105
Na_2HPO_4	1.31

0.4M Phosphate buffer, pH8.0

0.4M KH_2PO_4 (5.44 g/100 ml) was added to
0.4M Na_2HPO_4 (56.78 g/1,000 ml) until pH8.0 was reached.

0.07M Sodium acetate 0.05M NaCl, pH4.4

	g/l
NaCl	2.93
$NaCH_3COO$	5.74

2.2 Proteins

2.2.1 IgG from serum

IgG was prepared from rabbit, guinea pig and sheep sera by the method of Stevenson and Dorrington (1970). 60 ml of saturated ammonium sulphate in 0.2M Tris-HCl-0.01M EDTA, pH8.0 were added to each 100 ml of serum. After stirring for 15 minutes at room temperature the precipitate was collected by centrifugation at 3,000 g for 45 minutes and resuspended in the original serum volume in 0.2M TE8, pH8.0. The final precipitate was resuspended in 0.03M phosphate buffer, pH7.3 in 0.4 times the original serum volume or for sheep, the original serum volume and dialysed against this buffer. After dialysis, undissolved material was removed by centrifugation and the protein solution applied to a DEAE-cellulose (DE-32 grade; Whatman Biochemicals Ltd., Kent) column equilibrated with the

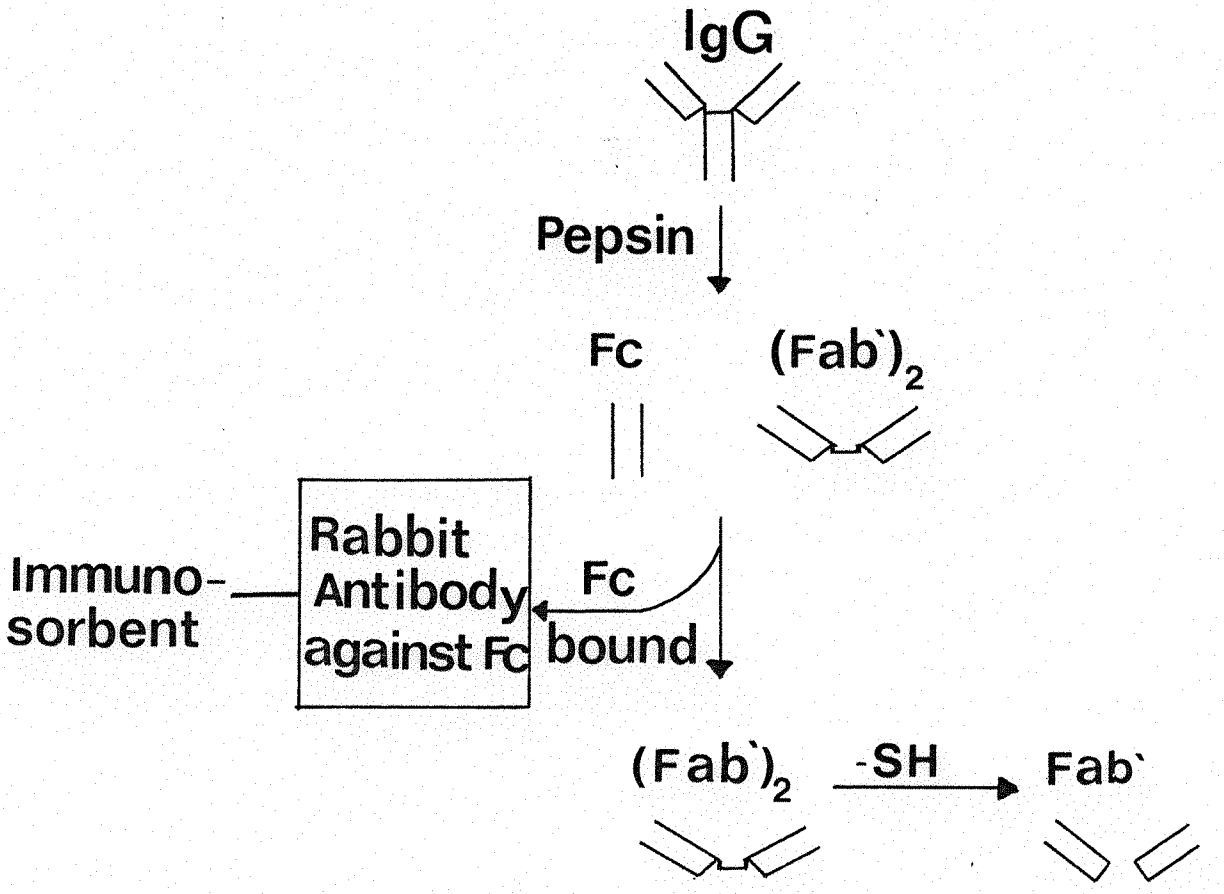


Fig. 2.1 Preparation of $(Fab'\gamma)_2$ and $Fab'\gamma$ fragments from IgG

phosphate buffer. The bed volume of the column was at least 5 times (rabbit and guinea pig) or 10 times (sheep) the original serum volume. Protein in the column effluent was monitored by absorption at 254 or 280 nm with a Uvicord (LKB Instruments Ltd., Surrey). IgG was collected and concentrated by ultrafiltration under nitrogen pressure using Diaflo membranes (PM10) in Amicon chambers (Amicon Ltd., Bucks.). Alternatively, concentration was carried out by pressure dialysis in 8/32" Visking tubing (Scientific Instrument Centre Ltd., London). To obtain a monodispersed solution IgG was chromatographed on Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden) and 7S IgG collected.

2.2.2 IgG_1 from serum

To prepare IgG_1 from serum 20 ml aliquots of serum were dialysed into 0.1M Tris 0.2M NaCl, pH8.0 and loaded onto an ACA-34 (LKB) column. The 7S peak was concentrated to the original serum volume and passed through a Sephadex G-150 column. After dialysis into 0.01M phosphate buffer, pH8.0 the protein was applied to a DEAE-cellulose column equilibrated in the same buffer. IgG_2 was unretarded and IgG_1 eluted with a gradient to 0.4M phosphate, pH8.0.

2.2.3 Fragments from IgG

The preparation of $(Fab'\gamma)_2$ and $Fab'\gamma$ is shown schematically in Fig. 2.1. IgG (or IgG_1) at 10 - 20 mg/ml in 0.07M sodium acetate 0.05M NaCl, pH4.4 was digested at 37°C for 18 hours by pepsin (Sigma Chemical Co., London) at 0.1 mg/ml. The digest was passed through a column of Sephadex G-100 or G-150 equilibrated with 0.2M Tris-HCl, pH8.0 to separate $(Fab'\gamma)_2$ and any undigested IgG from pepsin and small proteolytic fragments.

Residual IgG was removed from $(Fab'\gamma)_2$ by immunosorption (see Section 2.3) with rabbit anti-sheep $Fc\gamma$. This immuno-

sorbent was prepared with rabbit anti-sheep IgG previously absorbed with sheep Fab'γ leaving antibody activity to sheep IgG and Fcγ. If necessary $(Fab'\gamma)_2$ was recycled through the immunosorbent to obtain an uncontaminated preparation by the Ouchterlony precipitin test.

To prepare the Fab'γ monomer, $(Fab'\gamma)_2$ from the Sephadex column was reduced with 0.01M dithiothreitol (Calbiochem Ltd., Calif., U.S.A.) for 30 minutes and alkylated by the addition of iodoacetamide (recrystallized from ethanol) to 0.022M for 10 minutes at room temperature. Undigested IgG was removed using Sephadex G-100 and Fab'γ was shown by Ouchterlony analysis to be free of IgG.

2.3 Immunosorption

Antigen-linked Sepharose 4B (Pharmacia) was used to remove irrelevant antibodies from antisera and in the preparation of purified antibodies by specifically binding the required species which was subsequently eluted. Sepharose 4B linked to IgG from antisera could remove specific antigens from heterogeneous protein solutions.

Proteins were coupled to Sepharose 4B by the cyanogen bromide method of Axen, Porath and Ernback (1967). Before each definitive absorption, immunosorbent columns were washed with half a column volume of 0.5M NH_4OH and re-equilibrated with Tris-NaCl-azide, pH8.0. The procedure was modified during the course of this project and the molarity of this buffer was increased by a factor of 5. Serum (0.33 column volume) to be absorbed was applied to immunosorbent columns either directly or after ammonium sulphate fractionation. If the bound species was required it was eluted with 0.5M NH_4OH and collected on ice. It was immediately dialysed into cold high molarity Tris-NaCl-azide, pH8.0 and then into the more dilute buffer before removal of immune complexes on a ACA-34 (LKB) column.

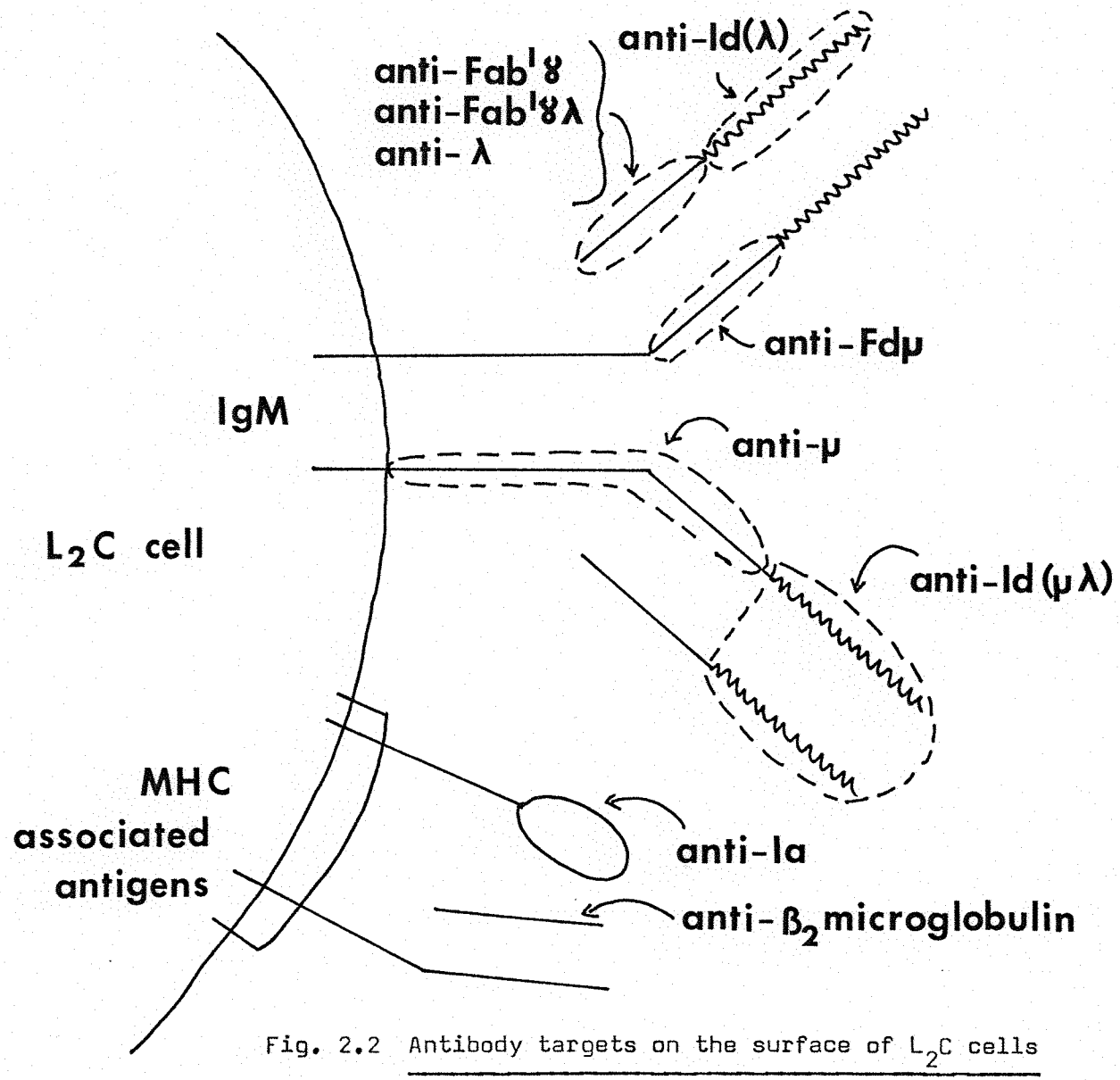


Fig. 2.2 Antibody targets on the surface of L_2C cells

MHC - major histocompatibility complex

TABLE 2.1

ANTISERA AGAINST GUINEA PIG ANTIGENS

Species immunized	Antigen	Antiserum absorbed with	Purified by elution from	Notation anti-	Reference
Sheep	Fab$^{\gamma}$Kλ from serum IgG		IgG	Fab$^{\gamma}$	Stevenson et al (1975a)
Sheep	Fab$^{\gamma}$λ from Fab$'^{\gamma}$Kλ absorbed with K chains		Normal light chains	Fab$^{\gamma}$λ	Hough et al (1978
Sheep	λ chains from L_2C leukaemic urine		Normal light chains	λ	) Stevenson, Elliott and Stevenson (1977b)
Sheep	λ chains from L_2C leukaemic urine	Normal light chains Normal urinary protein		Id(λ)	) Stevenson, Elliott and Stevenson (1977b)
Sheep	Fabμ from L_2C cell surface			Id(μλ)	Stevenson and Stevenson (1975)
Sheep	Fabμ from serum IgM	IgG	IgM	Fdμ	Hough et al (1978)
Sheep	IgM from serum	IgG			Hough et al (1978)
Sheep	β_2 microglobulin from urine of sodium chromate treated animals		Normal urinary protein	β_2 micro-globulin	Stevenson, Cleeter and Stevenson (1978)
Strain 13 guinea pig	Lymph node and spleen cells from strain 2			Ia	Shevach, Paul and Green (1972)
Rabbit	T cells from lymph nodes of strain 2	L_2C cells		T cell	Shevach, Green, Ellman and Maillard (1972)

TABLE 2.2

ANTISERA AGAINST HUMAN ANTIGENS

Species immunized	Antigen	Antiserum absorbed with	Purified by elution from	Specificity	Notation anti-
Sheep	Fabμ from normal serum IgM	IgG	IgM	Fdμ	Fdμ
Sheep	Fabδ from myeloma IgD	IgG		Fdδ	Fdδ
Sheep	Fab'γ from normal serum IgG		IgG	$\gamma K\lambda$	Fab'γ
Sheep	Fab'γ from normal serum IgG		λ chains	$\gamma\lambda$	Fab'$\gamma(\lambda)$

Antisera were raised using standard methods for immunization (Herbert, 1973).

2.4 Antibodies

Antisera were raised at the Tenovus Research Laboratories. A list of antigens used and the antibodies isolated from antisera is given in Tables 2.1 and 2.2. The antigens on the cell surface to which these antibodies are directed are shown in Figure 2.2. Where antibody was not purified by affinity chromatography, the IgG fraction was prepared as described previously. Normal sera were absorbed with guinea pig IgG or globulins and/or human IgG before use as controls containing no antibody activity. Rabbit anti-sheep IgG and rabbit anti-sheep Fab'γ, were absorbed with guinea pig globulins and purified on a column of Sepharose 4B coupled to sheep IgG. Purity of proteins was checked by Ouchterlony analysis. The specificity of antibody in binding to cells was determined using fluorescein isothiocyanate conjugates. The direct technique for immunofluorescent staining of the surface of L_2C cells has been described by Stevenson et al (1975a) and the adaptation for the indirect technique by Hough et al (1978).

In this thesis when the term antibody is used without further qualification it refers to antibody directed to a defined region of the immunoglobulin molecule.

2.5 Cellular Methods

2.5.1 Sterility

For use in cellular work all protein solutions were dialysed into PBS, pH7.3 and filtered (0.22 µm, Millipore Corporation, Mass., U.S.A.). All media was initially sterile and other solutions were filtered (0.8 µm, Millipore filter). Semi-sterile procedures were adequate for short term cultures up to 24 hours. Preparation of cells and other cellular techniques were performed near to a bunsen flame on the open bench. As an extra precaution a laminar flow cabinet was often used. All glassware

was thoroughly cleaned.

2.5.2 Media

Eagle's minimal essential medium with Earle's salts in 0.02M HEPES buffer (MEM), non-essential amino acids (NEAA), L-glutamine (GLN) and foetal calf serum (FCS) were obtained from Flow Laboratories Ltd., Ayrshire, Scotland. FCS was decomplemented at 56°C for 30 minutes before use. "Crystamycin" containing penicillin (100,000 units/ml) and streptomycin (100,000 µg/ml) was from British Drug Houses, Chemicals Ltd., Dorset, U.K.

MEM-NEAA

MEM containing NEAA, 1%.

Culture medium

MEM containing: NEAA, 1%
GLN, 2 mM
penicillin, 100 units/ml
streptomycin, 100 µg/ml
FCS, 10%

Leucine-free medium (Gibco Bio-Cult, Glasgow, Scotland) was supplemented similarly to give leucine-free culture medium.

Serum-free culture medium

MEM containing: NEAA, 1%
GLN, 2 mM
penicillin, 100 units/ml
streptomycin, 100 µg/ml

Low ionic strength medium

Von Boehmer and Shortman (1973) described the composition of this medium. Essentially it consisted of a pH7.2 buffered balanced salt solution made iso-osmolar by the addition of sorbitol (0.2M) and glucose (0.025M).

2.5.3 Preparation of cells

2.5.3.1 L_2C

Tenovus was provided with the L_2C leukaemia of strain 2 guinea pigs in 1972 by Dr. E. Shevach of the National Institutes of Health, Washington, U.S.A. In the Tenovus colony, the tumour has a doubling time of about 21 hours. Intraperitoneal inoculations of 5×10^6 cells into an 800 g animal results in death about 12 days later with a white cell count of about 300,000/µl blood.

Leukaemic cells were obtained from near-terminal animals exsanguinated by cardiac puncture under ether anaesthesia. The blood was collected into 0.125 volumes of 3.5% sodium citrate, pH7.4 and the cells separated and washed by a modification of the methods described by Eady, Hough, Kilshaw and Stevenson (1974). The red cells were separated by sedimentation with 0.33 volume of 5% Dextran T500 (Pharmacia) in PBS, pH7.3 in a measuring cylinder for 30 minutes at 37°C in a water bath. White cells from the plasma layer were collected and spun at 1,000 g for 15 minutes. The cells were resuspended in PBS, pH7.3 and subjected to one of the following washing procedures:

(1) An equal volume of 5% bovine serum albumin (BSA) (Armour Pharmaceutical Co. Ltd., Sussex) in PBS, pH7.3 was introduced beneath the cell suspension. The cells were sedimented through the gradient at 1,000 g for 10 - 15 minutes and the procedure repeated. The cells were washed once more in PBS, pH7.3 and suspended in MEM-NEAA at 37°C for 15 minutes to remove the last remnants of bound plasma IgG (Eady et al, 1974). The cells were sedimented at room temperature and washed in the cold with MEM-NEAA.

(2) If a cell preparation completely free of red cells was required, cells resuspended in PBS, pH7.3 were spun at the lowest possible speed (approximately 300 g) on a MSE bench centrifuge. The majority of L_2C cells sedimented after 10 minutes at this speed leaving red cells in the supernatant. This

washing procedure was repeated 4 - 6 times, ie. until the pellet was red-cell free. Cells were resuspended in MEM-NEAA for a 15 minutes incubation at 37°C, sedimented and washed as in (1). Viabilities of cells washed either way were regularly ≥ 99%.

2.5.3.2 Normal guinea pig lymphocytes

Strain 2 or strain 13 guinea pigs weighing approximately 400 g each were used. The cervical, inguinal and mesenteric nodes were dissected out, rinsed in MEM and placed in MEM on ice. Nodes were gently teased and pushed through a mesh tea-strainer with the plunger of a plastic syringe. The cells were sucked into a syringe using a large serum needle and filtered through glass wool soaked in MEM. The resulting single cell suspension was subjected to different procedures subsequently:

(1) This method was based on the observation (Von Boehmer and Shortman, 1973) that in media of low ionic strength, damaged cells aggregate and adhere to the surface to a much greater extent than do viable cells. The cell pellet was resuspended continuously in low ionic strength medium (10 ml for approximately 8×10^8 cells pooled from 2 animals) for a few minutes until the inside of the plastic pipette (Nunc, Denmark) become coated with coagulated dead cells. The suspension was quickly filtered through MEM-soaked glass wool and diluted with MEM. Cells were washed 3 times in MEM. The viability was regularly increased from approximately 60% to over 90% after treatment with low ionic strength medium but recovery (50%) was low in terms of viable cells.

(2) Cells pooled from 2 animals were resuspended in 2 ml MEM and layered onto 5 ml Ficoll-Paque (Pharmacia). After centrifugation at 1,500 g for 7 minutes at room temperature, the interface cells were washed and resuspended in MEM-NEAA before incubation with rabbit anti-T cell plus fresh

rabbit complement at 37°C for 45 minutes. The treated cells were washed in MEM and again layered onto 2 ml Ficoll-Paque to remove cells killed by antibody and complement. This technique was being developed in our laboratory by F.K. Stevenson and A. Pindar during the course of this project and the proportion of non B cells in the cell preparations was high. Normal lymph node cells generally contain 15 - 20% B cells and these preparations were estimated by immunofluorescence to contain 60% B cells. The T cell-depleted lymph node cells were ≥ 90% viable after treatment with Ficoll-Paque.

2.5.3.3 CLL

CLL cells were prepared from blood by 2 methods:

(1) Dextran sedimentation followed by washing on BSA gradients as for L_2C cells with an extended incubation time of 30 minutes at 37°C.

(2) An aliquot of blood was layered onto Ficoll-Paque (2:1 v/v and spun at 2,500 g for 20 minutes at room temperature. The cells at the interface were pipetted out and diluted by at least 50% with PBS, pH7.3 and spun at 1,000 g for 5 - 10 minutes. They were then washed twice in PBS, pH7.3 and once in MEM-NEAA. The cells were incubated for 30 minutes at 37°C, spun and washed once more. Viabilities of cells washed either way were regularly ≥ 99%.

2.5.3.4 Viability

Cell viability was tested by ability to exclude trypan blue. The cell suspension was mixed with an equal volume of 0.5% trypan blue in saline (Flow Laboratories). After exposure to the dye for 1 - 2 minutes, the cells were put into a Neubauer counting chamber (Arnold R. Horwell Ltd., London) and scored for staining.

2.5.3.5 Cell concentration

Cells were counted using a Coulter Counter X1B (Coulter Electronics Ltd., Beds.). 50 µl or 5 µl cell suspension were added to 25 ml "Isoton" (Coulter Electronics) and 6 drops of "Zaponin" (Coulter Electronics) were added to lyse contaminating red cells which are more readily susceptible to "Zaponin" than white cells.

CHAPTER 3
MIGRATION OF CELLS ON PLASTIC SURFACES

3.1 Introduction

L_2C cells actively migrate *in vitro* and the extent of migration can be quantitated by the capillary tube migration technique. The spontaneous migration can be inhibited by antibody (Stevenson and Stevenson, 1975) and in the current project a more detailed study of this effect was carried out. The importance of cross-linking surface Ig was investigated with $(Fab'\gamma)_2$ and $Fab'\gamma$ from antibody. Some comparison was made with migration of L_2C cells and of normal guinea pig lymphocytes

In a consideration of the mechanism of migration inhibition, soluble factors released by L_2C cells were sought. Normal guinea pig lymphocytes which are capable of producing MIF can be stimulated to do so with the mitogen, Con A (Pick and Kotkes, 1977). This made it possible to use a conventional MIF as a positive control with which to compare the properties of L_2C cells.

As movement of cells involves the cytoskeleton of micro-tubules and microfilaments some demonstration of the role of the cytoskeleton in L_2C cell migration was attempted.

3.2 Materials and Methods

3.2.1 Capillary tube migration

3.2.1.1 L_2C cells

Since red cells tended to interfere with the assay, cell suspensions free of red blood cells were prepared as described in 2.5.3.1. Washed cells were suspended at 5×10^7 cells/ml in MEM-NEAA to give the appropriate sized cell pellet in capillary tubes (Hughes, 1972). Micro-haematocrit capillary tubes, 100 µl capacity (Hawkesley, Sussex) were filled with 80 µl cell suspension and spun in a haematocrit head on a MSE bench centrifuge at 1,000 g for 10 minutes at 4^oC. Cell pellets were used within

30 minutes. Each capillary was cut at the cell-medium interface with a Kingcut blade (Jencons Scientific Ltd., Herts.), and the cell-pellet end attached with silicone grease (Edwards, Sussex) to the base of a culture well in a Sterilin S25 plastic plate (Sterilin Ltd., Middlesex). The agent to be tested was made up to 1 ml with culture medium and 0.5 ml doubling dilutions were made in 3 ml plastic tubes (Luckham, Sussex). The tubes containing the dilutions were then warmed to 37°C in a water bath and 0.4 ml of the appropriate solution was added to the migration well immediately after the cell pellet had been anchored. The well was sealed with silicone grease and a glass coverslip. The time between filling capillary tubes and sealing wells was kept to a minimum to ensure optimal migration (Hughes, 1972). Migration plates were left at room temperature until all were prepared and then transferred to a standard laboratory incubator at 37°C with a humid atmosphere.

Migration was significant after 1 hour at 37°C and measurements were usually made after 1.5-2 and 18 hours. To measure migration, the cell fan formed by the migrating cells was projected with a standard photographic enlarger onto graph paper of uniform thickness, the image traced, cut out and weighed. The magnification (5x) was constant throughout all experiments. As migration of L_2C cells was consistent and uniform, duplicate wells for each dilution were adequate. Results are expressed in migration units which represent mg of graph paper.

3.2.1.2 Normal guinea pig lymphocytes

T cell-depleted lymph node cells from normal guinea pigs were prepared as described in 2.5.3.2. Cells were suspended at 7.5×10^7 cells/ml in MEM-NEAA and drawn into capillary tubes. The migration technique was the same as for L_2C cells except that, because the normal cells migrated more slowly, measurements were made after 16 - 18 hours incubation at 37°C.

