TECHNICAL ASPECTS OF THE PRODUCTION AND GROWTH OF HYBRIDOMAS

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(Technische aspekten van de produktie en groei van hybridomen)

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CHAPTER I

INTRODUCTION

This thesis deals with factors influencing the production and growth of hybridomas. The hybridoma technique concerns fusion of antibody-forming cells to appropriate tumor cells to produce hybrid cells that can grow continuously and can serve as an unlimited source of homogeneous antibodies with specificity for one particular antigen.

Conventional antisera used in medicine and basic science are usually obtained by immunization of animals. There are, however, certain inherent limitations in the use of these antisera. First, it is impossible to reproduce precisely any given antiserum; each antiserum is broadly specific and usually contains antibodies to many different antigenic determinants (Brodsky et al., 1979; Ledbetter and Herzenberg, 1979). Even a small polypeptide molecule such as insulin has a variety of antigenic sites against which antibodies may be directed (Wu et al., 1986). Secondly, for any well-defined antigenic site a broad variety of antibodies can be raised with differences in affinity, valency and (sub)class, all of which may affect the functional activity of the antibody. Thirdly, conventional antisera are limited by both the quantity and titer of antibody that can be obtained.

The hybridoma technique, introduced by Köhler and Milstein (1975), enables the production of homogeneous antibodies directed to an individual antigenic determinant. In this technique antibody-secreting spleen cells, usually derived from mice or rats immunized with a particular antigen, are fused to a cancerous type of plasma cell known as a plasmacytoma, preferably one defective in immunoglobulin secretion and preferably originating from the same species as the antibody-secreting spleen cells. The hybrid cell (hybridoma) so formed produces a single type of antibody molecule of its spleen cell parent and continually grows and divides like its plasmacytoma cell parent. Using appropriately drugmarked plasmacytoma cells and selective media, the plasmacytoma cells and spleen cells that did not fuse with each other, can be eliminated. By limiting dilutional cloning, large numbers of hybridoma cells which had been derived

from a single antibody-forming cell, can grow in culture. The progeny of such a cloned hybrid cell were found to produce a monoclonal antibody - that is, an antibody of a single (sub)class and specificity, whose physical, chemical and immunological properties are constant and directed to a single antigenic determinant. Unlimited amounts of antibody can be produced from such hybridoma cells, either as ascites in mice or as culture supernatants of cells grown in tissue culture. Such hybridoma cells can be frozen and stored indefinitely and can easily be distributed to laboratories and hospitals.

Technical aspects of the fusion technique are reviewed in Chapter II.

Chapter III pays special attention to the growth of hybridomas. It reviews the literature with regard to growth promoters and possible causes for the loss of specific antibody production in culture. Subsequent chapters deal with the experimental work performed for this thesis.

One of the problems that arise in the production of hybridoma antibodies is that an antibody-producing clone may be outgrown by non-antibody producing clones. When this is prevented and the hybridoma is successfully rescued, another problem may be encountered, namely, the hybridoma may stop secreting the antibody and thus will be lost to subsequent use. Chapter IV pays attention to these particular aspects of monoclonal antibody production. It describes experiments aimed to investigate the influence of the ratio between the number of plasmacytoma cells and the number of immune spleen cells on (a) the efficiency of hybridoma formation, (b) the purity of hybridoma clones and (c) the loss of antibody production following fusion.

In order to increase the efficiency of hybridoma production it is important to use feeder cells and growth factors that can increase the cloning efficiency and the proliferative activity. Feeder cells from spleen and thymus, macrophages and human endothelial culture supernatant (HECS) can increase the cloning efficiency (Astaldi et al., 1980). Many investigators use these stimulators in the production of hybridomas. We have attempted to find more efficient stimulators, and tested a combination of dextran sulfate and lipopolysaccharide, macrophage supernatant, and human umbilical

cord serum. Their stimulating capacity was compared with that of HECS, which is known as one of the best factors available yet. These factors proved to be satisfying stimulators and no significant difference in the number of clones after fusion was observed between these stimulators and HECS. When grown in limiting dilution at one cell per well, all stimulators, except macrophage supernatant, proved to have the same stimulating capacity. This part of the study is described in Chapter V.

A new hybridoma growth stimulator, Hy-clone calf serum in combination with red cell lysate, is described in Chapter VI. In Chapter VII the stimulating factor in the red cell lysate, hemin, is determined. This stimulator proved to have the best growth promoting activity of all stimulators tested.

Finally, Chapter VIII reviews the present experimental data in the context of those reported in the literature.

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CHAPTER II

TECHNICAL ASPECTS OF THE PRODUCTION OF HYBRIDOMAS

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Review Article

Improved Fusion Methods. IV. Technical Aspects

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Introduction

Köhler and Milstein (1975) introduced a new technology to obtain a homogeneous antibody directed against individual (single) epitopes on an antigen. They fused antibody secreting spleen cells from immunized mice with murine plasmacytoma cells so that hybridomas were formed. These hybrid cells continually grew and divided, like their plasmacytoma cell parent, and produced monoclonal antibodies. The procedure involves several distinct steps: (1) Immunization; (2) Choice of myeloma cell lines; (3) Fusion of immunogen-primed lymphocytes with myeloma cells; (4) Culture and growth in selective medium; (5) Mass production of antibody in vivo and in vitro.

Many articles and reviews have appeared concerning the principles and problems of the technique and the uses of monoclonal antibodies (Melchers et al., 1978; Kennett, 1979; Milstein et al., 1979; Fazekas de St.Groth and Scheidegger, 1980; Goding, 1980; Kazmar and Fathman, 1980; Kennett et al., 1980; Milstein, 1980; Schröder, 1980; Secher, 1980; Diamond et al., 1981; Yelton and Scharff, 1981; Davis et al., 1982; Diamond and Scharff, 1982; Galfré and Milstein, 1982; Damjanov and Knowles, 1983). However, a large number of heterokaryons never reach mitosis or fail to complete normal mitosis, because of chromosal abnormalities. Very little attention is paid to these processes by immunologists.

The purpose of this paper is to review possible improvements in the hybridoma technique suggested by a review of the literature. The analysis of membrane fusion and synkaryon formation, as well as the stability of hybrid cells, will be discussed.

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(1) Immunization

Antibody production by hybridomas is determined by B lymphocytes. Antibody producing B cells may be obtained: (a) by immunization in vivo; (b) by immunization in vitro; or (c) through fusion of splenic or peripheral blood cells without pre-immunization.

(a) Immunization in vivo

Antibody producing B cells are obtained by immunization of mice or rats. Immunization may be achieved with whole cells as immunogens. Cell surface antigens are highly immunogenic when presented on whole cells. Fusion may be performed either 4 days after priming intravenously or after immunization intraperitoneally and boosting intravenously 2 weeks later. According to Lake et al. (1979) the latter method provides more antibody-producing B cells than the former. Purified antigens, chemically modified antigens or synthetic antigens are also used for immunization. The different immunization procedures and their advantages are reviewed by Damjanov and Knowles (1983).

To improve the antibody response, the antigens are emulsified in complete or incomplete Freund's adjuvant (Stahli et al., 1980). Improvement may also be obtained when specially selected mice strains are used. Boumsell and Bernard (1980) demonstrated that the high responder strain of mice selected by Biozzi et al. (1970) gave rise to larger numbers of antibody secreting hybridomas than the low responder strain.

(b) Immunization in vitro

A different procedure for obtaining specific antibody producing B cells is immunization in vitro. Butler et al. (1983) demonstrated that when human peripheral blood cells are stimulated in vitro, more antigen specific hybridomas are formed than when blood cells from immunized healthy volunteers are used. The methodology and recent advances in the field of in vitro stimulation are reviewed by Reading (1982).

(c) Fusion of splenic or peripheral blood cells without pre-immunization

Human lymphocytes from tumor bearing or virus infected subjects may be fused to produce specific human-human or human-mouse hybridomas. Lymphocytes are obtained from the peripheral circulation, from lymph nodes draining the tumor site or the tumor itself, or from tumor-like seminomas or gliomas heavily infiltrated with lymphocytes. This technique of hybridoma production yields markedly less success than fusions with antigen primed spleen cells (Olsson et al., 1983). One reason for this may be the occurrence of very few dividing cells, as these are the cells that probably give rise to viable hybridomas.

(2) Myeloma Cell Lines

The experimenter is faced with the problem of isolating a small number of hybrid cells from a large number of myeloma cells. This problem can be solved by using a

selective medium which favors the growth of hybrid cells, while killing or inhibiting deficient myeloma cells.

(a) Selection techniques

Differentiated lymphocytes which grow poorly or not at all in vitro, form proliferating hybrids when fused with more active myeloma cells. Such hybrids can be isolated by the use of semi-selective media in which selection is derected only against the rapidly proliferating parent. One such method for specific selection of hybrid cells from parental cells is based upon carriage by the latter of genetic markers in the form of nutritional requirements and drug resistance.

Growth of cells in culture can be inhibited by ouabain. Ouabain is a specific inhibitor of the Na^+-K^+ activated ATPase of the plasma membrane, the enzyme responsible for the active transport of K^+ into the cell and the extrusion of Na^+. The sensitivity of cells of different species is not the same; for example, murine cells are less sensitive than human cells. This phenomenon was utilized by Kozbor et al. (1982b). They fused murine myeloma cells with EBV transformed human B cells. The human cells, being more sensitive, were killed in selective medium containing ouabain. Also, mutant cells resistant to ouabain have been isolated from established cell lines and used for hybridization (Baker et al., 1974).

Other mutant cell lines are selected by the 2 cytotoxic guanine analogues, 8-azaguanine (AG) and 6-thioguanine (TG) (Szybalski et al., 1962; Littlefield, 1964, 1966). Cellular resistance to these synthetic purine analogues is mediated primarily by a reduction or loss of hypoxanthine phosphoribosyltransferase (HPRT). The predominant phenotype of stable cellular variants resistant to high levels of either AG or TG is thus HPRT deficiency, which in turn renders the cells sensitive to the hypoxanthine, aminopterin and thymidine (HAT) medium (Szybalski et al., 1962).

When 2 permanent cell lines are used for fusion, a different selection system is required (Jha and Ozer, 1976; Kozbor et al., 1982a). One of the parental cells must be a double mutant, HPRT negative and resistant to a chosen concentration of ouabain. When this mutant is fused with another cell type sensitive to the same concentration of ouabain, the resulting hybrids are able to grow on HAT medium containing ouabain. Both the parental cell types die because of their defects.

A third selection mechanism exploits deficiency of the enzyme adenine phosphoribosyltransferase (APRT), assigned to the murine chromosome 8. When APRT deficient cells are cultured in selective medium containing azaserine, de novo purine synthesis will be blocked. Taggart and Samloff (1983) described a myeloma cell line, FOX-NY, isolated as a spontaneous mutant of NS-1. This is a double mutant, both HPRT negative (chromosome X) and APRT negative (chromosome 8) (Kozak et al., 1975). These cells are fused with spleen cells from Robertsonian 8.12 (Rb (8.12)) mice, in which the active heavy chain Ig locus on chromosome 12 and a selectable enzyme marker locus APRT on chromosome 8 are genetically linked. In selection medium containing aminopterin to block the HPRT pathway, or azaserine to block the APRT pathway the parental myeloma cells die because of their deficiency. When the selection medium contains hypoxanthine or adenine the hybridomas survive. However, hybridomas selected by the APRT pathway proved to be more numerous

and more stable than those selected by the HPRT pathway. Non-antibody-producing APRT deficient hybridomas that arise by segregation of the 8.12 translocation chromosomes containing the APRT genes and the active heavy chain immunoglobulin gene, are eliminated in selection medium containing azaserine and adenine.

(b) Myeloma cell lines

Permanent growth in vitro of hybridomas is determined by fusion with myeloma cells. Initially myelomas making immunoglobulin heavy and light chains were fused with spleen cells. Under these conditions a hybridoma would make 10 distinct Ig molecules and the specific antibody would comprise only 1/16 of the total Ig (Köhler et al., 1978a; Shulman and Köhler, 1978). By selection techniques, improved myeloma cell lines were obtained. Hybridomas making only specific antibody require a tumor cell fusion partner that itself makes no Ig but can be fused with spleen cells to obtain hybrids secreting only the specific antibody. A number of myeloma cell lines of mouse and rat origin suitable for this purpose is available and listed in Table I. The murine cell lines were derived from BALB/c mice. Merwin and Algire (1959) were the first to induce plasmacytomas in BALB/c mice by insertion of millipore diffusion chambers. A tumor, MPC-11, was generated, and from this tumor a 6-thioguanine resistant plasmacytoma cell line was derived (Margulies et al., 1976).

Another 8-azaguanine resistant murine plasmacytoma cell line, P3K, successfully

TABLE I

MOUSE AND RAT MYELOMA LINES USED FOR CELL FUSION

Name	Parental tumor	Derived from	Phenotype		Authors
			Ig	Drug resistance	
Mouse lines					
4TO.2	MPC-11	45.6TG 1.7	IgG2b (K)	6-Thioguanine 1.0 mM ouabain	Margulies et al., 1976
P3-X63/Ag8	MOPC 21	P3K	IgGl (K)	8-Azaguanine	Köhler and Milstein, 1975
NSI/1.Ag4.1	MOPC 21	P3-X63/Ag8	(K) Non-secreted	8-Azaguanine	Köhler et al., 1978b
FOX-NY	MOPC 21	NS-1	None	8-Azaguanine 2,6-Diaminopurine	Taggart and Samloff, 1983
SP2/0	MOPC 21	P3-X63/Ag8	None	8-Azaguanine	Shulman et al., 1978
X63-Ag8.653	MOPC 21	P3-X63/Ag	None	8-Azaguanine	Kearney et al., 1979
Rat lines					
Y3-Agl.2.3	SR 210	210.RCY3.Agl	(K)	8-Azaguanine	Galfré et al., 1979
IR938F	SR 210	Y3.Agl.2.3	None	8-Azaguanine	Bazin, 1983

grown in cell culture, was derived from a mineral oil induced MOPC 21 tumor (Horibata and Harris, 1970).

Ileocecal immunocytoma tumor cells, R210, of the Lou/Ws1 rat strain are readily transplantable in isologous hosts without losing their capacity for immunoglobulin production (Bazin et al., 1972). It is from this rat tumor that Querinjean and Milstein (1972) developed a tissue culture line, 210.RCY3.Ag1, resistant to 8-azaguanine.

Rat-rat hybrids are a better alternative than mouse-mouse hybrids because the rat spleen is the major if not sole site of antibody production after intravenous injection of antigen (McKearn et al., 1980). The final recovery of positive clones from early hybrid cultures appears to be easier with rat lines (Milstein et al., 1980). Also the yield of serum and ascitic fluid is about 10 times better in tumor bearing rats than mice (Galfré et al., 1979, 1980; Choo et al., 1980).

Certain human myeloma cell lines have been described as suitable for human-human cell hybridization. Human myeloma cell lines yield much lower hybridization frequencies than mouse hybridomas (Vaughan et al., 1976; Olsson et al., 1983). There may be several reasons for this. Thus the mouse P3 cell line has been cultured and selected for many years and is a well established, good growing cell line. Human cell lines are much younger. Also, most human myeloma cells do not grow as fast as mouse myeloma cells. Recently a review of human hybridomas appeared (Kozbor and Roder, 1983).

(3) Fusion of Immunogen-Primed Lymphocytes with Myeloma Cells

In the hybridoma technique, cultures with different growth rates are fused and heterokaryons are formed from all combinations of G1, S, G2, and mitotic cells. After cell fusion, synchronization of DNA synthesis is essential for the survival of hybridomas. Nuclei in interphase enter mitosis with almost perfect synchrony (Rao and Johnson, 1970). Fusion of mitotic cells with interphase cells result in a precocious attempt of the interphase nucleus to enter mitosis. The chromatin of the interphase nucleus condenses into chromosome-like structures, sometimes with a fragmented appearance, and the nuclear membrane disappears. This phenomenon was called premature chromosome condensation (PCC) by Johnson and Rao (1970). PCC occurs within 10 min after fusion. The prematurely condensed chromosomes are either randomly segregated into the daughter nuclei and/or remain as fragments of chromatin in the cytoplasm which are likely to be eliminated during subsequent mitoses. Prematurely condensed chromosomes that are carried through one mitosis are not observed later than 48 h following fusion (Rechsteiner and Parsons, 1976). Rao and Johnson (1972) found that in heterophasic fusions very few of the heterokaryons formed with mitotic cells gave rise to viable synkaryons. PCC occurs in: (a) virus infected cells (Nichols, 1963; Nichols et al., 1964, 1965; Stich et al., 1964; Norrby et al., 1965; Heneen et al., 1970; Obara et al., 1975; Sandberg, 1978); (b) leukemia cells (Hittelman et al., 1979); or (c) mitogen stimulated cells (Hittelman and Rao, 1976; Hanks et al., 1982).

