PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST ANTIGENS OF THE HUMAN ERYTHROCYTE

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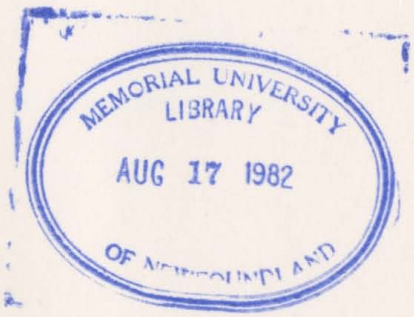

Production of Monoclonal Antibodies Against Antigens of the Human Erythrocyte

by

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ABSTRACT

The objectives of this program were to establish, in Newfoundland, the recently described method for making monoclonal antibodies by cell fusion, to explore various aspects of the technique and to attempt to produce lymphocyte hybridomas secreting monoclonal antibodies against human erythrocyte antigens.

Fusion, with the aid of polyethylene glycol, of spleen cells from an immunized mouse with mouse plasmacytoma cells, may result in formation of hybrid cells. Such hybrids, which contain a B lymphocyte nucleus from the spleen cells, may make monoclonal antibody.

Tests showed the plasmacytoma cells grew well in medium RPMI 1640 with 10% fetal calf serum from a concentration of 2.5×10^4 to 4×10^5 cells per ml. but viability declined rapidly if cultured longer. Polyethylene glycol was found to be toxic to these cells but a suitable concentration was found which allowed both cell fusion and survival of the resulting hybrids. Preliminary testing of the mouse response to red cells (RBC) of various species showed that the standard Jerne plaque technique gave good results with sheep RBC but no plaques with human RBC, even though serum antibody titres were satisfactory. Techniques were later established for detection, in culture fluid, of mouse immunoglobulin (inhibition of the antiglobulin test) and of specific anti-RBC antibody (agglutination).

After 35 fusions and various modifications, the method began to produce hybridomas. Altogether in a series of seven successful fusions 152 colonies were produced with 82 colonies secreting mouse immunoglobulin and 4 of those secreting anti-human RBC antibody.

Of nine species of red cells tested, the only ones to be agglutinated by the four culture supernatants were human RBC. Red cell samples from 87 unrelated humans were screened and all were agglutinated by each of the four culture supernatants. Using 2-mercaptoethanol all four antibodies behaved as if they were Ig M. It was concluded that these monoclonal antibodies were species specific and not detecting alleles.

It is evident from these and other published studies that monoclonal antibodies with allelic specificity may be less easy to obtain than expected.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

REVIEW OF PERTINENT LITERATURE

INTRODUCTION

The development of the cell fusion technique came about through contributions from several fields notably genetics in which nuclear control of cells was under investigation. Shortly thereafter the need for a reproducible antibody reagent as an immunological tool was filled by adapting the cell fusion technique to the mouse B lymphocyte and a mouse myeloma cell line. This produced a hybridoma cell line which secretes monoclonal antibody.

I Antisera

Studies of the antibody repertoire of inbred mice indicate a range of between 1 - 5 x 10^7 uniquely different antibody producing cells.[1a,1b] Between 1,000 and 8,000 different antibodies appear to be able to recognize a single hapten. Yet when the conventional response to antigen is analysed only 5-10 antibody species are seen, representing probably a random sample of the antibody repertoire.[1a] Considering the circumstances the difficulty in reproducing reagents against a particular antigen becomes clear. For instance when a mouse is immunized with xenogeneic cells, between 10 and 100 separate molecules will be recognized. Against each antigenic determinant the mouse may produce a number of distinctly different antibodies. Thus thousands of different antibodies may be produced in response to immunization. The resulting antiserum may be absorbed with appropriate cells, or the mouse may be immunized with allogeneic cells in which case the heterogeneity will be

decreased. Even under these conditions the sera have three major drawbacks: (1) though specific the serum is still heterogeneous; (2) amount of sera is strictly limited; (3) the particular antiserum is not reproducible even when obtained from the same mouse.

The possibility of producing a monoclonal antibody of high titre, available in large quantities and on a continuing basis has become a fortunate reality through immortalization of antibody producing cells by fusion with an established plasmacytoma line.

II Historical Aspects of Cell Fusion

Multinucleate cells: spontaneous fusion and virally induced fusion.

Multinucleate cells in vertebrates were first described over a hundred years ago by Müller[2] who observed them in tumors. Virchow[3] noted their presence in a variety of normal tissues and both inflammatory and neoplastic lesions. Multinucleate cells in pathological lesions were described by many researchers in the last century. It is very probable that some of the inflammatory lesions in which multinucleate cells were observed in the last century were caused by viruses although the viral aetiology of these conditions was not recognized until much later. The earliest reports of multinucleate cells in lesions which can be certainly identified as of viral origin appear to be those of Luginbühl (1873)[4], Unna (1896)[5], and Warthin (1931)[6], who described such cells at the periphery of smallpox pustules, skin lesions of chicken pox, and the tonsils of patients with measles, respectively. Following the introduction of tissue culture methods by Harrison[7]

in 1907, numerous observations were made on cell fusions in cultures of animal tissues. By the 1950s many examples of virally induced fusions in tissue culture began to appear in scientific literature[8,9,10,11,12]. That fusion could occur spontaneously as an event of low frequency in cultures of discrete cells was first recognized by Barski, Sorieul and Cornefert[13] in 1960 who isolated a hybrid line from a mixed culture of two mouse cell lines. Many hybrid lines have since been isolated. Where the formation of a hybrid depends on spontaneous fusion the number of hybrid cells formed is small and varies from one system to another. However Okada (1958)[14] demonstrated that animal tumor cells in suspension could be rapidly fused together to form multinucleate giant cells by high concentrations of HVJ virus (also known as Sendai virus), a member of the para influenza group. The next step in cell fusion was undertaken in a collaborative effort between Harris and Watkins (1965)[15] using the observations of Okada. In their experiment Harris and Watkins used inactivated Sendai virus as fusagen demonstrating that this could promote fusion not only between similar cells but also between cells of different species such as avian erythroblasts and human cultured cells resulting in viable hybrids. This development provided the tool for investigation into the control of genetic information within the cell through nucleo-cytoplasmic relationships[16]. Of equal importance, interspecies hybrids between human and mouse cells contributed information which permitted human gene products to be assigned to particular chromosomes. At this time the immunological value of this tool had not yet been realized.

Fusagens

The viability of hybrid cells may be jeopardized by infection with the viral fusagen, therefore the virus is killed with ultra violet light before being used as the fusing agent. Ultra violet light inactivated Sendai virus has thus provided a general method for fusing together differentiated and undifferentiated cells from various species and even different orders of vertebrates[17]. This fusagen has now been extensively used.

Chemically induced fusion

Recently it has been shown that polyethylene glycol (PEG) can be used to induce fusion between animal cells[18,19]. Qualitatively, fusion using PEG is indistinguishable from that induced by Sendai virus but due to ease in preparation and stability of PEG fusagen as well as elimination of the danger of incorporation of the viral genome of the inactivated Sendai virus[20a] into the fused cell, the chemical fusagen PEG has replaced Sendai virus. It is worth noting that Sendai virus is not very effective in bringing about fusion of mouse lymphocytes[20b] whereas PEG works very well.

III Early Fusion Experiments

By the early 1970s hybridization studies had supplied valuable information in many biological systems but offered little promise for immunologists. Nonetheless, the plasmacytoma induced in mice and adapted for growth in tissue culture, was often a partner in fusion experiments. In 1973 Cotton and Milstein fused two such immunoglobulin producing cells and found that the resulting hybrid cells secreted immunoglobulin of both parental types.[21]

Shortly thereafter Köhler and Milstein (1975)[22] undertook fusion of normal lymphocytes with cells of a myeloma line. The spleen, rich in lymphocytes, was used as the source of one parental line in the fusion. Following immunization of an animal, proliferation of lymphocytes produced antibody against the immunogen; in this case sheep erythrocytes. Remarkable results followed fusion of the lymphocytes with myeloma cells. The use of Littlefields selective medium[23] subsequent to exposure to the fusagen allowed survival of only those cells which had fused. In this experiment growth of cells in selective medium was exhibited in 4 out of 10 petri dishes containing Sendai virus treated cells. These were taken as independent hybrid lines probably each derived from a single fusion event. Those in which anti-sheep erythrocyte activity was evident were subcultured and almost all the clones of the derived purified line proved capable of lysing sheep erythrocytes. These hybrids of normal activated lymphocytes and the tumor cell MOPC 21 produced both anti sheep erythrocyte antibody and MOPC 21 myeloma protein.

The possibilities extended by this crucial experiment are immense: a plasma cell demonstrating a particular specificity of antibody can now be perpetuated through cell fusion with a continuous cell line and production of that antibody in unlimited quantity has now become possible.

Myeloma variants

Sublines and variants of the myeloma were developed.[24] Studies of myeloma cells have shown that variants which lose the capacity to secrete immunoglobulin arise spontaneously with a frequency of the order of

10^{-3} - 10^{-4} per cell generation[25,26]. Through appropriate detection and isolation techniques, rare variant myeloma cells secreting specific macromolecules can be obtained[27]. The cell line used in Köhler and Milstein's notable experiment, P3-X63-Ag8 secretes the MOPC-21 protein an immunoglobulin of the class γ, κ. In a later experiment by them[28] a non-secreting variant of the MOPC-21 myeloma was used: NS1/1-Ag4-1 which does not express heavy chain immunoglobulin.

Subpopulations

Although the fusion of mouse myeloma cells to antibody forming spleen cells has already been effective in producing monoclonal antibodies, many of the technical problems have not been solved and some of the fundamental questions have not been answered. It is not yet clear whether fusion occurs in a specific subpopulation of cells within the myeloma line and of lymphocytes within the stimulated spleen, although it is clear that heterogeneity within these cells in terms of cell cycle and differentiation states exists, indicating a potential for such restrictions. It seems likely that the activated B lymphocyte in its blast stage is the preferred partner for fusion with the myeloma cell, since small resting B cells could not be fused to yield hybrid lines.[29]

IV Biological Action of Polyethylene Glycol (PEG)

PEG efficiently fuses mammalian cells over a wide range of molecular weights. Nonetheless, the exposure time and concentration of PEG are critical to the viability of the fused cells. Studies of

optimal conditions have been carried out with various molecular weights of PEG, concentration of and exposure time to PEG.[30] Addition of dimethyl sulfoxide to fusagen was found in this study to dramatically increase the production of viable multinucleated cells over a range of concentrations of PEG.

Membrane changes[31]

Increasing fluidity of cell membranes is common to treatment with PEG, Sendai virus and lysolecithin. Extensive fusion of cultures occurs 2-4 hours following exposure to PEG. At this time coalescence of the membranes has occurred leading to incorporation of 2 or more nuclei in the resulting multinucleate cell. Although multinucleate cells produced by cell fusion may under favorable conditions remain alive for several weeks, their continued reproduction is conditional upon the formation of daughter cells that contain a single nucleus. When cells contain more than one nucleus irregular and abortive mitosis becomes more common. In cells where asynchrony of DNA synthesis persists the incidence of mitosis is greatly reduced; when it does occur chromosomes of the lagging nucleus may undergo disruption (chromosome pulverization) and ensuing maldistribution of chromosomes may be lethal.

Nuclear rearrangement[31]

If the fused cell enters mitosis, the nuclear envelopes disappear and the chromosomes become condensed. Very little is known about spindle formation and the mechanism of chromosome distribution to hybrid daughter

cells. Many fused cells abort at this stage or within the next few generations. In the small number that form daughter nuclei, the process may be achieved in a variety of ways but all of them mediated by the fusion of the individual nuclei in the multinucleated cell. This fusion takes place at mitosis. In binucleate cells synchronous mitosis of the two nuclei is commonly associated with the formation of a single spindle. All the chromosomes become aligned along a single metaphase plate, and division of the cell produces two mononucleate daughter cells that contain within one nucleus the chromosomes from both parental cells. Other forms of mitosis are also seen in which there is a less equal representation of parental chromosomes.

For the hybrid to evolve into an established cell line, at least one parent has to contribute a capacity to proliferate. The genotype of the hybrid depends in a striking manner on the species relatedness of the parents. Intraspecific hybrids retain more than 90% of the sum of parental chromosomes and remain remarkably stable over long periods.

V Selective Medium

Folic acid antagonists

Folic acid is a compound made up of the pteridine nucleus, p-aminobenzoic acid, and glutamic acid. The folic acid co-enzymes are specifically concerned with biochemical reactions involving the transfer of the single carbon moiety in the folic acid cycle and participate in synthesis of purines, thymine and methyl groups. Maximal inhibitory action is obtained when an amino group is substituted for the hydroxy

group on position 4 on the pteridine nucleus.[32] Thus aminopterin (4-amino folic acid) is the most potent folic acid inhibitor yet discovered. Another antagonist is amethopterin (4-amino-10-methylfolic acid; metho trexate). The interference of the antimetabolites occurs in the reduction of folic acid to the tetrahydro compound. Reduction is a necessary preliminary to the carriage of the one carbon moiety. Thus folic acid antagonists are extremely potent competitive inhibitors of the reductase reaction H_2 folate → H_4 folate.

In tissue culture it has been found that aminopterin blocks the synthesis of nucleic acids, presumably by preventing the reduction of folic acid to the tetrahydro derivative the form that is a major donor to the formyl (singly carbon) to the purine ring. The participation of the folic acid co-enzymes in reactions leading to synthesis of purines and methylated pyrimidines of DNA emphasize the fundamental role of folic acid in growth and reproduction of cells. Such inhibited cells fail to complete their mitoses; they do not progress from metaphase to anaphase because of failure in the synthesis of nucleoprotein - a synthesis which is essential to chromosome reduplication.

Purine salvage pathway

Some tissues within mammalian organisms, are dependent upon exogenous purines or purine ribonucleotides[32,33]. The salvage of these preformed purine compounds occurs by phosphoribosylation of free purine bases by specific enzymes requiring diphospho ribose phosphate (PPribose-P) as the ribose phosphate donor. The enzyme capable of phosphoribosylating hypoxanthine and guanine with PPribose-P to yield inosine monophosphate

(IMP) and guanine mono phosphate (GMP) respectively is hypoxanthine guanine phosphoribosyltransferase (HGPRT). Mammalian cells also have active salvage pathways for converting the pyrimidine nucleoside thymidine to its nucleotide. The enzyme involved is thymidine kinase (TK).

In the event that folic acid synthesis is blocked through administration of the antimetabolite aminopterin, addition of exogenous hypoxanthine and thymidine will bypass the blockade. The conversion of these bases progresses to DNA synthesis through a series of steps the first of which is catalysed by the enzymes HGPRT or TK. Thus cells with normal levels of HGPRT can circumvent the effects of the folate antagonist aminopterin if sufficient amounts of exogenous hypoxanthine are available, whereas those cells with a deficiency of this enzyme will not survive in a medium supplemented with aminopterin even when exogenous hypoxanthine is present.

The plasmacytoma line is a deliberately derived variant of the original cell lines producing HGPRT and is one in which the enzyme HGPRT is lacking. Therefore in tissue culture medium containing HAT (hypoxanthine, aminopterin, thymidine) these cells will stop dividing and will die. The selective medium developed by Littlefield[23] utilizes this characteristic to kill off the unfused myeloma cells in the fusion mixture.