3.2.1.3 Peritoneal exudate cells (PEC)

Peritoneal exudate cells (PEC) from guinea pigs were used as a source of macrophages for assaying MIF activity. For an experiment, 2 animals (strain 2 or strain 13) were each injected intraperitoneally with 24 ml sterile light liquid paraffin oil as specified in the British Pharmaceutical Codex 1963 (density 0.83 - 0.87 g/ml) warmed to 37°C. 2 - 3 days later the PEC were harvested. The animals were killed by cervical dislocation and a midline incision in the abdominal skin was made. 50 ml of MEM containing 0.5 ml preservative-free heparin (1,000 units/ml, Weddel Pharmaceuticals Ltd., London) were injected through the muscle layer into the peritoneal cavity. An incision was made in the muscle layer and the peritoneal exudate collected with a plastic pipette (Nunc, Denmark) into siliconized (Repelcote, Hopkins and Williams, Essex) 40 ml glass centrifuge tubes. After centrifugation at 1,000 g for 10 minutes the paraffin oil could be removed. The cells were then washed 3 times in MEM. Contamination with red cells was avoided by atraumatic injection using smaller animals, not larger than 450 g. If the cells appeared to clump, the aggregated cells could be removed by filtering the washed cell suspension through glass wool soaked in MEM in a glass funnel.

PEC $\geq$98% viable were used at 3.5×10^7 cells/ml in the migration system which was as described for L_2C cells. PEC migrated more slowly than L_2C cells and migration measurements were made after 16 - 18 hours incubation at 37°C. The effects of different batches of FCS on migration were tested and a batch selected in which PEC migrated well. There was no difference between migration in 10% FCS and in 15% FCS. Although macrophage migration is usually reported to have been performed in medium containing sodium bicarbonate, macrophages migrated well in culture medium containing HEPES buffer. To overcome problems with clumping of macrophages, the cells from the 2 guinea pigs were harvested separately. A migration plate was set up immediately and 2 - 3 hours later there was sufficient migration

to be able to see whether the PEC were migrating satisfactorily and the better population selected for the experiment.

3.2.2 Culture supernatants from L_2C cells

To prepare supernatant for testing in the migration assay, L_2C cells were cultured at 10^7 cells/ml in serum-free culture medium in conical flasks with gentle rotary agitation at 37°C. If antibody or normal sheep IgG was to be added it was present during the entire incubation period. After the culture, usually 16 - 18 hours, cell viability was regularly over 80%. The cells were removed by spinning at 1,000 g for 30 minutes at 4°C. Some aliquots of culture supernatant were tested without concentration or dialysis while others were concentrated and dialysed into PBS or fresh serum-free culture medium. Both pressure dialysis and membrane filtration (Amicon PM10 retaining molecules > 10,000 M.W.) were tried for concentration, the Amicon method being preferred in later experiments since it was quicker. All aliquots were filtered (0.22 µm Millipore filter) before being tested in the migration system. Supernatants were kept at 4°C and tested as soon as possible, always within 2 days, in case a loss of activity was occurring. For migration studies, 10% FCS was added to aliquots and doubling dilutions made in fresh culture medium.

In experiments where antibody was to be removed from the supernatants, a 30 ml column of Sepharose 4B coupled to rabbit anti-sheep IgG was used. The column did not fractionate a 3 ml mixture of ribonuclease (M.W. 13,700) and ovalbumin (M.W. 43,500), the estimated molecular weights of various macrophage MIFs being in this range. The void volume was 20 ml and both proteins eluted in one peak in the next 40 ml. The capacity of the column was determined with normal sheep IgG, 20 mg in 3 ml were applied to the column and 19.7 mg were retained. With a column calibrated in this way 30 ml of supernatant containing 15 mg sheep antibody concentrated to 3 ml and dialysed into PBS were applied to the column equilibrated with PBS at room temperature. The first 40 ml after

the void volume were collected and reconcentrated before being dialysed into fresh serum-free culture medium. The volume collected from the column was increased when unconcentrated supernatants before or after dialysis were applied. There was no IgG in the concentrated column effluent detectable by an Ouchterlony precipitin test.

3.2.3 MIF-containing culture supernatants from normal guinea pig lymphocytes

MIF was generated by pulse exposure of guinea pig lymphocytes to Con A (Pick and Kotkes, 1977). The method was based on the ability of originally non-adherent lymphocytes to attach to the flask surface in the presence of Con A. The Con A-induced lymphocyte "monolayer" could be easily rinsed allowing the removal of free Con A. The Con A-pulsed lymphocytes were cultured for 24 hours in serum-free culture medium and supernatants containing MIF but free of Con A were obtained.

Normal lymph node cells from strain 2 or strain 13 guinea pigs were treated with low ionic strength medium as described in 2.5.3.2 and cultured at 10^7 cells/ml in serum-free culture medium. Cells were cultured in disposable tissue culture flasks (Nunc, Denmark) of 25 cm^2 surface area for 5 ml of cell suspension or 75 cm^2 for 15 ml. Con A (Sigma, London) was diluted from a stock solution in MEM at 5 mg/ml and added to the cells to give a final concentration of 10 µg/ml. The flasks were incubated at 37°C for 2 hours by which time the cells had firmly adhered to the surface and the Con A supernatant poured off. The "monolayer" was then rinsed 5 times with 5 ml (small flask) or 15 ml (large flask) of MEM-NEAA warmed to 37°C. The original amount of fresh serum-free medium was added and flasks incubated at 37°C for 18 - 24 hours. Control flasks were incubated at 4°C. After incubation the supernatants were spun at 1,000 g for 30 minutes at 4°C. The supernatants were then concentrated by membrane filtration (Amicon, PM10) and dialysed into fresh medium. They were then filtered (0.22 µm, Millipore filter) and supplemented

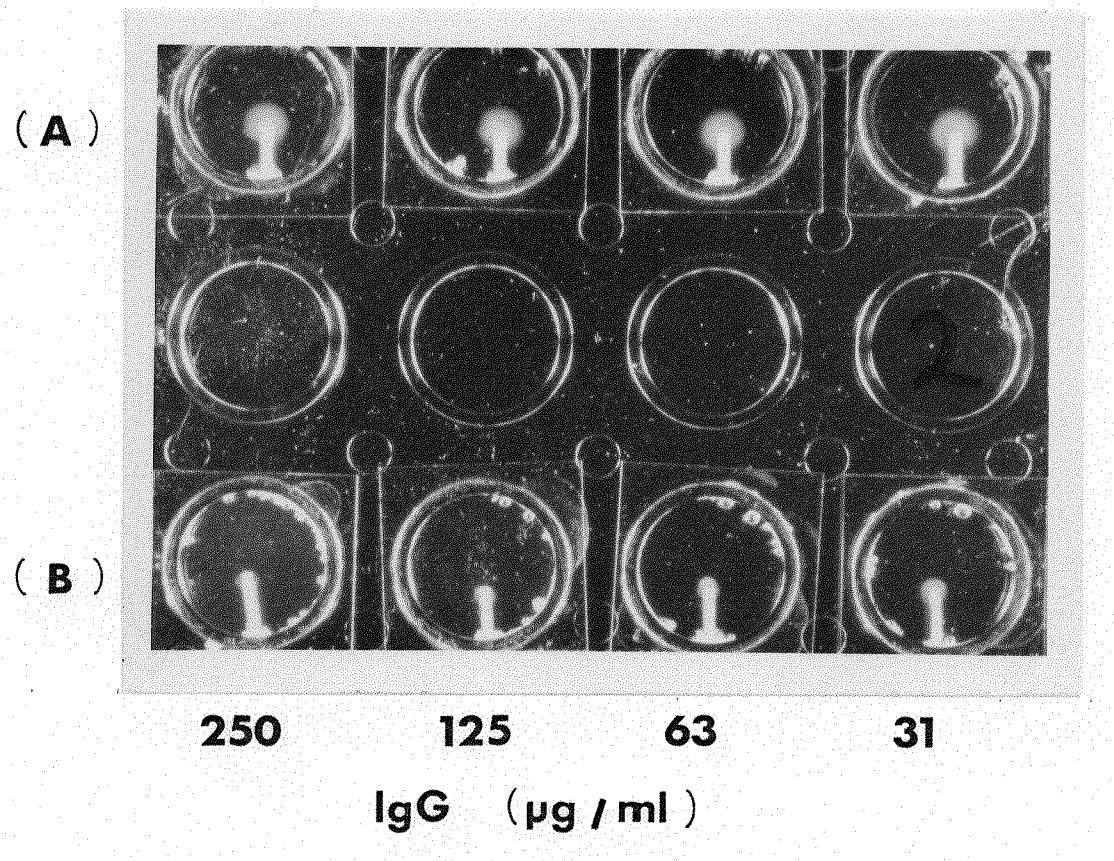


Fig. 3.1 Effect of antibody on migration of L_2C cells

L_2C cells were allowed to migrate into culture medium containing doubling dilutions of anti-Fab'γλ (B), or normal sheep IgG (A). Migration was photographed at 18h.

with 10% FCS before use in the migration assay. The cells were released from flasks with α-methyl-D-mannoside (5 mM) to check viability which was regularly over 90% in all flasks.

3.3 Results

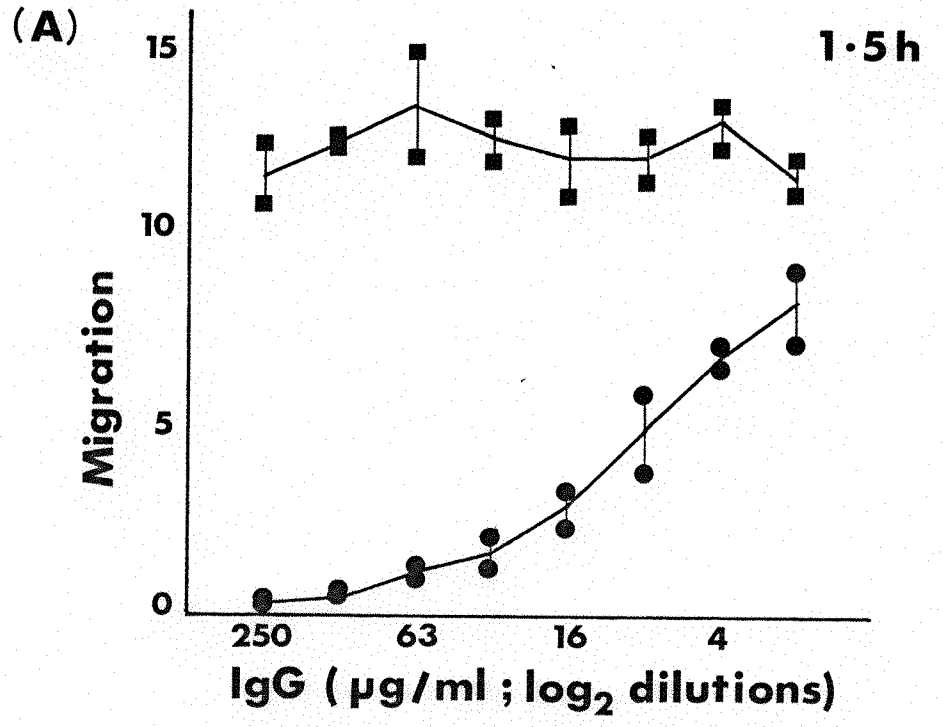
3.3.1 Effects of various reagents on migration of L_2C cells

3.3.1.1 Anti-immunoglobulin

Antibody inhibited the spontaneous migration of L_2C cells out of capillary tubes. An example of a migration plate demonstrating inhibition of L_2C cells by antibody is shown in Figure 3.1. Figure 3.2 displays the results of an experiment in which L_2C cells were allowed to migrate into culture medium containing antibody directed to the constant region of the λ chain, anti-Fab'γλ (Fig. 2.2). The extent of inhibition by antibody was more pronounced at 1.5 hours (A) than at 18 hours (B), a feature regularly observed. Normal sheep IgG had no inhibitory effect on migration and in fact was slightly stimulatory. Anti-λ and anti-Fab'γλ both acting against the same cellular antigenic targets had identical effects on migration. Anti-Id(λ) against the variable region of the light chain produced weaker but similar time and concentration-dependent inhibition (Fig. 3.3). After 18 hours, compared with uninhibited cells (85%) the viability of cells in the presence of anti-Fab'γλ (.25 mg/ml) was lower (61%) probably because the latter were restricted in their use of nutrients in the medium.

3.3.1.2 Fragments of anti-immunoglobulin

Bivalent $(Fab'\gamma)_2$ from antibody retains the ability to inhibit migration as effectively as intact antibody. Monovalent Fab'γ which is unable to cross-link surface Ig had no effect on migration. Fragments were used at 0.6 times the protein concentration of intact IgG so that the same number of Fab binding sites was being


(A)
1·5h
15
10
5
0
Migration
250
63
16
4
IgG (µg/ml ; log2 dilutions)

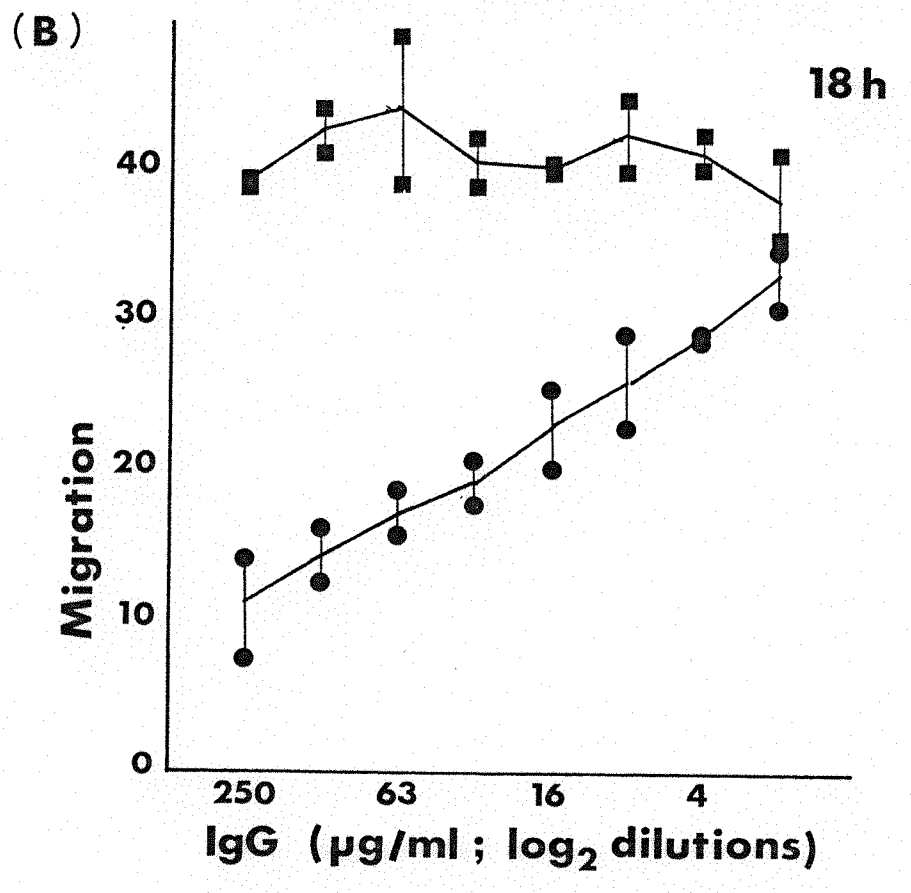
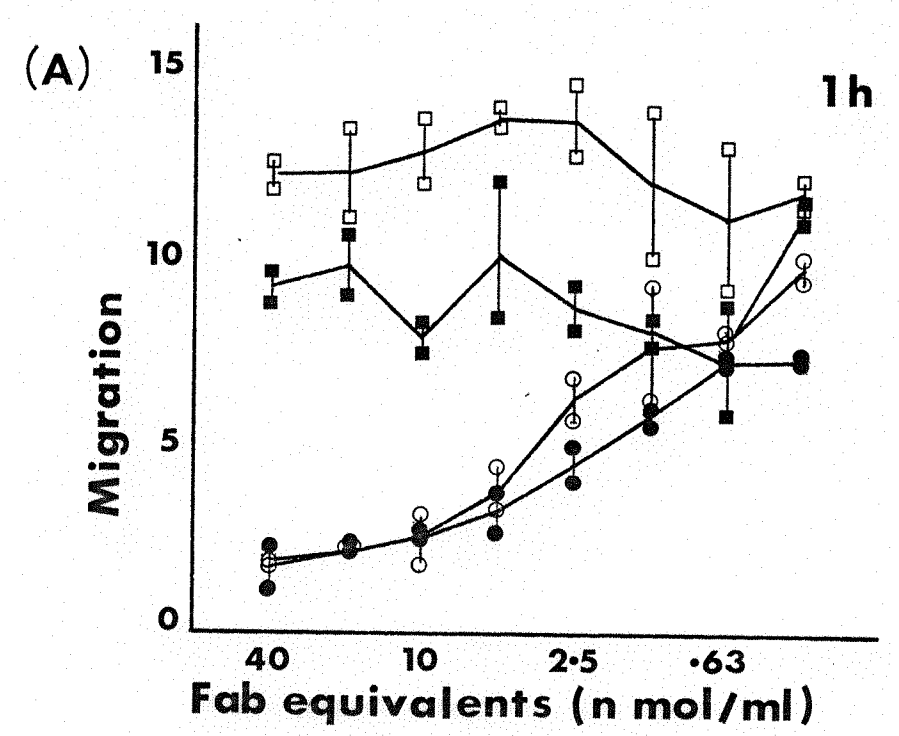

(B)
18h
40
30
20
10
0
Migration
250
63
16
4
IgG (µg/ml ; log2 dilutions)

Fig. 3.2 Effect of antibody on migration of L_2C cells

L_2C cells were allowed to migrate into culture medium containing doubling dilutions of anti-Fab'$\gamma\lambda$ ● or normal sheep IgG ■ . Migration was measured at (A) 1.5h and (B) 18h.


(A)
1h
Migration
15
10
5
0
40
10
2·5
·63
Fab equivalents (n mol/ml)

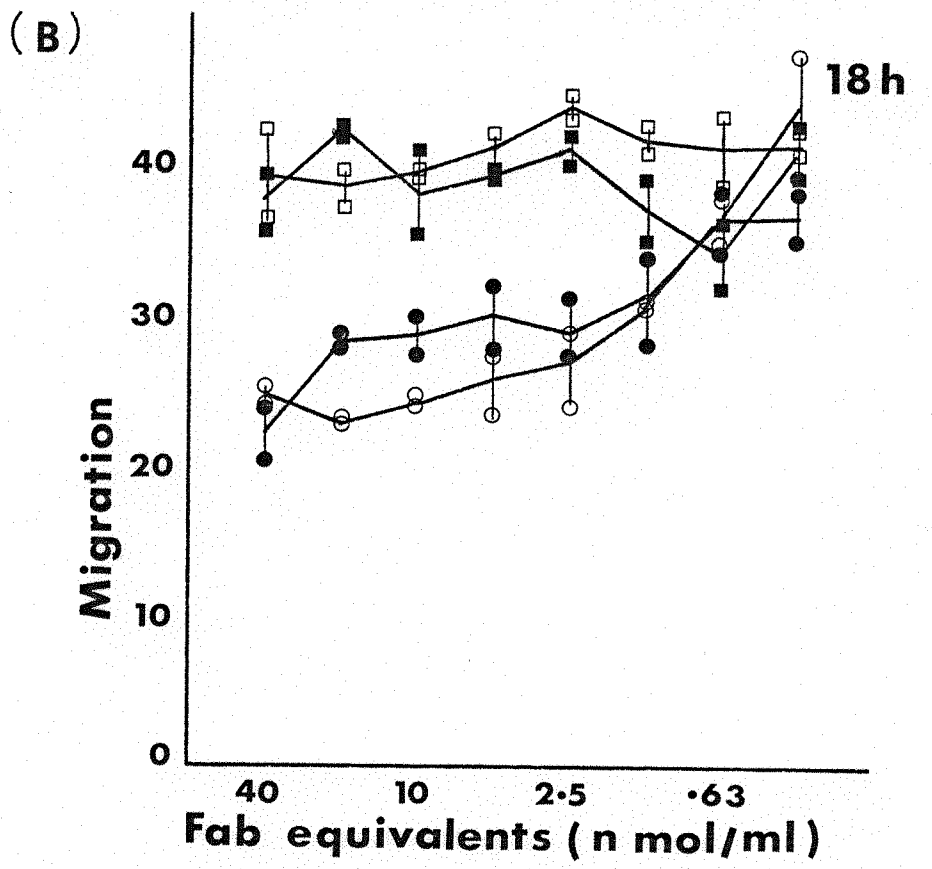
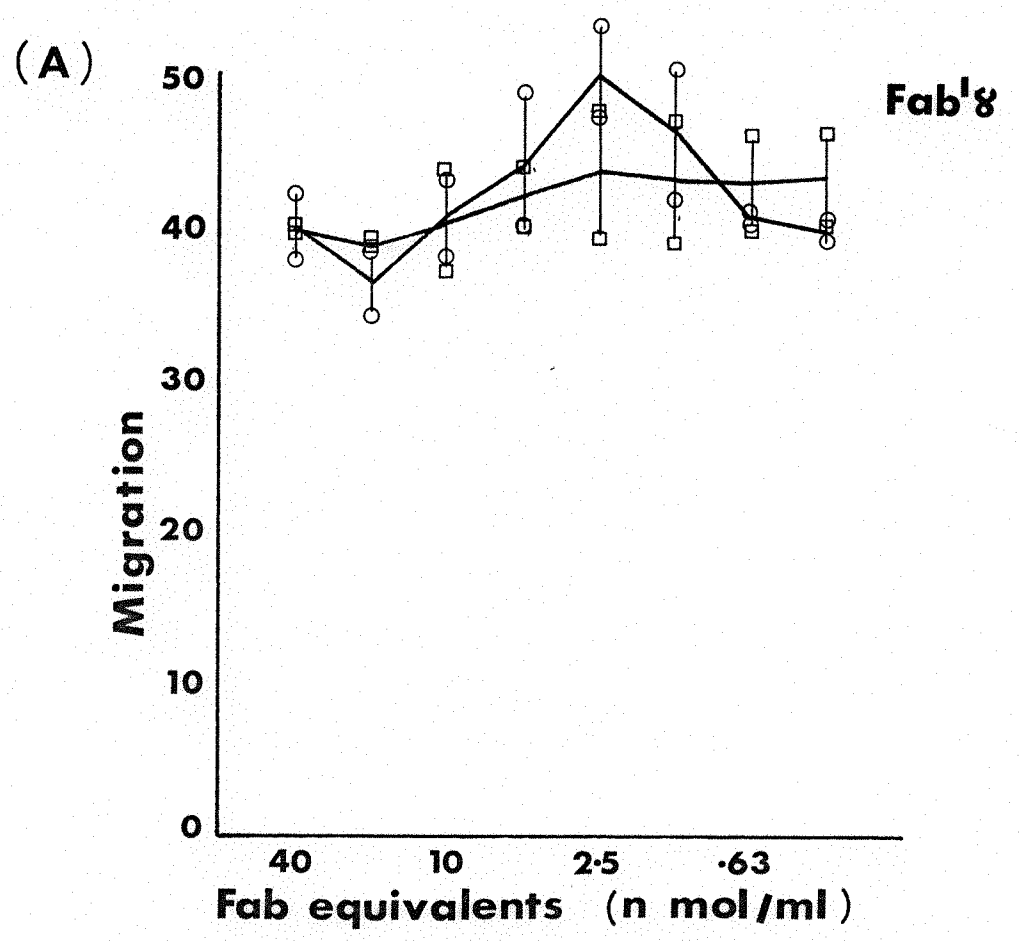

(B)
18 h
Migration
40
30
20
10
0
40
10
2·5
·63
Fab equivalents (n mol/ml)

Fig. 3.3 Effect of $(Fab'\gamma)_2$ from antibody on migration of L_2C cells

L_2C cells were allowed to migrate in culture medium containing doubling dilutions of $(Fab'\gamma)_2$ from anti-Id(λ) o , anti-Id(λ) ● , $(Fab'\gamma)_2$ from normal sheep IgG □ or normal sheep IgG ■ . Migration was measured at (A) 1h and (B) 18h.


(A)
Fab'γ
Migration
50
40
30
20
10
0
40
10
2·5
·63
Fab equivalents (n mol/ml)

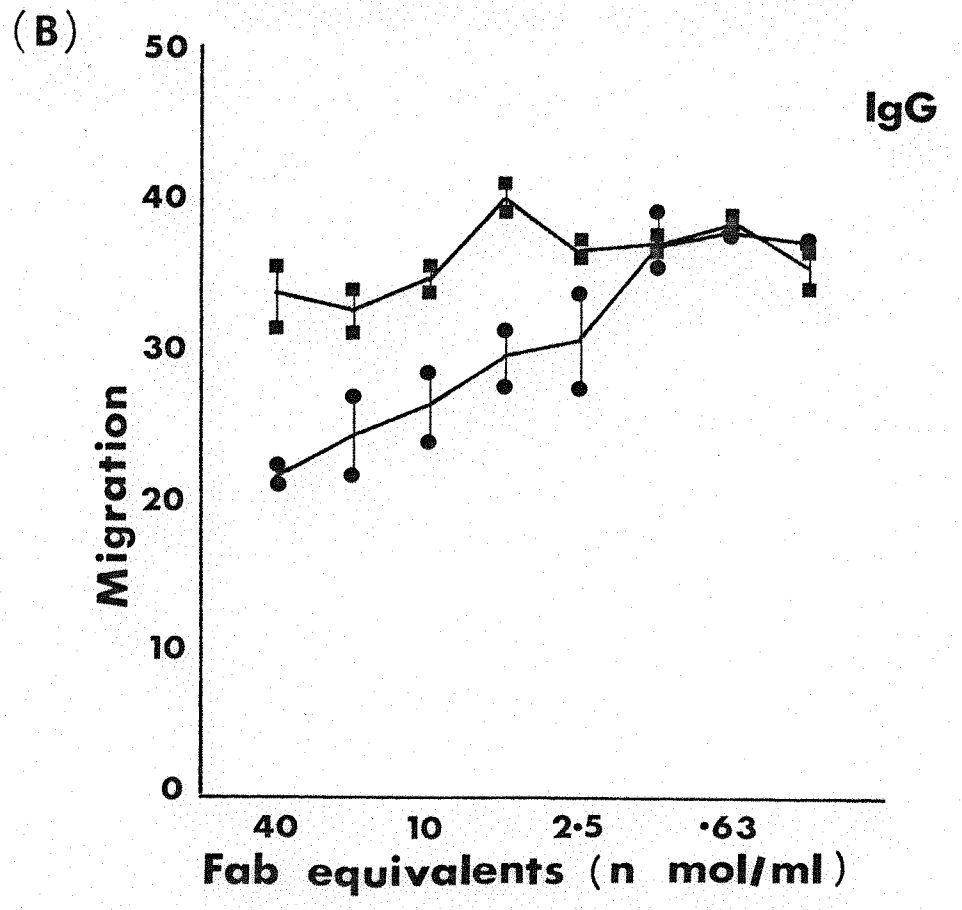

(B)
IgG
Migration
50
40
30
20
10
0
40
10
2·5
·63
Fab equivalents (n mol/ml)

Fig. 3.4 Effect of Fab'γ from antibody on migration of L_2C cells

L_2C cells were allowed to migrate into culture medium containing doubling dilutions of (A) Fab'γ from anti-Id(λ) ○ or Fab'γ from normal sheep IgG □ , (B) anti-Id(λ) ● or normal sheep IgG ■ . Migration was measured at 18h.