(a) Different fusion methods

It has become clear that somatic cell hybrids are an important source of specific cellular products that cannot be obtained from short-term primary cultures. To produce hybridomas, myeloma cells and spleen cells are washed in Hanks' BSS free of serum, because serum delays cell adhesion (Maroudas, 1975). After centrifugation, liquid is removed by decanting the supernatant and the pellet is loosened by tapping the tube.

Initial experiments on hybridoma formation were performed with Sendai virus. Kao and Michayluk (1974) then introduced a chemical fusogen, polyethylene glycol (PEG), an agglutinating agent for plant protoplasts which leads to cell fusion. One year later Pontecorvo (1975) investigated the use of this agent with mammalian cells. As compared with UV inactivated Sendai virus, PEG increases the number of hybrid cells in myeloma fusions (Gefter et al., 1977) because Sendai virus has fewer agglutination points on the membrane of B cells.

The incidence of PCC induced PEG is comparable with that obtained with inactivated Sendai virus (Lau et al., 1977). An increased incidence of PCC with inactivated virus is not expected. Fusion activity is not associated with infectivity; the virus retains fusing ability after complete inactivation.

Most cell fusions are now performed with PEG. The number of colonies is reported to be increased when PEG is sterilized by filtering instead of autoclaving (Kadish and Wenc, 1983). The optimal concentration of PEG is about 50% and the optimal molecular weight of PEG depends upon the fusion technique used. With monolayers, MW 1000 proved to be optimal and with cells in suspension MW 4000 will do better (Davidson et al., 1976; Steplewski et al., 1976; Fazekas de St.Groth and Scheidegger, 1980).

The yield of hybrid colonies is reported to increase when Ca^{2+} is omitted from the medium for at least 15 min after fusion with PEG (Schneiderman et al., 1979). Sharon et al. (1980) showed that hybridization frequency is highly dependent on the pH of the PEG solution used for fusion and on the cloning medium. Fusing metaphase with interphase cells results within 30 min in 1 or 2 phenomena in the resulting binucleate cells: either PCC of the interphase nucleus or formation of a nuclear envelope around the metaphase chromosomes. The frequency of either occurrence is strongly dependent on environmental pH. At pH 6.6–8.0 PCC predominates, at pH 8.0–8.5 nuclear envelope formation predominates. It appears that a pH that favors that part of the normal cell cycle that includes prophase also favors PCC in interphase-metaphase cells; a pH that favors the part of the cycle that includes telophase and normal nuclear envelope formation also favors membrane formation in interphase-metaphase cells. The frequencies of PCC or nuclear envelope formation in multinucleate cells further depend on the metaphase-interphase ratio (Obara et al., 1973, 1974). Maximal numbers of clones are obtained when the PEG solution used for fusion is at pH 8.0–8.2.

Membrane fusion consists of 2 distinct stages. Cell agglutination, during which the plasma membranes of adjacent cells are brought into close proximity, and the formation of cytoplasmic bridges between cells. These stages are followed by osmotic cell swelling and heterokaryon formation.

Sometimes 10% dimethylsulfoxide (DMSO) is added to the PEG solution (Norwood et al., 1976; Rabinovitch and Norwood, 1981). DMSO, which is fusogenic over a prolonged incubation period (Ahkong et al., 1975) but nonfusogenic on short incubation (Klebe and Mancuso, 1981), is reported to enhance fusion efficiency when added to PEG. Fazekas de St.Groth and Scheidegger (1980) reported no improvement in the number of hybridomas on addition of DMSO. However, the reliability of their results may be questioned because both fusion frequencies and growth rate were very poor compared with our results (Westerwoudt et al., 1984). Care must be taken not to expose cells to DMSO in medium containing Hepes since DMSO allows the buffer to gain entry to the cells with resultant toxicity (Kennett, 1979).

When PEG is added at a temperature of 4°C, only agglutination takes place without cell swelling and fusion. At this temperature fusogens have no harmful influence. Both PEG and DMSO markedly decrease the surface potential of biological membranes (Maggio et al., 1976) and cause charge neutralization of the cell membrane. PEG is slightly negatively charged in aqueous solution. It is hydrophilic, and water is bound to it in solutions at a concentration at least 35% by weight. Dehydration appears to play a role in PEG-mediated fusion (Knutton and Pasternak, 1979; Krähling, 1981).

One minute after exposure of the cells to PEG lipid probes spread from one plasma membrane into the other (Wojcieszyn et al., 1983). Membrane protein intermixing does not occur in all systems undergoing fusion. There is no intermixing between human erythrocytes and cultured cells such as human fibroblasts, monkey kidney cells or hamster kidney cells (Wojcieszyn et al., 1983). One minute later the PEG is diluted in serum-free medium to a concentration of 1–2% at 37°C and the preparation kept at this temperature for 15–30 min. About 4 min after exposure to PEG, small cytoplasmic bridges can be observed with an electron microscope (Maul et al., 1976; Clark and Shan, 1978; Knutton, 1979). In fibroblast monolayers each cell sends out pseudopodia to touch other cells (Pontecorvo et al., 1977).

Intercytoplasmic communication begins 1 to 3 min after dilution of PEG (Wojcieszyn et al., 1981). Water-soluble cytoplasmic proteins do not diffuse from one cell to the other until the PEG is removed. Cytoplasmic mixing is optimal at 37°C and is complete at 4 h (Rabinovitch and Norwood, 1981; Wojcieszyn et al., 1981, 1983). Cell clumping is maximal within 30 min (Steplewski et al., 1976) and cytoplasmic mixing is almost complete after 40 min (Rabinovitch and Norwood, 1981). At a temperature of 37°C cell fusion is induced (Knutton, 1979). Cell fusion is poor at lower temperature (Maul et al., 1976; Steplewski et al., 1976). Following fusion the cells are spun down and resuspended in selective medium.

A totally different fusion technique, electric field-induced fusion, was recently introduced (Bischoff et al., 1982; Vienken and Zimmermann, 1982). This method has the special advantage that yields are high and that the fusion process can be followed under the microscope, so that the resulting hybridoma cells can easily be identified and removed. The use of deficient myeloma cells is thus not necessary.

(b) Cross-species hybridization

Interspecies hybridizations have been achieved in rat-mouse and human-mouse hybridomas. The fusion efficiency, and the functional and karyotypic stability of rat-mouse hybridomas are equal to those of mouse-mouse hybridomas (Howard et al., 1979).

The usual pattern of chromosome segregation in interspecies human-mouse somatic cell hybrids is retention of mouse and loss of human chromosome. However, Minna and Coon (1974) fused an actively dividing human fibroblast cell line with mouse bone marrow and spleen cells, and found that the majority of the resulting hybrid cells retained human chromosomes while losing mouse chromosomes. Stable hybridomas are reported by Kozbor et al. (1982b) who fused mouse myeloma cells with Epstein-Barr virus (EBV) transformed human lymphocytes. It is clear that loss of chromosomes and of the consequent antibody production after fusion is not specific to human cells but inherent in the slow-growing participant, whether of human or of non-human origin.

Another difficulty with human-mouse interspecies hybridomas is that they cannot be grown in parental or BALB/c strains. An alternative solution is growth in nude mice (Noeman et al., 1982). Nude mice are deficient in thymus dependent immunological functions. Since immunological rejection of heterologous tissue grafts depends on the T cell mediated immunity of the host, the nude mouse is unable to reject implanted cells from a genetically non-identical donor. Freedman et al. (1976) demonstrated that the passage of human, mouse, rat, rabbit, Chinese hamster or Syrian hamster cell lines in nude mice does not result in loss or modification of cell-specific markers. However, certain human cell lines do not grow in nude mice (Nilsson et al., 1977). Noeman et al. (1982) reported growth and antibody production in hybridomas derived from fusions of rat splenic lymphocytes and mouse myeloma cells in nude rats irradiated one day before inoculation.

(4) Culture and Growth in Selective Medium

To obtain a satisfactory yield of hybridomas, both selective medium and a growth promoter are essential.

(a) HPRT selective medium

After fusion, the cells are immediately cultured in hypoxanthine (H) 5 mM, aminopterin (A) 1 mM, and thymidine (T) 0.8 mM medium enriched with a growth promoter. Fluctuations in the pH and temperature of the cultures should be avoided (Kennett, 1979), and the plates should be put in the incubator as soon as possible. Once the cultures are in the incubator the door should not be opened for at least 2 days. Occasional opening of the door during this period leads to a growth retardation of about 2 days. Thus, when HAT medium is added 24 h after fusion, this causes gross shock and harms the cells. Similar findings are reported by Fazekas de St.Groth and Scheidegger (1980).

All myeloma cells not fused with spleen cells are dead 1 week after fusion. At this

time an equal volume of hypoxanthine (H) and thymidine (T) medium, without growth stimulator, is added. About 10 days after fusion, the supernatants of wells with clones are examined. Examination should be repeated at least 3 times because of loss of antibody production early after fusion. Three to 4 weeks after fusion, the number of hybrid cells has increased by about 2×10^7. Some can be frozen and some injected into mice explained in a previous publication (Westerwoudt et al., 1984).

(b) Growth promoters

The best growth promoters so far known are Hy-clone calf serum (Greiner) and human umbilical cord serum (Westerwoudt et al., 1983a, b). When serum is used as growth promoter it is advisable not to renew the medium frequently, since in human umbilical cord serum (personal observations), normal human serum (Curtiss and Edgington, 1976) and calf serum (Harrington and Godman, 1980) non-cytotoxic compounds inhibiting proliferation are present. Daily addition of serum causes inhibition rather than a stimulation of cell proliferation.

(5) Mass Production of Antibody In Vitro and In Vivo

The antibody yield of hybridomas in culture medium is rather low (1–100 μg/ml). Such concentrations can be detected by very sensitive test methods. More conventional test methods require milligram quantities obtained by injecting the hybridomas into BALB/c mice.

(a) Cell culture

Stringent culture conditions are important prerequisites for success with hybrids if continued production of immunoglobulin for long periods is to be achieved. Overgrowth because of delayed transfer of the cells induces chromosome loss (Bengtsson et al., 1975). If the pH is too high, there is little or no cellular multiplication, and the protein content per cell increases 2–5-fold over a period of 10–16 days. On restoration of pH to the optimal range, this slowly reverts to normal concentration and cellular multiplication is restored. However, the time required for this 'recovery' is a function of the time for which the cells have been kept in alkaline medium; 5 days after 24 h exposure to pH 8.3 and 11 days after 72 h exposure. There is little growth, whether in terms of cell number or cell protein at low pH. Optimal growth takes place at pH 7.5–7.6 (Ceccarini and Eagle, 1971).

Chromosome stability and antibody production over a period of many years are desirable. Mouse hybridomas meet these requirements when recloned 2 or 3 times a year. There is extensive chromosome loss in human hybrids in the first 3–8 weeks after fusion (Olsson et al., 1983).

(b) Ascitic fluid

When hybrid cells are transferred from in vitro to in vivo conditions they undergo transformation from immature to mature plasma cells. Moreover, secretion of monoclonal antibody is more active in hybridomas from ascites tumors than from

hybrid cells in vitro (Renau-Piqueras et al., 1983). Potter et al. (1972) showed that in BALB/c mice pre-injected with mineral oil before transplantation tumor formation is enhanced compared with mice not pre-injected. This observation has led to the practice of pre-injecting mice with pristane before injection of hybridomas. According to Hoogenraad et al. (1983), pristane pre-injection followed after 10 days by injection of 5×10^5 hybridoma cells is optimal, 100% of the mice developing ascites tumors and the concentration of monoclonal antibody in the ascitic fluid being maximal.

Stability of Hybridomas

Different factors, including genetic control of mitosis, asynchronous DNA replication, premature chromosome condensation, genic disharmony between parental genomes and disturbances in the control of protein metabolism have been thought to effect elimination of chromosomes in hybrid cells. Faulty interaction between chromosomes and the spindle fibers may also be a reason why chromosomes are lost. In polykaryons, multipolar spindle fibers do not interact normally with all the chromosomes and unequal distribution of chromosomes to daughter cells may result.

(a) Chromosome stability

One of the factors that causes elimination of chromosomes in hybrid cells is PCC, mentioned above.

After cell fusion, factors in metaphase cells are capable of initiating biochemical and morphological events in interphase nuclei (Matsui et al., 1972). Factors for PCC induction are present in the cytoplasm of the mitotic cells (Ikeuchi and Sandberg, 1970; Sunkara et al., 1980). Factors present in the mitotic cells bring about breakdown of the nuclear membrane and condensation of the interphase chromatin into chromosomes, a process similar to that which occurs during initiation of mitosis (Matsui et al., 1971; Sunkara et al., 1983). Sunkara et al. (1983) showed that inhibition of polyamine synthesis reduces the ability of mitotic cells to induce chromosome condensation in interphase cells on fusion. There is probably an essential role for polyamines during chromosome condensation in mammalian cells.

These observations suggest that the number of clones formed after fusion is dependent both on the stage of differentiation and on the occurrence of PCC. Probably the only stage suitable for fusion is interphase. Mitotic cells fuse equally with interphase cells and cause PCC in many cases. Because loss of chromosomes is inherent to the slow-growing cell at fusion, mitotic cells inducing PCC will be found among the myeloma parent cells. These cells could be eliminated by means of a monoclonal antibody against mitotic cells (Davis et al., 1983).

PCC does not occur only at the moment of cell fusion, but also at a later stage. This may particularly be the case when malignant cells or mitogen stimulated cells are used as fusion partner.

Several drugs are known to reduce PCC. Spermidine, and some compounds carrying a negative charge, for example EDTA, 33258-H and *p*-fluorophenylalaline,

inhibit PCC (Rao and Johnson, 1971; Marcus and Sperling, 1979; Sunkara et al., 1981). However, when drugs are used to improve hybridization frequencies, the fusogen should be chosen with care. Trypsin or colcemid in combination with PEG does not induce cell fusion. Colcemid in combination with PEG increases cytoplasmic mixing (Wojcieszyn et al., 1981). Both drugs in combination with UV-inactivated Sendai virus enhance the fusion index (Ikeuchi et al., 1971; Vaughan et al., 1976; Hansen and Stadler, 1977).

Elimination of chromosomes by other mechanisms has also been reported. Chromosomes left at the metaphase plate are lost from the daughter cells because they get trapped in the midbody between the cells (Peterson and Berns, 1979). There is an explanation of the mechanics of this. The nucleolus is the site of rRNA synthesis and ribosome assembly and the rDNA is physically located herein. In a wide variety of interspecies hybrids 1 of the 2 complementary rRNA genes is preferentially expressed. Recessive rRNA genes fail to organize nucleolar structure and fail to produce mature rRNA (Elicieri and Green, 1969; Croce et al., 1977). Human-mouse hybrid cells, which show preferential elimination of human chromosomes, synthesize only mouse rRNA, but synthesize a variety of human proteins. Thus a number of different residual human chromosomes are active under these conditions (Elicieri and Green, 1969; Bramwell and Handmaker, 1971). In rodent-rodent interspecies hybridomas, which show no preferential elimination of chromosomes of one species, both sets of rDNA genes remain active even at the synkaryon stage (Elicieri, 1972; Kuter and Rodgers, 1975). This compatibility in rodent-rodent hybrids might also be expected in hybrid cells of the same species.

The time of inactivation of human rRNA does not depend on the extensive loss of human chromosomes, but on the relative number of mouse and human chromosomes in the hybrid cells. Although there is some correlation between recessive rRNA genes and loss of chromosomes (Dev et al., 1979), it is not clear whether the directional loss of chromosomes is the cause or the result of this suppression. Huebner et al. (1977) concluded that during the transition from heterokaryon to hybrid cell, suppression of expression of species specific functions required for the replication of species specific viruses occurs in parallel with the direction of chromosome loss and suppression of rRNA genes.

Stability of antibody production

Brown et al. (1980) and Levi and Dilley (1978) reported satisfactory antibody production from hybridomas of murine myeloma cell lines and human B leukemia cells which synthesize immunoglobulin but fail to secrete it. Raison et al. (1982) described a fusion of mouse myeloma line NS-1 and human tonsillar lymphocytes stimulated in vitro with pokeweed mitogen. They obtained a mouse-human line that continued to secrete human IgG for a period of 2 years in culture. This line was recloned several times to give a panel of secreting subclones. The clones that ceased antibody production could be restimulated with lipopolysaccharide at a concentration of 10 μg/ml to secrete human IgG.

Köhler et al. (1978b) and Köhler (1980) demonstrated that immunoglobulin chain loss is random only in lines with an excess of active light chain genes over heavy

chain genes. In all other combinations preferential heavy chain loss was observed. He postulated that free immunoglobulin heavy chain is toxic for the cells. Loss of antibody production is probably not due to loss of the antibody producing chromosomes. However, at the moment there are no selectable markers to select the human chromosome 14 which carries the genes for human immunoglobulin heavy chains. Recently EBV transformed B cells were fused with mouse X63 myeloma cells. The hybridomas continued to produce antibody up to 6 months (Kozbor et al., 1982a).