VI Development of Plasmacytoma Lines

The induction of plasma cell tumors has been employed for well over twenty years. It is a remarkable and fortunate experimental fact that plasma cell tumors can be produced in unlimited numbers in the

highly inbred BALB/c strain of mice by relatively simple procedures such as the intraperitoneal injection of mineral oil.[34] The basis for formation of tumor cells in response to mineral oil injection is not fully understood, but a number of factors have been described; foremost amongst these is the unique genetic susceptibility of the inbred BALB/c strain of mice. A second factor of interest is the abnormal peritoneal environment created in treated BALB/c mice. In most cases of an ascites tumor bearing mouse, the tumor cells characteristically produce a single type of immunoglobulin as all tumor cells are derived from a single highly proliferative malignant myeloma cell, hence the term monoclonal antibody is used to describe its secreted product. While the great majority of such plasmacytomas are monoclonal when assessed by immunological means they nevertheless originate within normal cells. The abnormal proliferative capacity probably develops only in a single cell but with this proliferative advantage soon make up the bulk of cells within the ascitic fluid.

Plasma cell tumors have been most useful in providing a source for large quantities of homogeneous immunoglobulin, since most of the tumors are relatively easy to transplant in syngenic hosts. These transplantable tumors attain large size, often more than one third of the body weight of the affected mouse. Large quantities of immunoglobulin can be obtained from the serum and the ascitic fluid or light chains from the urine of the mouse. Stability of the plasmacytoma is good and it maintains production of the characteristic immunoglobulin over many generations. The oldest immunoglobulin producing tumor X5563

has been in nearly continuous passage since 1957 and still produces the same immunoglobulin.[35]

H.A.T. sensitivity

Subclones arising from low level spontaneous mutation will not proliferate faster than other cells in the absence of selective pressure. Applying such pressures allows one to select subclones that exhibit interesting and useful variations. There is a high correlation between resistance to the purine analogue 8-azaguanine and the absence of the enzyme HGPRT.[36] Therefore the HGPRT defective myeloma cells can be selected for through resistance to 8-azaguanine in a single step selection. Cloning myeloma cells in 3 µg/ml 8-azaguanine results in survival of resistant clones only, which will subsequently not survive in HAT medium.[36] Similar selection procedures can be used to isolate variant myeloma cells defective for the enzyme thymidine kinase (TK). Thymidine kinase utilizes exogenous thymidine to overcome the central aminopterin block in much the same way that HGPRT utilizes hypoxanthine.[32,33] Such variants as the TK^- myeloma are selected for by their resistance to 30 µg/ml of the pyrimidine antagonist 5'-bromodeoxyuridine (BUdR)[37]. These particular traits are stable in the absence of selective pressure.

The significance of the presence of drug sensitivity synonymous with a particular cell is great, for administration of the drug can be used to eliminate this cell selectively. Selection for the genetic markers can be employed in various cell types not restricted to the plasmacytoma.

Further selection

The occurrence of spontaneous mutation is a low frequency event in cell cultures. In the absence of selective pressures, such variants will not normally be obvious, yet subcloning and characterization of these subclones unveils this clonal diversification. The spontaneous mutation of plasmacytomas in tissue culture was examined by Coffino and Scharff (1968)[38] leading to their isolation of nonsecreting, nonproducing variants. Subclones isolated from the culture were examined by isoelectric focusing of labeled products. Varients showing the loss of a heavy chain were revealed in the IEF screening by the absence of immunoglobulin bands.

P3-NSI-1-Ag4-1

The myeloma cell line NSI-1 was developed for use in fusion experiments by Kohler and Milstein.[39] The myeloma cell line is of BLAB/c origin and does not grow in selective medium (HAT^{23}). It does not synthesize the heavy (γ_1) chain, but does produce light (κ) chain which, however, is not secreted. A variant line P3-NSI-1-Ag4-1 (short name NS-1) resistant to 20 µg/ml of 8-azaguainine, dying in HAT medium, was isolated and used for fusion by Kohler and Milstein.

The NS-1 myeloma line was obtained through Dr. Elizabeth Simpson of the Clinical Research Center in Harrow U.K. and is from the original line of Dr. C. Milstein. The NS-1 myeloma cell was used in my early fusion experiments.

MOPC 315.43

The myeloma line MOPC 315.43, used in the hybridization experiments reported on in much of this study was obtained through Dr. K.C. Lee

from Dr. T. Mosmann both of the Department of Immunology of the University of Alberta. MOPC 315 is a plasmacytoma derived from a tumor induced in the BALB/c mouse and which secretes immunoglobulin with α heavy and λ light chains. Isolation of subclones subsequently produced subline 315.26 which synthesizes no α heavy chain protein but does make and secrete λ light chains. This subline was grown in 6-thioguanine (6-TG, 1 µg/ml) to select a resistant nutant.

Clone 315.32 was isolated and found to be able to grow in the presence of 6-TG (10 µg/ml) but unable to grow in HAT medium[23] suggesting that the enzyme function of hypoxanthine phosphorysosyltransferase (HGPRT) had been lost. All subsequent variants derived from 315.32 remained 6-TG resistant, HAT sensitive.

Subclone 315.34 was derived in which the λ chain is synthesized but not secreted. The added trait of ouabain resistance was selected for by growth in medium containing ouabain.[43] Subclone 315.43 developed in a medium supplemented with ouabain at a level cytotoxic to the wild type population. This variant remained HAT sensitive and produced but did not secrete light immunoglobulin chains. The nonsecretion is due to a structural mutation of the immunoglobulin chain; therefore when hybridized with a lymphocyte this chain is still not secreted - no contaminating light chain is found in the hybrid's product. The resulting subclone developed for use in the lymphocyte hybridoma experiments was a nonsecreting plasmacytoma resistant to ouabain and sensitive to HAT.

Method

Gently mix the suspension of myeloma cells and under sterile conditions transfer 0.1 ml to a 3 ml test tube. Add 0.1 ml of a 0.2% trypan blue solution in P.B.S. to test tube. Count must be done between 2 and 10 minutes following trypan blue addition. Agitate the tube to mix solutions and place a small drop under the cover slip of the hemocytometer and focus under low power of the microscope. Count the total number of cells and the stained cells in each of the four corner groups of 16 large squares of the hemocytometer grid. There should be 10-50 cells total (cell density 5×10^4 - 2.5×10^5) and 5-10% stained cells is acceptable (non viable cells). In the case of spleen cells, the cells must first be diluted 1:100 PBS, and in converting the count to cells/ml, dilution factor is 100 and conversion factor is 2.5×10^5.

Conversion of Count to Cells/ml

area counted	$4 \times 1\ mm^2$
depth	$\times .1$ mm
dilution factor	$\times 2$
$10^3\ mm^3 = 1\ cm^3$	$\times 10^3$
conversion factor	$\times 5 \times 10^3$

Thus a cell count of 28 is converted to $28 \times 5 \times 10^3 = 1.4 \times 10^5$ cells/ml.

III Freezing Myeloma Cells and Hybrid Cells

Materials and Equipment

Freezing machine

Centrifuge

Nunc tubes

DMSO 20% in RPMI - 15% FCS

RPMI - 15% FCS

Method

Set freezing machine on 4°C. Centrifuge cell suspension at 900 g for 2 minutes. Resuspend cell button in 10 ml RPMI - 15% FCS at 1 x 10^6 cells/ml. Add 10 ml of cell suspension to 10 ml DMSO solution. Mix and put 1 ml of mixture into 20 Nunc tubes. Put vials into freezing machine and set to freeze to -30°C. Leave for 2-3 hours. Transfer vials to liquid nitrogen.

Thawing Cells

Materials

Nunc tubes containing frozen cells

15 ml screw cap test tubes

RPMI - 15% FCS ice cold

RPMI - 15% FCS room temperature

Method

Take nunc tubes from liquid nitrogen and thaw in 37° bath. As last ice crystal melts put cells on ice. Transfer the 1 ml cell suspension into 10 ml ice cold medium. Centrifuge at 900 g, for 2-4 minutes to wash cells. Remove supernatant and resuspend cells in medium at room temperature. Centrifuge cells at 900 g for 2-4 minutes. Remove supernatant and resuspend cells in 10 ml of medium 15% FCS - cell density should be ~ 4 x 10^5/ml. Count cells and adjust density if necessary. Put cells in culture vessel with loose cap in CO_2 incubator at 37°C.

IV 1° Immunization of Mice

Materials

Antigen: washed human RBC, 20% v/v in PBS in 10 ml test tube

10 ml syringe

23 gauge needle

BALB/c mouse

Method

Priming of BALB/c mouse prior to fusion: IgM antibody is desirable. Inject i.p. 0.2 ml of the 20% RBC suspension into an adult BALB/c mouse. Hybridization of the mouse's spleen cells with myeloma cells is performed 4 days later.

V Preparation of Cell Suspensions from Mouse Spleen Cells

Materials

BALB/c adult mouse

2 pairs sterile sissors

3 pairs sterile forceps

Cork board

Pins

EtOH 70% in water

Petridish (sterile)

Serum free RPMI (RPMI(0)) chilled, 1% F/S

2 x 21 gauge needles (sterile) 1½" long

2 x 5 ml syringes

10 ml pipette (sterile)

Hemocytometer

Test tube

WBC diluting fluid

White blood cell diluting fluid: working solution

glacial acetic acid	2.0 ml
H_2O	98.0 ml
Methylene blue	1 drop (1 drop ≃ 25 µℓ)

Method

Immunologically primed mice are anesthetized or killed by cervical dislocation. Pin mouse on cork board. Wash abdominal area generously with 70% ethanol. Using 1 set of sterile sissors with the help of forceps, cut through skin longitudinally on abdomen ensuring not to pierce peritoneum. Pull back flap of skin on your right hand side of 2" incision and pin to cork board. With second set of sterile instruments cut peritoneum longitudinally ensuring not to touch skin of mouse with sterile instruments. Holding the flap of skin on your right hand side the spleen is visible. With the third pair of sterile forceps grip the adhering connective tissue beneath the spleen and lift out the spleen. Transfer spleen to petridish containing 5-10 ml of chilled RPMI(0). Remove any connective tissue from spleen. Attach 21 g needle to syringes. Partially withdraw needle from cover and bend it against the cover to put a 90° bend in the needle. Using 2 such needles cells can be squeezed from the spleen like a toothpaste tube. Discard outer cover of spleen. Draw cell suspension up and down in 10 ml pipette to break up lumps. Allow large lumps to settle for 1 minute and put 1 or

2 drops of cell suspension in test tube for counting. Using a RBC diluting pipette draw cell suspension up to the 0.5 mark. Draw WBC diluting fluid up to 100 mark. Mix well by inverting several times. Discard first 3 drops from pipette. Fill chamber of hemocytometer and count area of the 4 corner groups of 16 large squares on the hemocytometer grid. Conversion factor for count to cells per ml is 5×10^7. Resuspend spleen cells in petri dish and allow large lumps to settle for 1 minute. Being careful not to draw up large lumps, remove the required volume of spleen cell suspension with a 10 ml pipette.

VI Preselection of Mouse Spleen Cells Using poly-L-lysine

The rationale for preselection of mouse spleen cells is the knowledge that cells which are capable of binding antigen also secrete antibody to the same antigen. Thus the mouse lymphocytes should recognize and adhere to erythrocytes posessing the particular antigenic determinant against which they produce antibody.[44]

In negative selection the lymphocytes recognize species determinants and numerous blood group determinants on erythrocyte "type 2". Only lymphocytes which are not specific for any of these antigenic determinants are nonadherent and may be easily poured off the plate coated with type 2 erythrocytes.

These nonadherent cells are then positively selected. In positive selection the remaining lymphocytes recognize determinants for blood groups present only on erythrocyte "type 1" that are not common to erythrocyte "type 2". Pour off and dispose of nonadherent lymphocytes.

Thus the adherent lymphocytes are specific for RBC groups present on erythrocyte "type 1" which are not present on erythrocyte "type 2". The coated plate with adherent lymphocytes is treated with white cell diluting fluid to lyse erythrocytes and the remaining lymphocytes are retained for fusion.

Reagents

White Blood cell diluting fluid (WCDF)

poly-L-lysine: Stock solution 1 mg/ml (PLL)

Make up 0.2 ml/8 ml PBS

PBS

2 ml 1% freshly washed RBC in PBS of type 1

2 ml 1% freshly washed RBC in PBS of type 2

Equipment

2 plastic Falcon petri dishes

100 ml beaker 15 ml conical centrifuge tube

Centrifuge

Method

Pour 2 ml of diluted PLL into each of 2 petri dishes and leave at RT° for 15'. Pour off PLL and thoroughly rinse petri dishes with PBS. Pour 2 ml of 1% freshly washed RBC type 1(2) into petri dishes #1(2) and allow to settle and adhere for 30' during which time petri dishes may be gently agitated once or twice. Gently rinse off supernatant with PBS until free from loose RBC. (Surface tension will cause the PBS to peel back, removing the RBC coating, therefore the petri dish surface must be submerged at all times. Coated dishes may

be stored at 4°C for up to one week in PBS.) Aseptically remove spleen of mouse immunized with type 1 cells into 10 ml PBS and tease apart to free cells (see Methods: preparation of spleen cells). Pour spleen cells into petri dish #2 and allow to settle and adhere for 30' with one or two gentle agitations during this period. Gently rinse off nonadherent cells and retain in a 100 ml beaker. The nonadherent cells are allowed to settle or centrifuged so that volume of cell suspension may be brought down to 5 ml. Resuspend these nonadherent cells in 5.0 ml P.B.S. and pour onto coated dish #1. Allow to settle and adhere for 30' with one or two gently agitations. Gently rinse off and dispose of nonadherent cells. Leave 2-3 ml of PBS in petri dish. With a pasteur pipette remove the adherent lymphocytes and the RBC coating and pour into a 15 ml conical centrifuge tube. Add 10 ml of WBC diluting fluid and mix. 10 minutes is sufficient to rupture membranes of RBC. Spin cells at 900 g for 5 minutes. Decant supernatant. The remaining lymphocytes are specific for type 1 RBC antigens, theoretically.

VII Preparation of Immune Mouse Serum

Equipment

Immunization

Human erythrocytes, washed, 20% cells in PBS

1 ml syringe with 23 gauge needle

10 adult outbred mice

(Immunization against sheep RBC is carried out by the same method, substituting sheep RBC for human RBC.)

Collection of Serum

1 Scalpel; LP 3 tube

2 pairs sterile sissors

2 pairs sterile forceps

2 ml syringe with 21 gauge needle (sterile)

Cork board

Pins

5 ml centrifuge tube with cap (sterile)

70% EtOH for swabbing

2 ml vial for storage (sterile)

Centrifuge

Method

Immunization

Prepare 20% human erythrocytes in PBS fresh before each immunization. 1° immunization: Inject 0.2 ml of 20% human erythrocytes i.p. into each adult mouse. 2° immunization: 3-4 weeks later inject 0.2 ml of 20% human erythrocytes i.p. into the same mice. These mice are ready to be bled 4-6 days later.

Collection of Serum

4 days following the 2° immunization collect blood from the tail to determine if the antibody titre is sufficient. Wipe the tail vigorously with 70% alcohol, massage gently or expose to heat to dilate the vessels and with a sharp scalpel chop off approximately 1 cm. Several drops of blood may be obtained freely in this manner. Further manipulation of the tail - that is, attempting to squeeze the veins manually to obtain

more blood - should be avoided, since this dilutes the blood with tissue fluids. Allow blood to clot for 30' at 20°C and centrifuge to compact the clot. Remove the serum and test at doubling dilutions with an equal volume of 0.5% human erythrocytes for agglutination. If the titre of mouse serum is sufficiently high the mouse should be exsanguinated. To obtain maximal volume of blood, chloroform is preferred for anesthesia, since it causes dilation, first of the left atrium and later of the entire heart. Put the mouse into a beaker with a wire grid bottom under which a chloroform-moistened gauze swab is present. Close the top with a petri dish. At a deep level of anesthesia remove the mouse and pin taut by the feet, ventral side up on the cork board. Wash the chest area with 70% alcohol. Under sterile conditions open the chest wall. While the heart still beats, introduce the needle into the wall of the left ventricle and slowly withdraw the blood. Practice is essential to obtain the maximal amount of blood (1.5 - 2.0 ml) without clotting or hemolysis. Put the blood into the sterile centrifuge tube and allow it to clot for 30' at 20°C. (Kill the mouse by cervical dislocation.) Centrifuge the blood at high speed to compact the clot. Under sterile conditions remove serum with a sterile pasteur pipette and transfer to a sterile capped vial for storage at 4°C.