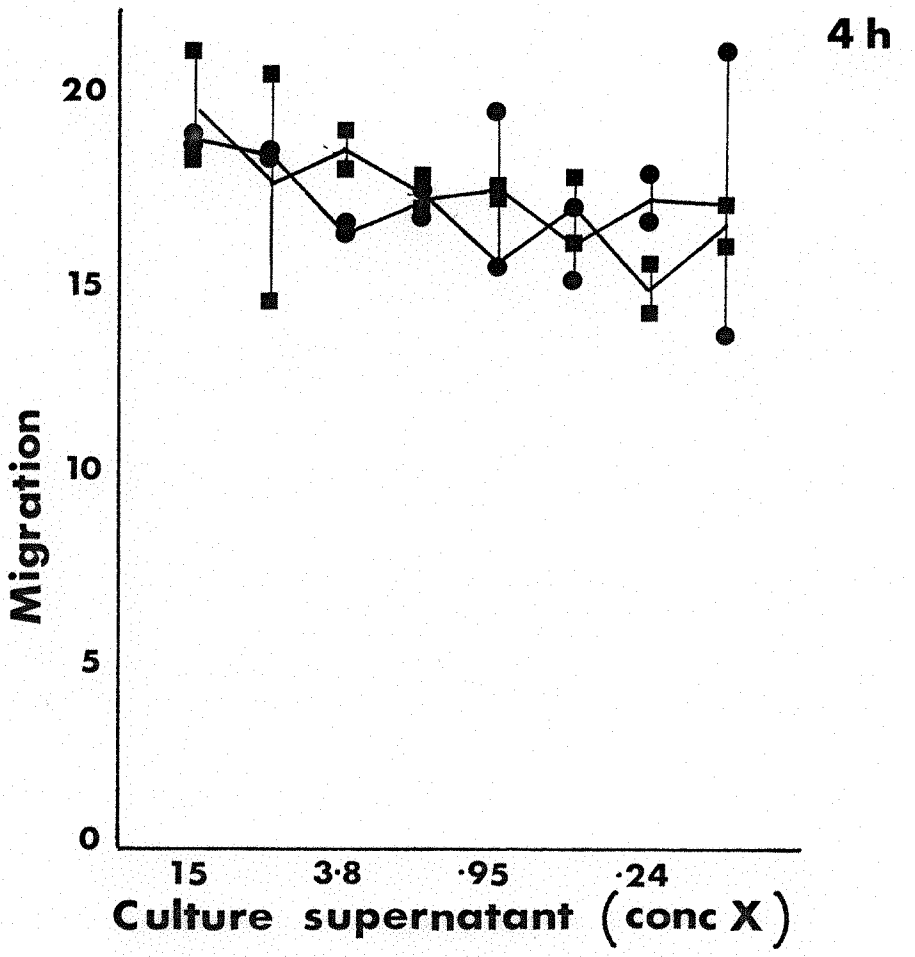


Fig. 3.5 Effect of antibody-free supernatant from L_2C cells cultured with antibody on migration of L_2C cells

L_2C cells were cultured at 10^7 cells/ml for 2h with anti-Id(λ) (1 mg/ml) ● , or without IgG ■ . The supernatant from the culture without IgG was reconstituted with anti-Id(λ) (1 mg/ml) and antibody was removed from both unconcentrated undialysed supernatants by immunosorption with rabbit anti-sheep IgG. The antibody-free supernatants were concentrated, dialysed into fresh medium and tested with doubling dilutions for effects on migration of L_2C cells. Migration was measured at 4h.

compared. The results in Figure 3.3 show that $(Fab'\gamma)_2$ from anti-Id(λ) inhibited migration of L_2C cells. The gradual diminution of inhibition with time was observed with $(Fab'\gamma)_2$. No inhibition was observed by Fab'γ from the same antibody (Fig. 3.4). Fab'γ and $(Fab'\gamma)_2$ from normal sheep IgG were non-inhibitory in these experiments. The greater migration in the presence of $(Fab'\gamma)_2$ from normal sheep IgG than intact IgG after one hour is probably due to a time difference in setting up plates.

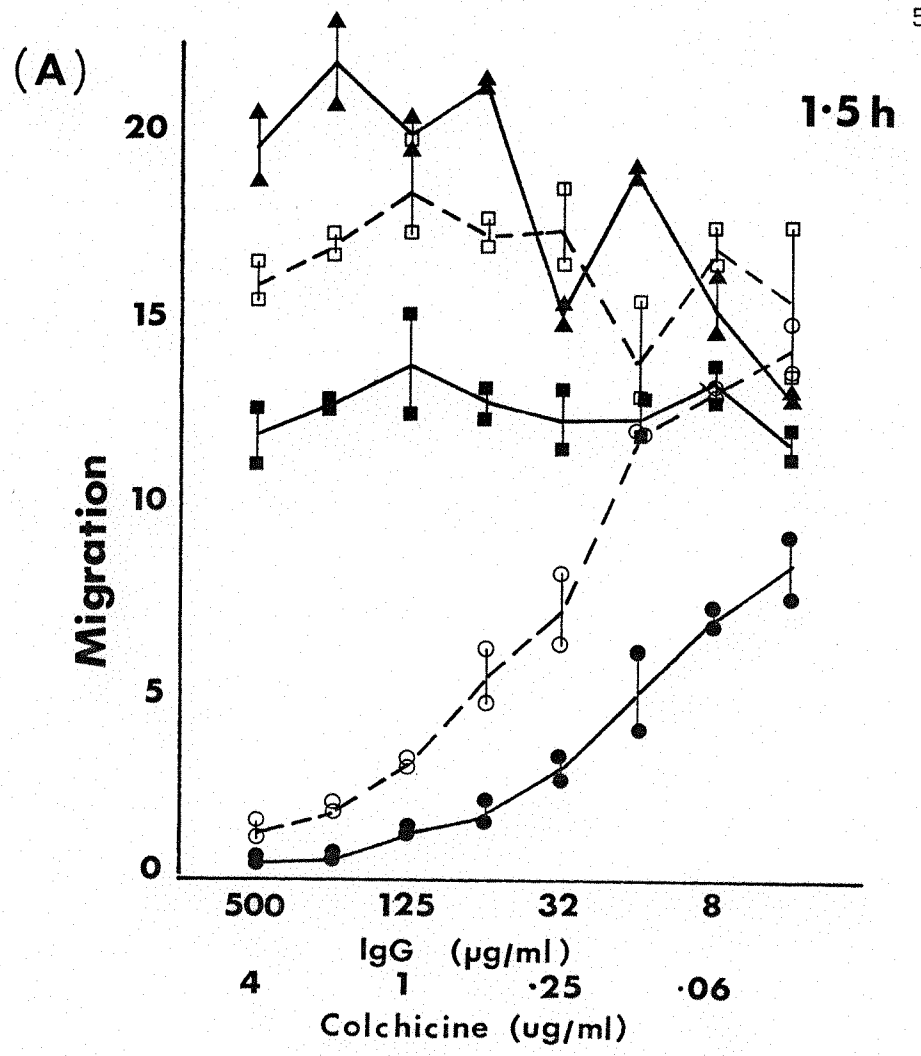
3.3.1.3 Culture supernatants from L_2C cells

Supernatants from L_2C cells cultured with antibody which was then removed had no effect on migration of L_2C cells themselves. The negative result in Figure 3.5 was obtained by incubating cells with and without antibody for 2 hours, migration inhibition by antibody being marked after 2 hours. The control supernatant was reconstituted with antibody and then the IgG removed from both supernatants. At 15 times the original concentration neither supernatant produced any inhibition.

3.3.1.4 Colchicine and cytochalasin B

To investigate the involvement of the cytoskeleton in migration of L_2C cells, two drugs were used. Colchicine, which prevents formation of microtubules by binding to tubulin sub-units (Deporter, 1978) had an enhancing effect on migration which was independent of the inhibitory effect of antibody. Cytochalasin B, which is known to interfere with microfilament function, inhibited migration.

Figure 3.6 shows the results of an experiment in which L_2C cells were allowed to migrate into wells containing colchicine (Sigma, London). 50% stimulation of migration by colchicine (10^{-5}M) was regularly observed after 1.5 hours (A). The effect was transient having disappeared by 18 hours (B). In the same experiment (Fig. 3.6), stimulation occurred in cells migrating


(A)
1·5 h
Migration
20
15
10
5
0
500
125
32
8
IgG (µg/ml)
4
1
·25
·06
Colchicine (ug/ml)

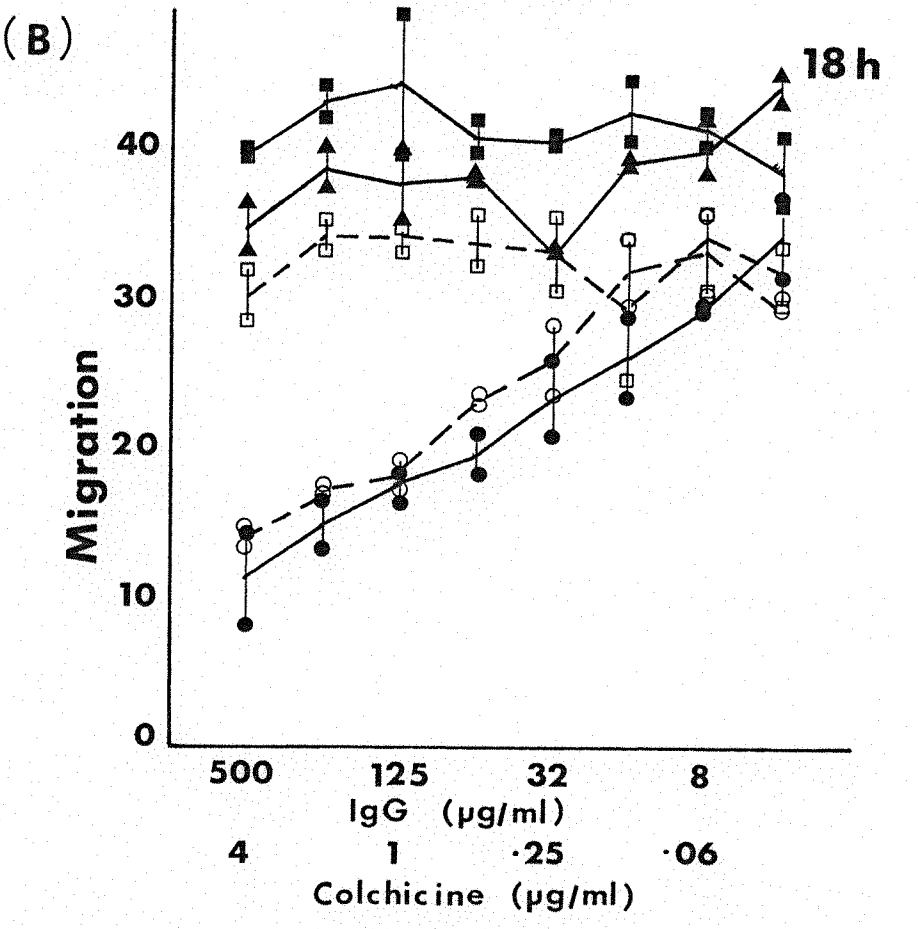

(B)
18 h
Migration
40
30
20
10
0
500
125
32
8
IgG (µg/ml)
4
1
·25
·06
Colchicine (µg/ml)

Fig. 3.6 Effect of colchicine on antibody-induced inhibition of migration of L_2C cells

L_2C cells were allowed to migrate into culture medium containing colchicine (10^{-5} M) and doubling dilutions of anti-Fab'$\gamma\lambda$ ○ or normal sheep IgG □ ; doubling dilutions of colchicine ▲ , anti-Fab'$\gamma\lambda$ ● or normal sheep IgG ■ . Migration was measured at (A) 1.5h and (B) 18h.

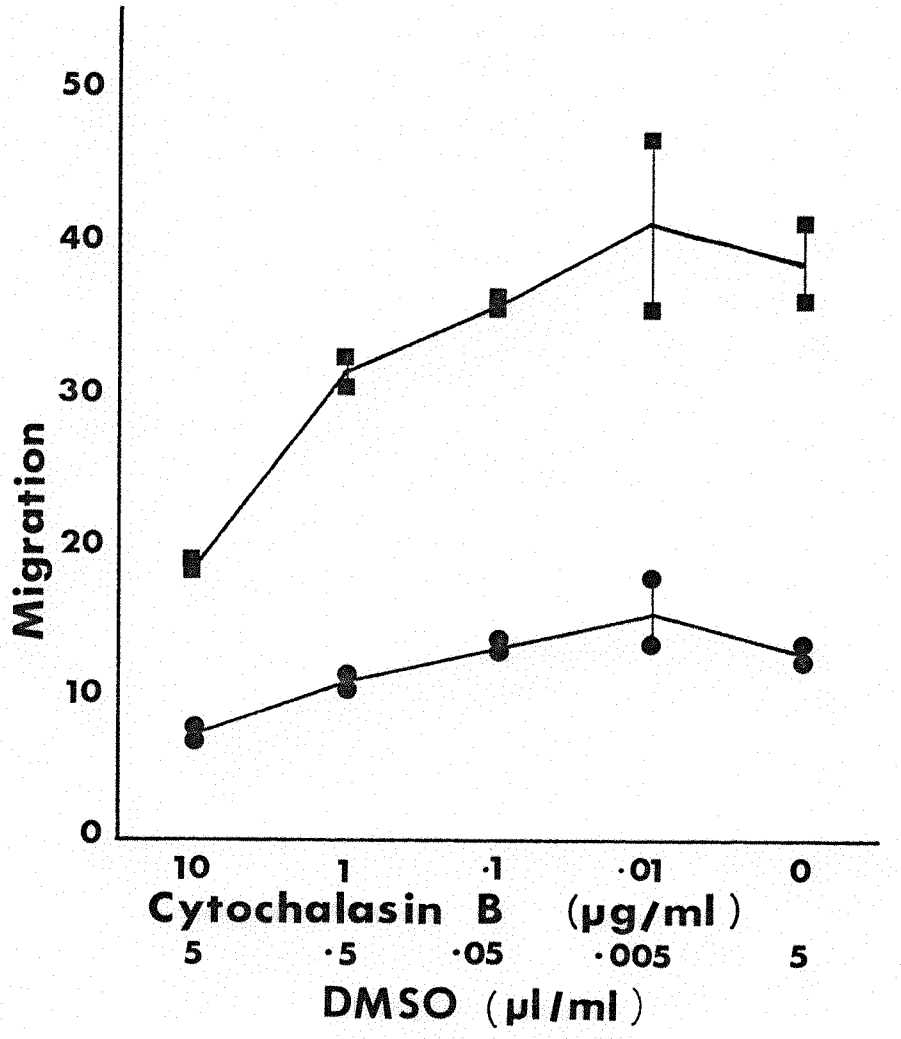


Fig. 3.7 Effect of cytochalasin B on migration of L_2C cells

L_2C cells were allowed to migrate into culture medium containing 10 fold dilutions of cytochalasin B and dimethylsulphoxide (DMSO). Migration was measured at 2h ● and 18h ■ .

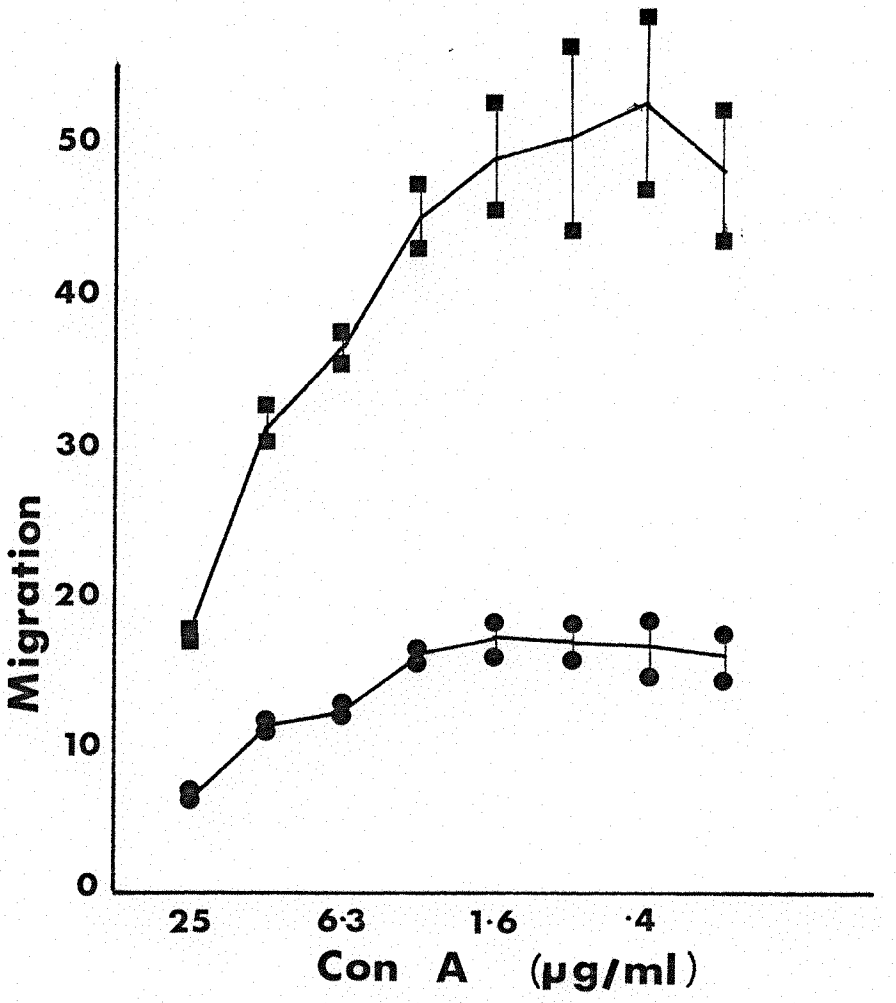


Fig. 3.8 Effect of Con A on migration of L_2C cells

L_2C cells were allowed to migrate into culture medium containing doubling dilutions of Con A. Migration was measured at 2h ● and 18h ■ .

into wells containing a constant concentration of colchicine (10^{-5}M) and doubling dilutions of antibody. The inhibition by antibody was not totally removed by the effect of colchicine. Similar results were obtained with anti-Fab'$\gamma\lambda$ and anti-Id(λ). Identical effects were seen when L_2C cells were exposed to colchicine (10^{-5}M) for 30 minutes at 37°C before capillary tubes were filled. In this case, it was observed that there was no further increase in stimulation with a higher concentration of colchicine (10^{-4}M) and that the extent of migration was independent of colchicine concentration in migration wells.

The effect of cytochalasin B on migration became insignificant between 0.1 and 0.01 µg/ml (Fig. 3.7). These concentrations were found to inhibit protein synthesis (see 4.3.1.6), perhaps due to inhibitory effects on transport of glucose into the wells (Lin, Lin and Flanagan, 1978), so no conclusion could be drawn specifically concerning the role of microfilaments in migration of L_2C cells.

3.3.1.5 Con A

L_2C cells were inhibited by concentrations of Con A (Fig. 3.8) effective in inhibiting migration of other tumour cells (Friberg et al, 1971). However there was no evidence that Con A was effective at subagglutinating levels. Visible agglutination of L_2C cells by Con A in a microtitre plate showed concentration-dependence similar to that seen in the inhibition of migration by Con A.

3.3.2 Effects of antibody on migration of normal guinea pig lymphocytes

T cell-depleted lymph node cells (60% B cells) from normal guinea pigs migrated poorly compared with L_2C cells. The bulk of cells remained close to the cut end of the capillary and the thin layer of migrating cells which constituted the cell fan consisted of residual T cells as it could be inhibited by anti-T cell antibody. Anti-Fab'γ had no effect on the migrating cells.

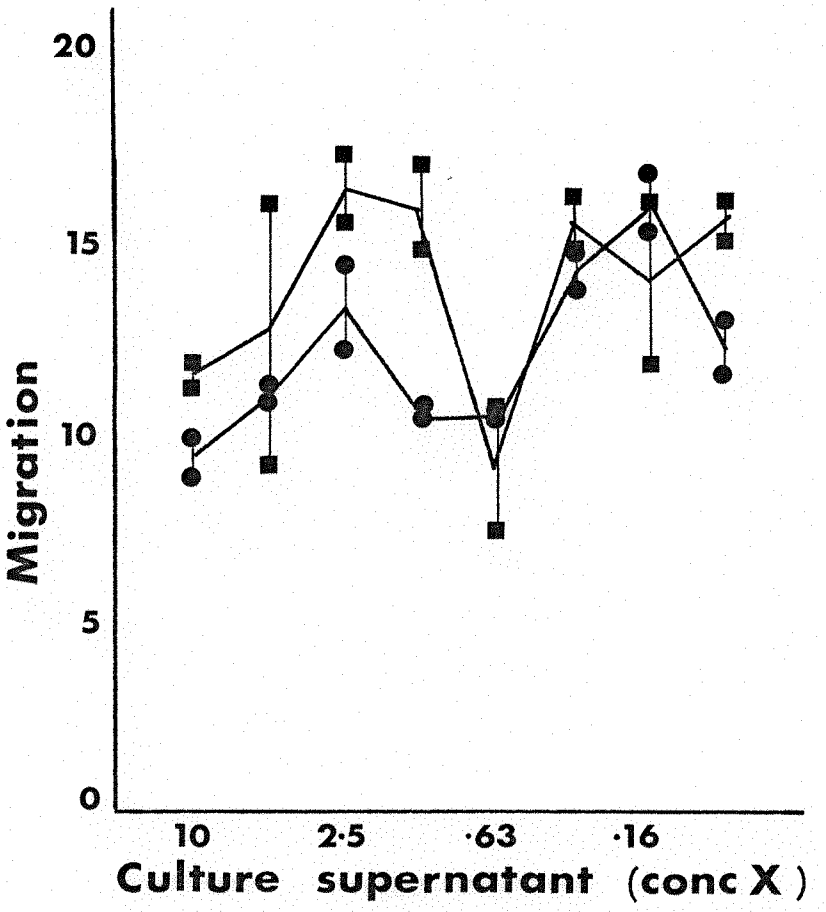


Fig. 3.9 Effect of antibody-free supernatant from L_2C cells cultured with antibody on migration of guinea pig PEC

L_2C cells were cultured at 10^7 cells/ml in serum-free culture medium for 18h with anti- λ (0.5 mg/ml) ● or without IgG ■ . The supernatant from the culture without IgG was reconstituted with anti- λ (0.5 mg/ml). Both supernatants were then concentrated 10 times, dialysed into PBS and antibody removed by immunosorption with rabbit anti-sheep IgG. The antibody-free supernatants were reconcentrated, dialysed into fresh medium and tested with doubling dilutions for effects on migration of guinea pig PEC. Migration was measured at 18h.

3.3.3 Effect of culture supernatants on migration of PEC

3.3.3.1 L_2C cells

L_2C cells did not release a demonstrable macrophage MIF either spontaneously or after culture with antibody. In initial experiments antibody was not removed from culture supernatants before they were tested for effects on migration of PEC and evidence was obtained of the antibody itself having some effect on the migration. This irregular effect of the antibody, unexplained but possibly associated with the macrophage Fcγ receptor made it essential to remove it from the supernatants before they were tested for effects on migration of PEC.

Spontaneous release of MIF was sought in the supernatants of L_2C cells cultured in medium alone, or in the presence of normal sheep IgG (1 mg/ml). In neither case did the supernatant show any inhibitory effect on the migration of PEC.

Antibody was removed by affinity chromatography from supernatants of cells cultured for 18 hours with antibody, and from control supernatants to which antibody had been added after the removal of the cells. Despite the irregularity of the graph (Fig. 3.9) it can be seen that at 10 times the original concentration the inhibition achieved by supernatant from cells cultured with antibody did not exceed the 20% level required for significance in this technique (Bloom and Bennett, 1971). This experiment was repeated several times with the omission of the reconstituted control to see if any definite inhibition of PEC migration could be demonstrated but no significant positive results were obtained with anti-λ, anti-Fab'$\gamma\lambda$ or anti-Id(λ). Concentration of supernatants with a Diaflo membrane retaining molecules > 1,000 M.W. (Amicon, UM2) made no difference to results.