Some Comments on the Production of Human Hybridomas

Hybridization frequencies between human-mouse cells are low and between human-human cells even lower. The fusion procedure with human B cells needs much more care and there are some differences in procedure, compared with mouse-mouse hybridomas.

Little is known about resistance to the fusion inducing effect of PEG. PEG dissolved in Hepes buffered medium is extremely toxic to human diploid cells (Norwood et al., 1976). Media should be buffered with $NaHCO_3$ only.

In the presence of PEG, human leukocytes clump together. This could explain the observations reported by Cote et al. (1983) that fusions with mouse myeloma cells produce far more clones than fusions with human myeloma cells. Clumping may be reduced if the leukocytes are dispersed among an equal number of rodent cells and if the PEG exposure time is kept very short (Davidson and Gerard, 1976). Butler et al. (1983) describe optimized conditions for mouse-human hybridomas. They demonstrated that the ratio 2 human blood lymphocytes: 1 mouse myeloma cell is optimal for number of wells with antibody producing hybridomas. A better method to make human-mouse hybridomas appeared to be that described by Brahe and Serra (1981). Fusions are done with cells cultured in monolayers, to obtain a cell ratio of 1 myeloma to 1 B cell. In monolayers, bikaryons prevail at low cell densities while most multinucleated cells have more than 2 nuclei at high cell densities (Lanfranchi and Marin, 1981).

In mouse-mouse hybridomas the growth of hybridomas that retain the spleen cell donated heavy chain Ig gene is promoted (Taggart and Samloff, 1983; Westerwoudt et al., 1984). Cloning shortly after fusion is not essential. However, mouse-human hybridomas at a ratio of 1 human to 2 mouse cells, with a largely diminished human chromosome complement, outgrow hybridoma cell lines with a ratio of 1 human to 1 mouse cell. With the latter, slowly growing hybrid cells should be cloned at an early stage of growth. They may contain hybrid cells with a large complement of human chromosomes (Chen, 1979).

Murine Cells as Source for Hybridomas

Production of mouse hybridomas should be performed with care, since A-oncorna viruses as well as C-oncorna viruses are reported in hybridoma and myeloma cells

(Bartal et al., 1982; Vicklicky and Bartek, 1982; Weis, 1982; Rudolph et al., 1983). However, till now, no infection has been reported.

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CHAPTER III

FACTORS AFFECTING PRODUCTION OF MONOCLONAL ANTIBODIES

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Factors Affecting Production of Monoclonal Antibodies

By REGINE J. WESTERWOUDT

When "nonsecretor" myeloma cells are fused with splenic lymphocytes, the hybrids produce a single immunoglobulin, thus demonstrating an ability inherited from the mouse splenic lymphocyte, and proliferate without limit, which is the contribution of the tumor cell. Hybridization followed by clonal expansion of the antibody-producing hybridoma makes possible the subsequent mass production of antibodies reactive with a single antigenic determinant. Although this technique seems rather simple, a drawback frequently encountered during the procedure is loss of antibody production. Some knowledge concerning the mechanism of action of hybridization is essential to understand this problem.

Polyethylene Glycol (PEG)-Mediated Fusion

Little is known of the mechanism by which PEG operates, but it is likely that its effect on cell membranes is complex. When antioxidants and/or polymerization agents added to commercial PEG are removed, PEG becomes nonfusogenic when used at 1-min incubation time.[1-3] Wojcieszyn and co-workers[3] showed that membranes are brought together at closely opposed contact regions and lipid probes spread from one cell membrane to the other in the presence of both fusogenic or nonfusogenic PEG. Therefore, they assumed that the coordinate action of two distinct components is necessary and that the fusion stimulus is provided by the additives contained in commercial PEG. However, PEG purified by dialysis or recrystallization is not uniformly nonfusogenic, because hen erythrocytes fused following a 15-min incubation with purified PEG.[2]

Synkaryon Formation. A number of biological, physical, and chemical processes which affect cell surfaces and cell aggregation are known to result in formation of polykaryocytes. Cells at all stages of the cell cycle fuse with each other when exposed to PEG. Because of chromosomal abnormalities, a large number of polykaryons never reach mitosis or fail to complete a normal mitosis. Polykaryocytes can remain viable without dividing for several weeks under favorable conditions. However, contin-

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ued survival of most hybrid cells requires the formation of daughter cells with a single nucleus (synkaryons).[4]

Polykaryons containing nuclei of more than one stage are said to be heterophasic, whereas those which contain nuclei from only one stage of the cell cycle are classified as homophasic.

Synchronization of Nuclei in Interphase. The mechanisms responsible for synchronization have been analyzed by Rao and Johnson[5,6] by fusing cells in different phases of the cell cycle with each other. They found that in multinucleate cells containing only G_1 nuclei, all nuclei started DNA synthesis at the same time. In heterophasic G_1/S nuclei of HeLa cells, stimulating substances from the S phase cytoplasm migrate into the G_1 nucleus and DNA synthesis is induced. The greater the proportion of S nuclei, the faster DNA synthesis is induced in the G_1 nuclei. Nuclei in G_1 or G_2 do not inhibit the initiation of DNA synthesis in nuclei entering S phase. The nuclei of these multinucleate cells subsequently enter mitosis with almost perfect synchrony.[5]

The rate and pattern of DNA synthesis of genomes in mouse–Chinese hamster hybrids are regulated autonomously. The S periods of the hybrids are identical to that of the mouse parent, which has the longest S phase and appears to remain constant with time. However, hamster chromosomes complete DNA synthesis considerably earlier than most mouse chromosomes.[7]

Fusion between Mitotic and Interphase Cells. Problems associated with synchronization between mitotic and interphase cells result in a phenomenon called premature chromosome condensation (PCC). PCC is dependent on two events in the post-G_2 mitotic nuclei: (1) the normal condensation of chromosomes within these nuclei, and (2) the breakdown of the nuclear envelopes around these chromosomes.[8] When there is no induction of PCC in mitotic–interphase fused cells the mitotic chromosomes do not complete division, but form micronuclei which synthesize neither DNA nor RNA during the next cycle and are probably genetically inactive. However, in some M/G_1 fusions there is no induction of PCC and mitotic chromosomes can undergo division with one of the daughter cells inheriting the G_1 nucleus. PCC induction is described in more detail by other authors.[9,10]

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PCC and pH. Hybridization frequencies are elevated when the fusion is performed at pH 8.0.[11] The same pH induces nuclear envelope formation and suppresses PCC.[12,13] An elevated pH during the first days after fusion also improves the fusion frequency. Presumably, in this period viable hybrids are being formed from the multinucleated heterokaryocytes.[14]

Chromosome Analysis of Hybrid Cells

Chromosome analysis plays an important part in the discovery of hybrid cells. Intraspecific mouse hybrid cells may contain a total chromosome number which is very close to the sum expected if two cells, one of each parental type, have fused. Interspecific hybrids may show extensive chromosome elimination. This phenomenon, referred to as chromosome segregation, frequently involves the preferential elimination of chromosomes of one species, while the chromosomes of the other species are selectively retained.

Chromosome Stability. About 24 hr after fusion of a permanent mouse cell line and a diploid human cell line, the majority of the hybrid cells contain the complete genome of both parents. The loss of chromosomes increases markedly during the following days.[15] Analysis of hybrid cell lines that show karyotypic instability suggests that cells which eliminate chromosomes from the slower growing parent are at a growth advantage.[16-18]

Chromosome Segregation. Chromosome loss is moderate in mouse–mouse and mouse–rat lymphocyte hybrids, but extensive in mouse–human and mouse–rabbit lymphocyte hybrids.[19] It was found that chromosome segregation in mouse–mouse T-cell hybrids was greater than in rat–mouse B-cell hybrids. This suggests that the chromosome segregation is not merely a species-specific event, but also depends on the parental cells or cell lines used.

Chen[20] concluded that human chromosome loss in man–rodent cell hybrids appears random and the number of lost chromosomes varies

[11] J. Sharon, S. L. Morrison, and E. A. Kabat, *Somatic Cell Genet.* **6**, 435 (1980).
[12] Y. Obara, H. Yoshida, L. S. Chai, H. Weinfeld, and A. A. Sandberg, *J. Cell Biol.* **58**, 608 (1973).
[13] Y. Obara, L. S. Chai, H. Weinfeld, and A. A. Sandberg, *J. Cell Biol.* **62**, 104 (1974).
[14] C. M. Croce, H. Koprowski, and H. Eagle, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1953 (1972).
[15] J. Jami and S. Grandchamp, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 3097 (1971).
[16] Y. Matsuya, H. Green, and C. Basilico, *Nature (London)* **220**, 1199 (1968).
[17] F. T. Kao and T. T. Puck, *Nature (London)* **228**, 329 (1970).
[18] L. J. Donald, H. S. Wang, and J. L. Hamerton, *Somatic Cell Genet.* **8**, 105 (1982).
[19] J. Schröder, M.-L. Sutinen, and H. A. Suomalainen, *Hereditas* **94**, 77 (1981).
[20] T. R. Chen, *Cytogenet. Cell Genet.* **23**, 221 (1979).

widely. Others, however, showed that chromosome loss is not random.[19,21] Survival of hybridomas in selective culture medium is possible when the human chromosomes carrying enzymes necessary for growth in such media (chromosome 17, thymidine kinase; chromosome X, hypoxanthine–guanine phosphoribosyltransferase[22]) are retained. It proved that mouse–human B-cell hybrids preferentially retain human chromosome 14, which carries the human immunoglobulin heavy-chain gene,[23] while mouse–human T-cell hybrids retain human chromosome 6, which carries several genes involved in the immune response.[24] Rushton[25] demonstrated by linear regression analysis of human chromosomes that segregation is concordant and nonindependent. Certain groups of human chromosomes appeared more frequently in the surviving hybrid cell line than others.

While environmental conditions can affect karyotypic stability, they do not explain the differential stability of different lines maintained under similar conditions. Karyotypic stability may be an inherent characteristic of each hybrid line, in that it depends on the integration of the two parental genomes. A balanced hybrid genome capable of survival in culture does not depend on the fusogen used.[26]

Segregation Reversal Genes on the X Chromosome. When the direction of chromosome loss in mouse–Chinese hamster hybrids was compared with the direction of segregation of the same hybrids, to which an additional murine X chromosome was introduced at the time of fusion, it was found that the addition of this X chromosome reversed the direction of chromosome segregation; that is, it led to loss of mouse chromosomes. The reversal in chromosome segregation is mediated by factors located on the X chromosome called segregation reversal genes. In the absence of a foreign X chromosome, mouse–Chinese hamster hybrids uniformly lose Chinese hamster chromosomes. In contrast, hybrids containing a Robertsonian translocation between an additional mouse X chromosome and Chinese hamster chromosome 16 switch their segregation and show loss of mouse chromosomes. This ability is not an *in vitro* acquired or transformation-related property.[27,28] Efforts to control the direction of chromo-

[21] G. Martin and L. Pugliatti-Crippa, *Exp. Cell Res.* **70,** 253 (1972).

[22] C. M. Croce, A. B. Knowles, and H. Koprowski, *Exp. Cell Res.* **82,** 457 (1973).

[23] C. M. Croce, M. Shander, J. Martinis, L. Cicurel, G. G. D'Ancona, and H. Koprowski, *Eur. J. Immunol.* **10,** 486 (1980).

[24] H. A. Suomalainen, R. A. Goldsby, B. A. Osborne, and J. Schröder, *Scand. J. Immunol.* **11,** 163 (1980).

[25] A. R. Rushton, *Cytogenet. Cell Genet.* **17,** 243 (1976).

[26] H. S. Wang, V. Niewezas, H. R. de S. Nazareth, and J. L. Hamerton, *Cytogenet. Cell Genet.* **24,** 233 (1979).

[27] D. D. Pravtcheva and F. H. Ruddle, *Exp. Cell Res.* **146,** 401 (1983).

[28] D. D. Pravtcheva and F. H. Ruddle, *Exp. Cell Res.* **148,** 265 (1983).

some segregation in mouse–Chinese hamster hybrids by irradiation of the murine parental genomes prior to fusion have met with little success.[29]

Interspecific Hybrids

Evolutionary divergence seems responsible for some events occurring during hybridization. From a number of publications it is evident that rodent–human hybrids lose chromosomes derived from one of the parental cells[30–32]; that is, chromosomes of the slower growing parent are either partly or completely lost after fusion. This is in contrast with rodent–rodent hybrids, in which chromosome loss hardly occurs.[33–35]

Aberrations at the Nucleic Acid Level. When human chromosomes are preferentially segregated, only mouse 28 S ribosomal RNA (rRNA) but no human 28 S rRNA is produced.[36] This happens even if human acrocentric chromosomes, where the nucleolus organizer regions (NOR) (containing the genes for rRNA) are located, are retained. The Ag–As silver staining method, which preferentially stains the NOR, only detects the 18 + 28 S rRNA gene sites which are active in the preceding interphase.[30,37,38] In these mouse–human hybrid cells only mouse, but no human 45 S, rRNA precursor gene sites are detected.[39] Results of some investigators suggested that nucleolar RNA synthesis was not suppressed during the first 48 hr in the nuclei of either species in any of the heterokaryons formed by fusion of human and mouse cells.[40] It is generally accepted that the suppression of rRNA synthesis occurs after synkaryon formation and is completed within a few days, the rate depending on the parental cell types and the ratio of their genomes.[41,42] Recently, Kaplan

[29] J. A. Marshall Graves, *Exp. Cell Res.* **125,** 483 (1980).
[30] O. J. Miller, D. A. Miller, V. G. Dev, R. Tantravahi, and C. M. Croce, *Proc. Natl. Acad. Sci. U.S.A.* **73,** 4531 (1976).
[31] C. M. Croce, *Proc. Natl. Acad. Sci. U.S.A.* **73,** 3248 (1976).
[32] C. M. Croce, A. Talavera, C. Basilico, and O. J. Miller, *Proc. Natl. Acad. Sci. U.S.A.* **74,** 694 (1977).
[33] G. L. Eliceiri, *J. Cell Biol.* **53,** 177 (1972).
[34] L. G. Weide, V. G. Dev, and C. S. Rupert, *Exp. Cell Res.* **123,** 424 (1979).
[35] O. J. Miller, V. G. Dev, D. A. Miller, R. Tantravahi, and G. L. Eliceiri, *Exp. Cell Res.* **115,** 457 (1978).
[36] G. L. Eliceiri and H. Green, *J. Mol. Biol.* **41,** 253 (1969).
[37] D. A. Miller, V. G. Dev, R. Tantravahi, and O. J. Miller, *Exp. Cell Res.* **101,** 235 (1976).
[38] R. Tantravahi, D. A. Miller, G. D'Ancona, C. M. Croce, and O. J. Miller, *Exp. Cell Res.* **119,** 387 (1979).
[39] R. P. Perry, D. E. Kelly, V. Schibler, K. Huebner, and C. M. Croce, *J. Cell. Physiol.* **98,** 553 (1979).
[40] J. S. Lipszyc, S. G. Phillips, and O. J. Miller, *Exp. Cell Res.* **133,** 373 (1981).
[41] C. J. Marshall, S. D. Handmaker, and M. E. Bramwell, *J. Cell Sci.* **17,** 307 (1975).
[42] V. G. Dev, D. A. Miller, M. Rechsteiner, and O. J. Miller, *Exp. Cell Res.* **123,** 47 (1979).

and Olstad[43] postulated that the complete cessation of NOR activity might occur soon after the hybridization. Chromatin condensation is not found to accompany inhibition of rRNA synthesis,[44] but "Ag-NOR" proteins are involved in the process of decondensation of NOR chromatin.[45]

A second phenomenon occurring in correspondence with chromosome segregation is suppression of histones, the DNA chain elongation proteins. Human–mouse somatic cell hybrids segregating human chromosomes express only mouse histones.[46,47] However, it is not clear whether species-specific suppression of the production of histones occurs at the transcriptional level as with rRNA[39] or whether it occurs at some post-transcriptional level of control. It may be possible that selective segregation of recessive parent cell chromosomes in interspecific hybrid cells could be induced, in part, by the absence of the appropriate species-specific histones during chromosome replication in hybrid cell lines.

A third phenomenon is the inability of mouse–human hybrids to replicate both species of mitochondrial DNA (mtDNA). mtDNA of the parent whose chromosomes are segregated is absent from the hybrid, even in the early stage when there is little chromosome loss.[48,49] The nucleus provides a major part of the information required for the synthesis of mitochondrial constituents. It is not yet established, however, whether a different mechanism is involved in the suppression of the genes required for mtDNA retention compared to the mechanism operating on the rRNA genes.

Fusion Frequency

Recently activated (probably dividing) B cells fuse preferentially compared to nonactivated B cells.[50,51] Activated peripheral blood cells (PBL) have very low fusion frequencies. This low fusion frequency (only 1 in 10 PBL are B cells) and the fact that only 5×10^{-4} cells produce a specific antibody are barriers to the hybridoma field.[52] The chance of obtaining a

[43] G. Kaplan and R. Olstad, *Exp. Cell Res.* **135,** 379 (1981).