VIII Preparation of H.A.T. and H.T. Mediums

Materials

Hypoxanthine

Aminopterin

Thymidine

NaOH 1N.

Method

Weigh accurately the approximate amounts of each compound in the table and dissolve in [wt (in mg) x f (in ml)] ml of solvent to give 1000X stock. (f = 1/molarity x M.W.) Store in -20°C freezer in dark as separate solutions.

Compound	M.W.	Convenient Approx. Wt.	f	molarity
Hypoxanthine	136.1	15 mg	0.074	1.0×10^{-1}
Aminopterin	440.43	1 mg	5.54	5×10^{-4}
Thymidine	242.23	30 mg	0.138	3.0×10^{-2}

N.B. Hypoxanthine requires ≃ 100 µl 1N. NaOH for solution

Aminopterin requires ≃ 50 µl 1N. NaOH for solution

Subtract these volumes from f x wt to get total ml water to be added to give desired molarity.

Take 0.1 ml of each solution and add 9.6 ml RPMI (DMEM) to make 10X Stock. (Omit aminopterin and use 9.7 ml RPMI (DMEM) for 10X HT stock).

For complete HAT (or HT). H:10^{-4} M; A:5×10^{-7} M; T:3×10^{-5} M

Fetal calf serum	2 parts
10X Stock solution	1 part
RPMI (or DMEM)	7 parts
2-Mercaptoethanol	5×10^{-5} M
Antibiotics	1%

IX Toxicity Testing of Mediums

It is necessary to ensure that the various mediums are correctly constituted and produce the desired effect on tumor cells and on normal cells. That is DMEM, HAT and HT should not be toxic to normal cells. DMEM and HT should not be toxic to tumor cells but HAT should be toxic to tumor cells. New batches of FCS should be tested to ensure they are not toxic to either cell type.

Equipment and Materials

Inverted phase contrast microscope

Light microscope

Linbro plate - 24 wells

2.0 ml pipettes

10 ml pipettes with 0.1 ml graduations

Mediums to be tested

HAT(10) medium

HT(10) medium

DMEM(10) medium

Fetal calf serum (FCS) 20% in DMEM

Method

Inject 1 ml thioglycollate broth i.p. into an outbred mouse. Several days later, remove macrophages from the peritoneum of the mouse and allow to establish themselves in 12 wells (rows 1 & 2) of the Linbro tray for several days prior to media testing. The macrophages should not be confluent for this test. Decant medium from these wells leaving only enough medium to just cover macrophages (approximately

0.1 ml). Into the 12 remaining wells add 0.1 ml of healthy myeloma cells adjusted to a density of approximately 1 x 10^6 cells/ml. To the first well in each of the four rows (column 1) add 2.5 ml of the first test medium: HAT(10). 2.5 ml of DMEM(10) is added to wells of column 3. FCS to be tested is made up as a 20% solution in DMEM and 2.5 ml are added to wells of column 4. Columns 5 and 6 may also be used to test mediums or FCS. Replace the cover on the Linbro plate and incubate at 37°C for 4 days. Observe the cells using the 40X power on the inverted phase contrast microscope. An example of the result of media testing is shown in TABLE II.

X Polyethyleneglycol M.W. 1000 (PEG 1000) Preparation, Toxicity Testing

The toxicity of PEG 1000 was tested on MOPC 315.43 cells 1.) with various concentrations of PEG and 2.) with various exposure times.

Materials

8 screw cap centrifuge tubes

2 x 2.0 mL graduated pipettes

24 x 3 ml test tubes

Test tube rack

Centrifuge

Light Microscope with 10X eye piece and 10X objective

MOPC 315.43 cells. 2-3 x 10^6 cells with high viability

RPMI(10) (RPMI with 10% FCS

RPMI(0) (serum free RPMI

PEG 1000 in RPMI in the following concentrations: 0,30,35,40,45,50, 55,60% v/v.

Method

Heat PEG until it becomes liquid. Meanwhile add to 8 screw cap centifuge tubes:

tube #	1	2	3	4	5	6	7	8
RPMI(0) ml.	2.0	1.4	1.3	1.2	1.1	1.0	0.9	0.8
PEG ml.	0.0	0.6	0.7	0.8	0.9	1.0	1.1	1.2
[PEG] in %	0	30	35	40	45	50	55	60

PEG is drawn into a 2 ml pipette. A bunsen flame may be lightly applied to the pipette to keep PEG liquid while dispensing. As soon as PEG is added mix by inverting tubes. Autoclave. Centrifuge cell suspension 900g x 5'. Decant supernatant. Make up to 12 ml with RPMI(0). Resuspend cells and measure 0.5 ml into each of 24 test tubes numbered A1 to A8, B1 to B8 and C1 to C8. Centrifuge tubes at 900 g. Tip each tube to decant supernatant. Arrange in rack: row 1: tubes A1-A8, row 2: tubes B1-B8, row 3: tubes C1-C8, row 4: PEG solutions 1-8. Place pipette with bulb in each of the 8 PEG solutions. Set timer and work quickly. Add 0.5 ml of PEG tube 1 to test tubes A1, B1, C1. Add 0.5 ml of PEG tube 2 to test tubes A2, B2, C2. Do the same with remaining PEG solutions. Resuspend cells by tapping test tube rack on bench. At 5' add 1.5 ml of RMPI(10) to all A tubes and centrifuge 5' at 900 g. At 10' centrifuge B tubes. Decant supernatant in A tubes and add 1.5 ml RPMI(10). Resuspend cells. A tubes are ready for analysis. Decant supernatant in B tubes and add 1.5 ml RPMI(10). Centrifuge again at 900 g for 5'. Decant supernatant from B tubes; add 1.5 ml RPMI(10) and resuspend cells. B tubes are ready for analysis. At 30'

centrifuge C tubes at 900 g for 5'. Decant supernatant and add 1.5 ml RPMI(10). Resuspend cells. Centrifuge 900 g for 5'. Decant supernatant and add 1.5 ml RPMI(10). Resuspend cells. C tubes are ready for analysis.

Viability of cells is determined by exclusion of trypan blue. Add 0.1 ml of cell suspension to 0.1 ml of 0.2% trypan blue. Refer to methods: counting of myeloma cells.

XI Fusion of Mouse Myeloma Cells and Mouse Spleen Cells

Protocol #1 (Modification of the procedure of Galfre and Milstein.[45])

Mediums

DMEM(20): DMEM with 20% Fetal Calf Serum

DMEM(0): Serum free DMEM (DMEM(0))

PEG is made up fresh for each fusion: weigh out approximately 2.0 g. PEG 1500 and autoclaved. When PEG cools to about 55°C, add an equal volume of DMEM(0).

HT medium: DMEM(20), supplemented with H & T only.

Cloning medium: DMEM(20) supplemented with HAT.

Method: Fusion

Prepare 10^8 spleen cells and put in conical centrifuge tube. (Method V: Preparation of cell suspensions from mouse spleen cells.) Add 10^7 myeloma cells. Wash 2X with DMEM(0). Remove supernatant. Add 0.5 ml PEG over 2' (0.1 ml every 20 seconds). Add 0.5 ml of DMEM(0) at the same rate. Add a further 5.0 ml DMEM(0) dropwise by doubling volume over every minute for 3 minutes. Fill tube with DMEM(0) Centrifuge for 5' at 900 Xg. Remove supernatant and resuspend pellet in DMEM(20). Make up to 100 ml with DMEM(20). Plate out 0.2 ml in each well of 4 x 96 well Linbro microtitre plates.

Method: Feeding

Remove approximately 1/3 to 1/2 of the culture medium from each well and replace with Cloning medium. Do this on alternate days from day 1 to day 19 following the fusion day. On alternate days from day 21 to day 27 replace the removed medium with HT medium. On alternate days from day 20 onwards replace removed culture medium with DMEM(20).

XII Fusion of Mouse Myeloma Cells and Mouse Spleen Cells

Protocol #2 (Modification of the Procedure I of Andersson and Melchers.[46])

Myeloma cells NS-1 are used in fusion experiments, taken at their exponential phase of growth. 1×10^7 cells used.

Spleen cells from a 1° immunized adult BALB/c are used.

Materials and Equipment

Centrifuge; 37°C water bath

Conical centrifuge tubes - 15 ml

Microtitre plates (Falcon) 96 well, flat bottom

Pipettes

Polyethylene glycol solution 45% v/v PEG, 15% v/v DMSO in PBS (PEG/DMSO)

Serum free DMEM (DMEM(0)) pH 8

Cloning medium DMEM/HAT with 20% FCS

Method

Pellet 10^7 myeloma cells and 10^8 spleen cells in a Falcon tube. Remove medium by suction. Bring temperature of cells up to 37°C and slowly add 1-2 ml of PEG/DMSO at 37°C (3') to cover the cell pellet.

Thereafter layer 10 ml of DMEM(0) pH 8 at 37°C slowly (10') above the PEG/DMSO solution. This mixture is spun at room temperature for 5' at 200 Xg, the supernatant removed by suction and the cells washed once with DMEM(0) pH 8. The cells are finally resuspended in full culture medium and plated in microtitre plates at 0.2 ml per culture. The culture medium is not changed until the appearance of clones of fused cells.

XIII Fusion of Mouse Myeloma Cells and Mouse Spleen Cells

Protocol #3 (Modification of the Procedure of Mosmann et al.[41])

1. Myeloma cells should be healthy and in exponential growth. The myeloma parent should preferably contain both a resistance and a sensitivity marker (eg. HGPRT negative and Ouabain resistant) to allow selection against both myeloma and spleen cell parents. (Although the spleen lymphocytes normally die out in tissue culture, growth of fibroblasts from the spleen cells can occasionally occur if ouabain is not used.)
2. Spleen cells are prepared as in Methods: Preparation of Cell Suspensions from Mouse Spleen Cells.
3. Count myeloma cells and spleen cells. Mix in a 50 ml centrifuge tube in the ratio myeloma:spleen of 1:10. Minimum number of cells suggested is 10^7 myeloma to 10^8 spleen cells.
4. Pellet cells at 900 Xg for 10'. Resuspend in 20 ml RMPI(0) Pellet cells at 900 Xg for 10'. Remove supernatant.

5. Tap tube gently to dislodge pellet. Add slowly with mixing 2 ml of PEG solution.
6. After 2', add dropwise, mixing continuously 20 ml RMPI(0). The addition should take 3'-5'.
7. Pellet cells by centrifugation at 900 Xg for 10'. Remove supernatant and dislodge pellet gently by tapping.
8. Add 5 ml RPMI(20). Do not completely resuspend pellet - leave as lumps.
9. Incubate with cap loose at 37°C for 1-3 hours. Dilute the fused cell suspension to a final concentration of 10^5 myeloma cells/ml in cloning medium.
10. Mix thoroughly and dispense 200 µl aliquots to a 96-well flat bottom Linbro tray; 2.5 ml aliquots in a 24-well Linbro tray or 5 ml aliquots in petri dishes.
11. Clones of fused cells will normally become apparent in the Linbro wells after 10-14 days. In a "good" experiment one might expect about one hybrid per well (96-well tray).
12. Selected clones can be grown up by transferring to fresh cloning medium B (containing no aminopterin). When the cells reach high density (1-2 x 10^5 cells/ml) they can be transferred to RPMI(20) and eventually RPMI(10) if growth continues.

XIV Cytocentrifuge Preparation of Fusion Products

Equipment and Materials

Cytocentrifuge

Glass slides

Cytocentrifuge blotting papers

Test tubes

Pipettes

Microscope

Haemocytometer

Giemsa stain

Fetal calf serum

Buffer

Trypan blue

Protex (Scientific Products Cat. # M7635)

Method

Count the cells to be prepared under the low power of the microscope, and dilute 1/2 in trypan blue 0.2%. Note viability, cell density and types of cells present. Adjust an aliquot of cell suspension to 1-3 x 10^5 cells/ml and add an equal volume of fetal calf serum and mix. Put four drops of this cell suspension (approx. 0.1 ml) into several wells of the cytocentrifuge. Centrifuge at 500 RPM for 5'. Air dry slides. Fix the slides in methyl alcohol for 2'-5'. Stain in Giemsa (fresh) for 7 minutes. Destain in buffer 30 seconds. Check intensity of stain under low power of microscope and destain for longer if necessary. Dry in air. Fix coverslip with Protex.

XV Inhibition of Antiglobulin Serum as a Method of Estimating Antibody Production in Hybrid Supernatant

Materials and Equipment

Centrifuge

Microscope

Microscope slides

Sheep erythrocytes

Immune mouse serum (antigen:sheep erythrocytes)

Anti mouse immunoglobulin (Gibco cat # 343-1 specificity: globulin portion of whole serum)

Universal flask

LP3 tubes or Linbro microtitre plates (round bottom wells)

PBS

Method

Preparation of Antigen Coated Erythrocytes (EA)

Sheep erythrocytes are coated as follows: wash sheep cells 3X. Mix 5.0 ml of a 1.0% suspension of sheep cells with 5.0 ml of a subagglutinating dilution of immune mouse serum (dilution used in this case 1/256). Allow adsorbtion of the mouse proteins to occur in overnight incubation at 4°C. Wash cells 3X and resuspended in PBS at pH 7.2 at 1×10^7 cells/ml. Check the cell suspension with the microscope to ensure no agglutination is visible. These sheep cells are now antigen coated, refered to as sensitized cells or EA.

Standardization of Antiglobulin Reagent

Anti mouse globulin was obtained commercially (Gibco #343-1). Prepare doubling dilutions of antiglobulin in 2 sets of 12 tubes (1 to 1/4096) with 20 µl final volume in each tube. Put PBS only in tube 13. Add 20 µl of sensitized sheep cells (EA) to each tube and mix. Allow to stand for 60' at room temperature. Observe the pattern of agglutination to determine the weakest dilution that produces strong agglutination. Refering to Table M-1 strong agglutination was seen in tube #6, a 1 in 32 dilution but agglutination was not strong in the next tube. It is desirable to use antiglobulin reagent of adequate strength to agglutinate 1 or 2 tubes beyond the test conditions, yet antiglobulin reagent of too strong a dilution may not be inhibited by small amounts of mouse globulin which may be found in hybrid supernatant. Therefore the antiglobulin reagent was diluted for use at the dilution two tubes before the end point. That is the dilution of tube #4, a 1 in 8 dilution.

Controls

The rationale of this test is the ability of mouse globulin to inhibit the agglutination of EA by the antiglobulin reagent. This is tested as follows. Make doubling dilutions in duplicate (20 µl each tube) of immune mouse serum - ten tubes. Put RPMI(20) in tube 11 and 12. To each of the tubes 1 to 11 add 20 µl of EA. Leave tubes undisturbed at room temperature for 60'. Observe the pattern of agglutination.

Table M-2 shows the outcome of such a test. From these results controls were obtained to be used in running tests of antiglobulin inhibition by hybrid supernatant.