Culture supernatants from L_2C cells pulsed with Con A as described in 3.2.3 did not inhibit migration of L_2C cells nor of normal guinea pig macrophages. Any inhibitory effects which were seen were due to residual Con A as they could be removed

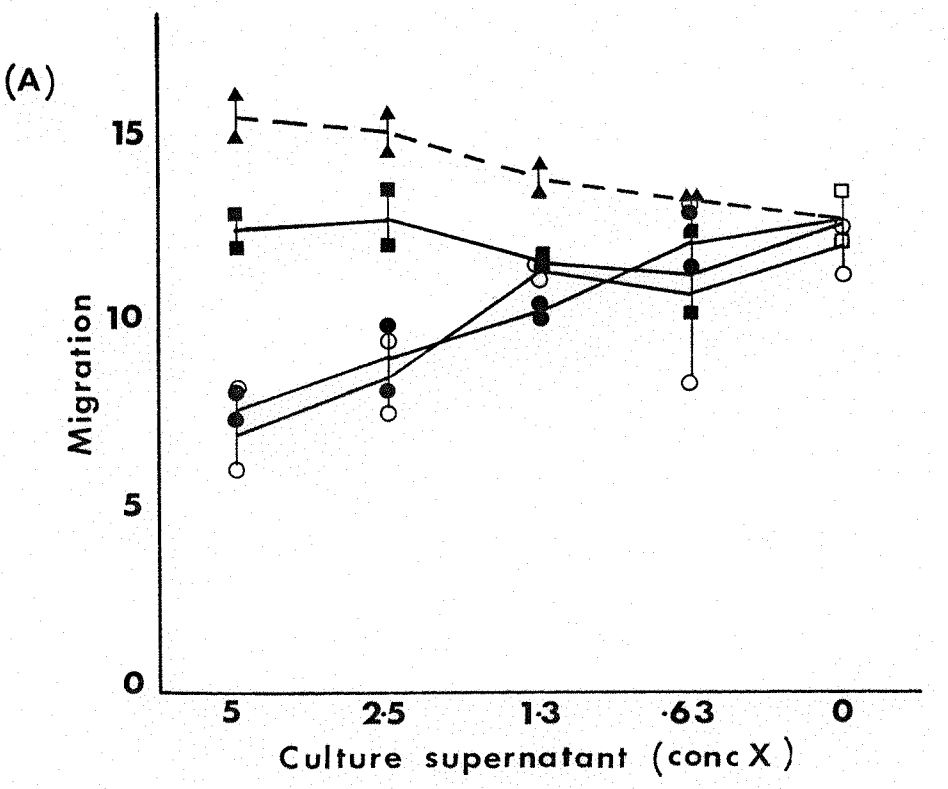
by the addition of α-methyl-D-mannoside to the culture medium.

3.3.3.2 Normal guinea pig lymphocytes

To ensure that MIF was not being lost on the Sepharose 4B-rabbit anti-sheep IgG column used to remove antibody from L_2C cell supernatants, positive control experiments were carried out. Normal guinea pig lymph node cells were stimulated with Con A to produce culture supernatants containing MIF activity which survived the procedure to which L_2C cell supernatants were subjected.

The supernatants from cells pulsed with Con A at 37°C and thereafter cultured at 4°C ("4°C supernatants") did not produce any inhibition of macrophage migration (Fig. 3.10). This made it possible to express the inhibition produced by supernatants cultured at 37°C ("37°C supernatants") as a migration index, migration in the presence of a "37°C supernatant"/migration in the presence of a "4°C supernatant". The averation migration index from 4 separate experiments for dialysed 5 fold concentrated supernatants was 0.62 ± 0.08. In 2 experiments, the addition of 100 mM α-methyl-D-mannoside to migration wells did not abrogate inhibition (Fig. 3.10A). 100 mM α-methyl-D-mannoside while having no effect on migration itself could completely remove inhibition of PEC migration by Con A at 25 µg/ml (Fig. 3.11).

Inhibitory "37°C supernatant" (10 ml) was passed down the Sepharose 4B-rabbit anti-sheep IgG column, concentrated and dialysed. The MIF activity was totally removed by the column (Fig. 3.10A). It was then found that normal sheep IgG (2 mg/10 ml) added to the "37°C supernatant" protected MIF against non-specific absorption on the column (Fig. 3.10B). "37°C supernatant" which had been down the column in the presence of normal sheep IgG was as inhibitory as an aliquot of the same supernatant which had not been absorbed. In this experiment residual IgG remaining after absorption would not matter as normal sheep IgG at 100 µg/ml (Fig. 3.10B) and up to 2 mg/ml in other experiments (results not shown) had no effect on PEC migration. It was concluded


(A)
15
10
5
0
Migration
5
2·5
1·3
·63
0
Culture supernatant (conc X)

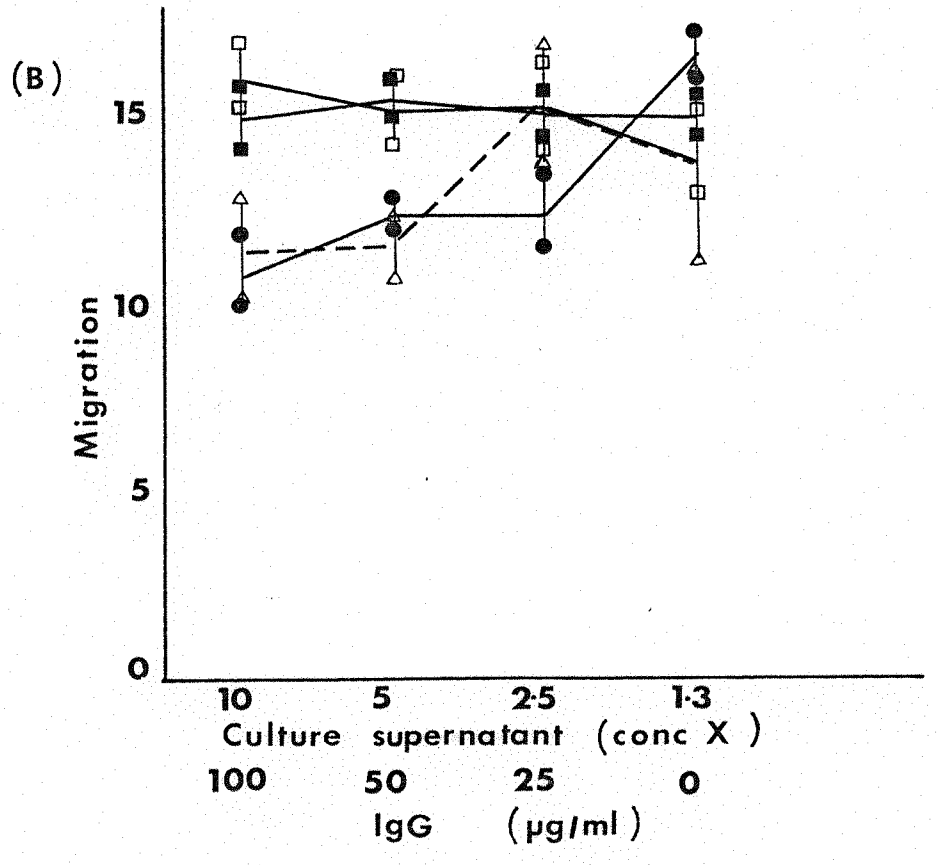

(B)
15
10
5
0
Migration
10
5
2·5
1·3
Culture supernatant (conc X)
100
50
25
0
IgG (µg/ml)

Fig. 3.10 Protection by sheep IgG of MIF activity in culture supernatants from normal guinea pig lymphocytes during immunosorption with rabbit anti-sheep IgG

(A) and (B) represent separate experiments in which MIF-containing "37°C supernatant" from normal guinea pig lymphocytes stimulated with Con A was absorbed with rabbit anti-sheep IgG in the absence of added IgG ▲ (A), and in the presence of normal sheep IgG (2 mg/10 ml supernatant) △ (B). Unabsorbed "37°C supernatant" ● , "4°C supernatant" ■ and "37°C supernatant" containing 100 mM α-methyl-D-mannoside in each migration well ○ ; fresh medium (A), or fresh medium and normal sheep IgG (B) □ . All supernatants were concentrated and dialysed into fresh medium (after immunosorption where applicable) before being tested with doubling dilutions for effects on migration of guinea pig PEC. Migration was measured at 18h.

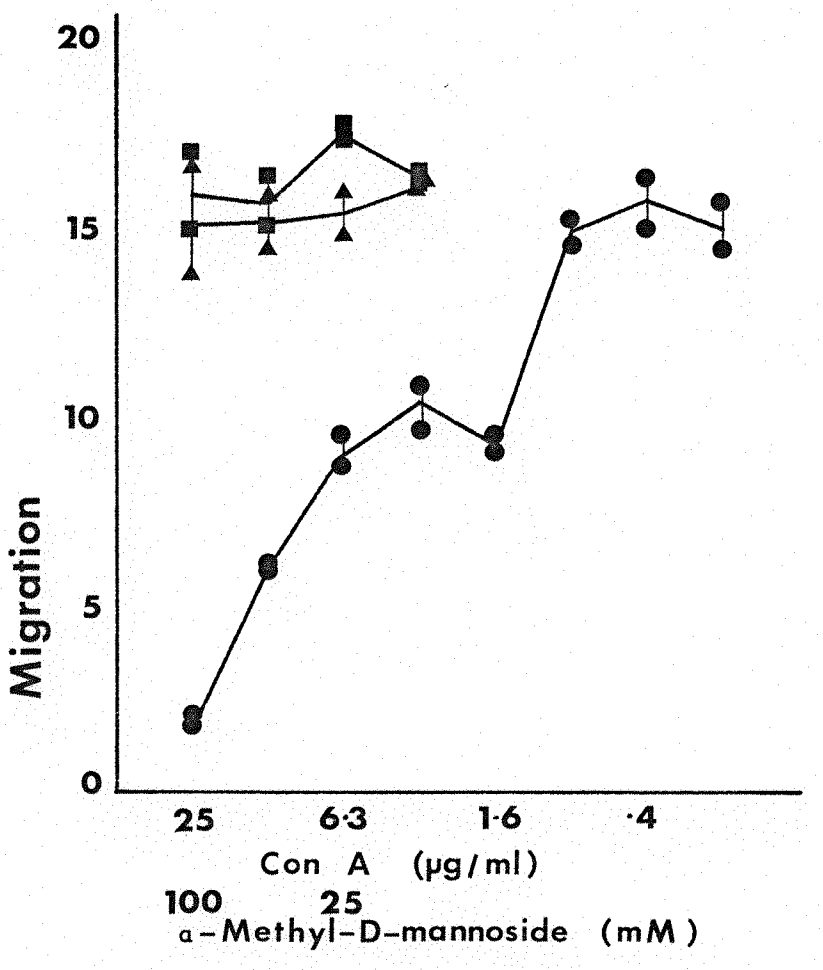


Fig. 3.11 Effect of Con A on migration of guinea pig PEC

Normal guinea pig PEC were allowed to migrate into culture medium containing doubling dilutions of Con A ● , Con A and 100 mM α-methyl-D-mannoside in each migration well ■ , α-methyl-D-mannoside ▲ . Migration was measured at 18h.

that if L_2C cells were producing MIF in significant amounts, it would have survived the affinity chromatography procedures to remove antibody.

3.4 Discussion

The migration of neoplastic lymphoid cells out of capillary tubes containing culture medium was described by Cochran (1971) and it was later shown that antibody could inhibit this migration (Cochran et al, 1972; Currie and Sime, 1973). Migration of L_2C leukaemic cells and its inhibition by antibody has been previously reported from this laboratory (Stevenson and Stevenson, 1975; Stevenson et al, 1977b). The present project has defined some of the requirements for inhibition of migration by antibody.

L_2C cells migrate rapidly and there was sufficient migration at 37^oC for measurements to be taken within 2 hours, which contrasted with the relatively poor migration by normal guinea pig lymph node cells in the same assay system. The spontaneous migration of L_2C cells was inhibited by antibody to surface Ig in the culture medium in a concentration-dependent manner. The relative inhibition was greater after 2 hours than after 18 hours migration, a finding which was experimentally useful in so far as saving time and avoiding problems of cell death were concerned.

The inhibition of migration of these blastoid cells contrasts with antibody-induced stimulation of B cell movement seen by Unanue et al (1972). The disparity may be explained by the fact that Unanue et al used mature cell populations. These observations of Unanue et al referred to stimulation of random motility in no one specific direction. Later, workers from the same laboratory (Ward et al, 1977) claimed that antibody could act as a chemotactic agent and stimulate directional movement. In our system, it is not clear whether the enlargement of the cellular fan which constitutes migration is a result of random

or directional movement by the L_2C cells.

When factors which may have been involved in inhibition of migration were considered, cytotoxicity did not appear to have a role, as L_2C cells could recover from inhibition to some extent. The phenomenon of escape has been observed in the inhibition of sensitized leukocyte migration by antigen (Brostoff, 1974) although as will be discussed later there is reason to believe that the mechanism of inhibition by antibody in the L_2C cell is quite different. It is very difficult to assess the role of agglutination in inhibition of migration. Previous work (Stevenson et al, 1977b) showed a lack of correlation between agglutinating and migration-inhibiting activities of different antibodies directed against L_2C surface Ig: at given concentrations rabbit anti-Id(λ) was the more strongly agglutinating whereas rabbit anti-λ was the more efficient at inhibiting migration. It is therefore unlikely that agglutination is the sole or even major mechanism involved.

An investigation of inhibition of migration by fragments of antibody revealed that $(Fab'\gamma)_2$ was as effective as intact IgG. Monovalent $Fab'\gamma$ from the same antibody did not inhibit migration. From these results it was concluded that to produce inhibition, a bivalent ligand able to cross-link surface Ig was required and the Fc piece, with at least a potential for involving $Fc\gamma$ receptors, was unnecessary.

Experiments with colchicine and cytochalasin B failed to give any clear indication of the roles of microtubules and microfilaments in antibody-induced inhibition of migration. Colchicine had a transient stimulatory effect on migration of L_2C cells which was independent of the inhibition of migration by antibody. Such observations might be related to reports (Unanue and Karnovsky, 1974; Schreiner and Unanue, 1975a) of a temporary increase in random motility of mouse normal B lymphocytes caused by this drug. It is not apparent how such

effects on cellular motility relate to the well known inhibition of microtubule polymerization by colchicine. Similarly the inhibitory effect on migration of L_2C cells by cytochalasin B cannot be attributed simply to its well known induction of microfilament dysfunction because of the complication that cytochalasin B even at low concentrations had an inhibitory effect on general protein synthesis.

It was concluded that the inhibition of migration of L_2C cells by antibody did not appear to depend on the release of a MIF-like substance. Moreover, when L_2C cells were cultured alone or with antibody no significant release of a soluble factor with the more conventional property of inhibiting macrophage migration could be demonstrated. The authenticity of the detection system using macrophages was verified by showing that they responded to a known source of MIF, the supernatant of Con A-stimulated normal lymphoid cells.

This negative result for L_2C cells does not accord with some studies on the release of MIF by B cells. Papageorgiou et al (1972) found that the 8 human lymphoid cell lines they examined, all of B cell origin, spontaneously released MIF which inhibited migration of human lymphoid cells and of normal guinea pig macrophages. Their paper included the observation that the migrating human lymphoid cells were inhibited by their own culture supernatant. As Papageorgiou et al found that non-lymphoid cell lines also produced MIF they suggested that this might be a common property of activated cells. However, L_2C cells which are activated cells do not produce a demonstrable MIF. The disparity in these results for neoplastic cell populations is reflected in reports on MIF production by normal guinea pig B cells. It has been reported that non-immune guinea pig normal B cells produce MIF after activation with PPD (Yoshida et al, 1973). On the other hand Bloom and Shevach (1975) concluded that T cell help was essential for MIF production by guinea pig normal B cells in response to PPD. From the latter

result it is possible that L_2C cells *in vitro* do not produce MIF in demonstrable quantities because they require assistance from other cells. Alternatively, L_2C cells may be arrested at a stage of differentiation where MIF release is not significant.

Our observations with Con A provide further evidence that inhibition of migration need not depend on the release of MIF. Con A was able to inhibit migration of L_2C cells but again our efforts to demonstrate a parallel release of MIF were unsuccessful. The possibility nevertheless remains that the mechanisms of inhibition by Con A and antibody are different.

There are two main conclusions to be drawn from these studies on migration. Firstly, in the L_2C cell, cross-linking of surface Ig transmits a signal to the cell which is responsible for inhibition of migration. Secondly, this inhibition of migration is apparently not due to the release of MIF-like material.

CHAPTER 4

PROTEIN SYNTHESIS IN NEOPLASTIC AND NORMAL LYMPHOCYTES

4.1 Introduction

After recognizing antigen on the B lymphocyte surface antibody may have a further biological effect on the cell as described in the General Introduction. L_2C cells actively synthesize proteins *in vitro* and the effect of antibody on this general metabolic process could be investigated. In a pure cell population provided from leukaemic blood the effects of antibody on one particular type of cell without additional stimuli from other cells can be observed. The requirement for cross-linking surface Ig by antibody in order to affect protein synthesis was investigated by means of $(Fab'\gamma)_2$ and $Fab'\gamma$ from antibody. The specificity of the effect of anti-Ig was determined using antibodies which could bind to other receptors, β_2 microglobulin and Ia antigens. The behaviour of L_2C cells in response to antibody was compared with that of normal guinea pig lymphocytes and neoplastic B lymphocytes from human patients with CLL.

A certain proportion of protein synthesized in a cell is Ig. In a small resting B cell, IgM represents 1 - 3% of radioactive leucine biosynthetically incorporated into cellular proteins (Melchers and Andersson, 1973). With antigenic stimulation the ratio of Ig synthesis to total protein synthesis will increase to reach a maximum seen in a plasma cell where as much as 40% of protein synthesis can be directed towards production of Ig for secretion. In the L_2C cell a significant proportion of Ig is synthesized for insertion into the membrane and it has been shown in this laboratory (Glennie, Stevenson, Stevenson and Virji, 1979) that antibody can inhibit the delivery of Ig to the cell surface. In this project the effect of antibody on the actual synthesis was investigated .

The antigenic targets of the antibodies used in the work described in this chapter are summarized in Figure 2.2.

4.2 Materials and Methods

4.2.1 Protein synthesis

4.2.1.1 Measurement with radioactive leucine

Protein synthesis can be measured by biosynthetic incorporation of radioactively labelled leucine into cellular protein. Leucine is a suitable marker for protein synthesis because it is an essential amino acid and is minimally metabolized by cells in growth medium (Neff, Ross, Bartholomew and Bissell, 1977). Cellular protein is generally harvested by precipitation with trichloroacetic acid (TCA). In the precipitate, newly synthesized protein is radioactively labelled and total protein content can be estimated by the method of Lowry, Rosebrough, Farr and Randall (1951). Rates of protein synthesis can also be measured by incorporation of radioactive leucine into cellular material which remains when cells collected on filter paper are lysed with water.

4.2.1.2 Culture conditions

Cells were cultured in suspension in sealed conical flasks with gentle rotary agitation at 37^{o}C. Some experiments were done with cultures standing without agitation. However, L_2C cells showed a tendency to sediment quickly and if plastic flasks were used, the cells adhered to the surface making it difficult to take accurate aliquots of cell suspension.

L_2C cells were cultured at a final concentration of 5×10^6 cells/ml in culture medium containing agents to be tested for their effects on protein synthesis and radioactive leucine (The Radiochemical Centre, Amersham). In initial experiments ^{14}C-leucine (300 mCi/mmol) was used at 0.5 µCi/ml and subsequently, ^{3}H-leucine (1 Ci/mmol) at 1 µCi/ml. Although MEM contains cold leucine at 0.4 µm/ml these levels of radioactive leucine were adequate for significant counts. For studies on culture

supernatant, cell and radioactive leucine concentrations were increased. To begin the incubation period, the final addition to culture flasks at 37°C was L_2C cells from a suspension at 10^8 cells/ml also warmed to 37°C in a water bath. During culture, flasks were opened in a laminar flow cabinet and samples of cell suspension were taken with a Finnpipette.

The same conditions were used for normal guinea pig T cell-depleted lymph node cells and CLL cells with increased levels of radioactive leucine to compensate for the smaller sizes and lower synthetic rates of these cells compared with L_2C cells. In addition CLL cells were usually cultured at 10^7 cells/ml.

4.2.1.3 Precipitation of cell protein with trichloroacetic acid (TCA)

Duplicate samples of cell suspension (0.25 or 0.5 ml) were taken from each culture flask and put directly into graduated glass centrifuge tubes (10 ml) on ice. After centrifugation at 1,000 g for 10 minutes at 4°C, the radioactive supernatants were removed and the cells washed with cold MEM (1 - 2 ml) which contains non-radioactive leucine. Cold 10% TCA (1 ml) was added to each of the cell pellets or 0.5 ml culture supernatant and after 30 minutes on ice, the tubes were spun at 3,000 g for 10 minutes at 4°C. The precipitates were washed twice with cold 5% TCA (1 - 2 ml) and once with ethanol : ether (2:1 v/v; 1 - 2 ml). Between washes, precipitates were dispersed by sonication. After the wash with organic solvent, precipitates were dried at 37°C for 18 hours or at 80°C for 2 hours. Then precipitates were digested in N NaOH (50 µl) at 37°C conveniently overnight and the solution diluted with water (200 µl) to 0.2N NaOH. For liquid scintillation counting 100 µl (10^6 cells) was transferred to an insert vial. The total protein content of the 0.2N NaOH solution could be estimated by the method of Lowry et al (1951).

4.2.1.4 Harvesting cell protein by water lysis

Duplicate or triplicate samples of cell suspension were taken from each culture and placed in flat-bottomed wells of a microtitre plate on ice. 100 µl (5×10^5 cells) aliquots were taken from each culture though this was increased with normal guinea pig lymphocytes and CLL cells. A multi automatic cell harvester (MASH) was used to transfer the aliquots to filter paper strips. The cells were then lysed with water by washing the cells 5 times with distilled water and the MASH. The filter paper was removed and dried at 37°C for 1 hour or at 80°C for 10 minutes. The imprinted discs containing material from lysed cells were transferred with forceps to insert vials.

4.2.1.5 Counting radioactivity in cell protein

Samples were counted in a liquid scintillation counter (Unilux II, Nuclear-Chicago, Illinois, U.S.A.) with channels set for counting ^3H or ^{14}C. Each vial was counted for 1 minute or 10 minutes if below 1,000 cpm. NaOH solutions containing radioactive cell protein were digested in 0.5 ml NCS tissue solubilizer (Hopkins and Williams, Essex) by shaking briefly at room temperature. 4 ml toluene scintillation fluid (toluene containing PPO at 6.6 g/l and Dimethyl-POPOP at 82.5 mg/l) recommended by suppliers of NCS were added. No quenching was observed due to the NaOH or NCS present. The filter paper discs were counted in 4 ml toluene scintillation fluid (toluene containing PPO (4 g/l) and POPOP (100 mg/l). The two scintillation fluids were interchangeable.

4.2.2 Immunoglobulin synthesis in L_2C cells

4.2.2.1 Immune precipitation

Radioactivity biosynthetically incorporated into Ig is determined by precipitation with specific antisera. In labelled

cells lysed with non-ionic detergent there may be very small amounts of radioactive Ig present and when combined with antibody, the complexes may remain soluble. Addition of an anti-antibody provides the necessary cross-linking to form an insoluble precipitate. The "sandwich technique" was described by Melchers and Andersson (1973) and was adapted to L_2C cells in this project to specifically precipitate Ig with sheep antibodies directed against guinea pig Ig (anti-Fab'$\gamma\lambda$ and anti-μ) and rabbit anti-sheep Fab'γ. It was necessary to establish that the sheep antibody was in large excess to ensure combination with all the radioactive Ig and that this complex was completely precipitated by the rabbit antibody. Some non-specific radioactivity was precipitated by a sandwich containing normal sheep IgG and rabbit antibody. During the development of this technique various ways of reducing this non-specific background were tried without significant success. The non-specific background represented approximately 50% of the total radioactivity precipitated by specific antibody.

4.2.2.2 Culture conditions

In order to incorporate sufficient ^{3}H-leucine into cells for Ig to be labelled in detectable amounts leucine-free medium is generally used (Melchers and Andersson, 1973). Leucine-free medium containing bicarbonate was not suitable for culture of L_2C cells. It was shown that L_2C cells at 5×10^6 cells/ml in leucine-free culture medium with added radioactive leucine providing 4 nmol leucine/ml did not have sufficient leucine to maintain growth. In fact the rate of protein synthesis decreased if leucine concentration was reduced below 0.4 μm/ml in medium containing bicarbonate.

It was decided therefore to use medium fully supplemented with leucine and increased levels of ^{3}H-leucine. In definitive experiments L_2C cells were cultured in the standard culture medium (HEPES) at 10^7 cells/ml with ^{3}H-leucine (> 100 Ci/mmol) up to 20 μCi/ml. No harmful effects on the cells were observed at this specific activity of ^{3}H-leucine.

4.2.2.3 Precipitation of sheep IgG by rabbit antibody

Before any experiments with radioactive cell lysates were done the amount of rabbit anti-sheep Fab'γ required to precipitate a given amount of sheep IgG was determined by a semi-quantitative Ouchterlony precipitin test. To perform this test antigen (1 mg/ml) in amounts forming an approximate geometric progression (10, 16, 25, 40, 63, 100μg) was added to antibody (400 μg, 0.2 ml). Precipitates were allowed to form and after centrifugation at 15,000 g for 10 minutes, supernatants were dispensed into wells (inter-well distance 3 mm) on an agar plate. Antibody was added to the appropriate wells from the original solution and antigen (5 μg) from a solution of 0.1 mg/ml. On the developed plate equivalence could be seen where there was no precipitin line formed with either antibody or antigen. Equivalence as defined by the semi-quantitative Ouchterlony precipitin test was between 6.3:1 and 4:1 (rabbit : sheep) for all three sheep IgGs, normal, anti-Fab'γλ and anti-μ. In experiments a ratio of 10:1 (rabbit : sheep) was used.