[44] F. J. Medina, M. C. Risueño, M. A. Sánchez-Pina, and M. E. Fernández-Gómez, *Chromosoma* **88,** 149 (1983).

[45] M. A. Sánchez-Pina, F. J. Medina, M. E. Fernández-Gómez, and M. C. Risueño, *Biol. Cell.* **50,** 199 (1984).

[46] K. Ajiro, A. Zweidler, T. Borun, and C. M. Croce, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5599 (1978).

[47] P. Hohmann, L. K. Hohmann, and T. B. Shows, *Somatic Cell Genet.* **6,** 653 (1980).

[48] B. Attardi and G. Attardi, *Proc. Natl. Acad. Sci. U.S.A.* **69,** 129 (1972).

[49] L. de Francesco, G. Attardi, and C. M. Croce, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 4079 (1980).

[50] J. Andersson and F. Melchers, *Curr. Top. Microbiol. Immunol.* **81,** 130 (1978).

[51] J. W. Goding, *J. Immunol. Methods* **39,** 285 (1980).

[52] R. H. Stevens, E. Macy, C. Morrow, and A. Saxon, *J. Immunol.* **122,** 2498 (1979).

specific hybridoma is of the order of 10^{-8}, and for unstimulated PBL even lower.[53,54]

Epstein–Barr virus (EBV)-transformed B cells, which have a doubling time of approximately 24 hr, are better fusion partners than resting PBL.[55] PBL from tetanus toxoid (TT)-immunized individuals, vaccinated with EBV, yielded cells which showed much higher frequencies of hybrid formation (36×10^{-7}) compared to nonstimulated PBL. Furthermore, a marked increase in immunoglobulin secretion was observed after hybridization while preselection of EBV subcultures for high anti-TT production prior to fusion resulted in a 5-fold increase in TT-specific hybridomas.[56]

Growth of Hybridomas

Four different growth stimulators for B-cell hybridomas are described and the efficiency discussed.

Lipopolysaccharides

Lipopolysaccharides (LPS) derived from the cell wall of various strains of *Escherichia coli* stimulate rodent B cells to divide and mature to immunoglobulin-producing B-cell stages. Lipid A, one of the three moieties of which LPS consists, possesses most of the polyclonal B-cell-stimulating activity. It is a mitogen for mature B lymphocytes from a variety of species including rodents, rabbits, chickens, cows, and hamsters, but not for human peripheral blood cells. Only 25–30% of the mature B cells of the spleen, the most prominent cells responding to LPS, generate antibody-secreting cells. Depending on the LPS concentration, two pathways of B-cell differentiation can be followed. At low doses, 20 μg/ml, the B cells differentiate into blast cells while at high doses, 100 μg/ml, the cells transform into plasma cells.[57] Furthermore, the efficiency of stimulation is definitely affected by the origin of the serum,[58,59] the density of the cell suspension, and the presence of accessory cells. In addition, LPS activity has been shown to be enhanced by dextran sulfate.[60]

[53] J. Davidson, S. Katzav, H. Ungar-Waron, Z. Eshkar, J. Haimovich, and Z. Trainin, *Mol. Immunol.* **19,** 893 (1982).

[54] J. Olsson, H. Kronstrøm, A. Cambon-de Mouzon, C. Honsik, T. Brodin, and B. Jakobsen, *J. Immunol. Methods* **61,** 17 (1983).

[55] D. Kozbor and J. C. Roder, *Immunol. Today* **4,** 72 (1983).

[56] D. Kozbor and J. C. Roder, *Eur. J. Immunol.* **14,** 23 (1984).

[57] D. Radoux, G. Goessens, and L. J. Simar, *Eur. J. Cell Biol.* **34,** 193 (1984).

[58] R. J. Westerwoudt, J. Blom, and A. M. Naipal, *Cell. Immunol.* **81,** 268 (1983).

[59] R. J. Westerwoudt, J. Blom, and C. M. H. Harrisson, to be published.

[60] J. Kettman and M. Wetzel, *J. Immunol. Methods* **39,** 203 (1980).

Preparation. Escherichia coli 055:B5 LPS, prepared by the Westphal technique, is dissolved in distilled water to a concentration of 5 mg/ml, and dextran sulfate (DxS; a sodium salt containing 17% sulfur; MW 500,000) is dissolved at a concentration of 4 mg/ml in distilled water. LPS, 0.1 ml, and DxS, 0.05 ml, are added to 10 ml of hybrid-selective medium.

Macrophage Supernatant

The influence of macrophages on host immune responses may be manifested directly via interaction with antigen, or indirectly through the synthesis and secretion of immunoregulatory molecules. Following appropriate stimulation *in vitro*, macrophages secrete effector molecules which are released into the culture medium. These highly potent secretory products may activate lymphocyte function. The macrophage-secreted effector molecules have been collectively defined as monokines. One monokine derived from monocytes that enhances *in vitro* plaque formation of murine spleen cells was named B-cell activation factor (BAF). Recently this monokine or BAF has been identified as interleukin I. Endotoxins have been demonstrated to be one of the most potent stimuli of macrophages leading to synthesis and secretion of monokines.[61]

Proliferation of B cells is sensitive to inhibition by the E-series prostaglandins (PGE). The production of PGE is increased by macrophages exposed to LPS, and is suppressed by the prostaglandin synthetase inhibitor, indomethacin.[62]

Preparation. BALB/c mice are injected with 1.5 ml thiogylcolate medium, and peritoneal exudate cells are harvested after 4 days. The peritoneal cavity is flushed with 4 ml of medium. The cells are cultured at a concentration of 1×10^5 cells/ml in culture medium supplemented with 20 μg LPS/ml and 0.05 μg indomethacin/ml. A stock solution of the latter is prepared by dissolving indomethacin in a minute amount of 95% ethyl alcohol and subsequent adjustment to 10 ml with PBS. Conditioned medium is harvested 2 days later and the cells removed by centrifugation. The conditioned medium is used at a 1 : 4 dilution in selective medium.

Human Endothelial Culture Supernatant

Vascular endothelial cells in culture are capable of producing a growth factor or factors. Activity, promoting proliferation or differentiation of B-cell hybridomas, has also been found in medium conditioned by human

[61] J. I. Kurland, *J. Reticuloendothel. Soc.* **24,** 19 (1978).
[62] J. I. Kurland and A. Bockman, *J. Exp. Med.* **147,** 952 (1978).

umbilical vein endothelium.[63] The nature of this mitogenic factor(s) is still unknown.

Preparation. Endothelial cells are obtained from human umbilical cord veins. The cord is severed from the placenta soon after birth, placed in a sterile container filled with cord buffer, and held at 4° until processing. The cord is inspected, and all areas with clamp marks are cut off. A cannula is inserted into the umbilical vein, and secured by clamping the cord over the cannula with an umbilical cord tie. Polyethylene tubing is slipped over the cannula and a syringe is connected. The vein is perfused with 100 ml of cord buffer to wash out the blood until almost all erythrocytes are removed. The other end of the umbilical vein is then cannulated with a blunt, hubless, needle shaft over which polyethylene tubing is slipped. Then 5–10 ml of 0.2% collagenase in Hanks'/HEPES buffer is infused into the umbilical vein, and the polyethylene tubing is clamped with hemostats. The umbilical cord, suspended by its ends, is placed in a water bath containing cord buffer and incubated at 37° for 20 min. After incubation, the collagenase solution containing the endothelial cells is flushed from the cord by perfusion with 30 ml of Hanks'/HEPES buffer. The effluent is collected in a sterile 50-ml conical tube. The cells are sedimented at 250 *g* for 10 min, washed once with 2 ml medium, and resuspended by trituration in 2.5 ml of fresh culture medium. The cell suspension is divided into one or more bottles. The bottles are incubated at 37° under 5% CO_2, and the medium changed after 4 hr. The cells are fed three times a week with a complete change of fresh culture medium. When the bottom of the bottle is well covered, the supernatant is harvested and called HECS (human endothelial culture supernatant).

Reagents

Cord buffer: 0.14 *M* NaCl. 0.004 *M* KCl, 0.001 *M* phosphate buffer (pH 7.4), 0.011 *M* glucose.

Medium: 30% human serum A, 2 m*M* gentamine, 10 m*M* glucose (end concentration), 50 μg/ml gentamicin, 70% medium 199 (Earle's).

Human Umbilical Cord Serum

Human umbilical cord serum (HUCS) increases the number of hybridoma colonies *in vitro*. The stimulatory effect is significantly greater than that of fetal or newborn calf serum. The stimulatory substances present in HUCS are still unknown. Compared to normal human serum, albumin concentration is decreased and the concentrations of most amino acids

[63] G. C. B. Astaldi, M. C. Janssen, P. Lansdorp, C. Willems, W. P. Zeylemaker, and F. Oosterhof, *J. Immunol.* **125,** 1411 (1980).

are increased.[64] Probably a growth-promoting factor produced by the endothelial cells, the same as in HECS, is also present in the serum.

Preparation. Placental blood is collected during delivery immediately after section of the umbilical cord. A pool of HUCS was made from 10–15 sera from healthy individuals. The serum is heat inactivated for 45 min at 56° and passed through a Millipore filter (0.45-μm pore size). Small aliquots are frozen at $-20°$.

Comparison of the Growth Promoters

Immediately after cell fusion hybridomas were dispensed into microtiter tissue culture plates with 10% fetal calf serum (FCS) or with 10% FCS and one of the four different growth promoters at a concentration of 10^5 spleen cells per well (Table I). As shown in Table I, it appears that with the addition of FCS and one of the growth promoters, both frequencies of spleen cells forming clones and of spleen cells forming antibody-producing clones are elevated. HUCS gives the most rapid growth of hybridomas, even at a concentration as low as 4%. It if is not available, a good alternative is LPS and DxS. HECS also is a good alternative, but it is quite laborious to produce. The frequency of antibody-producing clones cultured in macrophage supernatant is greater than with any other simulator. A disadvantage, however, is that it does not stimulate cells cultured separately in limiting dilution experiments. Figure 1 clearly shows that there is no significant difference between FCS and macrophage supernatant. However, LPS plus DxS, HECS, and HUCS increased the number of clones significantly. More details about the B-cell stimulators will be discussed elsewhere.[65]

Fusion Protocol

The manipulations of the fusion protocol have already been described in an earlier *Methods in Enzymology* volume.[66] There are, however, some additions.

It is my experience that the myeloma cells must be kept under good growing conditions and the medium changed three times a week. The day before fusion, medium is changed one additional time. Good culture conditions of the myeloma cell line improves the fusion frequency. I once

[64] M. H. Malloy, O. K. Rassin, and W. J. McGanity, *Biol. Neonate* **44,** 1 (1983).

[65] R. J. J. M. Westerwoudt, *in* "Hybridoma Formation: Mechanisms and Technical Aspects of Hybridoma Generation and Monoclonal Antibody Production" (A. H. Bartal and Y. Hirshaut, eds.). Humana Press, New York (in press).

[66] R. H. Kennett, this series, Vol. 58, p. 345.

TABLE I

EFFECT OF DIFFERENT FEEDER SYSTEMS ON RECOVERY AND YIELD OF ANTIBODY PRODUCTION BY HYBRIDOMA CELLS AFTER FUSION

		Wells with clones		Antibody-producing clones	
Stimulators	Wells plated[a] (and number of fusions)	Percentage of total plated	Frequency of spleen cells forming hybridoma clones[b] ($\times 10^5$)	Percentage of total plated	Frequency of spleen cells forming clones[b] ($\times 10^6$)
FCS	384 (4)	65	1.05	12	1.3
LPS + DxS	384 (4)	99	4.61	33	4.0
Macrophage supernatant	384 (4)	98	3.91	42	5.4
HECS	288 (3)	99	4.61	34	4.2
HUCA	384 (4)	99	4.61	33	4.0

[a] Concentrations of 10^5 spleen cells per well.

[b] Calculated from the logarithmic form of the zero term of the Poisson distribution ($-\ln Fo$) and the number of spleen cells per well, with the formula: ($-\ln Fo/10^5$ spleen cells).

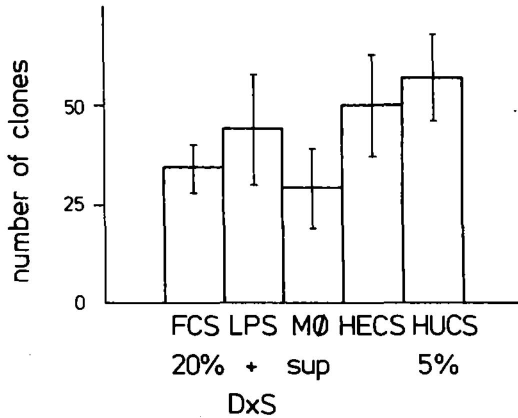

FIG. 1. A hybrid cell line cultured as single cells by limiting dilution to measure the influence of different feeder systems. Each column represents the mean of the number of wells containing hybrid clones (± SD) in four different experiments. Mϕ Sup, macrophage supernatant.

used a myeloma cell line from which the medium was changed fortnightly for a long period. The fusion frequency was very low, about 10% of what it should be. When the cells were kept under optimal culture conditions for 2 months, they grew well, but the fusion frequency was still very low.

Spleens from immunized mice are squeezed with tweezers. Myeloma cells and spleen cells are washed in Hanks' buffer or PBS and fused at a ratio of 1 : 10 using 1 ml PEG at 4°. Two minutes after the addition of PEG the cells are slowly diluted with 40 ml RPMI 1640 at 37° and kept at this temperature for 15–30 min. The cells are pelleted, resuspended in HAT medium enriched with a growth promoter, and distributed into microplates. The plates should be put in the incubator as soon as possible and kept at 37° for at least 3 days, without opening the door of the incubator. One week after fusion an equal amount of HT medium is added.

Although the hybridomas are cultured in HAT medium immediately after fusion and I find high fusion frequencies, Ege[67] reported that the early division products of heterokaryons are sensitive to HAT medium and that the cloning efficiency is increased if exposure to the selective medium is delayed until 5 days after fusion.

[67] T. Ege, *Cell Biol. Int. Rep.* 8, 599 (1984).

Reagents

Polyethylene glycol (PEG): 1 ml PEG 4000 and 0.1 ml dimethyl sulfoxide (DMSO) were added to 1 ml distilled water. The suspension was autoclaved 20 min at 120°.

Aminopterine, 4.4 mg, was diluted in 100 ml of 1 m*M* NaOH at 42°; 2 ml was added to 500 ml of culture medium and the pH adjusted.

Hypoxanthine, 340 mg, and thymidine, 100 mg, were diluted in 500 ml distilled water at 45°; 10 ml was added to 500 ml of culture medium.

Culture medium was supplemented with 1% nonessential amino acid solution, 1% sodium pyruvate, and 1% HEPES.

Antibody Production. Many investigators use the cell fusion technique to obtain monoclonal antibodies. One of the major problems encountered in this procedure is that clones that grow well and produce an apparent stable amount of antibody sometimes stop producing suddenly. One reason for loss of antibody production is inactivation or loss of the chromosome carrying the genes for heavy chains. To reduce this problem to a minimum the hybridomas should be kept under good growing conditions at an optimal pH. There is little growth either in terms of cell number or in terms of cell protein production at a low or high pH.[68] Also, loss of antibody production, mentioned by many investigators, may arise when more than one clone is present in a well. It has been postulated that a non-antibody-producing clone outgrows the antibody-producing clone.

Loss of Antibody Production. The stability of antibody-producing clones is investigated by limiting dilution experiments performed very soon after fusing the cells. Two weeks after fusion, the hybridomas from one antibody-producing clone are plated at one cell per well. Two weeks later, when the individual hybridomas of that clone also form clones so that the concentration of antibody in the well can be measured, supernatants are tested. This experiment is repeated with 36 different antibody-producing clones. In this way an estimation can be obtained of the percentage of clones that lose antibody production very soon after fusion. It appears that about half of the clones of which all hybridomas produce antibody in an initial stage lose this ability almost completely.

Overgrowth of Antibody-Producing Clones by Non-Antibody-Producing Clones. To investigate the overgrowth of antibody-producing clones by non-antibody-producing clones, three fusions were performed at five different concentrations ranging from a mean of 1.5 to a mean of 12 different clones per well. Two weeks after fusion, supernatants of wells

[68] C. Ceccarini and H. Eagle, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 229 (1971).

TABLE II
STABILITY OF ANTIBODY-PRODUCING HYBRIDOMAS WHEN MORE THAN ONE CLONE IS PRESENT IN THE WELL

Mean number of clones	Number of samples tested	Percentage of wells containing 90–100% pure Ab-producing clones
1.5	12	33
3	13	31
4.5	14	14
6	15	20
12	15	7

with clones were tested. From each concentration, clones from a number of wells showing antibody production were cultured by limiting dilution, and again antibody production from the individual hybridomas, grown to small clones, was tested. When at least 90% of the supernatants of the individual hybridomas contain antibody, the clone is considered to be pure. From these experiments it was calculated that 1 out of 7 hybrid clones produce specific antibody. If the antibody-producing clones are in a subordinate growth rate, certainly no antibody-producing clones would be detected at a mean of 3 or more clones per well. From the results of Table II it appears that at all concentrations, even at a mean of 6 or 12 different clones per well, there are antibody-producing clones without contamination of non-antibody-producing clones. Therefore, non-antibody-producing hybridomas are not favored over antibody-producing clones.