- immune mouse serum (antigen human erythrocytes) inhibited agglutination completely in tubes 1 to 7 (dilution 1 to 1/64). This control ensures that agglutination can be inhibited by small amounts of antibody. The dilution of tube 6, that is 1 in 32 dilution is used in all test runs to ensure that the system can be inhibited.
- RPMI(20) does not inhibit agglutination. This control ensures that culture medium in which hybrid cells are grown does not inhibit agglutination. A single tube of RPMI(20) in which EA and antiglobulin reagent are added is used in test runs as a control.
- RPMI(20) in the absence of an antiglobulin reagent shows no agglutination. This control ensures that EA are not being agglutinated due to something other than antiglobulin. A single tube RPMI(20) in which EA but no antiglobulin reagent is added is used in test runs on hybrid supernatant as a control.

XVI Ficoll Hypaque Separation of Viable Myeloma Cells

Materials

Ficoll Hypaque: density 1.082 Screw cap centrifuge tube

Myeloma cells: 1×10^7 cells (minimum) in serum free medium

Method

Layer approximately 2 ml of cell suspension on an equal volume of Ficoll Hypaque in the centrifuge tube. Following Centrifuge for 30' at 400-425 g. Using a pipette, remove cells from the interface. Viability ranges from 78-96%, and 85% of cells are recovered.

Table M-1

Agglutination of Sensitized Sheep Cells by Commercial Anti-Mouse Globulin

Anti Mouse Globulin:dilution		Sensitized Sheep Cells	Agglutination
"	1	present	+++
"	1/2	"	+++
"	1/4	"	+++
"	1/8	"	+++
"	1/16	"	+++
"	1/32	"	+++
"	1/64	"	+-
"	1/128	"	-
"	1/256	"	-
"	1/512	"	-
"	1/1024	"	-
"	1/2048	"	-
"	1/2096	"	-
-ve control	PBS	"	-

Table M-2

Inhibition of Agglutination of Sensitized Sheep Cells by Immune Mouse Serum

Tube#	Immune Mouse Serum	Dilution	Sensitized Sheep Cells (EA)	Antiglobulin Reagent 1/8 diln	Agglutination
1	present	1	present	present	-
2	"	1/2	"	"	-
3	"	1/4	"	"	-
4	"	1/8	"	"	-
5	"	1/16	"	"	-
6	"	1/32	"	"	-
7	"	1/64	"	"	-
8	"	1/128	"	"	+-
9	"	1/256	"	"	+++
10	"	1/512	"	"	+++
11 +ve Control	RPMI(20)		"	"	+++
12 -ve Control	RPMI(20)		"	Not present	-

RESULTS

TECHNICAL DEVELOPMENT

The hybridoma technique had not been set up in the province before this time so it was my task to develop it from its beginning. It was decided at the outset of the program not to plunge into the core experiment immediately but to save time later each component of the experiment was first established and practiced.

Elements of the experiment include:

(1) Optimizing conditions for growth of tumor cells

(2) Testing media and reagents before use

(3) Immunization of donor mouse

(4) Choice of inbred mouse strain

(5) Assays for antibody formation by hybridomas.

I Optimizing Conditions for Growth of Tumor Cells

Cells used

Cell line NS-1

The myeloma line used in the early research work was P3-NSI-1-Ag4-1. Its shortened name is NS-1.[39] This tumor cell line was obtained through Dr. Elizabeth Simpson of the Clinical Research Center in Harrow, U.K. and is from the original line of Dr. C. Milstein of MRC Institute for Molecular Research, Cambridge, England. This cell line was grown under the conditions given in the methods section.

Cell line MOPC 315.43.

In later experiments the mouse plasmacytoma MOPC 315.43 was used. This cell line[40,41] was obtained from Dr. T. Mosmann through Dr. K.C. Lee of the University of Alberta in Edmonton, Alberta. The conditions used for growth of this cell line were identical to that of NS-1 except that RPMI (Gibco Cat # 380-2400) was substituted for DMEM (Gibco Cat # 320-1885).

Effect of Cell Concentration on Viability

The viability of myeloma cells was investigated at various cell concentrations. A suspension of NSI myeloma cells shown by trypan blue exclusion to have a concentration of 3.7×10^5 cells/ml and viability of 94% was subcultured at various concentrations. These subcultures were examined at 24 hour intervals for viable cell concentration.

From the results - NS-1 cells concentration dependence GRAPH I - a number of conclusions were drawn:

1. Growth rate of the myeloma cells is not entirely predictable particularly when cell concentration is less than 2.5×10^4 cells per ml or greater than 4×10^5 cells/ml.
2. Exponential growth of myeloma cells occurs when the cell concentration is within the range of 2.5×10^4 cells/ml to 4×10^5 cells per ml.
3. The viability of the myeloma cells is highest when the cells are in their exponential growth phase.
4. Doubling time for the NS-1 cell line is 13 hours under the conditions attained during the exponential growth phase.

GRAPH I(a)

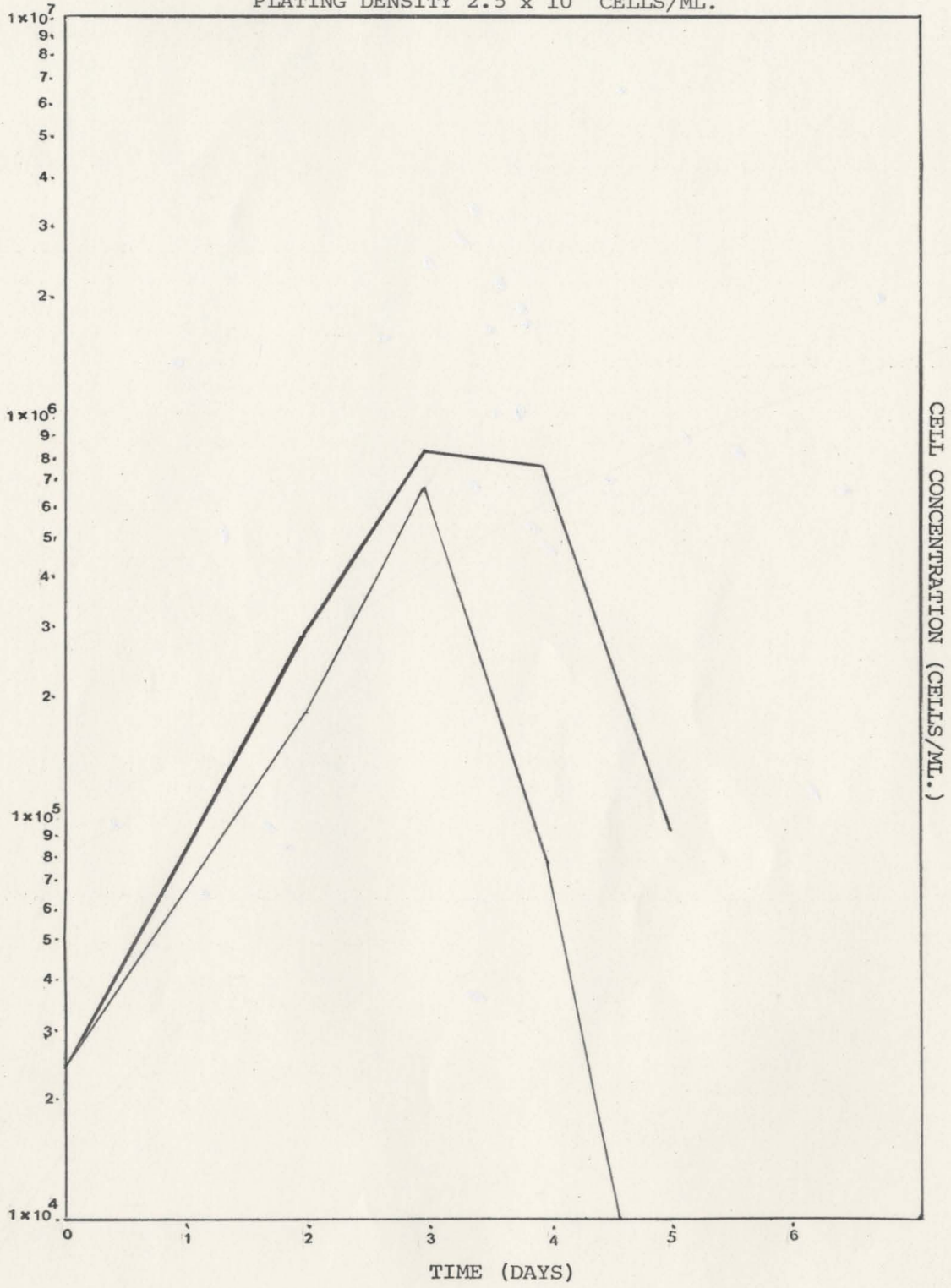
NS-1 CELL CONCENTRATION DEPENDENCE IN DUPLICATE CULTURES

PLATING DENSITY 1.25×10^4 CELLS/ML.

GRAPH I(b)

NS-1 CELL CONCENTRATION DEPENDENCE IN DUPLICATE CULTURES

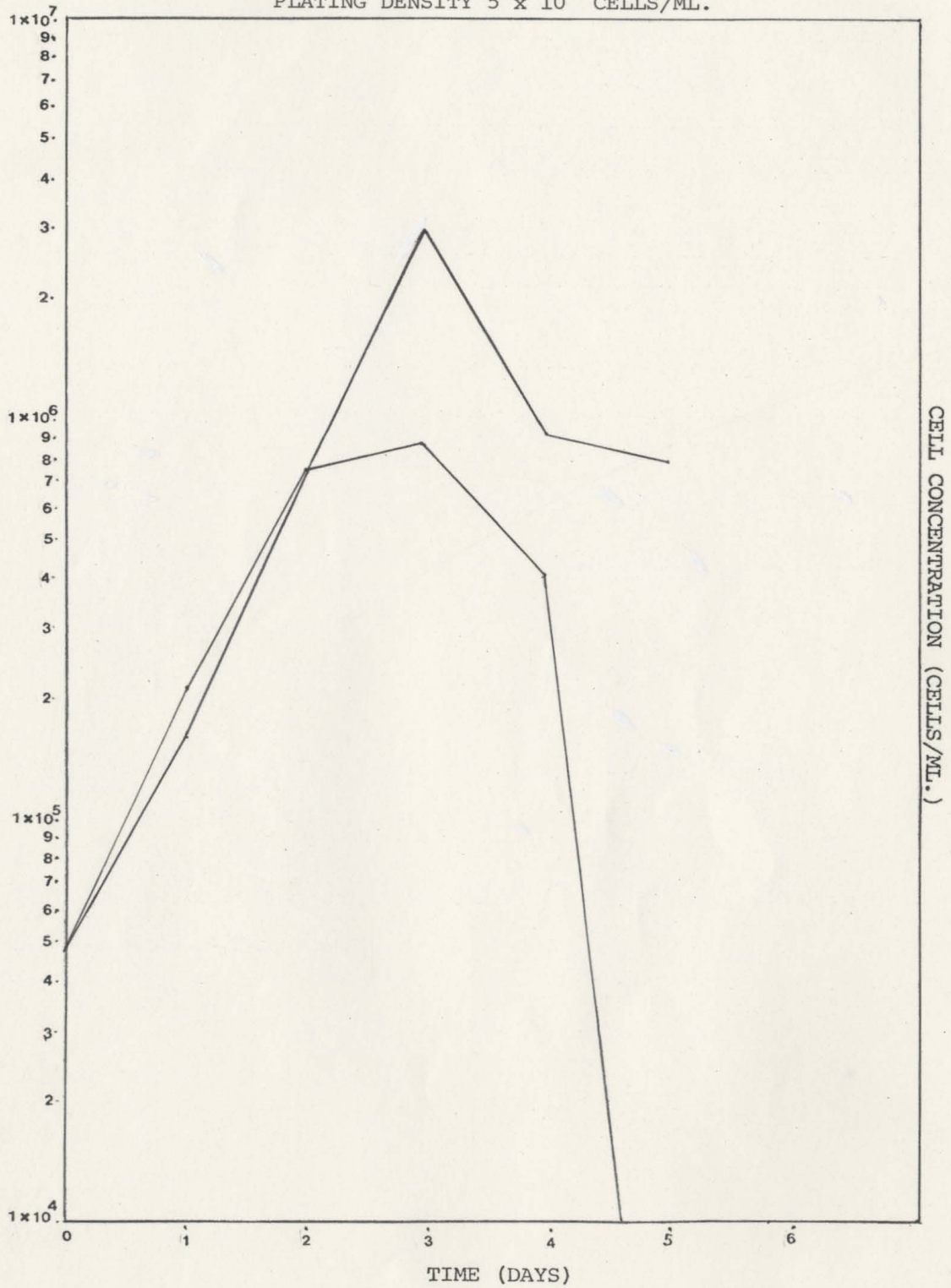
PLATING DENSITY 2.5 x 10^4 CELLS/ML.



GRAPH I(c)

NS-1 CELL CONCENTRATION DEPENDENCE IN DUPLICATE CULTURES

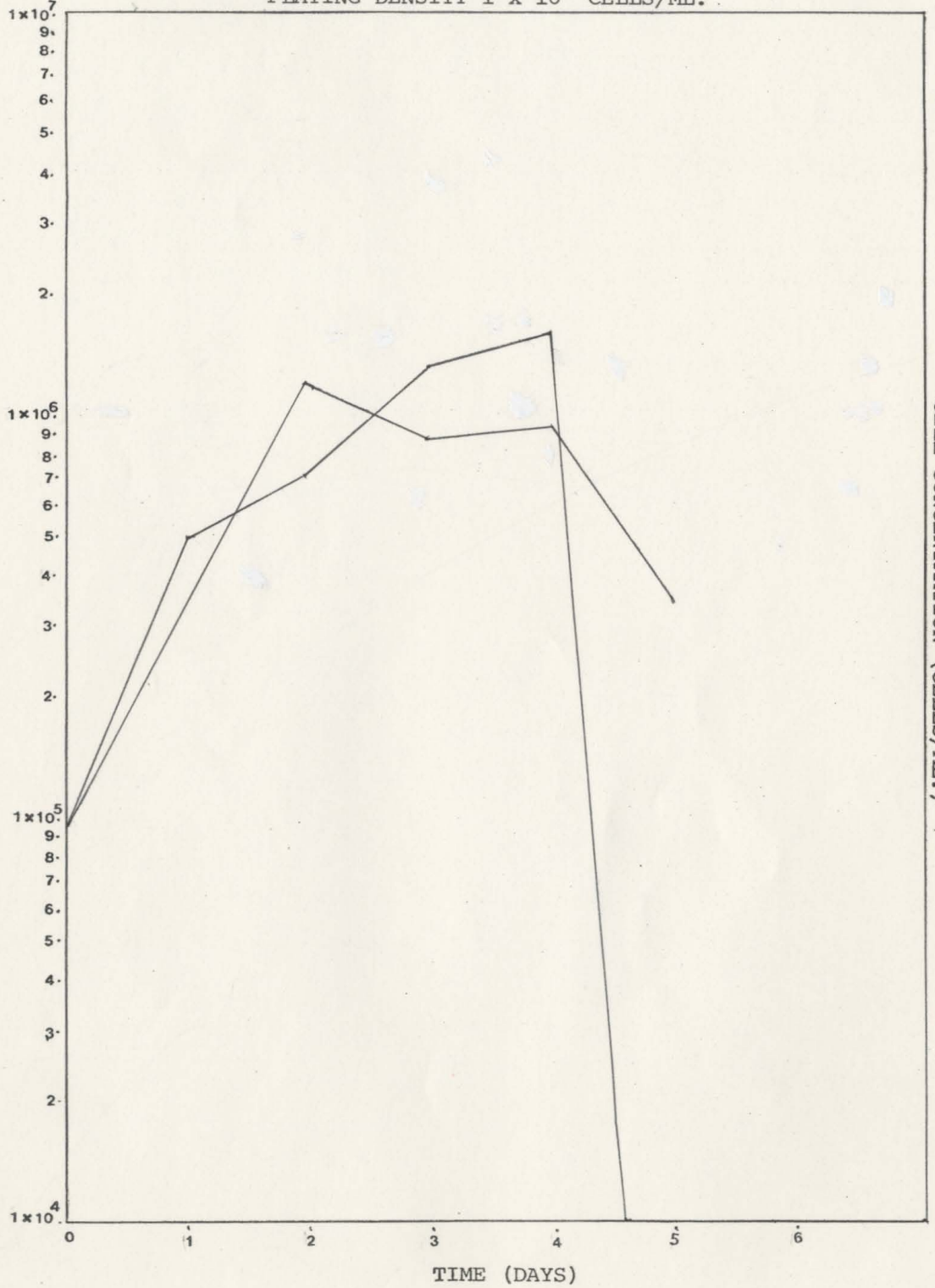
PLATING DENSITY 5 x 10^4 CELLS/ML.