4.2.2.4 Cell lysis with non-ionic detergent

One aliquot of cell suspension (2 - 2.5 ml) was taken from each of the cultures and placed in a graduated glass centrifuge tube (10 ml) on ice. All further manipulations up to the digestion of immune precipitates with NaOH were carried out at 0 - 4°C. After centrifugation at 1,000 g for 10 minutes the radioactive supernatant was removed and cells washed once with MEM (5 ml). The cells were lysed at 2.5×10^7 cells/ml in 1% Nonidet P40 in PBS containing iodoacetamide (0.2 M) and soybean trypsin inhibitor (10 μg/ml). After 30 minutes the lysate was spun at 30,000 g for 2 hours and then used immediately for precipitation of Ig and total protein. The lysates showed variable stability on storage but in general there was no opalescence within 24 hours. If lysates were frozen (-20°C) and thawed there was considerable precipitation which could be

removed by centrifugation. Immune precipitation on the thawed supernatant showed a great reduction (90%) in non-specific background but there was also a loss (50%) of specifically precipitated material. As no consistent pattern in losses was observed freezing and thawing of lysates could not be used as a means of reducing non-specific background.

4.2.2.5 Precipitation of radioactive immunoglobulin

An attempt was made to reduce non-specific background by performing a preliminary precipitation with an irrelevant antigen-antibody complex. However, this was found not to be significantly useful, therefore definitive precipitations were done directly. All precipitations were done in triplicate. 10 µg (50 µl) normal sheep IgG, anti-Fab'$\gamma\lambda$ or anti-μ were added to 100 µl (2.5×10^6 cells) aliquots of ^{3}H-leucine labelled cell lysate in small plastic tubes. Although 5 µg anti-Fab'$\gamma\lambda$ were shown to be sufficient for precipitation of all the labelled Ig in the 100 µl aliquot, the final sandwich precipitate formed was small and reproducibility among triplicates difficult. 10 µg anti-μ which was not purified antibody was also shown to be adequate to combine with all its radioactive antigen in a similar aliquot of lysate.

After 30 minutes 100 µg (50 µl) rabbit antibody were added to each tube. It was established that the radioactivity associated with all three sheep IgGs investigated was completely precipitated by rabbit antibody for ratios from 5:1 to 15:1 (rabbit : sheep). The precipitations were allowed to form overnight although 3 hours was shown to be sufficient to precipitate all labelled Ig.

4.2.2.6 Washing radioactive precipitates

Tubes were spun for 30 minutes at 15,000 g in an angle-head minifuge (Baird and Tatlock Ltd., London). The precipitates

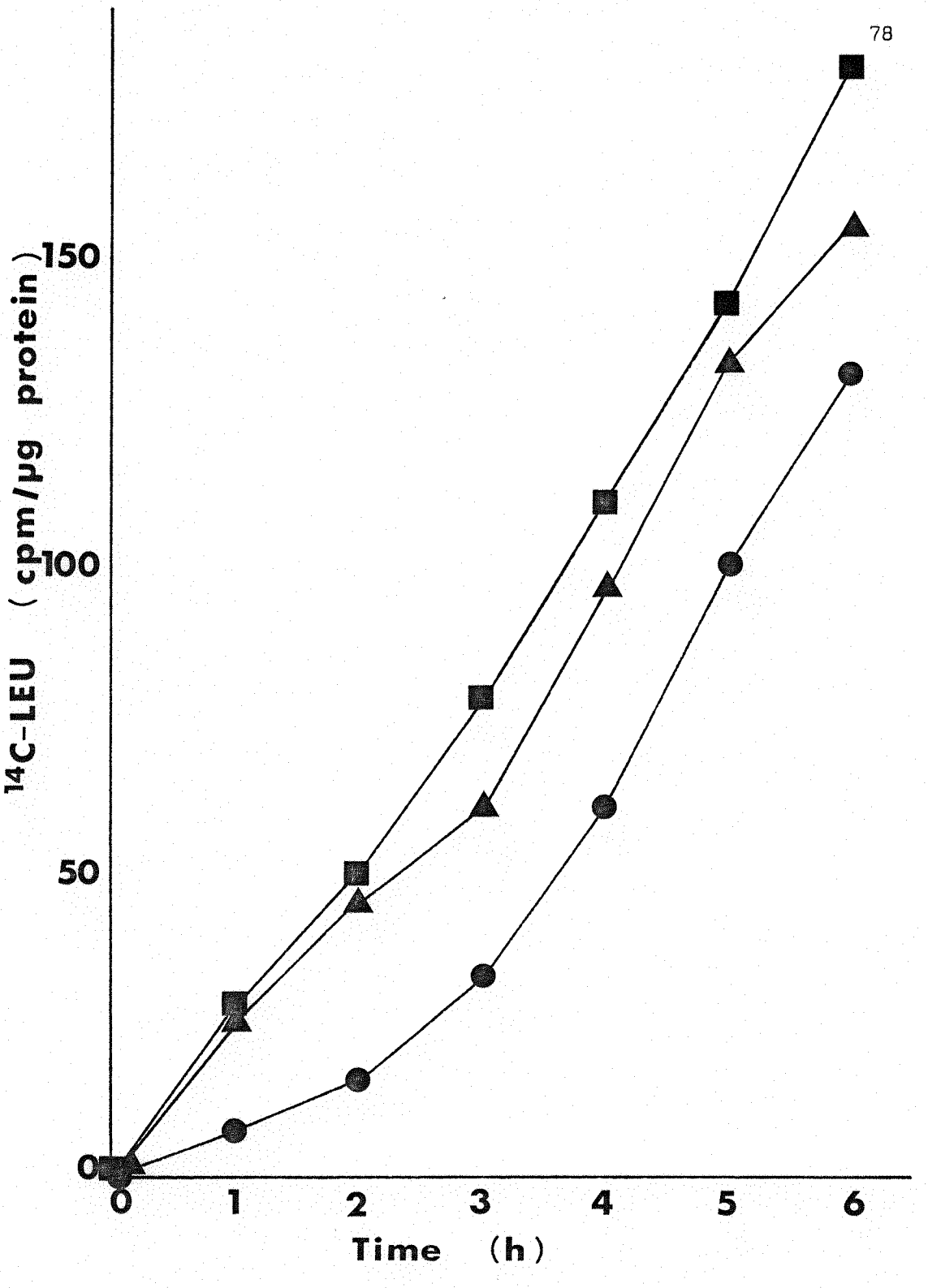


Fig. 4.1 Effect of antibody on protein synthesis in L_2C cells

L_2C cells were cultured at 5 x 10^6 cells/ml with ^{14}C-LEU(0.5 µCi/ml) and anti-Fab'γλ (0.25 mg/ml) ● , normal sheep IgG (0.25 mg/ml) ■ or PBS ▲ . 1 sample was taken from each culture at intervals to measure in duplicate ^{14}C-LEU and protein content in TCA precipitable cell material. Each point represents average cpm/average protein content.

were washed 3 times with 0.5% Nonidet P40, 1 mM leucine in PBS (2 ml). It was found that 3 washes with only 1 ml buffer were sufficient to reduce counts in washing media to an insignificant level. During washing, precipitates were resuspended with a pasteur pipette and centrifuged on the minifuge for 5 minutes. Dispersing precipitates between washes by sonication did not reduce non-specific precipitation. In one experiment, extensive dialysis of labelled lysate against PBS also did not reduce non-specific background.

4.2.2.7 Radioactivity in immunoglobulin

Washed precipitates were digested in N NaOH (20 µl) at 37°C for 1 hour before being transferred to insert vials and the tubes rinsed with 80 µl water. Samples were counted in NCS and toluene scintillation fluid. Results were expressed as the difference between counts in precipitates containing specific antibody and precipitates containing normal sheep IgG.

$$Ig = \bar{x}_{specific} - \bar{x}_{non\text{-}specific} \pm (S.E._{specific}^{2} + S.E._{non\text{-}specific}^{2})^{\frac{1}{2}}$$

4.3 Results

4.3.1 Effects of various reagents on protein synthesis in L_2C Cells

4.3.1.1 Anti-immunoglobulin

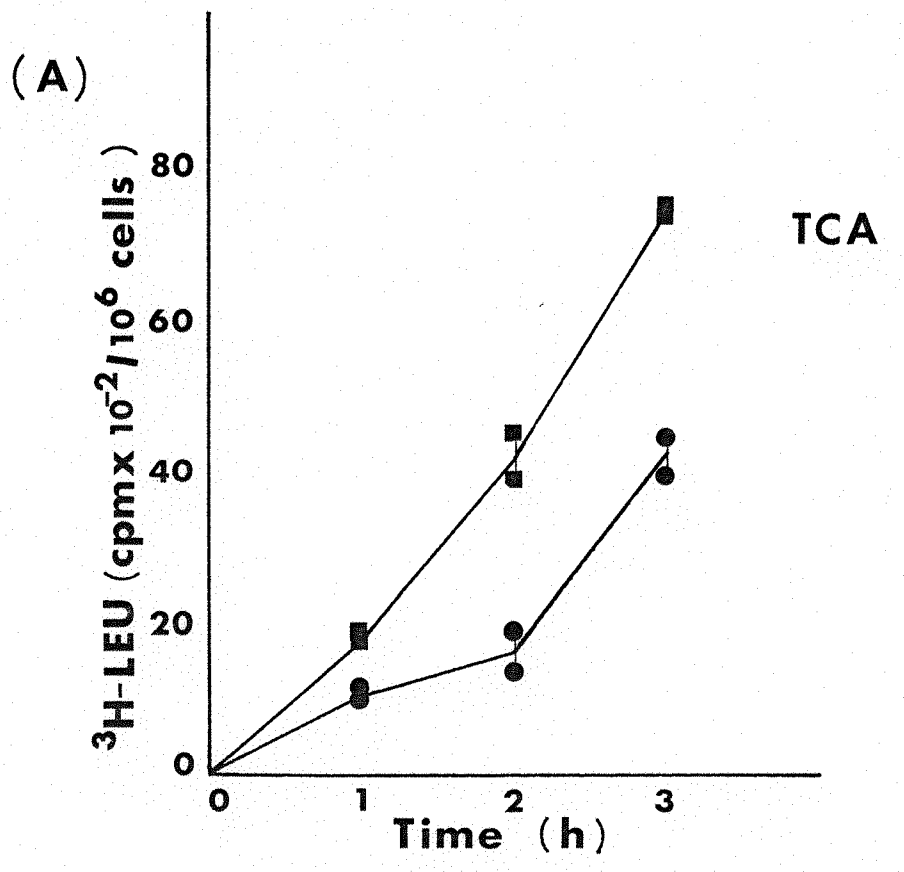
Antibodies to surface Ig always inhibited protein synthesis as measured by radioactive leucine incorporated into cell protein harvested with TCA or by water lysis. Unless stated otherwise, purified antibody directed to the constant region of the λ chain (Fig. 2.2) anti-Fab'γλ was used. Inhibition by antibody is expressed as a percentage, relative to the effect of normal sheep IgG. Incorporation of radioactive leucine in the absence of antibody was linear for up to 6 hours (Fig. 4.1) and in other experiments (Fig. 5.1) up to 8 hours. Some slight stimulation

by normal sheep IgG was repeatedly observed. In the experiment shown in Figure 4.1 inhibition by antibody (0.25 mg/ml) was 70% after 2 hours and 59% after 3 hours. Maximal inhibition was always attained within 2 hours after which it diminished to reach control values usually within 8 hours. Antibody did not affect cell viability which was 99% after 6 hours and 80% after 18 hours in all flasks.

In Figure 4.1 results are expressed as cpm/µg protein. The average protein content of TCA precipitable material in 10^6 L_2C cells over several experiments was 50 µg. Since each aliquot of cell suspension in an experiment contained the same amount of protein, results in subsequent experiments were expressed in cpm/number of cells. ^3H-leucine in TCA precipitable cell material was twice that in material harvested by washing cells on filter paper with water. The loss in counts was consistent as can be seen from the results (Fig. 4.2) of an experiment in which the two methods were compared. The "MASH method" was less time consuming and was adequate for investigating changes in rates of protein synthesis.

The release of a small amount of labelled protein to the culture medium by L_2C cells was not altered by antibody over the period of observation (Fig. 4.3).

Experiments with a wide range of concentrations of antibody showed that the degree of inhibition at a particular time was independent of the concentration of antibody above about 2 µg/ml. Figure 4.4 shows the results of an experiment in which inhibition at 1 hour was reduced to control levels between 0.125 and 0.031 µg/ml. There appeared to be a maximum response by the cells which is triggered by a critical amount of antibody. The actual percentage inhibition by antibody was variable for reasons which were not clear. Cells were used as fresh as possible.


(A)
^{3}H-LEU (cpm x 10^{-2}/10^6 cells)
80
60
40
20
0
0
1
2
3
Time (h)
TCA

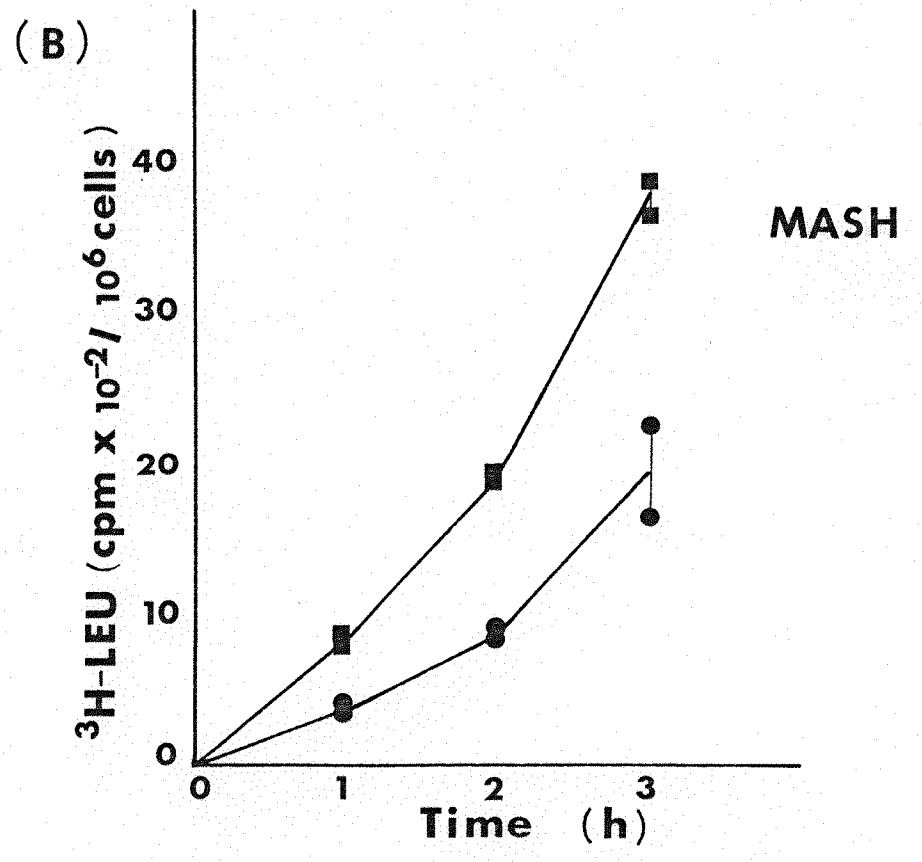
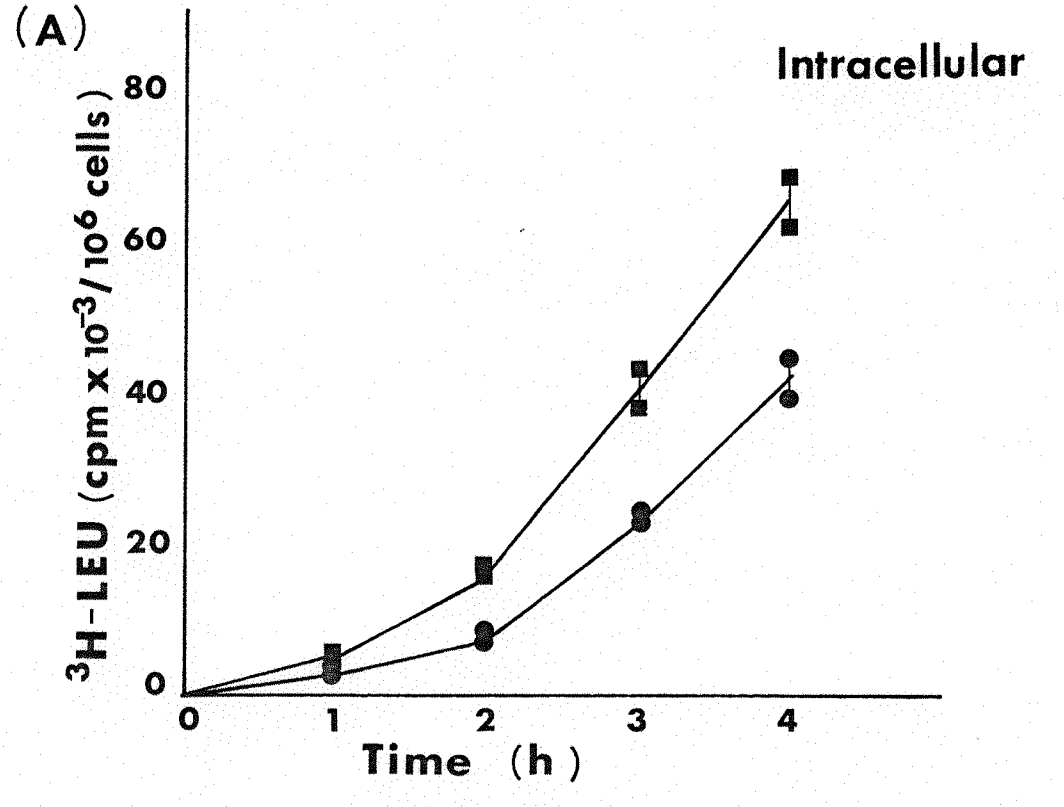

(B)
^{3}H-LEU (cpm x 10^{-2}/ 10^6cells)
40
30
20
10
0
0
1
2
3
Time (h)
MASH

Fig. 4.2 Effect of antibody on protein synthesis in L_2C cell material precipitable by TCA or water lysis

L_2C cells were cultured at 5 x 10^6 cells/ml with ^{3}H-LEU (1 µCi/ml) and anti-Fab$_{\gamma\lambda}$ (0.25 mg/ml) ● or normal sheep IgG (0.25 mg/ml) ■ . Duplicate samples were taken from each culture at intervals to measure ^{3}H-LEU in TCA precipitable cell material (A) using the MASH (B).


(A)
Intracellular
^{3}H-LEU (cpm x 10^{-3}/10^6 cells)
80
60
40
20
0
0
1
2
3
4
Time (h)

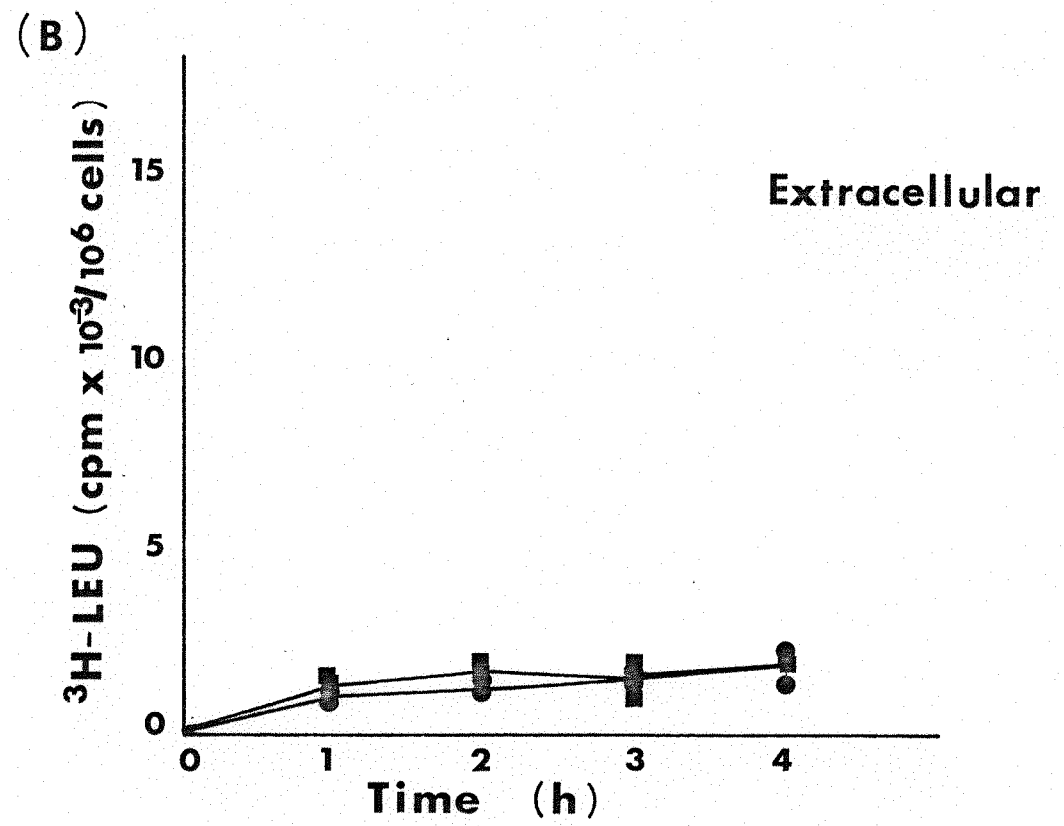

(B)
Extracellular
^{3}H-LEU (cpm x 10^{-3}/10^6 cells)
15
10
5
0
0
1
2
3
4
Time (h)

Fig. 4.3 Effect of antibody on ^{3}H-labelled protein released by L_2C cells

L_2C cells were cultured at 10^7 cells/ml with ^{3}H-LEU (20 μCi/ml) and anti-Fab'$\gamma\lambda$ (0.25 mg/ml) ● or normal sheep IgG (0.25 mg/ml) ■. Duplicate samples were taken from each culture at intervals to measure ^{3}H-LEU intracellularly using the MASH (A) and extracellularly by precipitation with TCA (B).

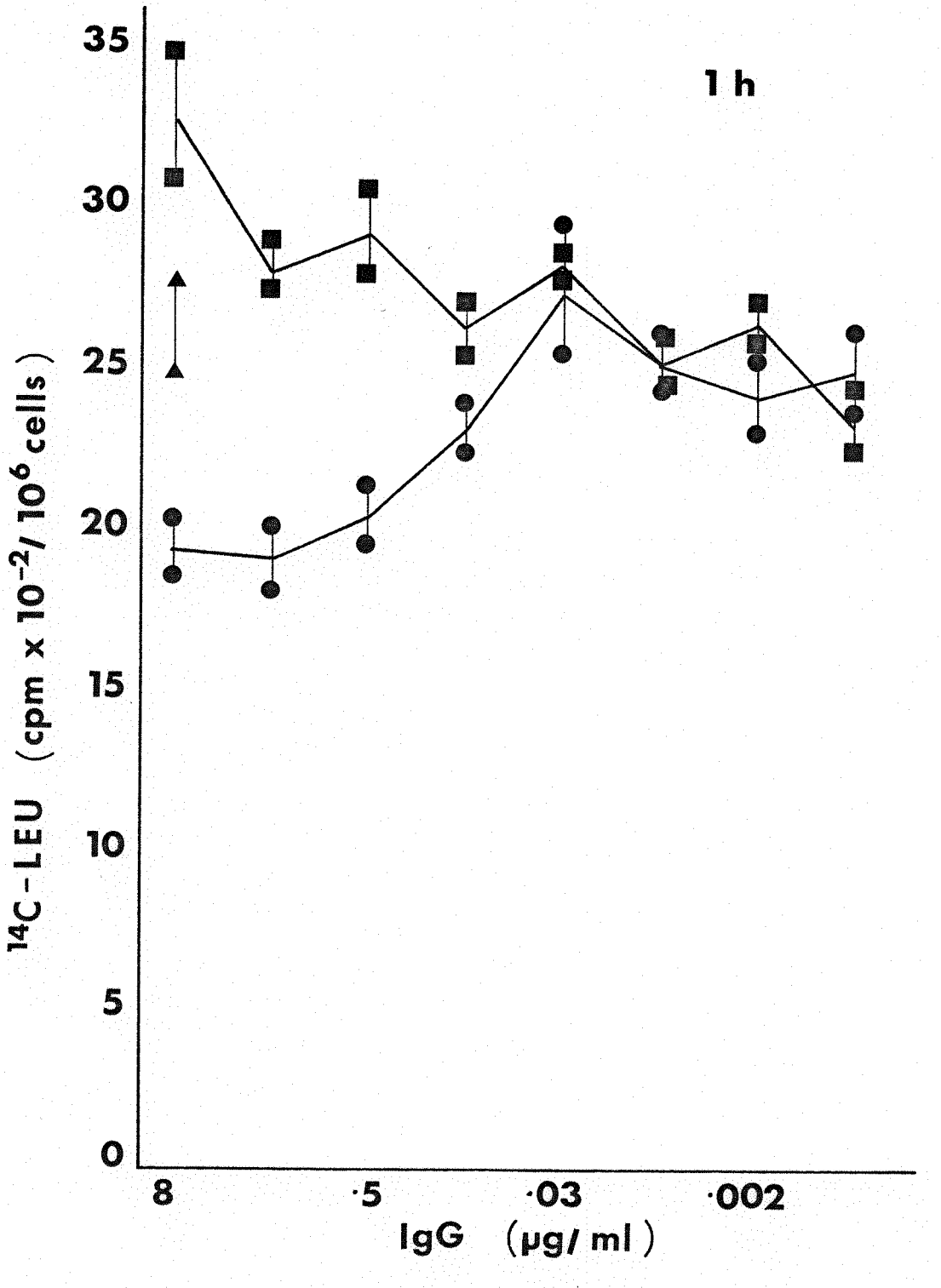


Fig. 4.4 Effect of antibody concentration on protein synthesis in L_2C cells

L_2C cells were cultured at 5 x 10^6 cells/ml with ^{14}C-LEU (0.5 µCi/ml) and anti-Fab'γλ ●, normal sheep IgG ■ or PBS ▲. At 1 h duplicate samples were taken from each culture to measure ^{14}C-LEU in TCA precipitable cell material.