Contamination of Antibody-Producing Clones with Non-Antibody-Producing Clones. The question now is whether there is any contamination with non-antibody-producing clones at high hybridoma concentrations after fusion. This was tested in the same series of experiments as described above. If at least one-third of the supernatants of the wells in limiting dilution do not contain antibody it is considered that the clones have lost antibody production, or that non-antibody-producing hybridomas are present, or both. Loss of antibody production due to chromosomal segregation or inactivation was calculated to be 40%. From the results presented in Table III it was established that with increasing mean number of clones per well the percentage of negative wells increases, until at a mean of 12 clones per well, the percentage of negative wells decreases. This is due to a higher number of antibody-producing

TABLE III
STABILITY OF ANTIBODY-PRODUCING HYBRIDOMAS WITH INCREASING NUMBER OF CLONES PER WELL

Mean number of clones per well	Number of samples tested	Percentage of Ab-negative wells (containing ≥33% Ab-negative clones)
1.5	9	44
3	11	55
4.5	8	63
6	8	75
12	9	56

clones per well rather than to a decreased number of non-antibody-producing hybridomas per well.

Comments

Cloning efficiency and maximal survival of hybridomas are important for the success of the fusion. During the whole process a number of events play a part: only binucleated hybridomas give rise to large colonies, no trinucleated or multinucleated cells give rise to continuously growing daughter cells,[67] and fusion of populations of cells which have been synchronized in the same phase of the cell cycle ensure maximal survival of the hybrids.[6,69] Also, Olsson and co-workers[54] have suggested that B lymphocytes should be in a certain stage of differentiation for successful hybridization. This may explain why the number of hybridomas depends on the number of spleen cells and not on the number of myeloma cells used for hybridization. Possibly, only those cells which are in interphase at the time of fusion can form stable hybridomas. It also appeared that suboptimal nutritional conditions led to asynchrony in DNA synthesis and mitosis in multinucleated cells.[70]

From the experiments described previously, it can be concluded that limiting dilution after fusion can be omitted. Similar observations have been made by others.[71] There is hardly any contamination by non-antibody-producing hybridomas. Stability of antibody production is depen-

[69] H. G. Coon, *J. Cell Biol.* **35,** 27A (1967).
[70] S. Ghosh, N. Paweletz, and I. Ghosh, *Chromosoma* **65,** 293 (1978).
[71] E. A. Klasen, personal communication.

dent on chromosome segregation. The stability of antibody-producing clones can be determined after freezing and thawing the hybridomas. Unstable hybrids stop antibody production and lose chromosomes after thawing.[28] Therefore if they do not withstand the freezing process, they cannot be stored and kept for long periods.

Acknowledgments

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CHAPTER IV

STABILITY AND PURITY OF HYBRID CLONES

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Improved Fusion Technique. II. Stability and Purity of Hybrid Clones

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Optimal conditions are defined for hybridoma formation between mouse spleen cells and mouse myeloma cells. The result of using different numbers of spleen cells in the fusion process is reported in 2 parts. Part I deals with the number of spleen cells in relation to hybridoma formation and antibody production. Part II treats of the purity of hybridoma clones and the loss of antibody production following fusion.

Part I. Two series of experiments show that when cell fusion is performed properly the total number of antibody producing clones is greater than in non-standard conditions. The yield of hybridomas obtained with a ratio of mouse myeloma to mouse spleen cells of 1 : 10 did not differ from that reported by De Blas et al. (1981). The number of hybridomas formed seems to depend mainly on the number of mouse spleen cells available. The most satisfactory yield of monoclonal antibodies is obtained under conditions producing growth in approximately 100% of the wells.

Part II. Two weeks after fusion a number of antibody producing clones were cultured in limiting dilution. Analysis of the hybridomas indicated that at least 40% of the antibody producing clones disappear during the first 3 weeks. Antibody producing hybridomas were as a rule not outgrown by non-antibody producing clones.

Key words: *ratio myeloma and spleen cells – hybridoma concentration – limiting dilution – outgrowth of non-antibody producing hybridomas – loss of antibody production*

Introduction

The cell fusion technique designed to obtain hybridomas producing monoclonal antibodies is widely used. Hitherto, only the effect of postfusion cell dilution on hybridoma yield has been studied (Fazekas de St.Groth and Scheidegger, 1980; De Blas et al., 1981). Antibody production, as compared with hyridoma growth, the crux of the whole technique, has not previously been studied. Further, little or no information exists on the homogeneity and stability of antibody producing hy-

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bridomas after cell fusion. Several authors have claimed that antibody producing clones may be outgrown by non-producers (Lemke et al., 1979; Goding, 1980). An experimental basis for this claim has not so far been established.

The results of our investigations reported here are considered on 2 parts. Part I deals with the fusion process itself and the formation of antibody producing hybridomas. Part II considers the stability, homogeneity and liability of monoclonal antibody producing hybridomas to be outgrown.

Material and Methods

A full account of the technical details is given in Westerwoudt et al. (1983). The following is a summary.

Culture medium

Hybridomas were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum, 1% non-essential amino acid solution (100 ×), 3% sodium bicarbonate (100 × 7.5%), 1% Hepes (100 × 1 M) (GIBCO, Grand Island, NY), 4 mM L-glutamine, 100 U/ml penicillin, 100 μl/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol. Concentrations of 5–10% human umbilical cord serum (HUCS) were added.

Cell fusion

BALB/c mice 6–12 weeks old were injected intravenously with 2×10^7 human peripheral blood lymphocytes. Four days later, the spleen was removed. Myeloma cells (Sp2/0-Ag 14) and spleen cells were fused at a ratio of 1 : 10 in 1 ml of 50% polyethylene glycol 4000 and 10% DMSO, for 1 min. The cells were resuspended in a selective medium consisting of 100 μM hypoxanthine with 16 μM thymidine (Fluka AG, Switzerland), 1 μM aminopterine (Serva, Feinbiochem., Heidelberg), in medium containing 5–10% HUCS. The cell suspensions were dispensed into 96-well microtiter tissue culture plates (Greiner, no. 655160), at different concentrations.

Microcytotoxicity assay

The tests were performed in triplicate as described by Westerwoudt et al. (1983). Ficoll-Isopaque isolated lymphocytes were labeled with carboxy-fluorescein diacetate, resuspended in McCoy's medium containing 20% fetal calf serum, and adjusted to a concentration of 2×10^6 cells/ml. To each well of a Terasaki microtest tray (Greiner, no. 653180) was added 1 μl hybridoma culture supernatant, and 1 μl of the lymphocyte suspension. Then after an incubation period of 60 min, 5 μl of pooled rabbit complement were added. After a further incubation period of 2 h the test was terminated by the addition of 5 μl of a 4 mM hemoglobin solution. Positive scores were allotted to wells showing 25% (or more) lysis of the total number of cells in the assay. The scores were subdivided into supernatants reacting with all lymphocytes (75–100% lysis, high lysis) and supernatants reacting with only some of the lymphocytes (25–75% lysis, low lysis).

Limiting dilution

Spleen cells dispensed at concentrations ranging from 0.5×10^5 to 4×10^5 spleen cells per well, were cultured for 14 days after fusion. For limiting dilution, 3–5 hybridoma clones, producing 'high lysis' antibody to peripheral blood lymphocytes (lysis of at least 75% of the target cells) were chosen from each of 3 fusions performed under standard conditions. The hybridoma samples were dispensed into single microtiter tissue culture plates at a mean concentration of about 1 cell/well. After approximately 14 days the wells with growth were counted, and each supernatant was tested with a microcytotoxicity assay. A maximum of 50 wells was tested. The mean number of clones per well was calculated and the data compared with the Poisson probability distribution (Lefkovits and Waldmann, 1979).

According to De Blas et al. (1981) the Poisson distribution may be used as a model for estimation of monoclonal growth. The average number of clones per well (μ) was calculated from the logarithmic form of the zero term of the Poisson distribution

$$\mu = -\ln F_0.$$

F_1 was calculated from the Poisson probability

$$F_i = \frac{(1-m) - \ln(1-m)^i}{i!m}$$

where:

$$m = \frac{\text{number of wells with growth (or antibody production)}}{\text{total number of wells plated}}$$

Linear regression analysis

Linear regression analysis was used to ascertain the correlation between the percentage of wells with clones and the number of spleen cells. Fitting of the data points was carried out by the least squares method, and r^2, the coefficient of determination (goodness of fit), was assessed in order to measure the variability given by a straight line regression.

Results and Discussion

For clarity the working procedure is set out in the form of a flow diagram in Fig. 1.

In the following part I concerns the procedure followed until day 9. In a number of fusion experiments about 5 different concentrations of spleen (and myeloma) cells were dispensed into microtiter tissue culture plates. At day 9 the wells were screened and the supernatants of wells with growth were tested in a microcytotoxicity assay.

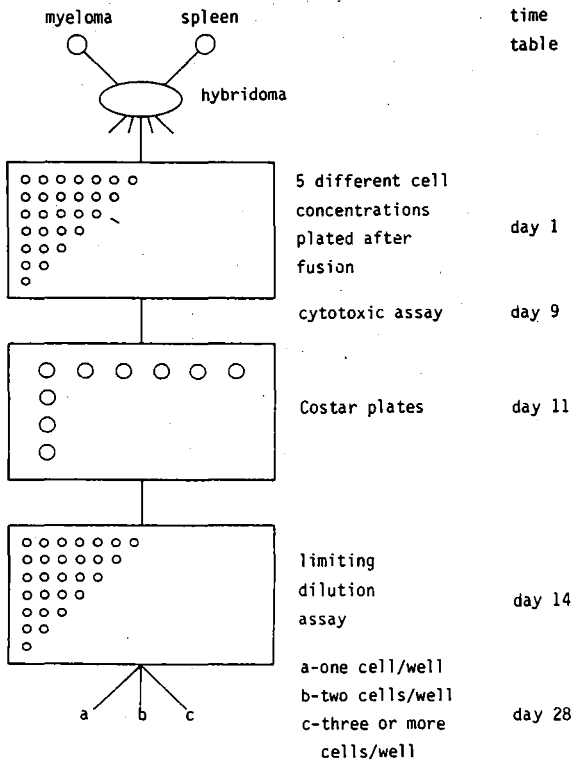

Fig. 1. Flow diagram for the establishment of stable clones. Myeloma cells are fused to spleen cells to form hybridomas. They are dispensed in microtiter tissue culture plates at different concentrations. At day 9 the percentage of wells with clones is calculated and the percentage of antibody producing clones is established. A number of antibody producing clones are transferred to Costar plates. At day 14 the clones are dispensed into microtiter tissue culture plates. Four weeks after fusion the percentage of wells with growing clones from each sample is calculated and antibody production is established.

The experiments in part II start at day 11. From each concentration 3–5 positive wells of 3 different experiments were chosen and cultured in limiting dilution at day 14 after fusion. Two weeks later the wells were screened and the supernatants of the wells with growth were tested in a microcytotoxicity assay. The average number of clones per well for each concentration was calculated and compared with the Poisson probability distribution. The results were tabulated in means of 1 cell/well, 2 cells/well and more cells/well.

Part I

The ratio of myeloma and spleen cells

It is important to know if the formation of hybridomas is mainly determined by the number of spleen cells or by the number of myeloma cells used for fusion. For

this reason we compared our results with the results of De Blas et al. (1981). In our experiments a ratio of 1 myeloma cell to 10 spleen cells was used for the fusion procedure. However the findings in Table I correspond very closely with the results obtained by De Blas et al. (1981), who used proportions of 1 : 2 and 1 : 5 myeloma cells to spleen cells. When the percentage of wells containing clones was plotted separately for the 3 experiments against the number of spleen cells, the slopes of the 3 regression lines were nearly identical. When the data for the different concentrations of spleen cells from the 3 different ratios from the De Blas and our results were combined, a coefficient of determination $r^2 = 0.91$ was obtained. Since inspection of the curve indicated that there were 2 components, the analysis was repeated with the $7.5–8 \times 10^4$ concentrations as a cut-off point. The amount of variability explained by the 2 regression lines was 98% and 91% respectively for lines A and B (Fig. 2). On the other hand, when the percentage of clones was plotted against the number of myeloma cells (Fig. 3), the slopes of the lines were different. To obtain the same number of clones with a ratio of 1 myeloma cell to 5 spleen cells, twice as many myeloma cells were required as with a ratio of 1 : 10. For 1 myeloma cell : 2 spleen cells, 5 times the number of myeloma cells are required for the same yield as for the 1 : 10 ratio.

Thus it appears that the success of hybridoma formation depends on the number of spleen cells used and not on the number of myeloma cells present. We therefore decided to use the spleen cell number as a measure of the success of hybridoma formation.

Suboptimal growth of hybridomas

Table I shows the results of 7 different experiments. Immediately after cell fusion, hybridomas were dispensed into microtiter tissue culture plates at 6 different cell

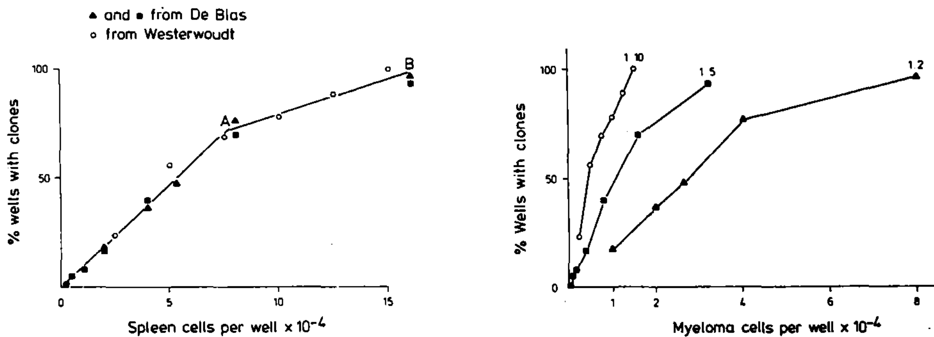

Fig. 2. Data from De Blas et al. (1981) and the present experiments. The different symbols represent the different ratios of myeloma (m) to spleen (s) cells: ▲ = 1m : 2s; ■ = 1m : 5s; O = 1m : 10s. The relation between percentage of wells with clones with the number of spleen cells is presented. The straight lines represent the results of linear regression analysis with the possible transition point at 7.5×10^4 cells/well. r^2 line A = 0.98, r^2 line B = 0.91.

Fig. 3. As Fig. 2, but comparing the percentage of wells with clones with the input number of myeloma cells. The ratios (1m : 10s) : (1m : 5s) : (1m : 2s) = 2 : 4 : 10.

TABLE I

GROWTH OF HYBRIDOMAS AT VARIOUS SPLEEN CELL DILUTIONS AND THEIR ANTIBODY PRODUCTION UNDER SUBOPTIMAL CONDITIONS

Spleen cells per well $\times 10^5$ (A)	Wells plated	Wells with clones			Antibody producing clones		
		Percentage (B)	Average clones per well [b] (μ)	Frequency of spleen cells forming clones [c] $\times 10^5$ (C)	% (D)	Average clones per well [b] (μ')	Frequency of spleen cells forming clones [c] $\times 10^6$ (E)
0.25	288 (3) [a]	23.7 ± 1.5	0.27	1.1	1.00	0.01	0.4
0.50	1056 (7)	56.2 ± 10.7	0.82	1.6	8.30	0.08	1.6
0.75	1056 (7)	69.0 ± 12.0	1.17	1.6	11.08	0.12	1.6
1.00	1056 (7)	78.6 ± 11.7	1.56	1.6	15.92	0.17	1.7
1.25	1056 (7)	88.7 ± 7.0	2.21	1.8	22.77	0.26	2.1
1.50	96 (1)	100	–	–	–	–	–

[a] Number of fusions in parentheses

[b] μ and μ' calculated from the logarithmic form of the zero term of the Poisson distribution ($\mu = -\ln F_0$)

[c] Calculated with the formula μ/A

TABLE IIa

GROWTH OF HYBRIDOMAS AT VARIOUS SPLEEN CELL DILUTIONS AND THEIR ANTIBODY PRODUCTION UNDER OPTIMAL CONDITIONS

Spleen cells per well $\times 10^5$ (A)	Wells plated	Wells with clones			Antibody producing clones		
		Percentage	Average clones per well [b] (μ)	Frequency of spleen cells forming clones [c] $\times 10^5$	% (B)	Average clones per well [b] (μ')	Frequency of spleen cells forming clones [c] $\times 10^6$
0.50	288 (3) [a]	77.3 ± 4.9	1.47	2.9	15.0	0.21	4.0
1.00	288 (3)	100	–	–	31.7	0.38	4.0
1.50	288 (3)	100	–	–	43.3	0.56	4.0
2.00	288 (3)	100	–	–	42.7	0.56	3.0
4.00	288 (3)	100	–	–	68.0 [d]	1.14	3.0

[a,b,c] See Table I.