GRAPH I(d)

NS-1 CELL CONCENTRATION DEPENDENCE IN DUPLICATE CULTURES

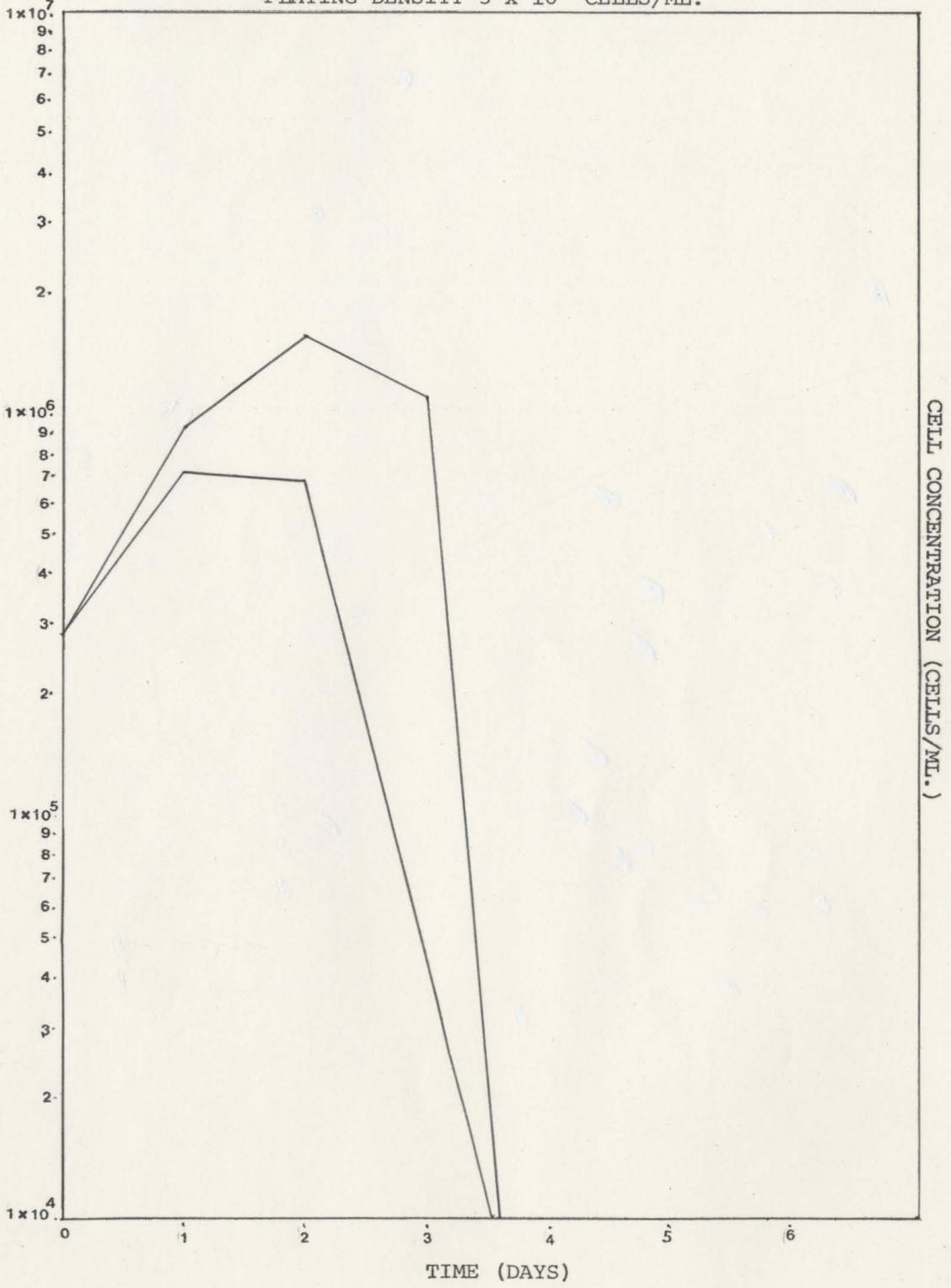
PLATING DENSITY 1 x 10^5 CELLS/ML.



GRAPH I(e)

NS-1 CELL CONCENTRATION DEPENDENCE IN DUPLICATE CULTURES

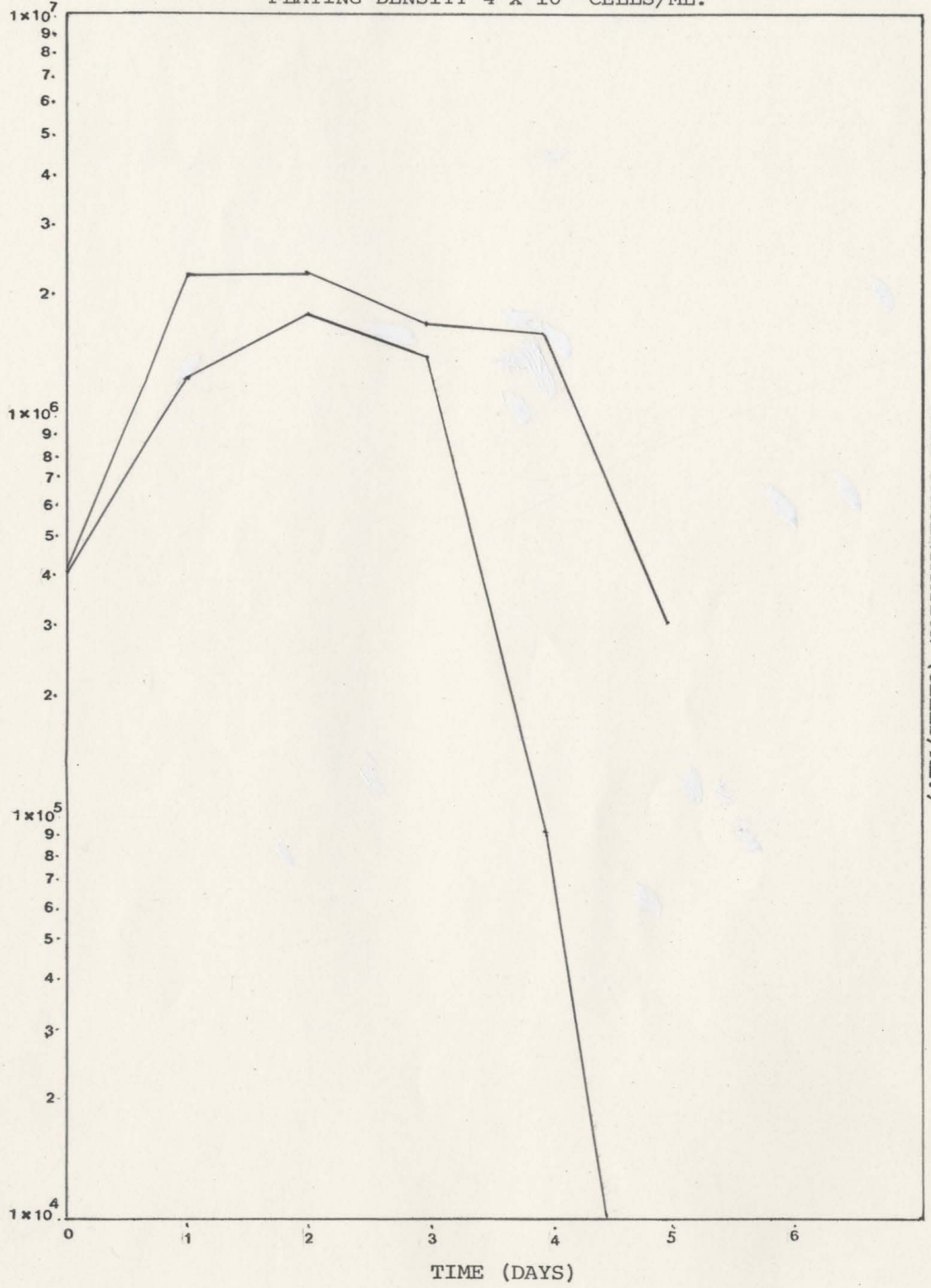
PLATING DENSITY 3 x 10^5 CELLS/ML.



GRAPH I(f)

NS-1 CELL CONCENTRATION DEPENDENCE IN DUPLICATE CULTURES

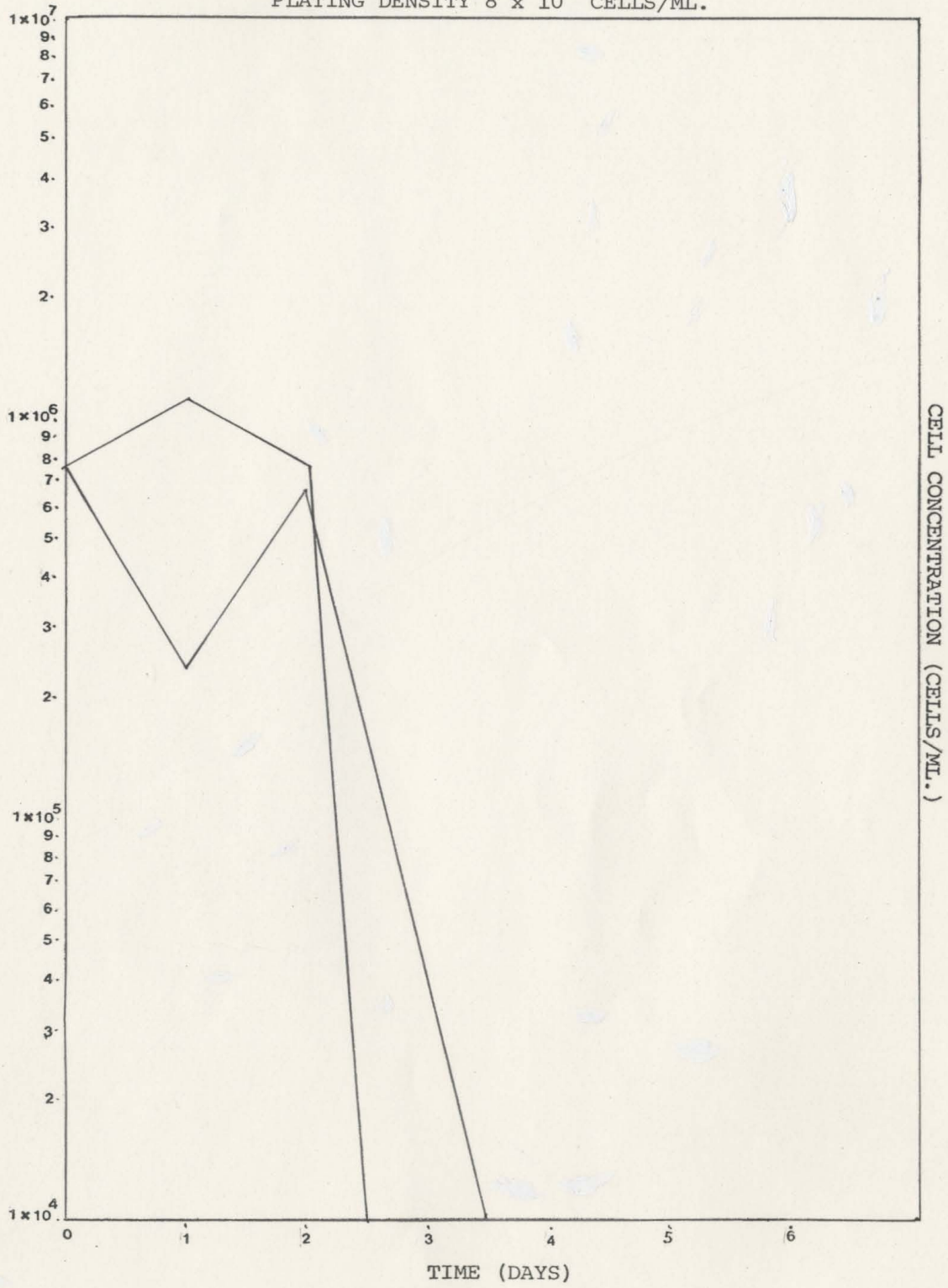
PLATING DENSITY 4 x 10^5 CELLS/ML.



GRAPH I(g)

NS-1 CELL CONCENTRATION DEPENDENCE IN DUPLICATE CULTURES

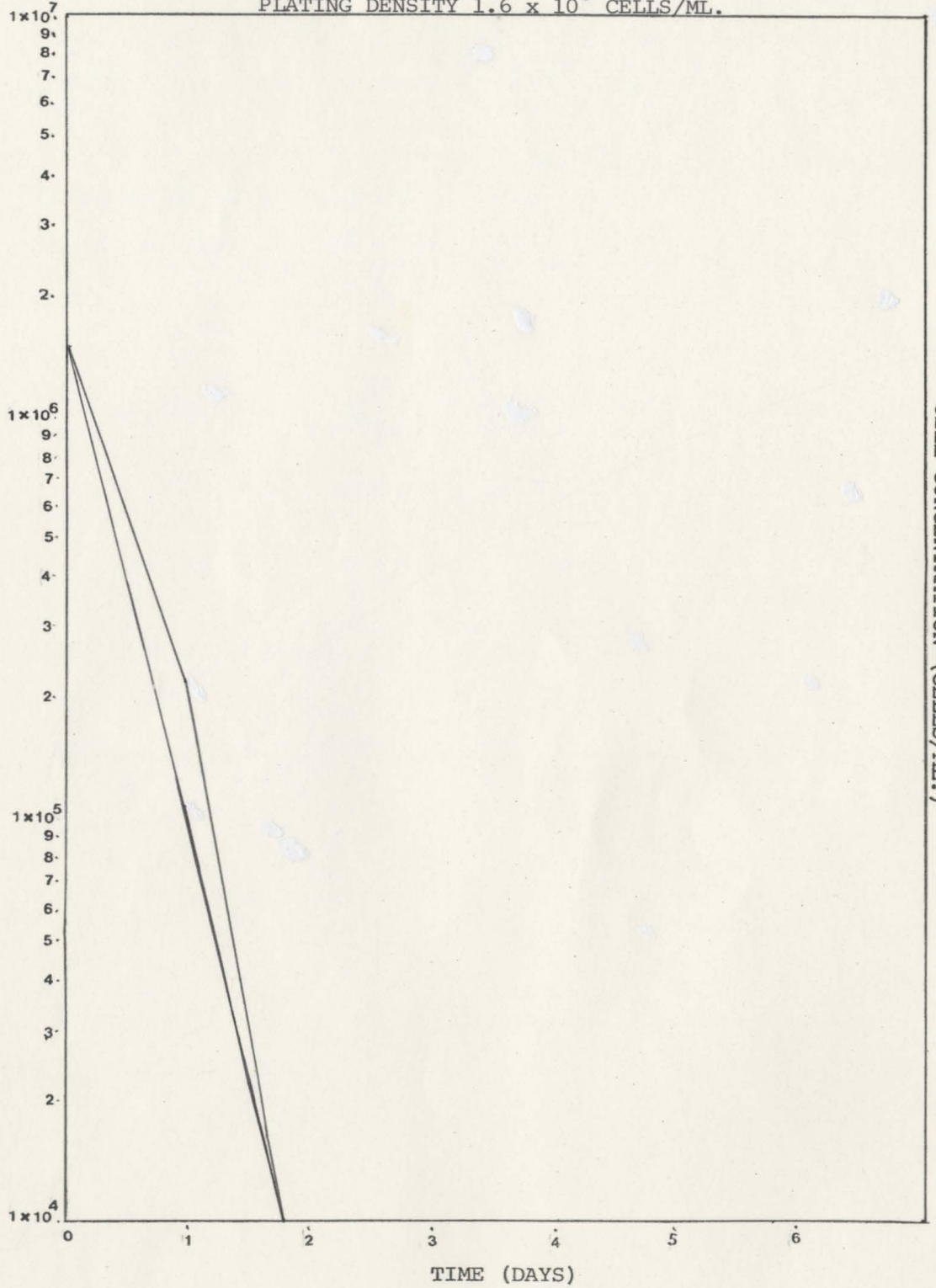
PLATING DENSITY 8 x 10^5 CELLS/ML.