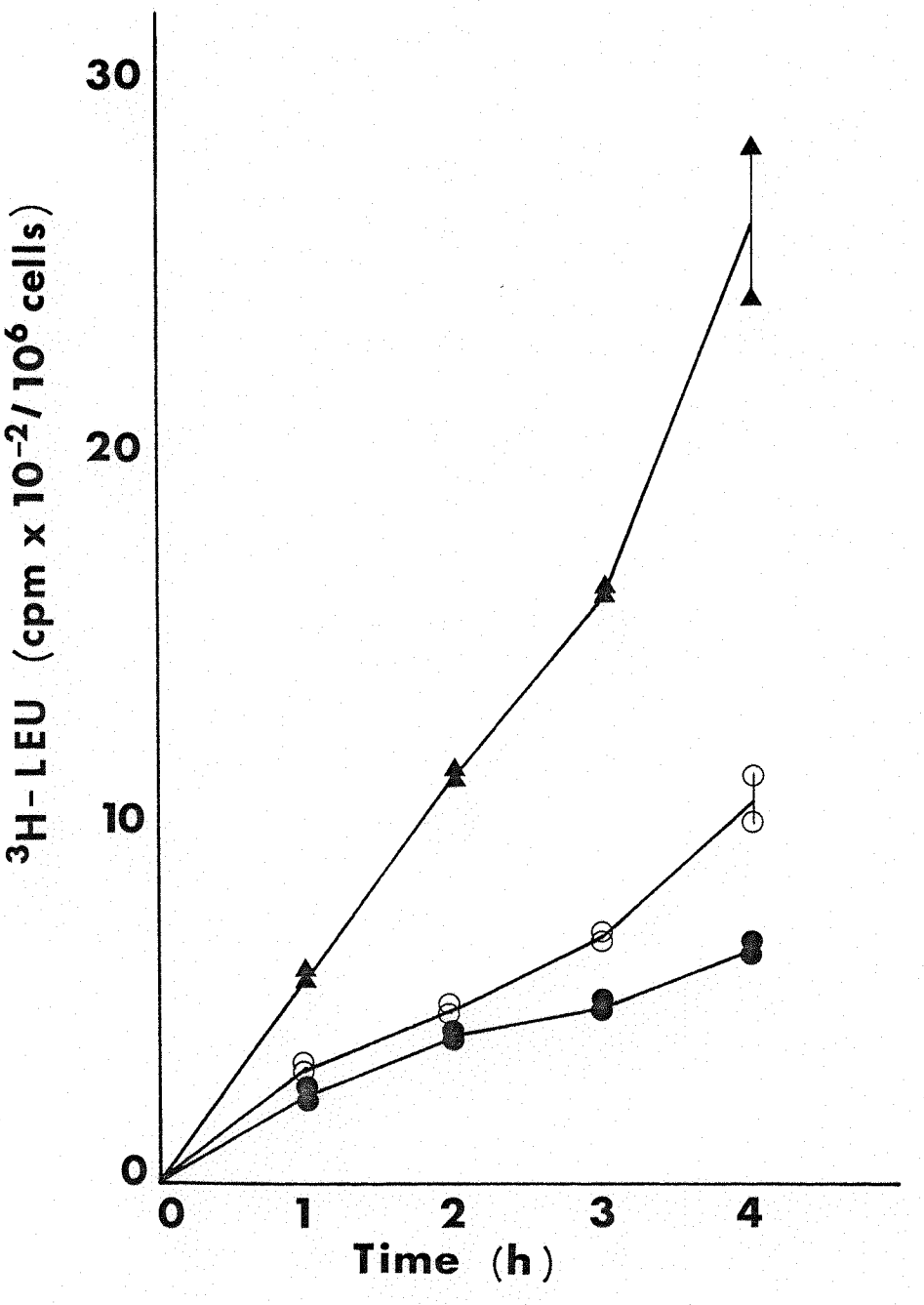


Fig. 4.5 Protein synthesis in L_2C cells after exposure to antibody for 30 min at 37°C

L_2C cells were incubated at 2.5 x 10^7 cells/ml for 30 min at 37°C with anti-Fab'γλ (0.25 mg/ml). Cells were washed 2x and cultured at 5 x 10^6 cells/ml with ^{3}H-LEU (1 μCi/ml) and anti-Fab'γλ (0.25 mg/ml) ● or PBS ○ . As a control, cells were treated similarly in medium ▲ . Duplicate samples were taken from each culture at intervals to measure ^{3}H-LEU using the MASH.

4.3.1.2 Time of exposure to anti-immunoglobulin

In experiments described above, antibody was present in the medium throughout the culture period. It was determined whether this was necessary, or whether a short exposure, followed by removal of antibody was equally effective. L_2C cells were incubated with antibody (0.25 mg/ml) for 30 minutes at 37°C. The cells were then washed twice in the cold with MEM and aliquots put into 2 separate flasks for culture with and without antibody (0.25 mg/ml). The results from one of 3 experiments are shown in Figure 4.5. The inhibition of protein synthesis in cells which had antibody removed from their culture medium was similar to that in cells which had antibody present all the time. Protein synthesis was not depressed in cells treated similarly with normal sheep IgG.

4.3.1.3 Antibodies to various determinants on immunoglobulin

Antibodies to various defined regions on surface immunoglobulin (Fig. 2.2) produced similar inhibitory effects on protein synthesis. The results from 3 separate experiments in which both the degree and kinetics of inhibition were comparable are summarized in Table 4.1. Antibodies to the variable region (anti-Id(λ)) and the constant region (anti-Fab'$\gamma\lambda$) of the λ chain produced identical results in the same experiment. Recognition of light chain was not critical as antibody to heavy chain determinants (anti-Fdμ) was effective. Antibody directed to idiotypic determinants on both the heavy and light chain (anti-Id($\mu\lambda$)) was also inhibitory.

4.3.1.4 Fragments of anti-immunoglobulin

Bivalent $(Fab'\gamma)_2$ from antibody was as efficient as intact IgG in inhibiting protein synthesis. Monovalent Fab'γ which is unable to cross-link surface Ig was considerably less effective in producing inhibition. Fragments were compared with IgG at the same molar concentrations of antibody binding sites. In the experiment shown in Figure 4.6 $(Fab'\gamma)_2$ (4 nmol/ml) from anti-Fab'γ

TABLE 4.1

EFFECTS OF ANTIBODIES TO VARIOUS DETERMINANTS ON IMMUNOGLOBULIN ON PROTEIN SYNTHESIS IN L_2C CELLS

Antibody	mg/ml	% inhibition
Anti-Fab'$\gamma\lambda$	.25	40
Anti-Id(λ)	1	40
Anti-Fdμ	.25	43
Anti-Id($\mu\lambda$)	.5	34

Inhibition of protein synthesis at 1 hour is expressed relative to protein synthesis in culture medium without added IgG. Anti-Fab'$\gamma\lambda$ and anti-Id(λ) were tested in the same experiment.

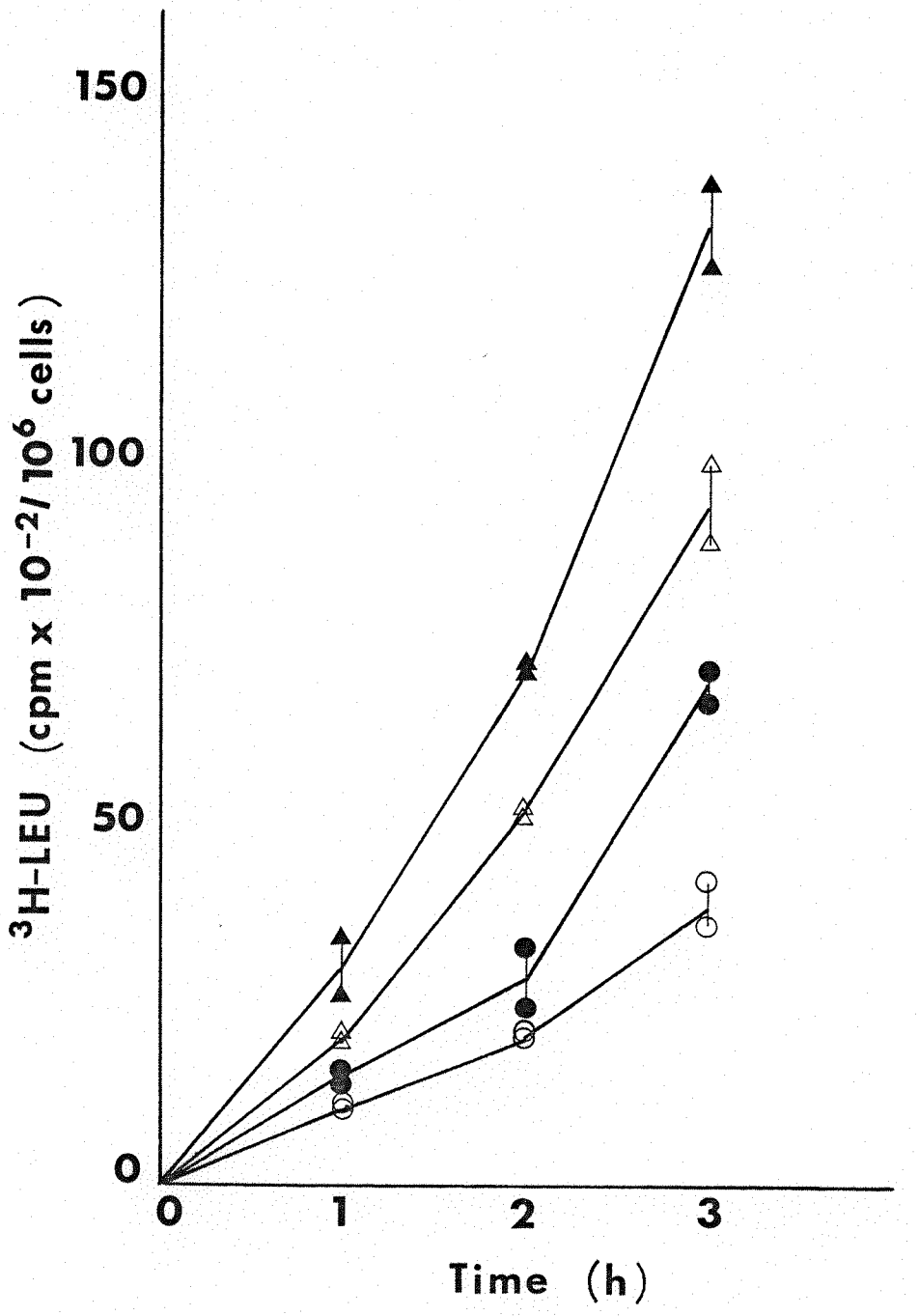


Fig. 4.6 Effect of $(Fab'\gamma)_2$ and $Fab'\gamma$ from antibody on protein synthesis in L_2C cells

L_2C cells were cultured at 5 x 10^6 cells/ml with ^{3}H-LEU (1 µCi/ml) and 4 nmol/ml of anti-$Fab'\gamma$ ● or its fragments $(Fab'\gamma)_2$ ○ or $Fab'\gamma$ △ or PBS ▲ . Duplicate samples were taken from each culture at intervals to measure ^{3}H-LEU in TCA precipitable cell material.

TABLE 4.2

EFFECTS OF FRAGMENTS OF ANTI-IMMUNOGLOBULIN ON PROTEIN SYNTHESIS IN L_2C CELLS

Antibody	nmol/ml	IgG	% inhibition $(Fab'\gamma)_2$	% inhibition $Fab'\gamma$
Anti-Fab'γ	4	59	72	38
	4	41		7
Anti-Id(λ)	40	30	45	
	8	33	51	21

Inhibition of protein synthesis at 2 hours is expressed relative to protein synthesis in culture medium without added IgG.

inhibited protein synthesis in a similar manner to the intact IgG. Slight inhibition was caused by Fab'γ from this antibody but there was no effect with another preparation of Fab'γ from anti-Fab'γ (Table 4.2). Fragments from anti-Id(λ) gave results (Table 4.2) which were consistent with those for anti-Fab'γ. $(Fab'\gamma)_2$ and Fab'γ from normal sheep IgG had no effect other than the slight stimulation seen with the intact IgG.

4.3.1.5 Antibodies to other surface molecules

Antibodies to molecules other than Ig on the cell surface did not affect protein synthesis. β_2 microglobulin and Ia antigens, which are associated with the major histocompatibility complex in guinea pigs were the particular targets investigated. Purified sheep anti-β_2 microglobulin (0.5 mg/ml) which binds to L_2C cells (Stevenson et al, 1978) had no effect on protein synthesis. In the same experiment purified anti-Ig (5 µg/ml) produced 39% inhibition at 1 hour. Similarly in the experiment shown in Figure 4.7 anti-β_2 microglobulin was non-inhibitory in contrast with anti-Ig at a 10 fold lower concentration. Neither IgG fraction (0.5 mg/ml) from guinea pig anti-Ia serum nor normal guinea pig IgG (0.5 mg/ml) affected protein synthesis (Fig. 4.7).

4.3.1.6 Colchicine and cytochalasin B

In chapter 3 it was found that colchicine enhanced and cytochalasin B inhibited migration of L_2C cells. The rate of protein synthesis was unaltered by colchicine but sensitive to inhibition by cytochalasin B. Exposure of cells to colchicine (10^{-5} M) for 30 minutes at 37°C before the addition of antibody and ^{3}H-leucine did not affect protein synthesis nor abrogate inhibition by antibody (Fig. 4.8). It can be seen from Figure 4.9 that low concentrations of cytochalasin B (1 µg/ml) inhibited protein synthesis. Cell viability was not affected by cytochalasin B within 4 hours.

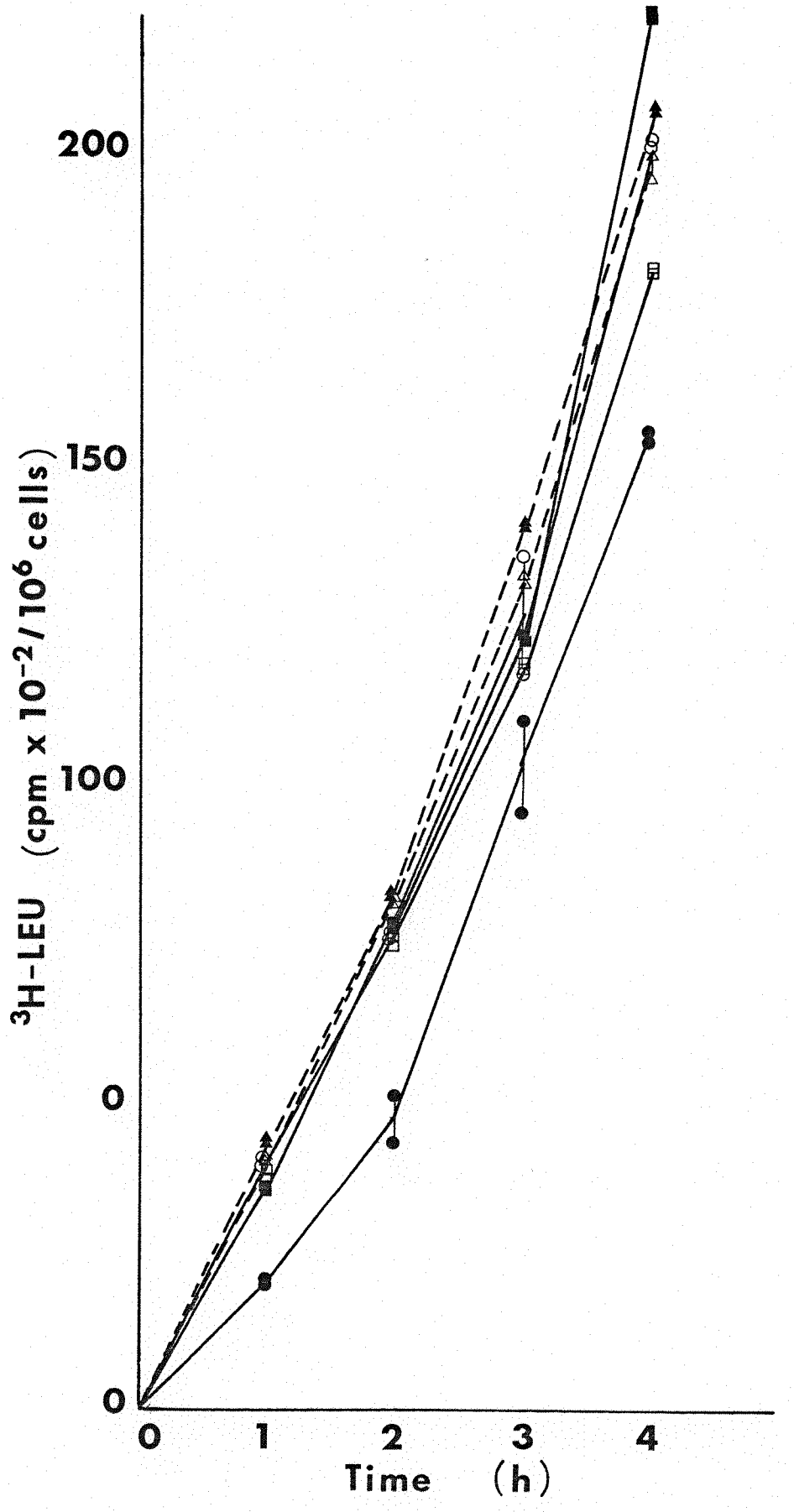

200
150
100
0
0
^{3}H-LEU (cpm x 10^{-2} / 10^6 cells)
0
1
2
3
4
Time (h)

Fig. 4.7 Effect of anti-β_2 microglobulin and anti-Ia on protein synthesis in L_2C cells

L_2C cells were cultured at 5×10^6 cells/ml with ^{3}H-LEU (1 μCi/ml) and anti-β_2 microglobulin (0.5 mg/ml) ■ , normal sheep IgG (0.5 mg/ml) □ , anti-Ia (0.5 mg/ml) ▲ , normal guinea pig IgG (0.5 mg/ml) △ , anti-Fab'γ χ(50 μg/ml) ● or PBS ○ . Duplicate samples were taken from each culture at intervals to measure ^{3}H-LEU in TCA precipitable cell material.

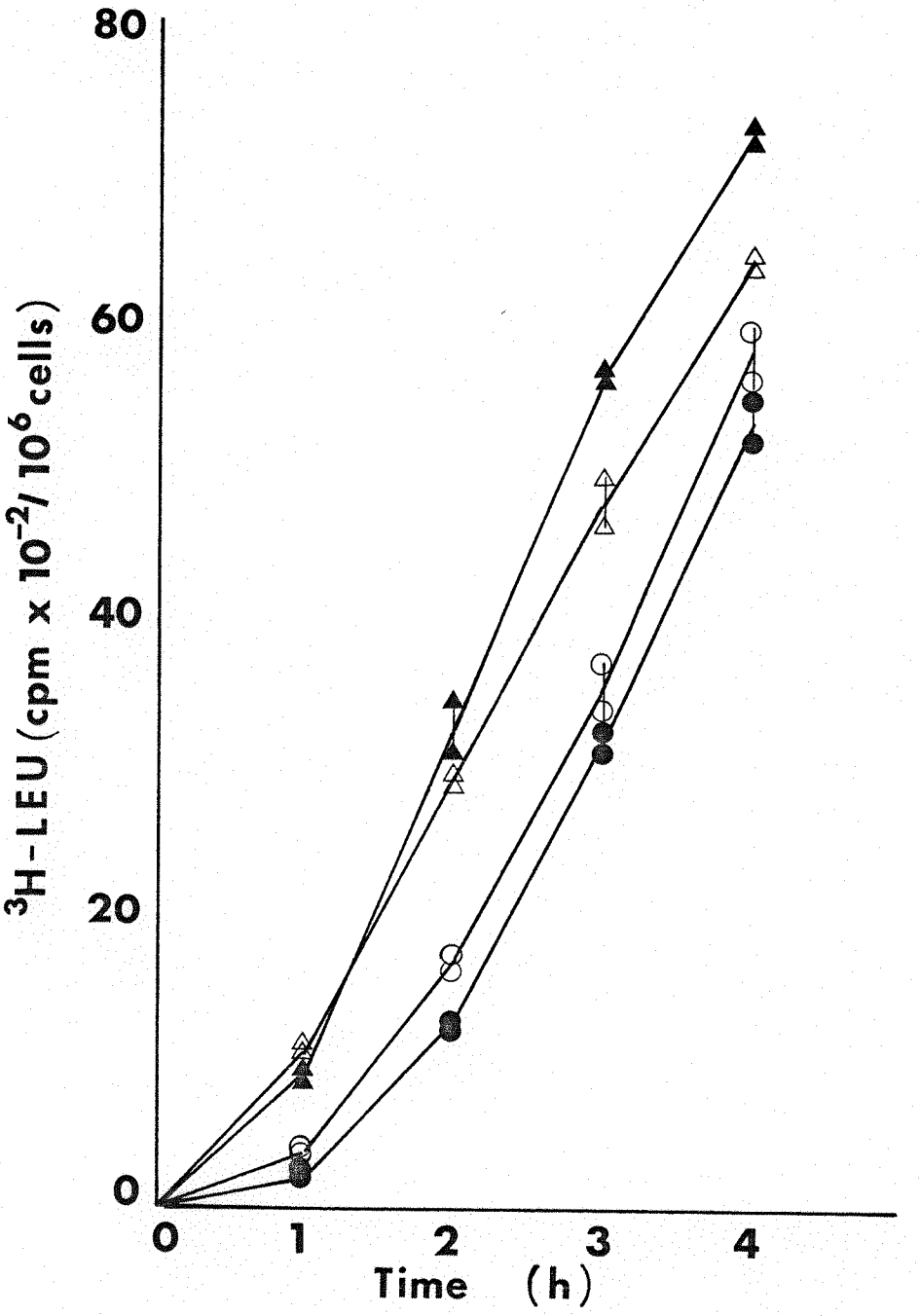


Fig. 4.8 Effect of colchicine on antibody-induced inhibition of protein synthesis in L_2C cells

L_2C cells were cultured at 5 x 10^6 cells/ml in culture medium with ^{3}H-LEU (1 µCi/ml) and colchicine (10^{-5} M) △ , colchicine (10^{-5} M) with the addition of anti-Fab'γλ (0.25 mg/ml) after 30 min ○ , anti-Fab'γλ (0.25 mg/ml) ● or PBS ▲ . Duplicate samples were taken from each culture at intervals to measure ^{3}H-LEU in TCA precipitable cell material.

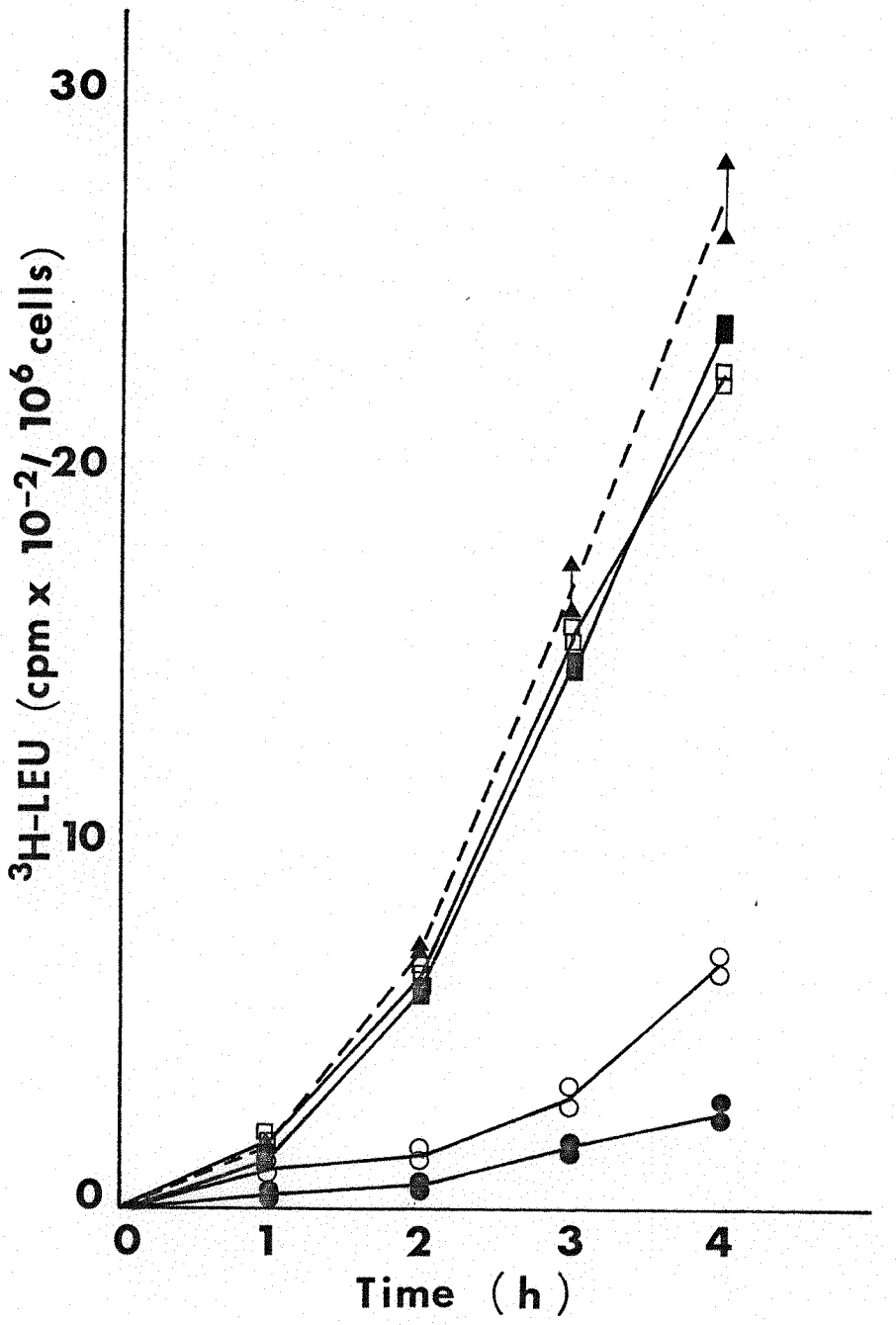


Fig. 4.9 Effect of cytochalasin B on protein synthesis in L_2C cells

L_2C cells were cultured at 5 x 10^6 cells/ml with ^{3}H-LEU (1 µCi/ml) and 10 fold dilutions of cytochalasin B (10 µg/5µl DMSO) at 10 µg/ml ● , 1 µg/ml ○ , 0.1 µg/ml ■ or 0.01 µg/ml □ ; DMSO (5 µl/ml) ▲ .