[d] In 2 'sham' fusions (spleen cells without myeloma cells) a mean of 20% of the wells proved to be slightly positive for antibody production, due to the high concentration of spleen cells. At a lower cell concentration the number of positive wells was less than 0.5%.

TABLE IIb

ANTIBODY PRODUCTION OF SAMPLES FROM TABLE IIa

Spleen cells per well $\times 10^5$	% Estimated by cytotoxic assay			Estimated monoclonal culture (F_1)	Calculated number of monoclonal cultures.[e] (d)	Calculated number of monoclonal high lysis samples [f]
	Antibody present (a)	High lysis (b)	Low lysis (c)			
0.5	15.0	6.7	8.0	90%	13.5	5.5
1.0	31.7	18.3	13.3	82%	26.0	12.7
1.5	43.3	29.3	14.0	74%	32.0	18.0
2.0	42.7	25.7	17.0	75%	32.0	15.0
4.0	68.0 [d]	49.0	19.2	54%	36.0	17.5

[d] See Table IIa.

[e] Calculated from $a \times F_1$.

[f] Calculated from d–c and represents the number of monoclonal cultures in the high lysis column.

concentrations. Column B represents the percentage of wells with clones compared with the total number of wells plated. The standard deviation did not exceed 20%. We therefore concluded that results of all 7 experiments were comparable with each other. Column C shows the frequency of spleen cells forming hybridomas. It appeared that at all concentrations except the first the frequency is very constant. From these values we conclude that for the formation of about 1.6 hybridomas, 10^5 spleen cells are necessary.

Column D shows the percentage of antibody containing wells compared with the total number of wells plated. The standard deviation was not calculated because the number of antibody producing spleen cells depends on the immunization procedure and the immune system of the mouse. Column E represents the frequency of spleen cells that form clones after hybridization. It is clear that for the formation of about 1.6 antibody producing hybridomas, 10^6 spleen cells are necessary.

When the frequencies of hybridomas and of antibody producing hybridomas formed after fusion were compared, it was calculated that approximately 1 out of 10 hybridomas produces antibody. The frequency of hybridomas was very low at the lowest concentration of spleen cells, and the frequency of antibody producing hybridomas was lower than could be expected from the ratio 1 : 10. At a concentration of 1.25×10^5 spleen cells per well there was an increased frequency of hybridoma clones and of antibody forming clones. Probably clonal interaction influences both growth and antibody production.

Optimal growth of hybridomas

Table IIa shows a series of 3 different experiments. In this series hybridoma formation and cell growth proved to be superior to the series shown in Table I. Under these conditions 1 out of 7 hybrid clones produced specific antibodies. The frequency of antibody producing clones was somewhat lower at the highest concentrations. A reason for this may be that hybridomas of different size were formed. Clones consisting of large cells had a lower proliferation rate than clones made up of small cells. We never saw large cells in wells with the 2 highest hybridoma cell concentrations. Probably small hybridoma cells outgrow at a very early stage large hybridoma cells. This means a loss of hybridoma clones among antibody producing clones.

Also the number of wells with clones was 1.3 times higher than in the first series of experiments (Table I). The frequency of spleen cells forming hybridoma clones was almost twice as high and the frequency of spleen cells forming antibody producing hybridoma clones was 2.5 times higher.

From these findings it is clear that the conditions in which such experiments are carried out must be optimal. Optimalization of hybridization and culture conditions will be discussed in a subsequent paper.

Relationship between spleen cells and antibody producing hybridoma clones

Fig. 4 shows the relationship between the number of spleen cells and the percentage of wells with antibody producing clones. In the experiments with suboptimal growth, wells with less than 2×10^4 spleen cells contain hybridoma clones, but antibody production is unlikely. This contrasts with results of experi-

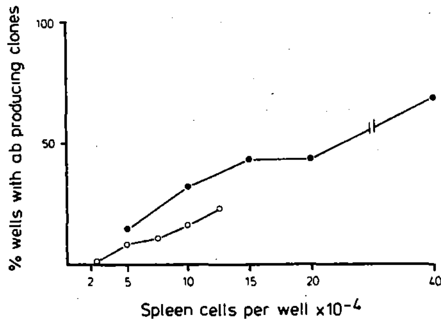

Fig. 4. Data from Tables I and IIa. The number of wells with antibody production is compared with the number of input spleen cells. O = Table I, columns A and D; ● = Table IIa, columns A and B.

ments with optimal growth. The amount of variability explained by the regression line through the 3 lowest concentrations (solid dots in Fig. 4) was 99%. When the regression line of these values was extrapolated, it intercepted the y-axis at the 2% value. This means that at any concentration of spleen cells antibody producing hybridoma clones may be formed. For this reason we concluded that our culture conditions were optimal.

Estimation of the number of antibody producing clones

This section deals with the specificity of monoclonal antibody producing hybridomas. The non-antibody producing clones are omitted from further consideration here.

The supernatants of the wells listed in Table IIa were tested for the presence of antibody. The positive wells were subdivided into those that contained antibodies directed against all target cells (lysis of at least 75% of the target cells, 'high lysis') and antibodies directed against a fraction of the target cells (lysis of 25–75% of the target cells, 'low lysis') (Table IIb). Wells with low lysis supernatants (Table IIb, column c) probably contained only a single clone of antibody producing hybridoma cells. The high lysis population (Table IIb, column b) showed an increased number of antibody producing wells with increasing spleen cell numbers. The low lysis population did not show an increase in the number of antibody producing clones, once the maximal number of wells with hybridomas (10^5 spleen cells/well) had been reached.

The percentage of wells with 1 antibody producing clone per well was compared with the Poisson distribution data (F_1). The lowest concentration of spleen cells (0.5×10^5) showed that 90% of the wells contained a single antibody producing clone. It can be seen from Table IIb (columns b and c) that almost half the clones occur among the high lysis group, and half among the low lysis group. Thus half the monoclonal antibody producing spleen cells generated in the mouse were high lysis and half low lysis.

When the number of wells with monoclonal antibodies was calculated for all other concentrations (Table IIb, column d), it was noted that values were about twice those in column c. It may be concluded that half the clones are from the high lysis fraction.

When a mixture of human cells is injected into a mouse to obtain a population of B cells activated against a subpopulation of the injected cells, it is of no advantage to exceed the maximal value of 10^5 spleen cells/well after fusion in order to obtain hybridomas yielding monoclonal antibodies directed at the human cell subpopulation.

Part II

This part of the paper concerns the limiting dilution tests performed 14 days after fusion. From the 3 different experiments shown in Tables IIa and b, and from each of the 5 different concentrations, cells of 3–5 hybridoma clones were chosen and dispensed at a mean dose of 1, 2 or more cells per well. In this way it was possible to test the stability of antibody producing hybridomas and the likelihood of their being outgrown by non-antibody producing clones.

Table III summarizes the results. To obtain an estimate of the mean number of clones per well (including both antibody producing and non-producing hybridomas) the values of the different concentrations were deduced from the first entry in Table III (column 2).

Our samples were not chosen at random. Only wells with hybridomas producing antibody of the high lysis type were selected. In this way, the chance that more than 1 antibody producing hybridoma clone was present was slightly increased, as compared with randomly chosen samples.

Column B (Table III) represents the estimated percentage of wells containing more than 1 antibody producing clone, calculated from the Poisson probability. The calculated number of wells containing more than 1 antibody producing clone is shown in the last column of Table III. The chance that a well containing more than 1 antibody producing clone was chosen for our limiting dilution experiments was, for the highest concentration, about 3 times that for the lowest concentration.

TABLE III

DATA FROM TABLE IIa AND b USED FOR THE INVESTIGATION OF THE STABILITY OF THE CLONES

Spleen cells per well $\times 10^5$	Mean number of clones per well [a]	Wells with antibody producing clones			
		Mean (A)	High lysis (%)	Multiclonal wells	
				Estimated [b] (B)	Number [c]
0.5	1.5	15.0	6.7	10%	1.5
1.0	3	31.7	18.3	18%	5.7
1.5	4.5	43.3	29.3	26%	11.3
2.0	6	42.7	25.7	25%	10.7
4.0	12	68.0	49.0	46%	32.0

[a] The first value is calculated from the logarithmic form of the zero term of the Poisson distribution, the others are deduced from the first.

[b] Calculated from $100\% - P_1$ (Table IIb).

[c] Calculated from $A \times B$.

TABLE IV

STABILITY OF ANTIBODY PRODUCING HYBRIDOMAS WHEN MORE THAN ONE CLONE IS PRESENT IN THE WELL

Spleen cells per well $\times 10^5$	Mean number of clones	Number of samples tested	90–100% of the wells with Ab producing clones
0.5	1.5	12	33%
1.0	3	13	31%
1.5	4.5	14	14%
2.0	6	15	20%
4.0	12	15	7%

Stability of antibody producing clones versus non-producing clones

Table IV shows the percentage of wells (in limiting dilution), containing 90–100% antibody producing clones. Values were chosen from the samples a, b and c (Fig. 1), from all 5 different concentrations of spleen cells.

It appeared that a maximum of 33% of the hybridomas tested were stable and did not contain non-antibody producing clones, when hybridomas were dispensed at a mean of 1.5 cells/well after cell fusion. Even when a mean of 3 clones/well was present, about the same percentage of stable and pure clones was reached. However, when the mean number of clones per well was increased the percentage of pure clones decreased. At a mean of 12 different clones per well, still 7% of the clones were not contaminated with non-antibody producing clones. It is thus clear that there is no tendency for non-antibody producing hybridomas to outgrow antibody producing hybridoma clones.

Loss of antibody production after fusion

To obtain the results shown in Table V, the values at the 2 lowest concentrations (spleen cell concentrations 0.5×10^5 and 1.0×10^5), were converted to values for 1 hybridoma cell/well after fusion and 1 cell/well after limiting dilution respectively

TABLE V

PERCENTAGE OF CLONES LOSING ANTIBODY PRODUCTION AT AN EARLY STAGE

	% Wells containing Ab producing clones [a]
Unstable hybridomas (0–50% Ab positive wells)	42%
Semi-stable hybridomas (50–99% Ab positive wells)	1%
Stable hybridomas (100% Ab positive wells)	57%

[a] 36 different hybridoma samples are tested.

(Fig. 1 samples a, b and c). It appears that about 40% of the hybridomas lost half or more of the antibody production during the first 3 weeks. It must be emphasised again that samples were not chosen at random, since only the high lysis hybridoma population was used for this test. With the low lysis population, a higher percentage of hybridomas would most probably have been found to lose their production capacity at this early stage.

The influence of non-antibody producing hybridomas on high concentrations of spleen cells

The data in Table VI is based on samples of different concentrations of hybridomas after fusion. Only samples were selected that were dispensed at a mean concentration of 1 cell/well after limiting dilution (Fig. 1 sample a). Wells containing hybridomas of which at least 33% did not produce antibody when tested 2 weeks after limiting dilution were considered negative because loss of antibody production was due to chromosomal instability. At the lowest concentration (0.5×10^5) the number of negative wells was about 40%, probably due to loss of antibody production at an early stage. At higher concentrations (1×10^5, 1.5×10^5 and 2×10^5), the percentage increased by about 10%, 20% and 30% respectively, due to an increasing amount of non-antibody producing clones. However the highest concentration of spleen cells after fusion showed a decrease in the number of negative wells. At this concentration, many wells contained more than 1 antibody producing hybridoma clone after fusion, which resulted in an apparent (but artificial) increase in stability. It therefore appears that with an increasing number of different hybridomas per well after fusion contamination with non-antibody producing hybridomas occurs, resulting in reduced purity of the clones.

Rapid fusion procedure

Loss of antibody production after cell fusion is not due to outgrowth of non-antibody producing clones, but rather to loss or inactivation of antibody producing chromosomes. With this in mind we decided that limiting dilution shortly after fusion would not be of any advantage.

We performed a fusion and at day 9 the supernatants were tested. Antibody

TABLE VI

STABILITY OF ANTIBODY PRODUCING HYBRIDOMAS WITH INCREASING NUMBER OF CLONES PER WELL

Spleen cells per well $\times 10^5$	Mean number of clones per well	Number of samples tested	0–33% Ab negative wells (%)
0.5	1.5	9	44
1.0	3	11	55
1.5	4.5	8	63
2.0	6	8	75
4.0	12	9	56

production was found in 78 of a total of 576 wells. Five days later the wells were examined a second time. Only 27 (35%) of the original positives still produced antibody. Three to 4 weeks after fusion 19 clones were still positive. Some of the hybridomas were injected in mice to produce ascitic fluid and the remaining cells were frozen. Ten of the ascitic fluids had a titer of more than 1:1000. The 10 clones were thawed, and limiting dilution performed. Two clones had ceased to produce antibody. In the other 8 clones antibody production was still present in 75–100% of the wells. These 8 clones continued to produce antibody for 3 months. These findings agree with those of Weissman and Stanbridge (1980), who investigated hybrid cell populations over long periods of time and found chromosome loss shortly after fusion and then gradual segregation during continued culture.

The reason why we found only 35% positive wells in the second cytotoxic test, instead of 60%, as in our earlier experiments, may have been because all clones, both low lysis and high lysis, were examined. Some low lysis clones may have lost antibody production a few days after fusion. In such cases the antibody concentration would be very low and although directed against the whole target cell population, would be able to lyse only part of the target cells. In a second test no antibody would be detected.

From the above results a number of conclusions may be drawn: Within the limits of our experiments, the number of hybridomas formed depends on the number of spleen cells available irrespective of the number of myeloma cells present. Hybridoma formation at either too low or too high concentrations of mouse spleen cells interferes with the optimal yield of antibody producing clones. A satisfactory yield of antibody producing clones in approximately 100% of the wells, was obtained under optimal conditions. Contamination by non-antibody producing clones was very limited, as appeared from the limiting dilution experiments. Loss of antibody production after cell fusion was not due to overgrowth of non-antibody producing clones. Fusions may be performed in a short time under the conditions described here, and are less expensive than when carried out the conventional way.

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CHAPTER V

HUMAN UMBILICAL CORD SERUM, A NEW AND POTENT GROWTH PROMOTOR, COMPARED WITH OTHER B CELL AND HYBRIDOMA ACTIVATORS

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Improved Fusion Technique. I. Human Umbilical Cord Serum, a New and Potent Growth Promoter, Compared with Other B Cell and Hybridoma Activators

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Accelerated proliferation of hybridoma cells was observed in the presence of human umbilical cord serum (HUCS). This had very strong growth-promoting activity, even at a concentration of 2%. A comparison was made between HUCS and other B cell growth promoters, such as lipopolysaccharide (LPS) and dextran sulfate (DxS), macrophage supernatant, and human endothelial culture supernatant (HECS). The growth-promoting effect of HUCS was superior.

Key words: *fusion technique — umbilical cord serum — growth promoter — hybridoma cultures*

Introduction

Cell-cell interaction is thought to play an important role in cultures containing low numbers of hybrid cells (Hämmerling et al., 1978; Lernhardt et al., 1978; Fazekas de St. Groth and Scheidegger, 1980). Astaldi et al. (1980, 1981) found that the induction of growth in single, isolated hybridomas is enhanced by the addition of human endothelial culture supernatant (HECS), which could substitute for feeder cells.

It has been shown that murine as well as human bone marrow cultures are stimulated to growth by both HECS (Quesenberry and Gimbrone, 1980) and human umbilical cord serum (HUCS) (Odavic and Beck, 1975).

We compared the stimulating capacity for hybridoma growth of HUCS, HECS

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and other B cell growth promoters. The influence of these 'factors' on thymidine incorporation, limiting dilution and number of antibody producing clones after fusion was investigated.

Material and Methods

Mice

BALB/c mice, 6–12 weeks old, were used for immunization.

Immunization

Mice were injected intravenously with 2×10^7 human peripheral blood lymphocytes. Four days later, the spleens were removed. Cells were isolated by gently teasing the spleen apart with tweezers.

Myeloma cell line

Sp2/0-Ag 14, a BALB/c non-secretory plasmacytoma line, provided by Dr. G. Köhler, was maintained in culture medium (without 2-mercaptoethanol) in an atmosphere of 5% CO_2.

Culture medium

The myeloma cell line and the hybridomas were cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), 1% sodium pyruvate (100 ×), 1% non-essential amino acid solution (100 ×), 3% sodium bicarbonate (100 × 7.5), 1% Hepes (100 × 1 M) (Gibco, Grand Island, NY), 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol.