GRAPH I(h)

NS-1 CELL CONCENTRATION DEPENDENCE IN DUPLICATE CULTURES

PLATING DENSITY 1.6×10^6 CELLS/ML.



5. When cells reach a maximum concentration of approximately 8×10^5 to 4×10^6 they rapidly begin to die off. The drop in concentration of viable myeloma cells may be exponential within 24 hours.

II Testing Media and Reagents Before Use

Fetal Calf Serum (FCS)

Toxicity of some batches of FCS to the myeloma cells indicate the need to screen FCS before use. This was done using 10% FCS plus culture medium for growing myeloma cells in a test in which properly functioning FCS and medium was used as a control. The growth rates in duplicate cultures of both batches of FCS with medium indicated if the FCS was toxic.

HAT Medium

Batches of HAT (Hypoxanthine (H) Aminopterin (A) and Thymidine (T)) and HT (Hypoxanthine (H) and Thymidine (T)) supplemented medium (Methods: Preparation of H.A.T. and H.T. Mediums) were tested on two types of cultures 1) Myeloma cells and 2) mouse peritoneal macrophages. Duplicate cultures were examined after 3 days at which time it was expected that the myeloma cells would have failed to grow in HAT but not HT and the macrophages should be healthy in both mediums. Data from one test appears in TABLE I.

These results are typical. It can be seen in TABLE I that NS-1 myeloma cells grown in HAT supplemented medium start dying off by day 3. Whereas NS-1 myeloma cells grown in HT supplemented medium proliferate, doubling nearly 3X in this period. This is similar to the growth observed in the control cultures.

TABLE I

Effect of Various Media on Cell Proliferation

MYELOMA CELLS

	in DMEM (10) (Control)		in HAT		in HT	
Day 1 Plating density	a) 4×10^4 cells/ml	b) 6×10^4 cells/ml	a) 4×10^4 cells/ml	b) 6×10^4 cells/ml	a) 4×10^4 cells/ml	b) 6×10^4 cells/ml
Day 3 Cell count	a) 2×10^5 cells/ml	b) 3.2×10^5 cells/ml	a) 2.1×10^4 cells/ml	b) 1.1×10^4 cells/ml	a) 2.7×10^5 cells/ml	b) 3.1×10^5 cells/ml

MOUSE PERITONEAL MACROPHAGES

	in DMEM (10) (Control)		in HAT		in HT	
Day 1	a) healthy subconfluent	b) healthy subconfluent	a) healthy subconfluent	b) healthy subconfluent	a) healthy subconfluent	b) healthy subconfluent
Day 3	a) healthy subconfluent	b) healthy subconfluent	a) healthy subconfluent	b) healthy subconfluent	a) healthy subconfluent	b) healthy subconfluent

Mouse peritoneal macrophages are not effected by HAT supplemented or HT supplemented media. The health and density of these cells remain unchanged following 2 days of growth in supplemented media, being indistinguishable from control cultures grown in unsupplemented medium.

Conclusion

The HAT medium tested inhibits proliferation of myeloma cells but has no detrimental effect on macrophages. HT medium tested shows no detrimental effect on myeloma cells or macrophages.

Polyethylene Glycol (PEG)

Higher concentrations of PEG and increased exposure time favour fusion of cells but increase toxicity to the cells.[19,30] Maximum PEG concentration and PEG exposure time were to be chosen that result in cell viability of not less than 80%. PEG was prepared in 7 dilutions and tested for toxicity against myeloma cells in 5, 10 and 30 minute exposures.

Method

Appears in methods section.

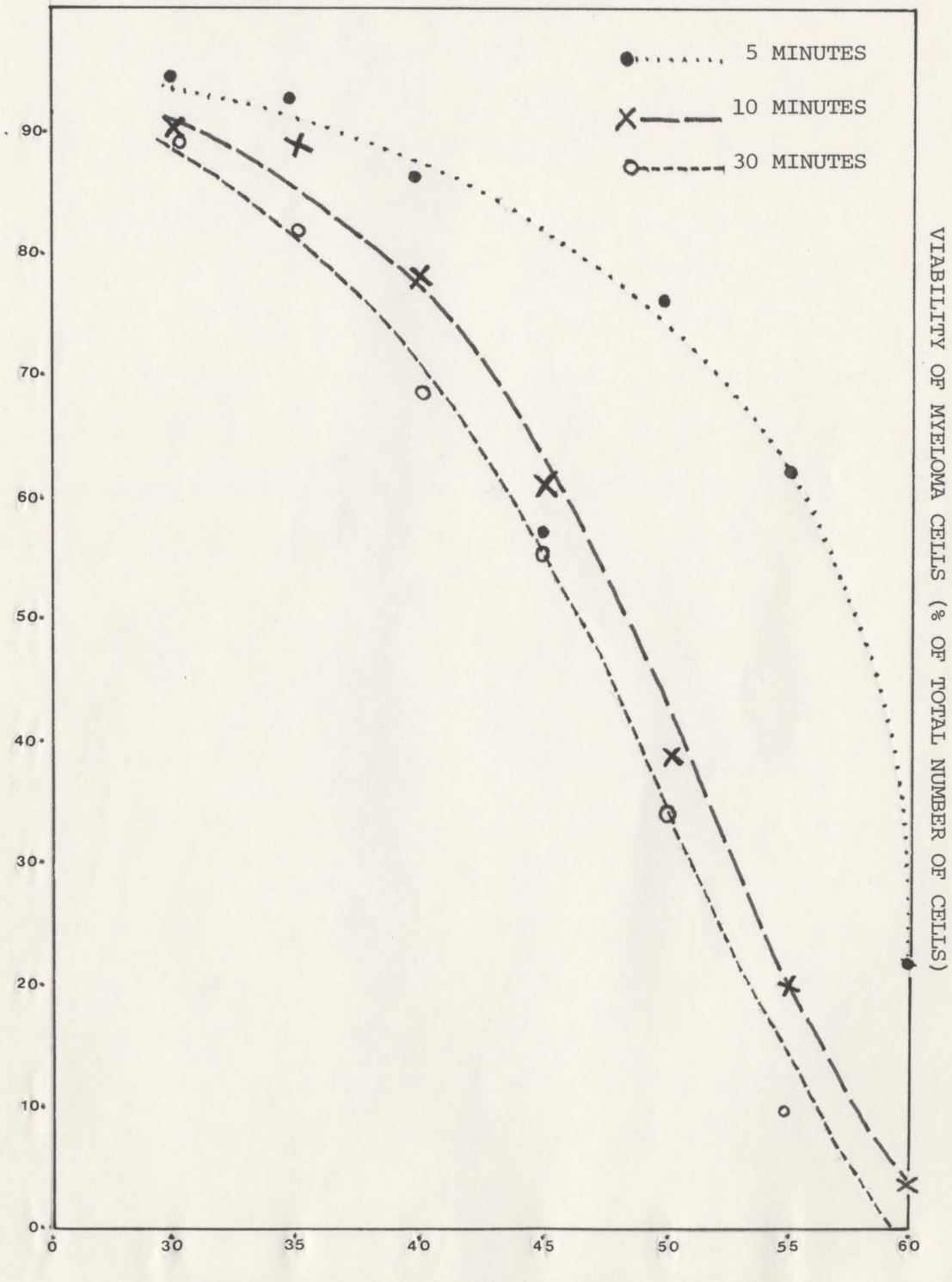
Results

As seen in GRAPH II increasing the time that myeloma cells are exposed to PEG or increasing the concentration of PEG decreases viability of the exposed cells. In 5' exposures of PEG up to a concentration of 45% over 80% of the cells remain viable compared to the viability of cells exposed to 0% PEG. Concentrations of greater than 45% PEG cause a substantial decrease in viability of the myeloma cells.

In a 10' or 30' exposure the viability of the myeloma cells exposed to 30% to 45% PEG drops considerably compared to cells exposed to 0% PEG.

GRAPH II

VIABILITY OF MYELOMA CELLS EXPOSED TO VARIOUS CONCENTRATIONS OF PEG FOR 5, 10 AND 30 MINUTES



Conclusion

5' exposure of cells to 45% PEG and 10' or 30' exposure to 35% PEG results in > 80% myeloma cell viability under the test conditions. The 5' exposure to 45% PEG was chosen to be used in all of the fusions carried out in this paper.

III Immunization of Donor Mouse

One factor which influences the yield of hybridomas of desired specificity is the prior immunization of the mouse whose spleen is to be used in the fusion. In order to discover the time of peak activity in the spleen after immunization a plaque assay was used. Several mouse strains were compared to find which ones made a satisfactory response to human RBC; the latter point is described in Section IV.

Method

The plaque method, based on that of Jerne is used[47], in which spleen cells are counted and applied with complement over a thin layer of RBC in gel. Appearance of hemolytic plaques indicates spleen cells which form antibody to RBC.

Results

TABLE II shows the plaque response by mouse spleen cells following immunization with sheep RBC. The number of plaques on days 3 and 4 post-immunization is not substantially more than the background level (the number of plaques produced by the spleen of a non immunized mouse), although the serum titre increases steadily. On day 5 peak activity is observed in the spleen in terms of highest number of plaques. Activity in the spleen in terms of number of plaques produced drops sharply after day 5 although the serum titre continues to rise. Peak activity occurs in the spleens of mice immunized with sheep RBC on day 5 post-immunization.

TABLE II

Analysis of Haemolytic Titre and Jerne Plaque Response to Sheep RBC in 3 Strains of Mice

BREED	ANTIGEN	INDICATOR	POST-IMMUNIZATION TIME (DAYS)	HAEMOLYTIC TITRE (SERUM)	PLAQUES/10^6
		SHEEP		1/4	0
C57	NOT IMMUNIZED	RED BLOOD		1/4	0
		CELLS		1/8	2
				1/2	0
C3H	" "	"		1/2	0
				1/4	0
				1/2	2
BALB/c	" "	"		1/2	1
				1/2	0
	SHEEP			1/2	0
C57	RED BLOOD	"	3	1/4	1
	CELLS			1/2	0
				1/2	2
C3H	"	"	3	1/2	1
				1/4	0
				1/8	1
BALB/c	"	"	3	1/2	2
				1/8	2
				1/32	1
C57	"	"	4	1/32	0
				1/32	1
				1/16	0
C3H	"	"	4	1/32	0
				1/16	0
				1/32	0
BALB/c	"	"	4	1/64	3
				1/16	0
				1/512	48
C57	"	"	5	1/1024	40
				1/512	29
				1/256	49
C3H	"	"	5	1/512	55
				1/512	43

continued . . .

TABLE II (continued)

BREED	ANTIGEN	INDICATOR	POST-IMMUNIZATION TIME (DAYS)	HAEMOLYTIC TITRE (SERUM)	PLAQUES/10^6
				1/512	41
BALB/c	"	"	5	1/1024	53
				1/512	61
				1/2048	41
C57	"	"	6	1/2048	10
				1/2048	11
				1/32	29
C3H	"	"	6	-	14
				1/2048	23
				1/1024	10
BALB/c	"	"	6	1/2048	17
				1/1024	9

Several attempts were made to produce similar results using human erythrocytes as an antigen but there were virtually no plaques detected. TABLE VII shows the plaque response by mouse spleen cells following immunization with human RBC. Although the serum titre is highest at day 6, (much the same as seen in mice immunized with sheep RBC - TABLE II) activity detected in the spleen remains unchanged from the background level.

Conclusion

It was decided to be guided in the present work by immunization schedules published in the literature which had been satisfactory for other antigens[48,49]. Four days after primary immunization the spleen was removed for the fusion.

IV Choice of Inbred Mouse Strain

Comparison of haemolytic titres were made in C3H, BALB/c and C57BL/6 strains of mice.

Method

Triplicate tests were carried out in each of the 3 strains of inbred mice. Each received one intraperitoneal injection of 0.4 ml of a 20% suspension of washed human or sheep RBC. Four days later blood was removed from the mouse by cardiac puncture, allowed to clot and the serum prepared by double dilutions with PBS. An equal volume of washed 3% suspension of the same type erythrocytes was added. One hour later the pattern of hemolysis was observed.

Results

Table II and III shows the hemolytic response of C3H, BALB/c and C57 strains of primary immunized mice against human and sheep RBC.

TABLE III

Analysis of Haemolytic Titre in Serum and Jerne Plaque Response of Spleen Cells to Human RBC in 3 Strains of Mice

BREED	ANTIGEN	INDICATOR	TIME (DAYS)	HAEMOLYTIC TITRE (SERUM)*	PLAQUES/10^6*
	NOT	HUMAN		1/2	2
C57	IMMUNIZED	RED BLOOD		1/2	0
		CELLS		1/2	0
				1/2	0
C3H	"	"		1/4	0
				1/8	1
				1/2	2
BALB/c	"	"		1/2	0
				1/4	0
	HUMAN			1/2	0
C57	RED BLOOD	"	3	-	0
	CELLS			1/2	2
				1/4	1
C3H	"	"	3	1/8	2
				1/16	0
				1/2	0
BALB/c	"	"	3	1/4	0
				1/2	0
				1/32	0
C57	"	"	4	1/32	0
				1/32	2
				1/16	0
C3H	"	"	4	1/32	2
				1/32	0
				1/32	1
BALB/c	"	"	4	1/64	0
				1/64	0
				1/512	0
C57	"	"	5	1/512	0
				1/2	2
				1/512	0
C3H	"	"	5	1/1024	2
				1/1024	0

* Each measurement represents the value for one mouse.

continued . . .

TABLE III (continued)

BREED	ANTIGEN	INDICATOR	TIME (DAYS)	HAEMOLYTIC TITRE (SERUM)*	PLAQUES/10^6*
				1/512	1
BALB/c	"	"	5	1/512	1
				1/256	3
				1/1024	0
C57	"	"	6	1/1024	0
				1/2048	0
				1/1024	1
C3H	"	"	6	1/1024	3
				1/2048	0
				1/2048	0
BALB/c	"	"	6	1/16	0
				-	0

* Each measurement represents the value for one mouse.