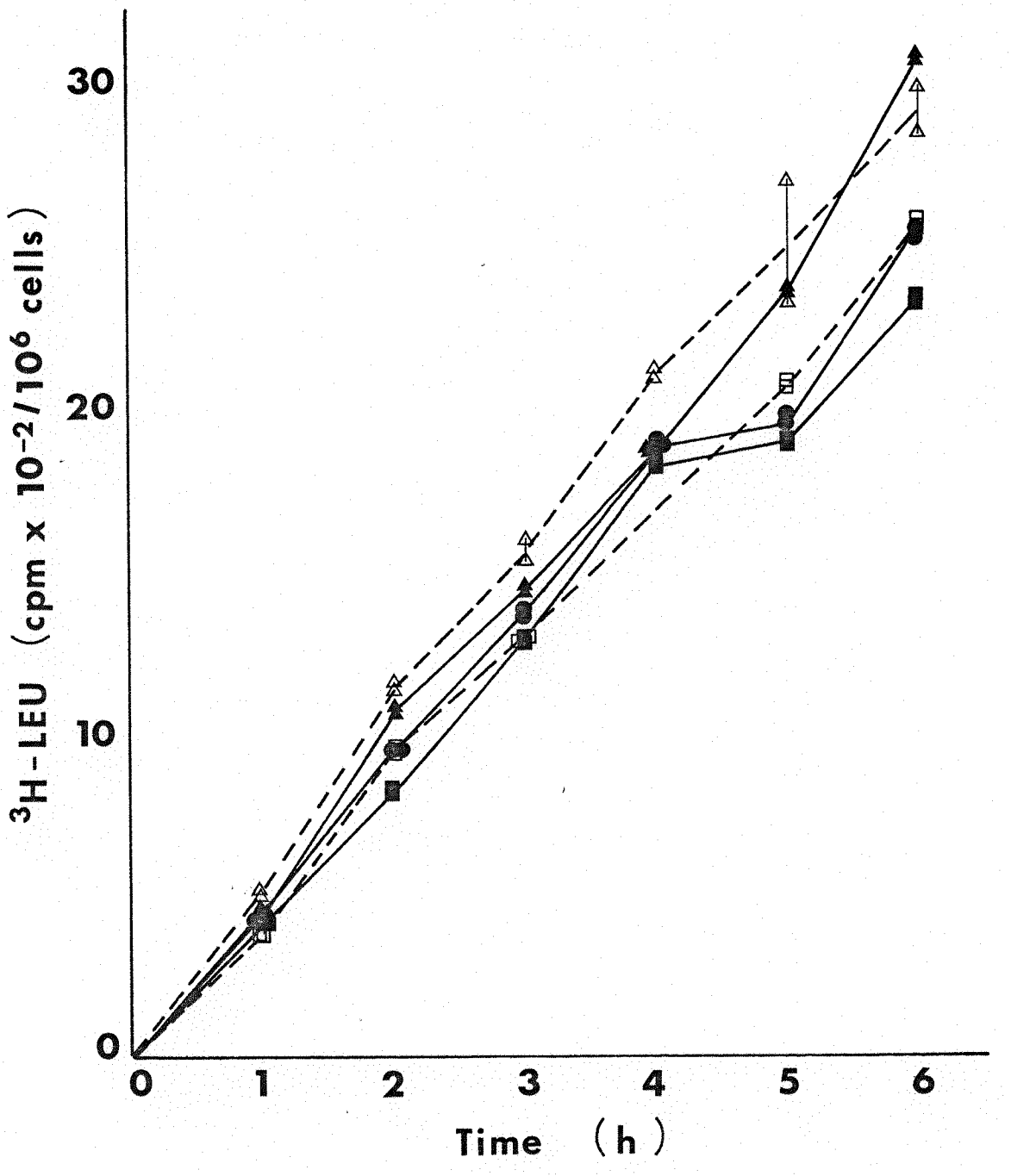


Fig. 4.10 Effect of antibody on protein synthesis in normal guinea pig lymphocytes

T-cell depleted lymph node cells (60% B cells) from normal guinea pigs were cultured at 3.8 x 10^6 cells/ml with ^{3}H-LEU (4 µCi/ml) and IgG (0.2 mg/ml) with or without 2-mercaptoethanol (50 µM); anti-µ ▲, anti-µ and 2-mercaptoethanol △, anti-Fab'γλ ●, normal sheep IgG ■ or normal sheep IgG and 2-mercaptoethanol □. Duplicate samples were taken at intervals to measure ^{3}H-LEU using the MASH.

4.3.1.7 Cycloheximide

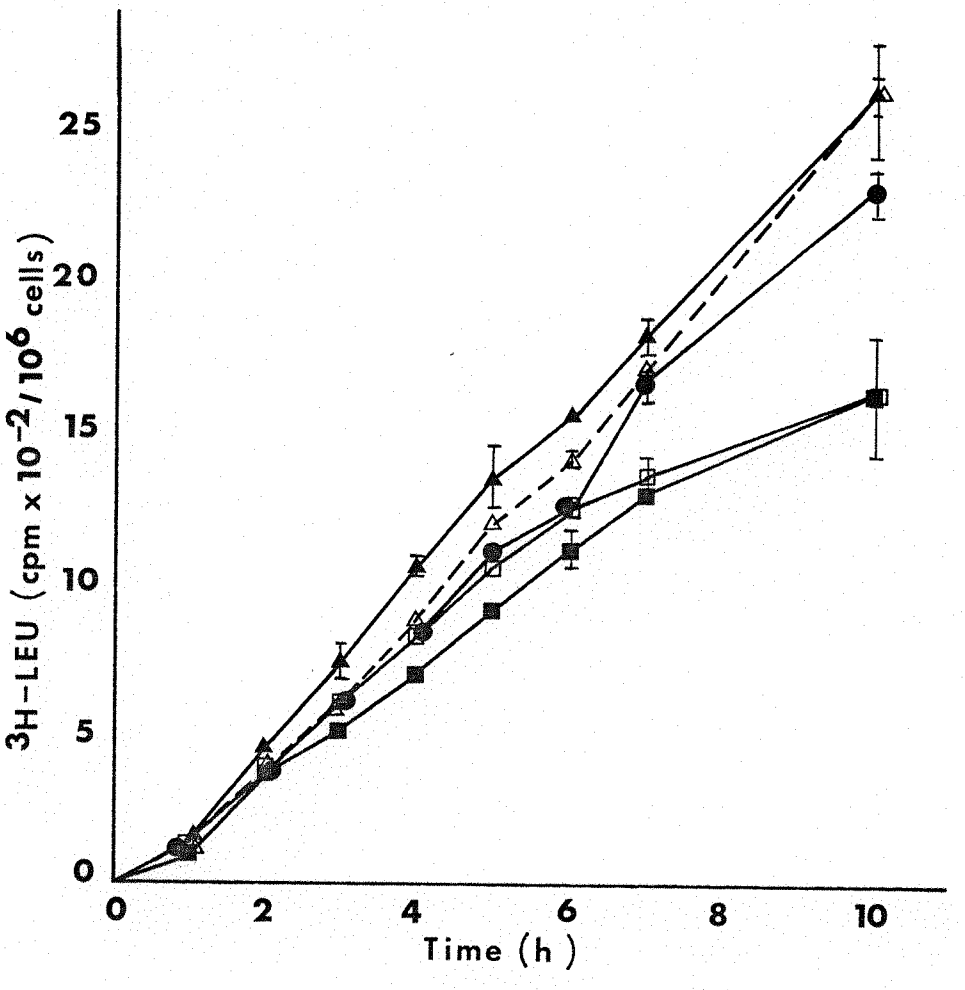
The effect of cycloheximide, a conventional inhibitor of protein synthesis differed from that of antibody. The presence of cycloheximide (10 μg/ml) in the culture medium immediately reduced protein synthesis to a very low level and no recovery from inhibition was observed within 3 hours.

4.3.2 Effect of anti-immunoglobulin on protein synthesis in normal guinea pig lymphocytes

Normal guinea pig T cell-depleted lymph node cells containing 60% B cells were prepared as described in 2.5.3.2. Antibody did not have any inhibitory effect on protein synthesis in these cells. In fact, in the presence of antibody protein synthesis in these cells was as great or greater than in absence of antibody. Figure 4.10 shows the results of an experiment in which antibody specific for μ chains enhanced protein synthesis. Anti-Fab'γλ which reacts with cells via the λ chain (Fig. 2.2) would not affect all B cells in a normal population and did not have any observable effect on protein synthesis. However, both anti-μ and anti-Fab'γλ maintained cell viability at 90% for 6 hours whereas it dropped to 85% in the presence of normal sheep IgG.

In Figure 4.10 it can be seen that the addition of 2-mercaptoethanol (50 μM) to the culture medium did not significantly increase the effect of anti-μ.

(A)


25
20
15
10
5
0
0
2
4
6
8
10
Time (h)
^{3}H-LEU (cpm x 10^{-2}/10^6 cells)

(B)

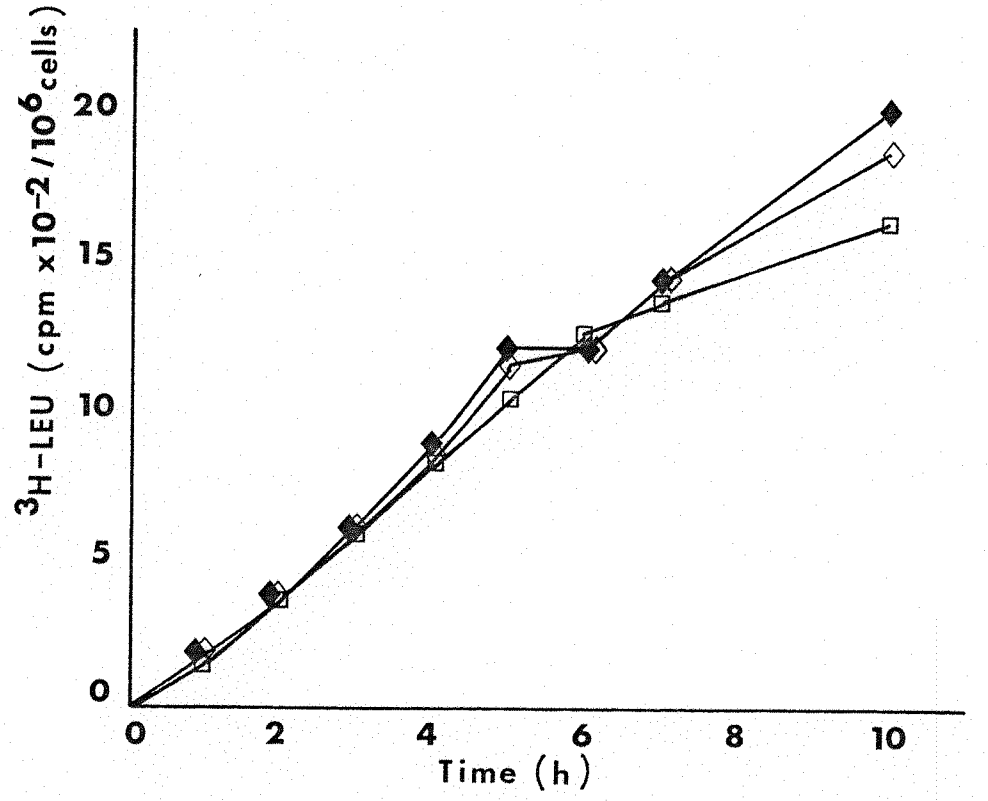

20
15
10
5
0
0
2
4
6
8
10
Time (h)
^{3}H-LEU (cpm x 10^{-2}/10^6 cells)

Fig. 4.11 Effect of antibody on protein synthesis in CLL cells

CLL cells were cultured at 10^7 cells/ml with ^{3}H-LEU (4 μCi/ml) and (A) anti-Fdμ (0.125 mg/ml) ▲ , anti-Fdδ (0.25 mg/ml) △ , anti-Fab'γ(λ) (0.125 mg/ml) ● , normal sheep IgG (0.25 mg/ml) ■ , (B) anti-β_2 microglobulin (0.25 mg/ml) ◆ , normal rabbit IgG (0.25 mg/ml) ◇ ; PBS □ . Duplicate samples were taken from each culture at intervals to measure ^{3}H-LEU using the MASH. (A) and (B) represent results from the same experiment.

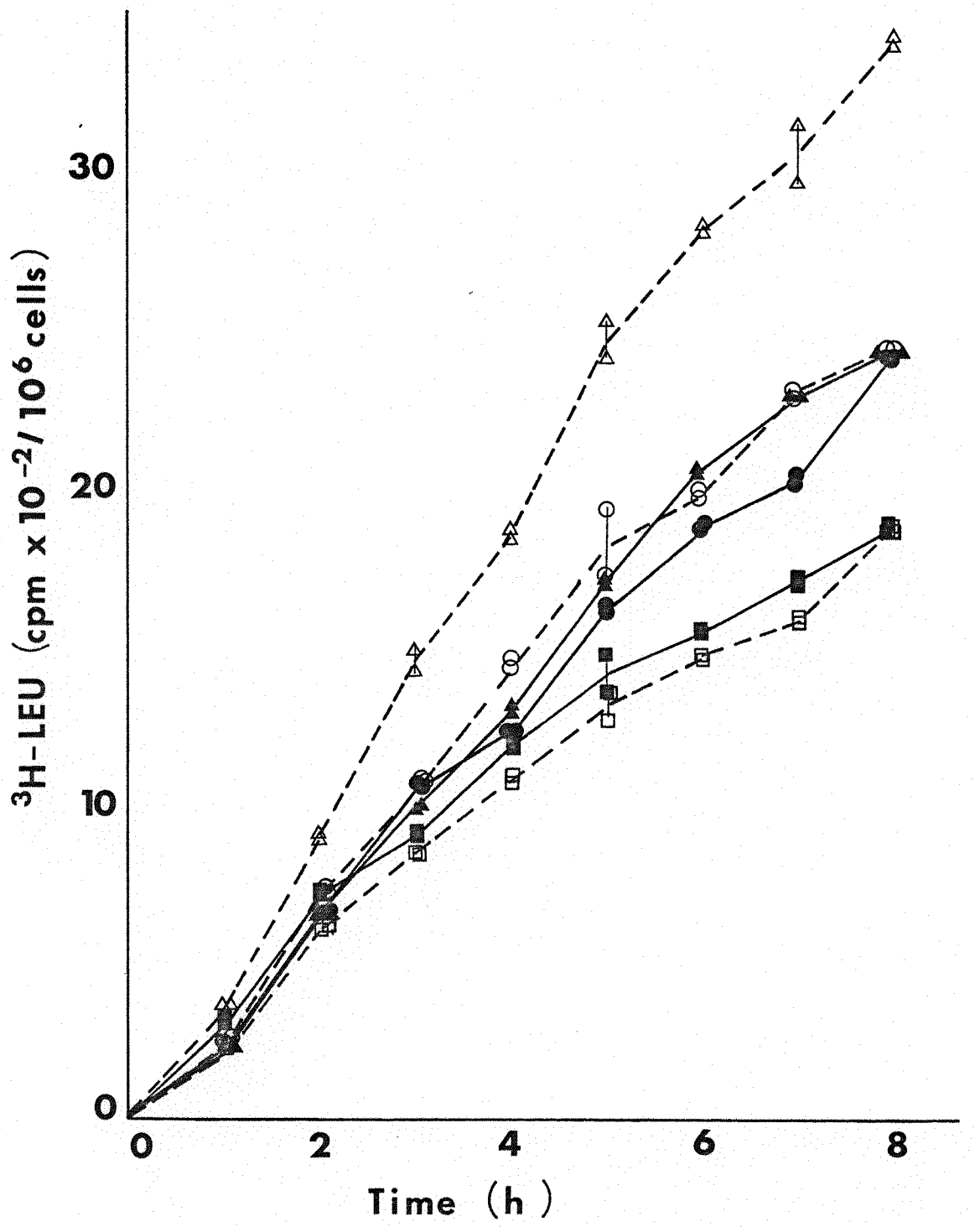


Fig. 4.12 Effect of antibody and 2-mercaptoethanol on protein synthesis in CLL cells

CLL cells were cultured at 10^7 cells/ml with ^{3}H-LEU (4 μCi/ml) and IgG (0.125 mg/ml) with or without 2-mercaptoethanol (50 μM); anti-Fdμ ▲, anti-Fdμ and 2-mercaptoethanol △, anti-Fab'γ(λ) ●, anti-Fab'γ(λ) and 2-mercaptoethanol ○, normal sheep IgG ■ or normal sheep IgG and 2-mercaptoethanol □. Duplicate samples were taken from each culture at intervals to measure ^{3}H-LEU using the MASH.

4.3.3 Effects of antibody on protein synthesis in CLL cells

Cells from 4 different human patients with CLL had IgM and IgD with κ or λ light chains on their surfaces. Antibody produced effects in these cells similar to those seen in normal guinea pig lymphocytes. The results from 2 experiments with cells from the same patient are shown in Figures 4.11 and 4.12. Anti-Fdμ, anti-Fdδ and anti-Fab'γ(λ) enhanced protein synthesis to much the same extent (4.11A). Cell viability was over 90% in the presence of anti-Ig and 80% in the normal sheep IgG control after a 24 hour incubation. Other CLL cells showed similar rates of protein synthesis and the same trends with antibody. With the cells from 1 patient known to have very little surface Ig, no effect of antibody was observed. Rabbit antibody to human β_2 microglobulin had no effect on protein synthesis above that of normal rabbit IgG (Fig. 4.11B).

There was an increase in the effect of anti-Fdμ when 2-mercaptoethanol (50 μM) was added to the culture medium (Fig. 4.12). This did not correlate with any change in cell viability.

4.3.4 Effect of anti-immunoglobulin on immunoglobulin synthesis

The effect of antibody on Ig synthesis was investigated to ascertain whether the inhibition of protein synthesis extended to Ig synthesis. It was found that Ig was included among proteins which had their synthesis inhibited by antibody. Figure 4.13 shows the results from an experiment in which the effect of antibody on the synthesis of Ig precipitable with anti-Fab'γλ and anti-μ was investigated. Inhibition produced by antibody in specific Ig synthesis (A) can be compared with that in total protein synthesis from the same experiment (B). Specifically precipitated Ig represented 1% of the radioactivity in TCA precipitable material in cell lysates. Anti-Fab'γλ

and anti-μ specifically precipitated similar amounts of radioactivity in cell lysates. From results in Figure 4.13A it can be seen that antibody inhibited the synthesis of Ig precipitable by anti-Fab'γλ and anti-μ to the same extent. In Table 4.3 the results of 3 similar experiments in which the effect of antibody on the synthesis of Ig precipitable with anti-Fab'γλ was investigated are presented. The depression of Ig synthesis is as great as that observed in total protein. At 4 hours the inhibition of Ig synthesis is greater than that of total protein. After 4 hours the radioactivity in non-specific precipitates increased disproportionately and obscured specific Ig synthesis.

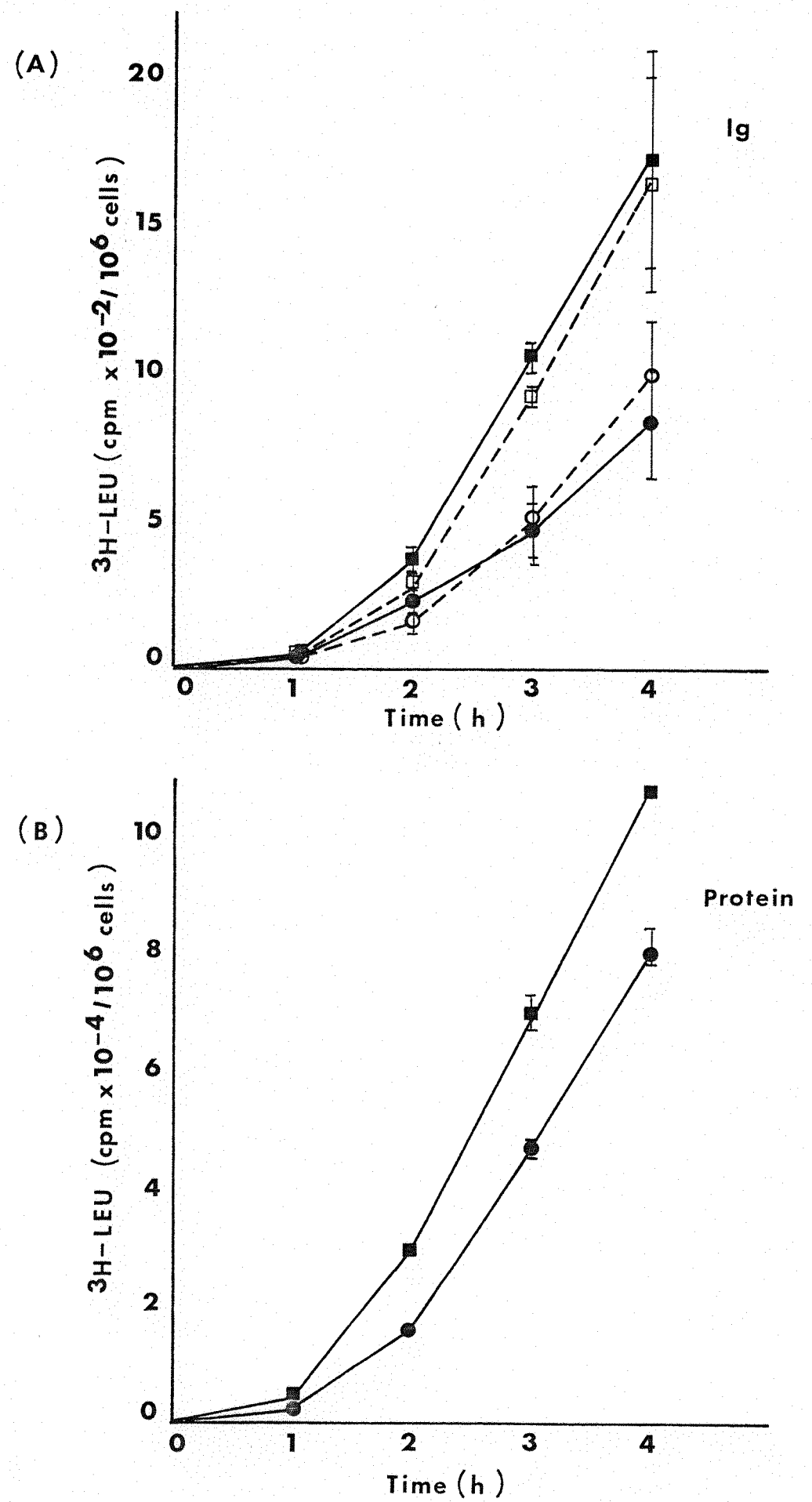

(A)
20
15
10
5
0
^{3}H-LEU (cpm x 10^{-2}/ 10^6 cells)
Ig
0
1
2
3
4
Time (h)
(B)
10
8
6
4
2
0
^{3}H-LEU (cpm x 10^{-4}/ 10^6 cells)
Protein
0
1
2
3
4
Time (h)

Fig. 4.13 Effect of antibody on immunoglobulin synthesis in L_2C cells

L_2C cells were cultured at 10^7 cells/ml with ^{3}H-LEU (20 μCi/ml) and anti-Fab'γλ (0.25 mg/ml) circles, or normal sheep IgG (0.25 mg/ml) squares. ^{3}H-LEU was measured in Ig (A) by precipitation with anti-Fab'γλ closed symbols, or anti-μ open symbols (non-specific counts were subtracted and error bars represent the S.E. of the difference between means of triplicate precipitations) and in protein (B) using the MASH (ranges of triplicates are plotted).

TABLE 4.3

EFFECT OF ANTIBODY ON PROTEIN AND IMMUNOGLOBULIN SYNTHESIS IN L_2C CELLS

	% inhibition		
Time (h)	Protein	Ig	Probability
1	49 ± 6.6	47 ± 9.2	Not significant
2	41 ± 8.7	44 ± 9.6	Not significant
3	30 ± 10.4	45 ± 3.3	Not significant
4	24 ± 7.3	57 ± 6.2	< .05

Inhibition by anti-Fab'$\gamma\lambda$ (0.25 mg/ml) is expressed as a percentage relative to the effect of normal sheep IgG (0.25 mg/ml). Results are the mean ± S.E. derived from the means of 3 experiments. Ig is defined as the difference between precipitation with anti-Fab'$\gamma\lambda$ and with normal sheep IgG. Protein was measured using the MASH.

4.4 Discussion

L_2C leukaemic cells which have the appearance of lymphoblastoid B cells actively synthesize proteins *in vitro* as indicated by the incorporation of radioactive leucine. When L_2C cells were cultured in the presence of antibody to various regions of surface IgM, protein synthesis was temporarily inhibited. Maximum inhibition could be achieved with low concentrations of antibody and no further inhibition was observed by increasing antibody concentration. This suppressive effect of antibody on protein synthesis was not observed in normal adult guinea pig lymphocytes nor in CLL cells. In fact, in these cells enhancement of protein synthesis was observed by treatment with antibody to surface Ig. This may have been due to protection of cell viability, ie. the antibody is to some extent acting as a growth factor.

The most reasonable explanation for variations in behaviour by the cell populations studied is a difference in maturity. The class of Ig expressed on the surface of a cell is related to the stage of differentiation (Vitetta and Uhr, 1975). It is believed that B cells first express IgM (Vitetta et al, 1975) and the mature antigen-sensitive lymphocyte may display both IgM and IgD (Vitetta and Uhr, 1976). The acquisition of IgD is believed to influence the way in which cells respond to antibody (Kettman et al, 1979). The absence of IgD on L_2C cells and its presence on CLL cells and normal adult guinea pig B cells may be the reason for differences seen in cell behaviour.

Evidence that the ability to respond to antibody in a particular way is dependent on the state of maturation of the B cell and not just the expression of IgD comes from the experiments of Sidman and Unanue (1978b). They found that DNA synthesis in B cells from young mice which bear mainly IgM was susceptible to inhibition by antibody whereas stimulation occurred in phenotypically similar IgM-bearing cells from adult mice.