Feeder systems

Dextran sulfate (DxS; Gibco, Grand Island, NY), and *Escherichia coli* 0055 : B5 lipopolysaccharide (LPS) prepared by the Westphal technique (Difco Laboratories, Detroit, MI), were used at concentrations of 20 μg/ml and 50 μg/ml respectively, according to the method of Wetzel and Kettman (1981a, b).

Macrophage supernatant was prepared according to the method of Kurland (1978). Peritoneal macrophages were cultured at a concentration of 1×10^5 cells/ml in culture medium supplemented with 20 μg/ml lipopolysaccharide (LPS) *Salmonella typhosa* (W 0901; Difco Laboratories, Detroit, MI), and an end concentration of 1.4×10^{-7} M indomethacin (IND; Sigma Chemical Co., St. Louis, MO). The latter was dissolved in 0.5 ml of 95% ethyl alcohol and subsequently diluted with 9.5 ml PBS. After 2 days the supernatant was harvested, and cells removed by centrifugation. The conditioned medium was used at a dilution 1 : 4 in culture medium.

HECS was provided by A.M. Wassenaar, Laboratory of Cell Biology and Histology, University Hospital, Leiden. Trypsinized endothelial cells were cultured for about 8 days in 30% pooled A + AB serum, Med 199 (Earle's) supplemented with 0.3 mg/ml glutamine, 5 μg/ml glucose and 5 μg/ml garamycin. HECS was used at a dilution 1 : 5 in culture medium.

Placental blood was collected during delivery immediately after section of the umbilical cord. HUCS was made by pooling 10–15 sera from healthy individuals. The serum was heat inactivated for 45 min at 56°C and passed through a millipore filter (0.45 μm pore size). Small aliquots were frozen at −20°C for a period not longer than 2 months and thawed immediately before use.

Cell fusion

Myeloma and spleen cells were fused at a ratio of 1 : 10 in 1 ml of 50% polyethylene glycol 4000 (Merck) in distilled water. The cells were slowly diluted with 40 ml RPMI 1640 medium, at 37°C. The cells were pelleted for 5 min at 250 × *g* and gently resuspended in 5 different aliquots of culture medium, containing different growth promoters, and selective medium, i.e. HT medium, consisting of 100 μM hypoxanthine and 16 μM thymidine (Fluka, Switzerland), and 1 μM aminopterine (Serva, Feinbiochem., Heidelberg). The suspensions were dispensed into 96 wells of microtiter tissue-culture plates (Greiner, no. 655160) at a concentration of 1.1×10^5 cells/well in 100 μl. After 7 days, an equal volume of HT medium was added to each well. Three days later, the supernatants of wells containing colonies of hybrid clones were tested with a microcytotoxicity assay (see below).

Limiting dilution

A hybridoma cell line, Bl 1, producing antibody to mononuclear cells, was cultured at a concentration of one cell/well, in 50 μl, in microtiter tissue-culture plates with the different growth promoters. After 6 days the number of clones was counted.

[^{3}H]thymidine uptake

Hybridoma cells were cultured in microtiter tissue-culture plates at a concentration of 50 cells/well with or without growth promoters. The cultures were radiolabeled after 2, 4, 6, 8 and 10 days with 0.5 μCi of [^{3}H]thymidine (TRA 310, spec. act. 2.0 mCi/mmol; Radiochemical Centre, Amersham) and harvested 16 h later with a multisample collector. Radioactivity was counted in a liquid scintillation spectrometer. The data were analyzed by the Wilcoxon method for statistical significance.

Microcytotoxicity assay

The test was performed in duplicate, according to Bruning et al. (1975). Lymphocytes were isolated by Ficoll-Isopaque gradient centrifugation. The cells were labeled with carboxy-fluorescein diacetate (Bruning et al., 1980) for 15 min at 37°C, washed once with PBS, resuspended in McCoy's medium with 20% FCS, and adjusted to 2×10^6 cells/ml. Terasaki microtest trays (Greiner, no. 653180), provided with medinol oil to prevent evaporation, were filled with 1 μl supernatant, and spun in a swinging bucket rotor at 250 × *g*. One microliter of the lymphocyte suspension was then added. After 60 min incubation at 20°C, 5 μl of pooled rabbit complement were added, and the trays incubated for a further 2 h at 20°C. Finally 5

μl haemoglobin solution prepared as described by Bruning et al. (1982) were added to quench fluorescence of the medium. Microtest trays were read with a Digital Equipment Corporation PDP 8/E computer, as described by Bruning et al. (1980). Positive scores were wells that showed 50% or more lysis in the assay.

Results

HUCS a growth promoter for hybridoma culture

HUCS was tested for its capacity to promote the growth of hybridoma cultures by [^{3}H]thymidine incorporation. As can be seen from Fig. 1, during the first 8 days the hybridoma grew significantly better in the presence of HUCS than FCS. From day 10, growth in HUCS diminished significantly, probably because the medium was exhausted.

Optimal concentration of HUCS

Because HUCS is not very readily obtainable, we tried to determine the lowest possible concentration that improved growth in 2 systems, namely cultures with isolated cells in limiting dilution, and [^{3}H]thymidine uptake experiments starting with 50 cells/well.

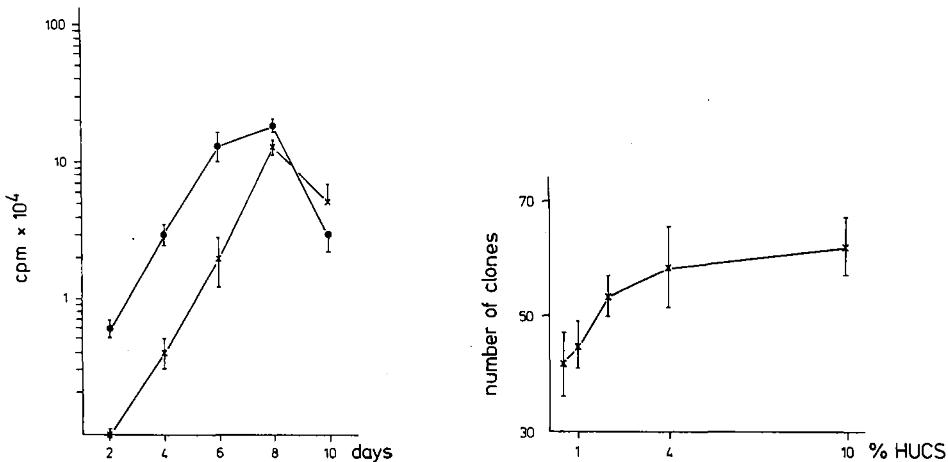


Fig. 1. Effect of HUCS on incorporation of [^{3}H]thymidine into hybridoma cells. Fifty hybridoma cells cultured in 5% HUCS (●——●) or 20% FCS (×——×). There is a significant difference on days 2, 4, 6 and 8 ($P = 0.01$). On day 10 the effect of HUCS was significantly lower ($P = 0.05$).

Fig. 2. A hybrid cell line cultured as single cells at limiting dilution in different concentrations of HUCS. There is a significant difference between 0.5% or 1%, and 2% HUCS ($P = 0.02$). Also between 2% and 10% HUCS ($P = 0.05$) though not between 4% and 10% HUCS. Each point represents the mean number of wells containing hybrid clones growing (±S.D.) in 4 different experiments. In each experiment 96 different wells from each sample were tested.

Various concentrations of HUCS were examined for growth promoting effect on isolated hybridoma cells (Fig. 2). From this and similar experiments we conclude that optimal growth promotion occurs already at a concentration of about 4% HUCS.

In other experiments, HUCS-stimulated hybridoma proliferation was measured by [^{3}H]thymidine incorporation every 2 days during 10 days of culture. Day 8 was found the best for examining the response by [^{3}H]thymidine uptake (data not shown). In 3 consecutive experiments we determined [^{3}H]thymidine incorporation (Fig. 3).

From Figs. 2 and 3, it can be seen that in both systems a concentration of 2% HUCS improved growth significantly better than 0.5% or 1%, and that growth with 4% HUCS is significantly better than with 2%.

The influence of 2-mercaptoethanol on hybridoma culture

The influence of 2-mercaptoethanol on isolated hybridoma cells in limiting dilution was investigated for 20% FCS and 10% HUCS. Fig. 4A shows that 2-mercaptoethanol though a potent stimulator of hybridoma cultures, does not enhance the growth stimulating effect of HUCS (Fig. 4B).

Comparison of different B cell stimulators in hybridoma culture

The growth promotion of a variety of B cell stimulators was investigated and compared with the growth promoting capacity of HECS. Fig. 5 shows [^{3}H]thymidine incorporation during a 10 day culture period. Significant differences are seen between the stimulating effects over the first 6 days of 20% FCS, LPS + DxS, 25%

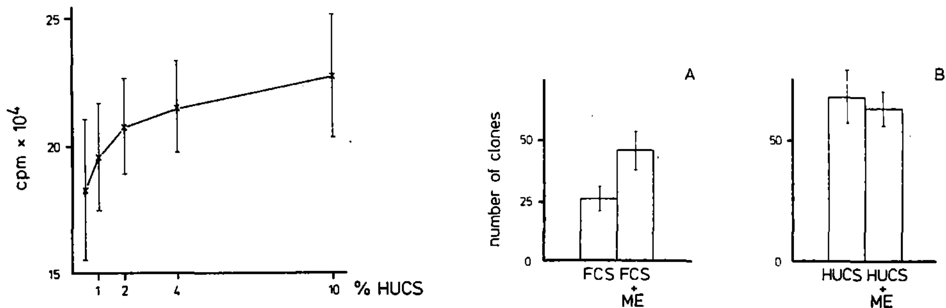
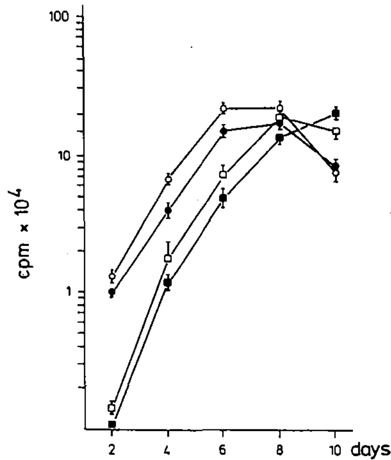


Fig. 3. Optimal concentration of HUCS determined by [^{3}H]thymidine incorporation on day 8. There are significant differences between 0.5% and 2% HUCS ($P = 0.05$) and 2% and 4% HUCS ($P = 0.05$). Results expressed as mean cpm of 6 determinations, (± S.D.) and representative of 3 separate experiments.

Fig. 4. A hybrid cell line was cultured as single cells in limiting dilution to measure the influence of 2-mercaptoethanol in 20% FCS and in 10% HUCS. After 6 days clones became evident. A, there is a significant difference between FCS with and without 2-mercaptoethanol ($P = 0.05$). B, there was no difference between HUCS with and without 2-mercaptoethanol. Each point represents the mean of the number of wells containing hybrid clones growing (± S.D.) in 3 different experiments. In each experiment 96 different wells from each sample were examined.



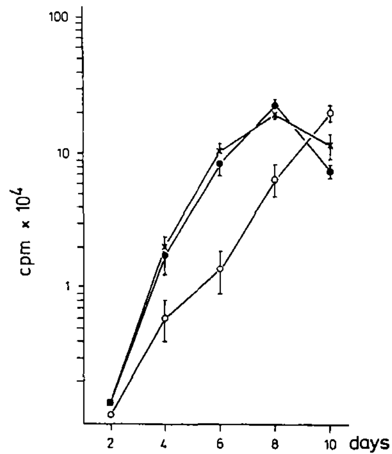


Fig. 5. Effect of different feeder systems on incorporation of [^{3}H]thymidine. Results were expressed as mean cpm of 6 determinations. Cells cultured at a concentration of 100 cells/well. Data show [^{3}H]thymidine incorporation for 20% FCS (■); 20 μg/ml DxS+50 μg/ml LPS (□); 25% macrophage supernatant (●); and 20% HECS (○).

Fig. 6. Comparison of 20% HECS (○——○) with 2% (×——×) and 10% HUCS (●——●) on the incorporation of [^{3}H]thymidine.

macrophage supernatant, and 20% HECS. On day 8 there is still a significant difference between HECS and the other growth promoters. Of all B cell stimulators HECS induced the highest proliferation.

Next we compared HUCS (at concentrations of 2% and 10%), with 20% HECS (Fig. 6). Although in HECS maximal growth was achieved by day 10 or later, growth was still significantly lower ($P = 0.1$) than maximal growth in 10% HUCS by day 8. There was no difference in the maximal uptake value in 2% HUCS on day 8 and the

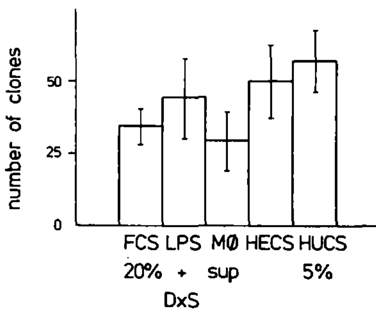


Fig. 7. A hybrid cell line was cultured as single cells in limiting dilution to measure the influence of different feeder systems. Each column represents the mean of the number of wells containing hybrid clones growing (±S.D.) of 4 different experiments.

TABLE I

EFFECT OF DIFFERENT FEEDER SYSTEMS ON RECOVERY AND YIELD OF ANTIBODY PRODUCTION BY HYBRIDOMA CELLS AFTER FUSION

Results were expressed as number of wells containing hybridoma clones growing over a total of 96 wells per sample.

Stimulators	Number of wells [a] containing growing hybridomas	Number of Ig producing wells [b]			
		I	II	III	IV
FCS	62 (57–68)	8	8	17	14
LPS + DxS	95	21	40	27	37
MØ. sup.	94	20	29	64	47
HECS	95	n.d.	24	35	38
HUCS	95	12	23	32	58

[a] Mean of 4 fusions. Between parentheses the ranges of numbers of growing wells.

[b] Hybridomas producing anti-lymphocyte antibodies 10 days after fusion in the 4 different fusions. Antibody production was measured by a microcytotoxicity assay.

maximal value in 20% HECS which occurred on day 10.

When tested in limiting dilution, the growth effect of all B cell stimulators gave results comparable with those obtained from measurements of [^{3}H]thymidine incorporation, except for macrophage supernatant, which gave the lowest values (Fig. 7).

Antibody production and recovery of hybrids after fusion by addition of different feeder systems

The effect of different feeder systems on the recovery of hybrids after fusion is shown in Table I. Only FCS gave low growth values. All other feeder systems, which were tested at a concentration of 10^4 myeloma cells/well, gave growth in almost 100% of the wells. Clones stimulated by macrophage supernatant were somewhat bigger than those stimulated by LPS + DxS. The best growing clones were those stimulated by HECS and HUCS.

The effect of the various feeder systems on the yield of antibody-producing hybrid cells was measured by microcytotoxicity assay on 4 repeats of the same kind of fusion. It was found that, FCS by itself, gave a far lower percentage of antibody-producing clones, in comparison to other feeder systems (Table I).

Discussion

In our laboratory HUCS proved to be more potent than HECS, and HECS more potent than the other feeder systems. With HUCS, 9 out of 11 experiments performed with [^{3}H]thymidine uptake showed maximal growth on day 8, 1 was maximal on day 6, and 1 maximal on day 10. In the case of HECS, 6 out of 12 experiments had maximal growth on day 8, and 6 on day 10 or later (data not shown).

To investigate whether HUCS should be added more than once, we performed 2 series of fusions. In 1 series, HUCS was added only at the initial stage. In the other series, we made a second identical addition of HUCS after 1 week, but saw no difference in growth rate during a prolonged subsequent culture period (data not shown). It is possible that HUCS, like HECS, contains a growth-promoting signal, as described by Astaldi et al. (1981), that induces hybridoma cells to proliferate. In addition to a growth promoting factor, HUCS also contains gamma-globulin which proved to be very effective in supporting lymphocyte growth, according to Yachnin and Raymond (1975). This may explain why HUCS is a more potent growth promoter than HECS.

Single hybridoma cells grown in separated wells were counted at a very early stage. In this way, wells containing only a few cells were ignored. When the same plates were examined one week later, there was no difference in the number of positive wells for all hybridoma stimulators, but there appeared to be a difference in proliferation rate.

According to Fazekas de St.Groth and Scheidegger (1980), macrophages are potent stimulators of hybridoma cultures. However, from our own experience is that when macrophages are added to incubation wells they not only devour dead cells, but also destroy the newly formed hybridomas, so that after a week or two clean wells remain with only intact macrophages in them. We therefore decided to use macrophage supernatant. In 5 of the 6 experiments tested with [^{3}H]thymidine incorporation, macrophage supernatant proved as good as, or better than LPS + DxS. However, when starting from 1 cell/well macrophage supernatant hardly promoted clonal growth. In this case feeder cells are possibly necessary to initiate proliferation.

Although HUCS appeared the strongest stimulator for hybridoma growth, antibody production was enhanced equally by all stimulators except FCS (Table I). This agrees with the findings of Astaldi et al. (1980), who report that the proportion of antibody-producing hybridomas remains similar in the presence of HECS or other feeder systems.