It can be seen that the humoral antibody response to both antigens, as examined in three strains of mice increases steadily from day 3 to day 6. The three mouse strains studied were not significantly different in terms of hemolytic titre observed on days 3,4,5 or 6 or in the case of non-immunized mice.

Conclusion

Choice of one strain of mouse could not be made based on these data. All strains are equal in terms of hemolytic response.

V Assays for Antibody Formation by Hybridomas

Two approaches were considered desirable (i) to assay for immunoglobulin (Ig) production by the hybridoma (ii) to assay for production of specific antibody to human red cells. It was expected that many more wells would contain mouse Ig than would contain specific anti RBC antibody.

Assay for mouse Ig

It was expected that amounts of Ig would be small, in the supernatants of initial cultures. Therefore an inhibition of the anti-globulin test was set up and its sensitivity tested. See Table IV.

Method

The method finally arrived at made use of a rabbit anti-mouse globulin antibody and sheep RBC sensitized with subagglutinating doses of a mouse anti-sheep RBC antibody that was obtained from nonabsorbed mouse sera from mice injected on 3 occasions with 0.4 ml of washed sheep erythrocytes.

Results

A panel of results given in TABLE IV show that this test is capable of detecting the immunoglobulin in normal mouse serum undiluted (b) through to dilutions of up to and including 1/64 (h). The control (a) in which saline is added to the rabbit anti-mouse globulin antibody shows no inhibition of agglutination of the sheep cells. The same result is seen in tests in which very small amounts of mouse serum are present; ie. dilutions of greater than 1/64 (i & j).

Conclusion

The wide range of immunoglobulin concentrations over which this test is sensitive makes it suitable for screening supernatants in which colonies are found.

Assay for specific anti-human RBC antibody

After preliminary trials it seemed that agglutination would be superior to haemolysis as an assay. The method used is conceptually simple and involved mixing 4 volumes of supernatant with 1 volume of washed 3% RBC suspension in a round bottom microtitre tray and incubating at 37° for one hour. These trays were then centrifuged at 3000 r.p.m. for 5' and then checked macroscopically for agglutination.

Results

Table V shows the results of this test using immune mouse serum (antigen human RBC) as a positive control and using supernatant from a well in which a hybridoma colony was present but no immunoglobulin was detected as the negative control. Strong agglutination was apparent when immune mouse serum was used to agglutinate the human erythrocytes. The

TABLE IV

Inhibition of Antiglobulin Test

Test #		Rabbit antimouse Ig antibody	Pooled normal mouse serum	Sensitized sheep cells	Agglutination of sheep cells
(control)	a.	present	0 (-ve control)	present	+++
	b.	"	1	"	-
	c.	"	1/2	"	-
	d.	"	1/4	"	-
	e.	"	1/8	"	-
	f.	"	1/16	"	-
	g.	"	1/32	"	-
	h.	"	1/64	"	-
	i.	"	1/128	"	+-
	j.	"	1/256	"	++

TABLE V

Test of direct agglutination assay for

anti-human antibody

Test solution	Direct agglutination of human RBC	
+ve control (immune mouse serum: antigen human RBC) dil'n. 1	+ve	(+++)
test sera:		
dil'n. 1/2	+ve	(+++)
" 1/4	+ve	(+++)
" 1/8	+ve	(+++)
" 1/16	+ve	(+++)
" 1/32	+ve	(+++)
" 1/64	+ve	(++)
" 1/128	-ve	(+-)
-ve control (tissue culture medium from wells containing no clones)	-ve	(-)

positive control of this assay utilized undiluted immune mouse serum. Dilutions of the serum from 1/2 to 1/64 gave a positive result, in which strong agglutination was seen. A dilution of 1/128 gave a negative result, in which agglutination was borderline (+-). Agglutination in the negative control was a definite negative (-).

Conclusion

Direct agglutination of human erythrocytes is a suitable assay to detect the presence of anti-human RBC antibody in supernatants of hybridoma colonies. Supernatants of hybridoma colonies do not appear to contain any other material which interferes with this test.

FUSION RESULTS

Thirty five attempts at fusion were carried out as shown in TABLE VI. As can be seen, apart from three minor productions of presumed hybridoma colonies the technique did not yield significant numbers of colonies until after 26 fusions had been completed.

During the earlier experiments various modifications of the method were tried:

Fusions 2-6 The myeloma cells were enriched for viable cells by a one step discontinuous density separation on Ficoll. This produced 92 - 95% viable myeloma cells for the fusion. Fusion 3b produced 5 presumed hybridoma colonies. (Refer to Methods XVI)

Fusions 3 & 5-12 A feeder layer of mouse peritoneal macrophages[50] in subconfluent amounts was used in an attempt to improve

culture conditions. In fusion 3a and b colonies resulted in the control wells (those without macrophages) but none resulted in those wells containing macrophages and so the use of the feeder layer was not considered advantageous.

Fusions 6-12 Cell selection procedures were performed on spleen cells before the fusion. This modification was abandoned due to indications in the literature that input of low numbers of cells prevented fusion with the protocol being used. (Refer to Methods VI)

In view of the fact that 17 fusion experiments had been performed in which only one produced hybridoma colonies, the problem of cell viability was given closer attention. Exposure to PEG as called for in the fusion protocol causes a drop in viability to approximately 80% of that of input cells (GRAPH II). Monitoring the viability of spleen and myeloma cells before, immediately after and 24 hours after the fusion was considered desirable. In most cases viability over the next 24 hours remained relatively unchanged, and in some cases dropped slightly.

Fusions 18-35 Monitoring of viability of cells was carried out in all fusions. In cases in which viability of input cells was below an acceptable level, that fusion experiment was abandoned. Referring also to TABLE VII it may be seen that viability of myeloma and spleen cells used in fusions 18 - 35 was not lower than 86% and 91% respectively. As the TABLE shows, in fusion experiments in which the viability remains high throughout and

TABLE VI

Fusion Experiments 1 - 35

Fusion #	Protocol*	Variations*	# of myeloma cells	# of spleen cells	# of colonies	# of Ig +ve by aggn. inhibition	# of Ab +ve by direct aggn.	additional comments
1	1	none, NS-1 cells	$1x10^7$	$1x10^8$	0			
2	1	NS-1, myeloma cells Ficolled giving 93% viability	$1x10^7$	$1x10^8$	0			
3	1	NS-1, myeloma cells Ficolled giving 92% viability	$1.5x10^7$	$1x10^8$				
a		NS-1, plated on macrophage feeder layer[50]			0			
b		NS-1, plated without feeder layer			5	0		
4	1	NS-1, myeloma cells Ficolled giving 95% viability	$1x10^7$	$1x10^8$	0			
5	1	NS-1, Ficolled: 96% viability spleen cells +&- selected; feeder layer used	$1x10^7$	$1x10^7$	0			

TABLE VI (cont'd)

Fusion #	Protocol*	Variations*	# of myeloma cells	# of spleen cells	# of colonies	# of Ig +ve by aggn. inhibition	# of Ab +ve by direct aggn.	additional comments
6	1	NS-1, Ficolled: 94% viability spleen cells +&- selected; feeder layer used	$1x10^7$	$7x10^6$	0			
7	1	none, NS-1	$1x10^7$	$1x10^8$	0			
8	1	NS-1 cells, spleen cells +&- selected; feeder layer used	$1x10^7$	$1x10^8$	0			
9	1	" "	$1x10^7$	$1x10^8$	0			
10	1	" "	$1x10^7$	$1x10^8$	0			
11	1	" "	$1x10^7$	$1x10^8$	0			
12	1	" "	$1x10^7$	$1x10^8$	0			
13	2	none, NS-1	$1x10^7$	$1x10^8$	0			
14	2	none, NS-1	$1x10^7$	$1x10^8$	0			
15	2	immunogen: sheep erythrocytes; NS-1	$1x10^7$	$1x10^8$	0			
16	2	none, NS-1	$1x10^7$	$1x10^8$	0			
17	2	none, NS-1	$1x10^7$	$1x10^8$	0			

TABLE VI (cont'd)

Fusion #	Protocol*	Variations*	# of myeloma cells	# of spleen cells	# of colonies	# of Ig +ve by aggn. inhibition	# of Ab +ve by direct aggn.	additional comments
18	3	MOPC 315.43 cells; viability monitored; no ouabain	$1x10^7$	$1x10^8$	8			cells lost through incubator failure
19	3	" "	$1x10^7$	$1x10^8$	2			cells lost through incubator failure
20	3	MOPC 315.43 cells; viability monitored; ouabain used	$1x10^7$	$1x10^8$	0			-
21	3	" "	$1x10^7$	$1x10^8$	0			-
22	3	" "	$1x10^7$	$1x10^8$	0			-
23	3	" "	$1x10^7$	$1x10^8$	0			-
24	3	" "	$1x10^7$	$1x10^8$	0			-
25	3	" "	$1x10^7$	$1x10^8$	0			-
26	3	" "	$1x10^7$	$1x10^8$	0			-
27 a	3	MOPC 315.43 cells; viability monitored; ouabain	$1x10^7$	$1x10^8$	0			possibility of ouabain interference
b		MOPC 315.43 cells; viability monitored; no ouabain			18	9	0	non specific Ab only

TABLE VI (cont'd)

Fusion #	Protocol*	Variations*	# of myeloma cells	# of spleen cells	# of colonies	# of Ig +ve by aggn. inhibition	# of Ab +ve by direct aggn.	additional comments
28	3	MOPC 315.43 cells; viability monitored; ouabain	$1x10^7$	$1x10^8$	0			-
29	3	" "	$1x10^7$	$1x10^8$	0			-
30	3	MOPC 315.43 cells; viability monitored; no ouabain	$1x10^7$	$1x10^8$	16	8	1	antihuman Ab
31	3	" "	$1x10^7$	$1x10^8$	43	24	1	antihuman Ab
32	3	MOPC 315.43 cells; viability monitored; no ouabain, immunogen: sheep erythrocytes;	$1x10^7$	$1x10^8$	0			
33	3	MOPC 315.43 cells; viability monitored; no ouabain, immunogen: human erythrocytes	$1x10^7$	$1x10^8$	28	17	0	non specific Ab only
34	3	MOPC 315.43 cells; viability monitored, no ouabain, immunogen: sheep erythrocytes	$1x10^7$	$1x10^8$	0			
35	3	MOPC 315.43 cells; viability monitored; no ouabain, immunogen: human erythrocytes	$1x10^7$	$1x10^8$	37	24	2	antihuman Ab

* The conditions used in the fusion experiments are described in detail in the methods section, and ref. 50.

following the fusion step the incidence of hybridoma colony formation is considerably higher than in those fusions in which the viability of the cells following fusion is low. Referring to TABLE VII, in 9 cell cultures in which mean viability of cells immediately after fusion is less than 70% (cultures 20-24, 26, 29, 32 & 34) no hybridoma colonies developed, whereas in the 10 cultures in which viability of cells immediately after fusion is 70% or higher (cultures 18, 19, 25, 27a, 27b, 28, 30, 31, 33 & 35) seven of these later developed hybridoma colonies.

Some further variations were used in fusions 18-35:

Fusions 20-27a Ouabain was added to the cultures following the fusion step. The use of ouabain to inhibit growth of fibroblasts from spleen cells resulted in a drop in viability of cells in the 24 hours following the fusion. As the growth of non-hybridised spleen cells had caused no problem and in view of the drop in viability, the use of ouabain was discontinued.

Fusions 18, 19 & 27b-35 Ouabain was not added to these cultures. The viability in these cultures remained high throughout fusion monitoring, and a large number of these fusion experiments produced hybridoma colonies. The high correlation between viability and hybridoma colony formation is considered significant.

TABLE VII

Viability Monitoring of Cells of Fusions 18 - 35

Fusion #	viability of* myeloma cells ($1x10^7$)	viability of* spleen cells ($1x10^8$)	mean viability of cells before fusion	mean viability of cells immediately after fusion	mean viability of cells 24 hours after fusion	hybridoma‡ colonies present
18	89%	94%	94%	71%	68%	+
19	90%	94%	94%	79%	72%	+
20	86%	92%	91%	23%	23%	-
21	89%	91%	91%	21%	24%	-
22	86%	95%	94%	48%	24%	-
23	87%	95%	94%	49%	23%	-
24	89%	95%	94%	51%	21%	-
25	86%	96%	95%	71%	54%	-
26	96%	91%	91%	69%	52%	-
27a	90%	91%	91%	73%	58%	-
b	90%	91%	91%	73%	69%	+
28	86%	93%	92%	70%	73%	-
29	90%	91%	91%	69%	50%	-
30	89%	91%	91%	71%	84%	+
31	86%	96%	95%	70%	79%	+
32	89%	95%	94%	68%	72%	-
33	90%	96%	95%	73%	79%	+
34	86%	96%	95%	51%	43%	-
35	89%	96%	95%	74%	81%	+

‡ refer to Table X

* fusion experiments were discontinued if the viability of either myeloma cells or spleen cells was below 85% or 90% respectively

Hybridoma incidence and quality

Several days after fusion small white pinhead size clumps became macroscopically visible in some of the wells. Examination of these with the inverted phase microscope showed colonies of spherical cells, confluent and several layers deep. Over the next few days the colonies became larger and the morphology of the cells remained the same. Ouabain can be used in fusions with MOPC 315.43 in order to kill unfused spleen cells some of which can survive in tissue culture. Although ouabain was not used the rapidly developing colonies were believed to be hybridoma colonies. The rapid proliferation of cells is characteristic of hybridoma cells, where as those spleen cells which survive and proliferate are commonly fibroblasts which are slow growing fusifoid or irregularly shaped cells.

In five out of nine fusions in the final (after fusion 26) phase of this work hybridoma cell colonies were noted in some of the wells. From their distribution in the wells it was concluded that there were 152 separate colonies present in 112 wells.

Evidence of immunoglobulin production:

Referring again to TABLE VI - it is observed that supernatants of 152 separate colonies were tested for their ability to inhibit agglutination of sheep cells sensitized with a sub-agglutinating amount of mouse serum. Of the supernatants of the 152 colonies, 82 are seen to inhibit agglutination. These are therefore considered to contain immunoglobulin produced by the cells of the colony.

Evidence of antibody production against the human RBC used for immunization:

Referring to TABLE VIII - assay for anti-human RBC antibody - it is seen that of 82 immunoglobulin containing supernatants only four agglutinated the human RBC that had been used for immunization. All other clone supernatants which were found to contain immunoglobulin by the inhibition of agglutination assay were tested for direct agglutination of the specific human RBC and found to be negative. The supernatants of the four positive assays are concluded to contain antibody directed at the specific human RBC surface antigen(s). The supernatants from these four clones were diluted to determine the strength of antibody present. From the table the titre of these supernatants range from 1/64 to 1/1024.

Three days later these supernatants were again tested for ability to agglutinate the specific human RBC. The volume of supernatant had been doubled by medium required to nourish the growing hybridoma colony, therefore with a dilution factor of 2 one can see if antibody formation had continued during the next 3 days. Consulting the table, it is seen that amount of antibody in terms of the titre has reached the same level in 3 cases and has increased in one case.