The inhibitory effect of antibody on re-expression in L_2C cells (Glennie et al, 1979) suggests that it behaves as an immature B cell (Raff et al, 1975; Sidman and Unanue, 1975).

The inhibitory signal by antibody to L_2C cells was delivered within 30 minutes at 37°C and the continuing presence of antibody to the culture medium produced no further inhibitory effect. This could be due to there being insufficient Ig molecules re-expressed (Glennie et al, 1979) for repeated interaction with antibody.

An investigation of the fragments of antibody capable of producing inhibition of protein synthesis in L_2C cells showed that bivalent $(Fab'\gamma)_2$ was as efficient as intact IgG. Monovalent $Fab'\gamma$ was considerably less effective in producing inhibition than bivalent antibody. As the inhibition of protein synthesis in L_2C cells was exquisitely sensitive to antibody, a slight effect caused by $Fab'\gamma$ preparation was probably due to residual IgG. Other biological effects of antibody on cells such as induction of DNA synthesis require a bivalent ligand (Weiner et al, 1976b). However, as $Fab'\gamma$ has been reported to inactivate immature B cells (Raff et al, 1975; Sidman and Unanue, 1975) and also to be endocytosed (de Petris and Raff, 1973) the possibility remains that $Fab'\gamma$ is capable of sending a signal to the cell interior.

The observation that $(Fab'\gamma)_2$ gave similar results to whole IgG suggests a lack of influence by the Fc piece in antibody-induced inhibition of protein synthesis in L_2C cells. This is consistent with the fact that they have no significant $Fc\gamma$ receptors (Stevenson et al, 1975a). The Fc piece by binding to the $Fc\gamma$ receptor has been implicated in regulating effects by antibody (Sidman and Unanue, 1976; Scribner et al, 1978). As whole antibody was used in experiments with CLL cells, Fc binding to $Fc\gamma$ receptors which are present on these cells may have been involved.

Cross-linking of other surface receptors, β_2 microglobulin and Ia antigens both associated with the major histocompatibility complex did not produce inhibition of protein synthesis in L_2C cells. Similarly, in CLL cells antibody binding to β_2 microglobulin had no effect on protein synthesis. Whether the inability of β_2 microglobulin and Ia antigen to convey the same signal to the cytoplasm as surface Ig is related to the fact that they fail to form transmembrane linkages after binding antibody or some other reason cannot be decided. It is not yet clear whether all receptors are able to form transmembrane linkages. Singer and co-workers (Singer et al, 1978) are firmly convinced of the generality of the phenomenon and describe the linkage of β_2 microglobulin to actin after antibody binding in human fibroblasts. Schreiner and Unanue (1977) maintain that only certain receptors form an association with actin excluding H2 (Braun et al, 1978a). Possibly, the specificity of the signal by anti-Ig relies on an efficient endocytotic mechanism with no or inefficient endocytosis of other surface complexes. Lack of endocytosis of β_2 microglobulin-antibody complexes has been observed in this laboratory (F.K. Stevenson, personal communication).

The transmembrane linkage is believed to involve linkage to microfilaments. As mentioned before (Chapter 3), experiments using cytochalasin B as an inhibitor of microfilament action were confounded by the observation that protein synthesis in L_2C cells is sensitive to inhibition by the drug. This suggests that cytochalasin B is exerting its effect on L_2C cells by some means other than causing microfilament dysfunction, possibly by inhibiting glucose uptake (Lin et al, 1978). Prevention of microtubule polymerization with colchicine had no effect on antibody-induced inhibition of protein synthesis in L_2C cells which is in accordance with the idea of antibody acting via a transmembrane linkage not directly involving microtubules.

The effect of antibody on cells may be altered by the addition

of 2-mercaptoethanol to the culture medium (Sidman and Unanue, 1978a; Sieckmann et al, 1978a). 2-mercaptoethanol can generate a factor in serum which enables antibody specific for μ to stimulate DNA synthesis and abrogate the inhibitory effect of antibody to μ, δ or light chain on LPS-induced mitogenesis (Sidman and Unanue, 1978a). In our experiments with CLL cells the addition of 2-mercaptoethanol to the culture medium resulted in a further increase in protein synthesis in the presence of anti-μ. The stimulatory effect was not seen with anti-Fab'γ(λ) and further investigation is required to determine the influence of IgD present on these cells and to correlate these effects on protein synthesis with those on DNA synthesis reported in the literature. The effect of anti-μ on normal guinea pig lymphocytes was not significantly increased by the addition of 2-mercaptoethanol to the medium but further work is required before conclusions can be drawn concerning these cells.

Synthesis of Ig in L_2C cells as measured by precipitation with specific antibody was inhibited by antibody as least as much as general protein synthesis. The percentage of protein synthesis represented by Ig (1%) compares well with values (1 - 3%) for B cells which do not actively secrete Ig (Melchers and Andersson, 1973). Antibodies specific for μ and for λ chains both precipitated the same amount of labelled Ig suggesting that there is very little free light chain in the cell. This could possibly be due to rapid secretion of excess light chain which has been detected in the urine of leukaemia-bearing animals (Stevenson et al, 1975b). The inhibition of Ig synthesis could possibly be more long lasting than the effect on general protein synthesis although the technique did not allow investigation of times beyond 4 hours due to a disproportionate increase in non-specifically precipitated material. In L_2C cells antibody inhibited the delivery of Ig but not of Ia antigen nor GPLA (B1) (another histocompatibility antigen) to the cell surface (Glennie et al, 1979). This could possibly be due to specific inhibition of Ig synthesis.

The main points to be gathered from this chapter are firstly, that cross-linking of surface Ig on L_2C cells can produce an inhibitory effect on protein synthesis not achieved by binding to other surface molecules, β_2 microglobulin and Ia antigens. The second point is that the effect of binding to the Ig receptor may not be the same in cells at different stages of differentiation.

CHAPTER 5
EFFECT OF ANTIBODY-DIPHTHERIA TOXIN CONJUGATE ON L_2C CELLS

5.1 Introduction

Antibody linked to a cytotoxic drug has potential use in the treatment of tumours. Antibody should provide the specificity lacked by the drug. Difficulties encountered in the generation of a tumour specific cytotoxic agent include preparation of a tumour specific antibody and a method of conjugating it to a drug which does not damage antibody binding sites. One approach to conjugation is to react antibody directly with drug. This approach was used by Dullens, de Weger, Vennegoor and Den Otter (1979) who found that a complex of antibody and chlorambucil had more anti-tumour effect than the combined effects of the unlinked components on a murine melanoma. To avoid damage to Ig which may be incurred by direct conjugation other attempts to generate a specific cytotoxic drug have been made by linking drug to a carrier and then to antibody. A conjugate of antibody and the drug p-phenylenediamine mustard linked by using polyglutamic acid as the bridge was more effective at suppressing tumour growth in mice than uncombined drug and Ig given together (Rowland, O'Neill and Davies, 1975). This group did not report the effect of conjugating the drug to normal IgG. Conjugation of antibody to diphtheria toxin by indirect methods was reported by Moolten, Capparell, Zajdel and Cooperband (1975). Some therapeutic effects by these antibody-diphtheria toxin conjugates against a virus-induced lymphoma in hamsters were observed. A method of conjugating antibody to diphtheria toxin by using chlorambucil as a drug carrier was developed by Thorpe, Ross, Cumber, Hinson, Edwards and Davies (1978). The conjugate of diphtheria toxin and anti-lymphocytic globulin was able to kill human lymphoblastoid cells at one-thousandth the dose necessary for free toxin. Furthermore, conjugation of antibody to normal IgG decreased the toxicity of diphtheria toxin for the cells.

Diphtheria toxin (M.W. 60,000) is produced by Corynebacterium diphtheriae which grows in the upper respiratory tract. Animals

which are susceptible including guinea pig are killed within a few days after injection of 100 ng or less per kilo. The mode of action of diphtheria toxin was described by Pappenheimer (1978). The molecule can be split into two fragments, A and B which have different functions. The A chain enters the cell and stops protein synthesis by catalysing the inactivation of the elongation factor which is required for translocation of the polypeptidyl-t-RNA along the ribosome. The B chain recognizes and interacts with specific receptors thought to be glycoproteins (Draper, Chin and Simon, 1978) that are present on sensitive cells including guinea pig lymph node cells (Proia, Hart, Holmes, Holmes and Eidels, 1979).

To produce an efficient specific cytotoxic agent the mechanism of conjugating antibody to diphtheria toxin must allow dissociation of the A chain from the B chain. Thorpe et al (1978) report a chemical coupling procedure which avoids the formation of intrachain bonds. In brief, anti-lymphocytic globulin was first conjugated at $4^{\circ}C$ to a mixed anhydride prepared from chlorambucil. The mustard groups of chlorambucil were activated by raising the temperature to 25 - $30^{\circ}C$ and the conjugate allowed to react with the toxin. After gel filtration on Sephadex G-150 a conjugate containing diphtheria toxin and antibody in a 1:1 molar ratio was isolated.

There may be difficulties in using antibody-diphtheria toxin conjugates in man because of the presence of antibodies. The possibility of substituting abrin, a plant toxin for diphtheria toxin is being investigated by the group at the Chester Beatty Research Institute (personal communication).

5.2 Materials and Methods

Sheep anti-Fab'$\gamma\lambda$ and normal sheep IgG prepared at this laboratory were conjugated to diphtheria toxin at the Chester Beatty Research Institute, London using the methods described by Thorpe et al (1978). Purified diphtheria toxin was provided by the Chester Beatty Institute. The concentrations quoted in this chapter refer to the concentration of diphtheria toxin present. Antibody and diphtheria toxin were present in conjugates in a 1:1 molar ratio.

The methods used to assay the effect of conjugates were similar to those described by Thorpe et al (1978). L_2C cells at 10^7 cells/ml were exposed to diphtheria toxin or conjugates in serum-free culture medium (without glutamine which is known to interfere with the cytotoxic action of diphtheria toxin (Kim and Groman, 1965)) for 1 hour at 37°C. Free cytotoxic agents were removed by washing the cells in the cold 3 times with MEM-NEAA (10 ml) and once in culture medium. The cells were then suspended at 5×10^6 cells/ml in culture medium for incubation with ^{3}H-leucine and protein synthesis was determined as described in Chapter 4. An aliquot of cells treated in the same way minus toxic agents provided a control culture. Cell viabilities were checked by trypan blue dye exclusion.

5.3 Results

5.3.1 Effect of diphtheria toxin on L_2C cells

Diphtheria toxin was able to kill L_2C cells. L_2C cells were exposed to diphtheria toxin for 1 hour at 37°C. After 8 hours subsequent culture with ^{3}H-leucine, protein synthesis in cells exposed to diphtheria toxin at 10^{-3} mg/ml was reduced by 70% compared with cells not treated with toxin. Significant cell death was not detected by trypan blue dye exclusion until 24 hours when cells were 15% viable compared with controls

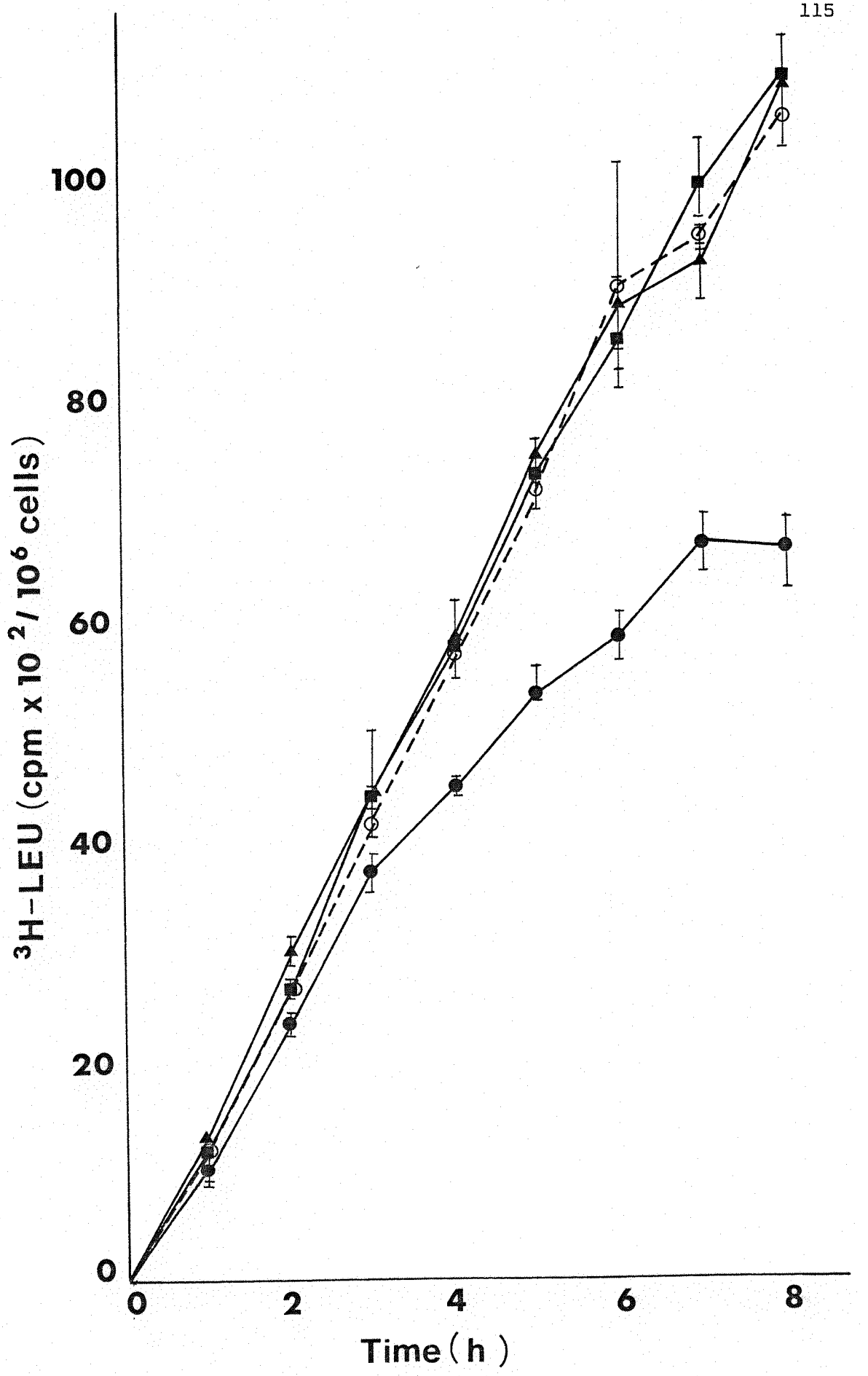

100
80
60
40
20
0
^{3}H-LEU (cpm x 10^2/ 10^6 cells)
0
2
4
6
8
Time (h)

Fig. 5.1 Effect of antibody-diphtheria toxin conjugate (5×10^{-5} mg/ml) on L_2C cells

L_2C cells were exposed to antibody conjugate ● , normal sheep IgG conjugate ■ , diphtheria toxin O (all at 5×10^{-5} mg/ml) or to PBS ▲ , for 1h at $37^{o}C$ and then cultured (time 0h) with ^{3}H-LEU. ^{3}H-LEU incorporation was measured using the MASH and the ranges of triplicate samples are plotted.

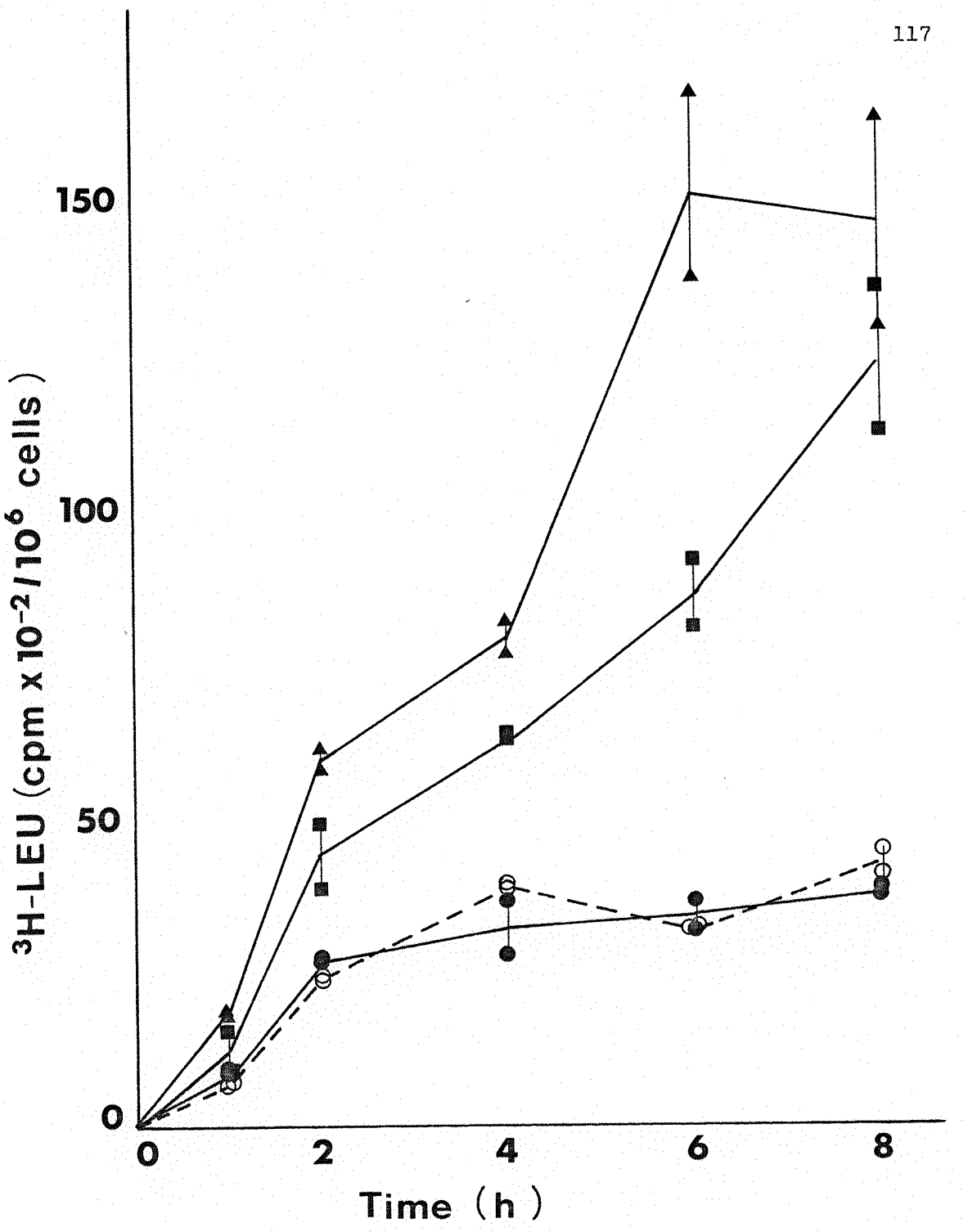

150
100
50
0
^{3}H-LEU (cpm x 10^{-2} / 10^6 cells)
0
2
4
6
8
Time (h)

Fig. 5.2 Effect of antibody-diphtheria toxin conjugate (10^{-4} mg/ml) on L_2C cells

L_2C cells were exposed to antibody conjugate ● , normal sheep IgG conjugate ■ and diphtheria toxin O (all at 10^{-4} mg/ml) or to PBS ▲ , for 1h at 37°C and then cultured (time 0h) with ^{3}H-LEU. ^{3}H-LEU incorporation was measured in TCA precipitated cell material from duplicate samples.

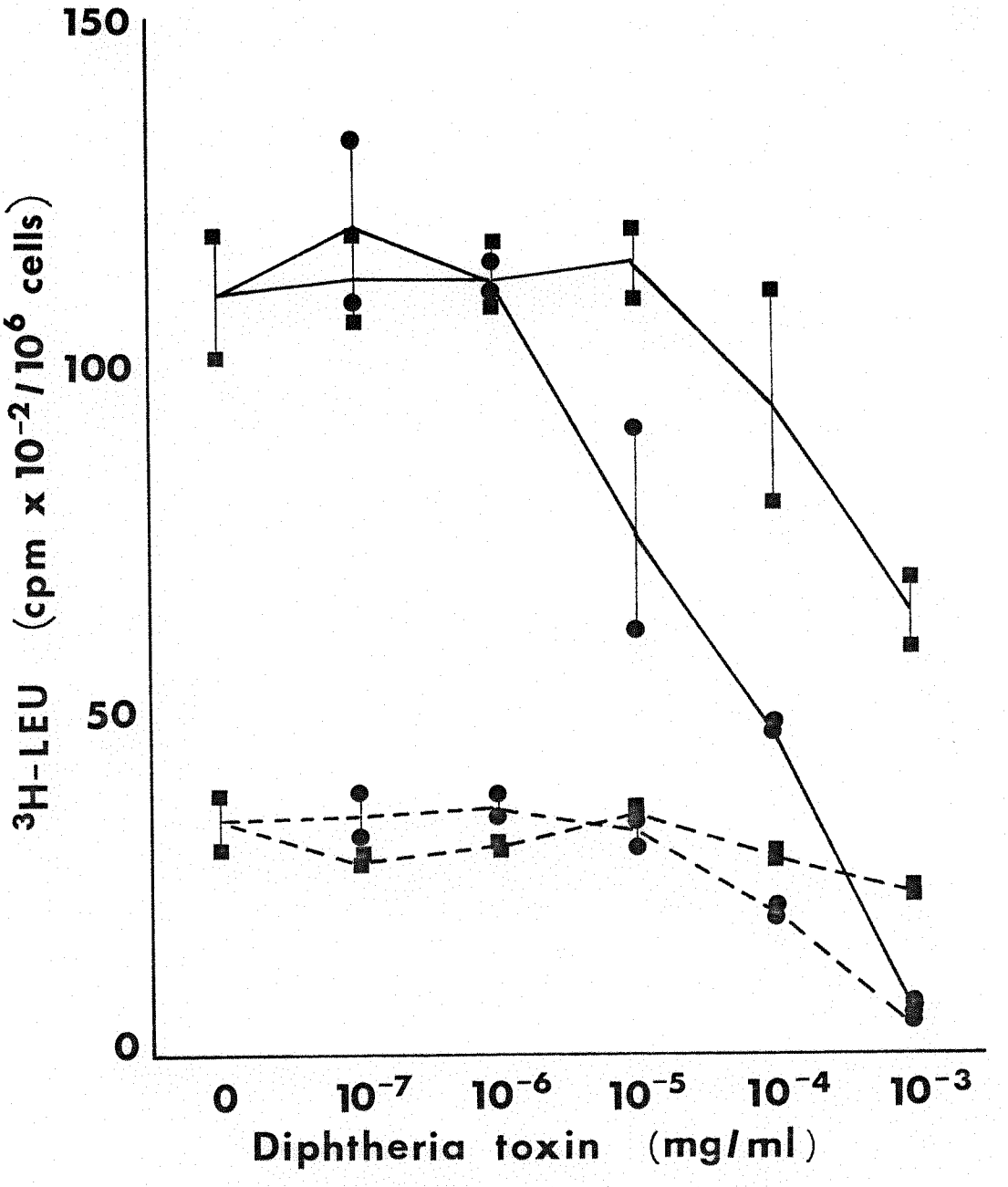


Fig. 5.3 Effect of antibody-diphtheria toxin conjugate concentration on L_2C cells

L_2C cells were exposed to antibody conjugate ● or normal sheep IgG conjugate ■ (at different concentrations) for 1h at 37°C and then cultured (time 0h) with ^{3}H-LEU. ^{3}H-LEU incorporation was measured in TCA precipitable cell material from duplicate samples at 2h - - - and 6h ——.

which were 87% viable. From a number of experiments it was established that the detrimental effects of diphtheria toxin on L_2C cells first became detectable when the concentration of toxin was raised from 5×10^{-5} mg/ml (Fig. 5.1) to 10^{-4} mg/ml (Fig. 5.2).

5.3.2 Effect of antibody-diphtheria toxin conjugate on L_2C cells

Antibody conjugated to diphtheria toxin was more effective at killing L_2C cells than a normal sheep IgG conjugate or free toxin. Figure 5.1 shows the results of an experiment in which antibody conjugate at 5×10^{-5} mg/ml produced a 33% reduction in protein synthesis after 6 hours culture with ^{3}H-leucine whereas normal sheep IgG conjugate and free toxin had no effect. At a higher concentration, 10^{-4} mg/ml (Fig. 5.2) free toxin and antibody conjugate produced a 77% reduction in protein synthesis at 6 hours. However, the normal sheep IgG conjugate had a considerably less toxic effect. The inhibition produced by different concentrations of antibody conjugates and normal sheep IgG conjugates is shown in Figure 5.3. Antibody conjugates were 10 - 100 times more effective than normal sheep IgG conjugates at killing L_2C cells. It should be noted that antibody alone had an inhibitory effect on protein synthesis but the effect was not cytotoxic (see 4.3.1.1).

5.4 Discussion

L_2C cells were susceptible to killing by diphtheria toxin as are normal guinea pig cells (Baseman, Pappenheimer, Gill and Harper, 1970). Conjugation of diphtheria toxin to antibody increased the toxicity of diphtheria toxin for L_2C cells. Conversely, the normal sheep conjugate was less toxic than free toxin. The differential between antibody conjugate and the normal sheep conjugate was 10 - 100 fold and although this differential was less than that of anti-lymphocytic globulin

conjugates (Thorpe et al, 1978) in our case the target antigen is well defined. The mechanism by which antibody conjugate facilitates killing of cells is not known. It is possible that antibody binds more efficiently to cells than diphtheria toxin. Alternatively perhaps internalisation of the A chain is promoted by attachment to antibody. The decrease in toxicity produced by conjugation to normal IgG may be due to steric hindrance of toxin-toxin receptor interaction, an explanation favoured by Thorpe et al (1978). On the other hand conjugation to IgG may damage the binding ability of diphtheria toxin.

From these preliminary observations it was concluded that the conjugation of diphtheria toxin to antibody generated a cytotoxic agent with a degree of specificity for L_2C cells which warrants further study with a view to its use <u>in vivo</u>.

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