Evidence for species specific antibody:

Red blood cells of several species of mammal plus a bird were tested against the supernatants of clones F30-D1, F31-A3, F35-A4 and F35-E4 for direct agglutination. Examining TABLE IX - Agglutination of cells of these various species - it is seen that supernatants from

TABLE VIII

Assay for Specific Anti-Human RBC Antibody

supernatant of clone #	direct agglutination of specific human RBC	titre (21 days post fusion)	titre (24 days post fusion)
F27b-A1	-		
-A9a	-		
-A9b	-		
-B3	-		
-C4b	-		
-L7	-		
-L9	-		
-M9	-		
-O7	-		
F30-B4	-		
-B6b	-		
-D1	+++	1/256	1/256
-G1c	-		
-N4a	-		
-N4b	-		
-N5	-		
-N6	-		
F31-A3	+++	1/1024	1/1024
-A8	-		
-D1	-		
-D3	-		
-D7	-		
-M4a	-		
-M4b	-		
-M5	-		
-M6	-		
-N6	-		
-O7	-		
-O9	-		

TABLE VIII (cont'd)

supernatant of clone #	direct agglutination of specific human RBC	titre (21 days post fusion)	titre (24 days post fusion)
F31-011	-		
-012	-		
-Q3	-		
-Q4	-		
-Q6	-		
-R3	-		
-R4	-		
-S3	-		
-S4	-		
-S6	-		
-T2	-		
-T3a	-		
F33-A1	-		
-A2	-		
-A3a	-		
-A3b	-		
-A3c	-		
-N1	-		
-012a	-		
-P3	-		
-P12	-		
-Q3	-		
-Q7a	-		
-S3	-		
-S6	-		
-S8b	-		
-T10	-		
-U8a	-		
-U8b	-		

TABLE VIII (cont'd)

supernatant of clone #	direct agglutination of specific human RBC	titre (21 days post fusion)	titre (24 days post fusion)
F35-A1	-		
-A2a	-		
-A4	+++	1/64	1/128
-A6a	-		
-A6b	-		
-A6c	-		
-C1	-		
-E4	+++	1/64	1/64
-E6	-		
-E7b	-		
-Ec	-		
-Ed	-		
-E11	-		
-F7	-		
-G8	-		
-H1	-		
-H2	-		
-H7	-		
-H8	-		
-H11	-		
-M1	-		
-S2b	-		
-T7a	-		
-T7b	-		
+ve control (immune mouse serum)	+++	1/64	1/64
-ve control (tissue culture medium from wells containing no clones)	-		

TABLE IX

Agglutination of RBC of Various Species of Animals

species of RB cells	agglutination of cells using supernatant of clone # F30-D1	F31-A3	F35-A4	F35-E4
goat	-	-	-	-
pig	-	-	-	-
mouse	-	-	-	-
dog	-	-	-	-
cat	-	-	-	-
rabbit	-	-	-	-
guinea pig	-	-	-	-
rat	-	-	-	-
sheep	-	-	-	-
chicken	-	-	-	-
+ve control (human)	+++	+++	+++	+++
-ve control (tissue culture medium from wells containing no clones)	-	-	-	-

these colonies were unable to agglutinate the cells of any of the species tested. The antibodies found in the four supernatants are therefore considered to be specific for antigen(s) found on the human RBC.

Evidence that the antibody is directed at a common surface antigen of the human RBC:

A panel of cells from 87 human volunteers was prepared to test the ability of the antibodies of the four clones for direct agglutination. The panel of volunteers was chosen at random and contained the common blood groupings listed in TABLE X. Refering to the table, it is seen that in all cases agglutination of the cells was caused by antibodies in each of the four supernatants. The antibodies present in the clone supernatants are therefore considered to be directed at an antigen which is likely to be common to most human red blood cells.

Evidence of the class of antibody:

The four supernatants which were considered to contain anti-human antibody were tested to determine the class of antibody. An equal volume of 0.1 molar 2-Mercaptoethanol was added to each supernatant and mixed. Following thirty minutes incubation at 37°C the solutions were tested for ability to agglutinate human RBC.

Results

Referring to TABLE XI - agglutination of HRBC (human red blood cells) following 2-Mercaptoethanol treatment of clone supernatant - supernatants from clones F30-D1, F31-A3, F35-A4 and F35-E4 no longer agglutinated human RBC, but the control, serum from hyper-immunized mice (therefore containing IgG) agglutinated human RBC.

TABLE X

Agglutination of RBC of a Panel of 87 Human Volunteers

	agglutination of cells using supernatant of clone #			
	F30-D1	F31-A3	F35-A4	S35-E4
agglutination of a panel* of RBC of human volunteers (87 individual tests)	+++	+++	+++	+++

*Antigens present on the surface of cells tested were: D,C,E,c,e,M,N,S,s,A,B, P,Lu^a,Lu^b, K,k,Le^a,Le^b,Fy^a,Fy^b,Jk^a,Jk^b,Kp^a,Kp^b.

Antigens present on the surface of the immunising cells were: D,C,c,e,N,s, K,k,Le^b,Fy^a,Fy^b,Jk^b,Kp^b.

TABLE XI

Agglutination of HRBC

Following 2ME Treatment of Clone Supernatant

Supernatant of clone #	F30-D1	F31-A3	F35-A4	F35-E4	+ve control (immune mouse serum)	-ve control (tissue culture medium)
Direct agglutination of human RBC	+ve	+ve	+ve	+ve	+ve	-ve
Direct agglutination of human RBC following 2ME treatment	-ve	-ve	-ve	-ve	+ve	-ve

Conclusion

The inability of the supernatants to agglutinate human RBC following treatment with 2-Mercaptoethanol indicates that antibody of the Ig M class is present in the supernatant of all four clones.

DISCUSSION

The approach used in the lymphocyte hybridoma investigation was to break down the experiment into small components and evaluate each one systematically. This approach is considered essential. Less than optimal conditions in one component of the experiment can result in complete lack of hybridoma formation. Thus when the experiment is carried out as a whole it is very difficult to identify the source of the problem and to eliminate it. The technical development section of the results deals with these components of the experiment.

The optimal conditions for growing the myeloma cells were arrived at through a process of controlled adjustment of growing conditions. The optimal temperature and choice of cell culture medium as indicated in the literature were accepted. In addition, an examination of the effect of cell concentration on growth of viable cells indicated the best feeding protocol for the cells. The exponential growth phase of the myeloma cell could thus be coincided with the intended day of fusion, ensuring a higher viability of myeloma cells. Problems of the probability of a mycoplasma infection in one strain of myeloma cell (NS-1) directed that the work should continue using the MOPC 315.43 line of myeloma cell. Consequently, some of the studies and conclusions drawn on the NS-1 cell work were used as a guide for MOPC 315.43 cells.

The next component considered - the danger of jeopardizing the viability of the cells through addition of toxic medium or reagents - necessitated a series of tests for evaluating toxicity. These tests indicated toxic batches of fetal calf serum. Also they ensured that

each batch of enriched medium was functioning properly. The tests for polyethylene glycol (PEG) investigated the toxic effect of various concentrations and exposure times of PEG on myeloma cells in an attempt to minimize the damage to the cells, but allowing exposure comparable to that directed in the fusion protocols. Cells exposed to PEG become quite fragile and must therefore be kept from overexposure. Following exposure to PEG it was found essential that the cells be handled very gently, allowing the membranes to regain their normal tenacity, over the ensuing 24 hours.

The immunization schedule is considered to be of prime importance for the production within the spleen of activated B cells - the favoured partner in cell fusion - and for increasing the level of and specificity of antibody production by these cells. Evaluation of the response of the spleen cells after various lag times, was attempted using the Jerne plaque assay. This assay was met with very limited success when the immunizing cell was of human origin, yet when the immunizing cell was the sheep RBC no such problems were encountered. The reason for this phenomenon is not clear. An article later discovered in which Cunningham[51] investigated the response of various species of animal in the Jerne plaque assay also shows the lack of response of human RBC in the Jerne plaque assay. Because of the non-response of the human RBC in this assay, I decided to be guided by the accepted immunization schedules for fusion work, found in the literature. Indications were that 4 days post 1° immunization favoured Ig M production, and that 4 days post-2° or multiple immunization favoured

Ig G production. The production of Ig M, a stronger direct agglutinin, was desired and therefore the schedule selected was a single immunization 4 days before fusion of the cells.

The choice of mouse strain to be used in cell fusion was initially based on the response each strain exhibited in the Jerne plaque assay using the SRBC (sheep red blood cells) as immunogen and as target cell. Little difference in the three strains was exhibited in terms of response in the Jerne plaque assay. Therefore other criteria were used in the choice of mouse strain. A hybridoma cell colony once established can be grown to large numbers inside the BALB/c mouse, as an ascites tumor. The BALB/c mouse is particularly well suited to this purpose[34] but immunosupression for ascites growth of foreign types of cells is necessary. The myeloma cell used is of BALB/c origin. If the second parental cell of the hybridoma is also of BALB/c origin, no immunosuppression is needed to grow the hybridoma colony as the ascites tumor in a BALB/c mouse. Although the ascites tumor growth of the hybridoma was not planned for this investigation, the ability of the hybridomas produced in this study to be easily adapted for ascites growth was considered worthwhile for possible future studies. Therefore the BALB/c strain of mouse was chosen for the fusion partner.

In many fusion experiments the use of ouabain appeared to prevent the development of hybrid clones. Ouabain is used to kill off unfused spleen cells. Although some of these cells occasionally develop into fibroblasts, this problem didn't arise in these experiments and spleen cells died off within several days of fusion. In the early stages of hybridoma colony formation low cell density can prevent growth of cells

in vitro. A feeder effect can be provided by many types of cells to overcome this deficiency. The growth supportive effect of macrophages has been successfully used in cloning under limiting dilution conditions in liquid culture as well as for soft agar cloning[50,52,53] Thymus cells and erythrocytes[54,55] have also been used to provide a feeder effect in cloning hybridoma cells. In my experiments it appears that in cultures untreated with ouabain extra assistance in terms of a feeder effect was provided by the surviving spleen cells during the first few days after fusion. The critical period when few hybrid cells have developed was therefore overcome and cloning efficiency increased in these cultures.

Direct agglutination of the immunogen, the human erythrocyte, provides a simple test for determining if the culture supernatant contains an appreciable amount of antibody specific for the human erythrocyte surface, presumably secreted by the hybridoma colony. Hybridomas which secrete antibody not specific for the immunogen arise if the parental B cell was not activated by antigens on the human erthrocyte surface. Inhibition of the antiglobulin test provides a method for determining wells in which there is specific or non specific antibody production by the hybridoma colonies. This test is capable of detecting small amounts of immunoglobulin and can therefore be used to indicate wells in which specific antibody production by hybridomas is low and might otherwise be missed by direct agglutination. Any wells which contain immunoglobulin by the inhibition test were tested by direct agglutination for the specific antibody. In case antibody production in these wells was a bit low due to hybridoma colonies

being small or slow in becoming established, they were retested by direct agglutination several days later. This insured that colonies of hybridoma cells established within 24 days of fusion producing antibody directed at immunizing human erythrocyte would be detected.

Of the 152 colonies of hybridoma cells, it was found that 82 secreted immunoglobulin. These results agree well with work by Milstein's group[28] in which immunoglobulin production by viable hybrids occurred in approximately 50% of the hybrids. Of the 82 bybridomas which secrete antibody only four were capable of agglutinating the human erythrocyte. The immunogen, the human erythrocyte has many surface molecules many of which could be the target for the immune response exhibited by the immunized mouse but in most species immune response appears to be preferential to certain antigens. High backgrounds to ABO antigens exists in the mouse, therefore to obtain antibody response to other antigens, more extensive fusion experiments would be required. In recent experiments by Mosmann et al[56] cell fusion using the spleen cells of a mouse immunized with type A human erythrocytes was carried out. Of a total of 74 hybrids that produced antibody to human erythrocytes 53 were specific for the A blood group. In fusions by this group a high response to both A and B antigens was found. This strong preference for a particular determinant can only be revealed by using the appropriate immunogen.

In my experiments antigens present on the surface of the immunizing cells were D,C,c,e,N,s,K,k,Le^b,Fy^a,Fy^b,Jk^b,Kp^b. The absence of A and B antigens prevents the same strong preferential response exhibited in the above mentioned fusion experiments. Antibodies produced by the

four hybridomas which agglutinate the immunizing erthrocytes also agglutinate all cells of the panel of 87 humans indicating the likelihood that the target antigen is common to most human RBC. It is possible that if a larger number of cell donors are tested a rare one may be found which is not agglutinated by one or other of the monoclonal antibodies. Considering the preferential response to the antigens of the ABO system, a response to the H antigen of this system is likely but would be revealed only by testing for agglutination of the rare cells of the Bombay phenotype which are lacking in A, B and H antigens. If the antibodies which agglutinate all cells of the panel do not agglutinate Bombay cells, the antibody is specific for the H antigen. As such a test was not performed, the conclusions reached is that the anti-human antibodies are directed at antigens common to must human erythrocytes.

In these experiments an allelic response was not demonstrated by any of the four human erythrocyte agglutinating antibodies. The likelihood of finding antibodies with allelic specificities would be increased if additional fusion experiments were undertaken. A small percentage of human erythrocyte agglutinating antibodies produced by resulting hybridomas would be expected to display allelic specificity, but this would necessitate more extensive fusion experiments not within the scope of this study. Another approach could also increase the chance of identifying allelic specificities: The use of a more extensive panel of cells could be employed in which allelic forms of all known public human erthrocyte antigens appear. With the small number of specific anti-human antibodies resulting from my fusion experiments the lack of an allelic response is not unexpected.

CONCLUSION

The cell fusion technique was thoroughly investigated and is currently being employed in this laboratory. Through the experiments four bybridoma colonies were established and grown for several weeks in tissue culture. Their products were analysed. It is evident from these experiments that even when the basic techniques have been mastered, the desired product of the hybridomas, namely antibodies with particular allotypic specificity are still going to be difficult to obtain.

The yield of hybridomas increased dramatically when monitoring of the cell viability was employed. In the interest of increasing this yield still further it is felt that other aspects of the pre-fusion considerations are worthy of refinement. That is the immunization schedule and also the time between the immunization and day of fusion. The schedules used in these studies can be examined to determine variations which yield the highest numbers of hybridomas. Even when the frequency of desirable hybrids is low, rapid screening for specific antibody production would allow large numbers of hybridomas to be practically handled. Such rapid screening techniques include direct and indirect agglutination of the target cells. Indirect agglutination using anti-mouse globulin has the advantage of increased sensitivity over direct agglutination tests and can still be carried out quickly on a large scale. It is felt that it would be wise to discontinue the antiglobulin inhibition test previously employed as this test is time consuming and superfluous where direct and indirect agglutination would serve to locate those clones secreting useful antibodies.

Another aspect of the lymphocyte hybridoma study is worth investigating. The use of the poly-L-lysine bound monolayer preparations of erythrocytes bearing appropriately bound antigen was tried but not pursued in these experiments. Selection of appropriate spleen cells using this approach should increase the percentage of hybridomas secreting the desired specificity of antibody. In continuing hybridoma experiments it would be wise to use pre-selection procedures such as this. A different approach to pre-selection which also should improve the success of obtaining functional hybridomas includes rosetting methods with appropriate antigens attached to sheep erythrocytes. Preselection of cells used in conjunction with fusion methods for small numbers of cells[42] is in my opinion the best direction for continuing experimentation, and would likely result in a larger percentage of the hybridomas secreting antibody with allotypic specificity.

An increasing number of laboratories are using monoclonal antibodies in research and for various biomedical applications. As the lymphocyte hybridoma techniques become widespread more complete data on optimal conditions for growth, fusion and cloning of cells, are being compiled. Applications of monoclonal antibodies are rapidly expanding and hold great promise in biomedical fields. Up to now continuous growth of human myeloma cells in vitro has remained a formidable problem. Success in this area of lymphocyte cultures would certainly open the way for obtaining human lymphocyte hybridomas producing human monoclonal antibodies - which may well revolutionize the clinical applications of monoclonal antibodies.

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