Acknowledgements

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CHAPTER VI

THE GROWTH PROMOTING ACTIVITY OF LIPOPOLYSACCHARIDE, DEXTRAN SULFATE AND RED CELL LYSATE ADDED TO HY-CLONE CALF SERUM

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CELLULAR IMMUNOLOGY 81, 268–275 (1983)

Improved Fusion Technique

III. The Growth Promoting Activity of Lipopolysaccharide, Dextran Sulfate, and Red Cell Lysate Added to Hy-Clone Calf Serum

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A new high quality young-calf serum, Hy-clone calf serum (HcCS), was tested for use in hybridoma culture. This calf serum alone had little growth promoting activity and was much inferior to fetal calf serum (FCS). Red cell lysate (RCL) used in combination with the young-calf serum showed very good growth promoting activity. Growth was increased about threefold over that in the presence of FCS. However, HcCS and RCL could not substitute for feeder cells when hybridomas were cultured as single cells under conditions of limiting dilution. It is thought likely that the potent growth promoting factor in red cell lysate is hemoglobin.

INTRODUCTION

The search for specific and potent growth promoters is central to antibody production in hybridoma cell cultures. It has long been known that different batches of FCS[2] have very different growth promoting activities (1), and that even the most active batch does not give truly optimal growth. Various investigators have sought alternative systems. One possibility is to grow hybridomas in the presence of feeder cells, either thymocytes (2, 3), spleen cells from unimmunized mice (4), or cells from the peritoneal fluid (5, 6). Other workers have tried additives such as human endothelial cell supernatant (7, 8) and human umbilical cord serum (HUCS) (9).

Synergy between lipopolysaccharide (LPS) and dextran sulfate (DxS) in B-cell mitogenesis has been mentioned in a series of publications (10–13). These mitogens in combination act to induce more B cells to grow than the sum of the induced growth by either LPS or DxS alone. In a previous publication (9) we showed that a combination of both stimulators promotes both growth and antibody synthesis in hybridoma cells, when FCS was used. Although the combination did not stimulate growth as well as HUCS (the best growth promoter found until now), there was no demonstrable difference in the number of antibody-producing clones.

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[2] Abbreviations used: DxS, dextran sulfate; FCS, fetal calf serum; HcCS, Hy-clone calf serum; HUCS, human umbilical cord serum; LPS, lipopolysaccharide; RCL, red cell lysate.

In the present communication we used LPS, DxS, and red cell lysate (RCL) in combination with a new serum, Hy-clone calf serum (HcCS). The serum is of high quality and collected from 15- to 17-week-old calves. Endotoxin levels are very low, 1 ng/ml. The hemoglobin level is so low as to be unmeasurable. The serum gives maximal growth of B-cell hybridomas in our hands.

MATERIALS AND METHODS

Sera. Three different sera were used: fetal calf serum (Batche Nos. K512010N, K117010D; Gibco, Glasgow, Scotland) and Hy-clone calf serum (Batch No. 200269; Greiner, Sterile Systems, Inc., Logan, Utah).

Culture medium. The hybridomas were cultured in RPMI 1640 medium, supplemented with 10% serum, 1% sodium pyruvate (100×), 1% non-essential amino acid solution (100×), 3% bicarbonate (100 × 7.5), 1% HEPES (100 × 1 *M*) (Gibco, Grand Island, N.Y.), 4 m*M* L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} *M* 2-mercaptoethanol.

Feeder systems. Dextran sulfate (Gibco) was used at a concentration of 20 μg/ml, and *Escherichia coli* 0055:B5 lipopolysaccharide (LPS) was prepared by the Westphal technique (Difco Laboratories, Detroit, Mich.) and used at a concentration of 50 μg/ml, as previously described (10).

Human umbilical cord serum was made by pooling 10 to 15 sera from healthy individuals, as described in a previous publication (9). It was heat inactivated for 45 min at 56°C and passed through a Millipore filter (0.45-μm pore size). This serum was then stored at −20°C for a period of no more than 2 months.

Red cell lysate was prepared from human erythrocytes. Heparinized blood was centrifuged for 10 min at 500*g*. The supernatant serum, heavily contaminated with thrombocytes and leukocytes, was removed. The remaining erythrocytes were washed twice in PBS. Then 1 vol distilled water was added to 1 vol packed cells, and after 5 min the cell debris was spun down at 700*g*. The lysate was diluted about 20 times and filtered through a Millipore filter (0.45-μm pore size). The final hemoglobin concentration of this stock solution was 42.0 μmol/liter, as measured by converting hemoglobin into cyanmethemoglobin, and reading the extinction at 540 nm in a Zeiss PM 6 spectrophotometer.

Limiting dilution. A hybridoma cell line from mouse myeloma (Sp2/0-Ag 14) and mouse (Balb/c) spleen cells were dispensed in microtiter tissue culture plates, such that on average single cells would be added to the wells. Three different growth promoters were tested; 10% HUCS, 10% HcCS supplemented with LPS, DxS, and 5% RCL, or HcCS with its supplements and 2×10^6 unimmunized mouse spleen cells/ml (4). Aliquots (50 μl) of the suspension were dispensed into 96 wells of the tissue culture plates. After 12 days the number of clones was counted.

[^{3}H]thymidine uptake. Hybridoma cells were cultured in microtiter tissue culture plates at a concentration of 50 cell/well with FCS or HcCS, in the presence or absence of mitogens. The cultures were radiolabeled after 4, 6, 8, and 10 days with 0.5 μCi of [^{3}H]thymidine (TRA 310, sp act 2.0 mCi/mmol; Radiochemical Centre, Amersham, U.K.). Cultures were harvested 16 hr later with a multisample collector. Radioactivity was counted in a liquid scintillation spectrometer. The data were analyzed using the Wilcoxon method for statistical significance.

RESULTS

Growth promoting activity of different batches of FCS. Six different batches of FCS were tested. One of which was thought to be especially active. In fact, all batches proved to have about the same growth promoting activity, except one. This one proved inferior to the others (Fig. 1). In an attempt to render the FCS more active we added 10% of the RCL solution to different batches of FCS. With good growth promoting FCS there was no significant difference between medium with or without RCL. With poor growth promoting FCS there was often a significant improvement in activity. We decided to pursue experiments with FCS of the best growth promoting quality.

Hy-clone calf serum in combination with LPS, DxS, and RCL. We introduced a new commercially available bovine serum: young-calf serum of high quality (HcCS). When used in hybridoma culture instead of FCS it proved to have very poor growth promoting activity by comparison of FCS (Fig. 2a). A combination of LPS + DxS improved hybridoma cell growth in the presence of FCS as well as that in the presence HcCS (Fig. 2b). However, when 10% RCL was added to the medium, HcCS showed a far better growth promoting activity than FCS (Fig. 2c). We also added RCL together with LPS + DxS to FCS or HcCS medium. HcCS proved significantly more active than FCS under these conditions (Fig. 2d).

Samples in Figs. 2a–d came from the same source and showed that a combination of HcCS and RCL had the most potent growth promoting activity. Supplemental addition of LPS and DxS did not alter the activity significantly.

Different concentrations of RCL. To investigate whether there is an optimal concentration of RCL for promoting proliferation, three different concentrations were

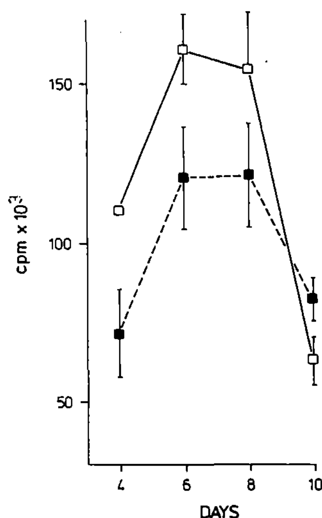

FIG. 1. Comparison of two different batches of FCS with poor (■) and good (□) growth promoting activity. There was a significant difference on Days 4, 6, and 8 ($P = 0.01$). Results were expressed as the mean cpm of six determinations (±SD).

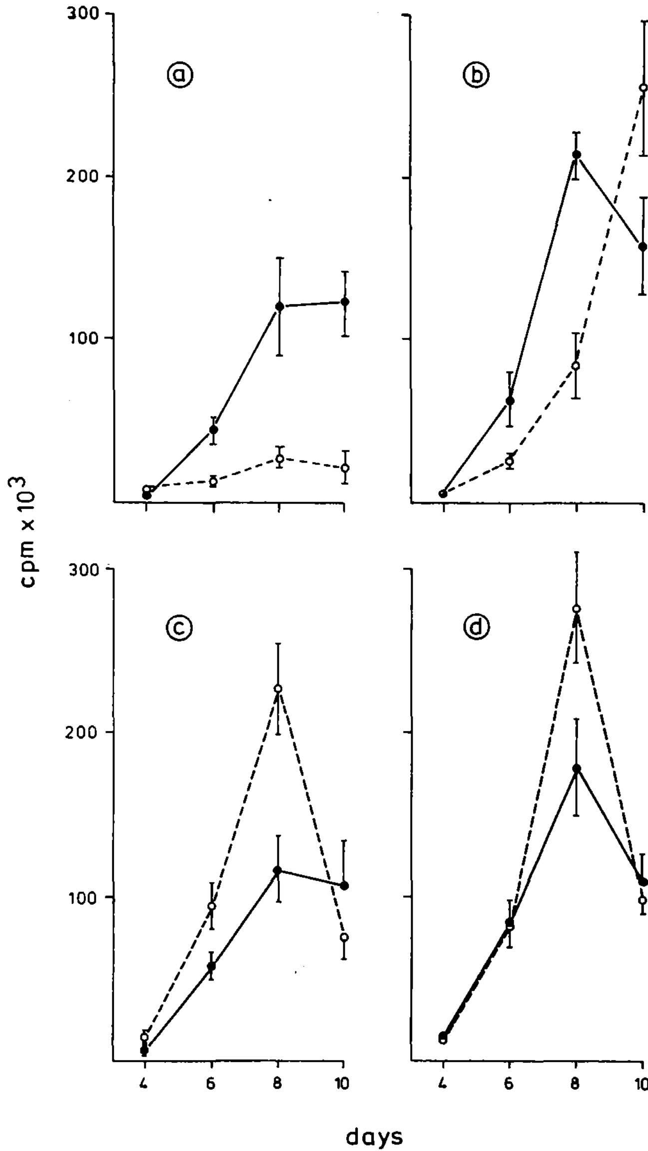

FIG. 2. Comparison of 10% FCS (●) and 10% HcCS (O). (a) Without any addition, HcCS showed poor growth promoting activity. (b) With the addition of LPS and DxS, proliferation was increased with FCS as well as with addition of HcCS. (c) The addition of 10% RCL increased proliferation by Day 6 and 8 ($P = 0.01$). (d) With the addition of LPS, DxS, and 10% RCL, enhanced proliferation was evident by Day 8 ($P = 0.01$). Samples were from the same experiment. Results were expressed as the mean cpm of six determinations (±SD).

used. Tests were done using 5, 10, and 20% RCL in combination with 10% HcCS. The three concentrations showed increased growth promoting activity but proliferation was significantly higher with 5% RCL compared to 10 or 20% RCL (Fig. 3).

Optimal hybridoma stimulator. From Figs. 2 and 3 it can be seen that HcCS in combination with LPS, DxS, and 5% RCL had enhanced growth promoting activity. We wished to find whether this combination would yield better results than 10% HUCS. Figure 4 showed that (HcCS + LPS + DxS + 5% RCL) had significantly better growth promoting activity than HUCS. So HcCS may be regarded as the best hybridoma growth promoter found hitherto.

Growth promoting activity of HUCS and HcCS on single cells. All experiments described until now were performed at the concentration of 50 cell/well. In a previous publication (9) we showed that although a growth promoter (macrophage supernatant) can stimulate when a certain minimum number of cells is present initially, stimulation did not necessarily occur when the experiment was performed starting with 1 cell/well. As can be seen in Fig. 5 there is no difference between HUCS and HcCS (+ LPS + DxS + 5% RCL), in experiments starting with a mean of 1 cell/well.

To discover whether feeder cells could influence hybridoma cell growth at low cell numbers when HcCS was used, we added 10^5 spleen cells/well. From Fig. 5 it is clear that enhanced growth promotion was obtained.

The influence of HcCS on fusion. HUCS and (HcCS + LPS + DxS + 5% RCL) were used in a fusion experiment. Samples were tested in 288 wells in microtiter tissue culture plates for both media. Cells were dispensed at concentrations of 10^5 spleen cells and 10^4 Sp2/0-Ag 14 myeloma cells per well. Supernatants were tested in a microcytotoxicity assay following the procedure described earlier (9). There was no difference in either the number of growing wells or the number of antibody-producing wells in each case. Hybridomas cultured in both sera proved to be equally stable after a prolonged culture period.

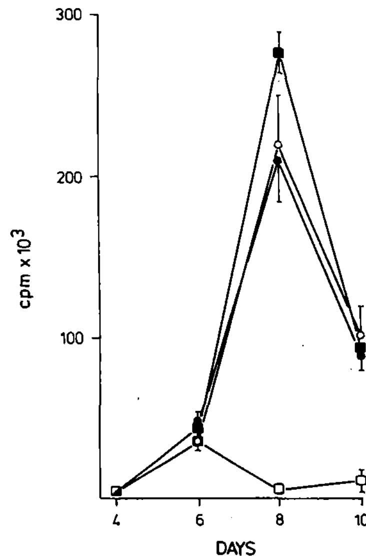

FIG. 3. The optimal concentration of RCL. There was a significant difference by Day 8 between 5% RCL and both other concentrations ($P = 0.01$). (□, 0% RCL; ■, 5% RCL; O, 10% RCL; ●, 20% RCL.) Results were expressed as the mean cpm of 6 determinations (±SD).

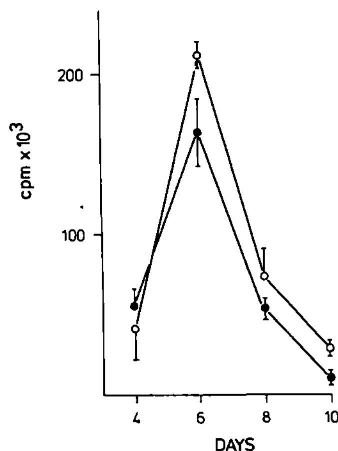

FIG. 4. Comparison of 10% HUCS (●) and 10% HcCS + LPS + DxS + 5% RCL (○). There was a significant difference by Day 6 ($P = 0.01$). Results were expressed as the mean cpm of six determinations (±SD).

DISCUSSION

When thymocytes were used as feeder cells, antibody production and the number of clones measured in B cells or hybridomas was enhanced (13, 18). However growth promotion was not caused by a thymus factor (10), but probably by cell–cell interaction. Macrophages or a macrophage cell line (10, 19) was found to stimulate B-cell growth when an inactive FCS was used. In our experiments (Fig. 5), added spleen cells gave enhanced growth. It is not clear whether growth promotion was caused by cell–cell contact as postulated by Andersson *et al.* (20), or whether macrophages among the spleen cells caused growth promotion. The latter supposition is likely, as HcCS alone

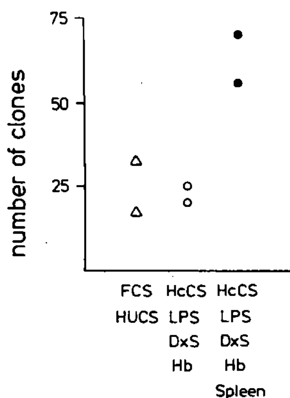

FIG. 5. Limiting dilution starting from the average expected number with 1 cell/well. There was no difference between 10% HUCS and 10% HcCS + LPS + DxS + 5% RCL. Addition of spleen cells to the latter serum showed far better growth than without feeder cells. Each point represents the number of wells containing growing clones in two different experiments.

(like some FCS batches) gave only very poor growth stimulation. Addition of human endothelial cell supernatant or human umbilical cord serum to Hy-clone calf serum gave a lower level of proliferation than when these two growth promoters were added to FCS.

Dextran sulfate, a mitogen for human T lymphocytes (14), was found to increase B-cell division (15). It activated DNA synthesis at a rather early stage of differentiation, but only marginally induced the cells as high rate antibody producers (16).

LPS from gram-negative bacteria, was found to activate B cells. It promoted the proliferation of B cells and resulted in the development of a large number of antibody-producing cells (15, 16). Prestimulation with DxS greatly enhanced B-cell responses to LPS. In fact, cells highly activated with DxS, can be reactivated 4 days later with LPS, whereas LPS-activated cells cannot (16). The synergistic response of LPS + DxS to proliferation and antibody production in spleen cells was not diminished by the depletion of T cells, or by the blocking of their activation. Therefore the synergistic response was not T cell dependent (17). We did not find any difference in growth promotion when inactive FCS was used instead of active FCS.

When LPS or a combination of LPS + DxS was added to hybridoma cultures, an increase in the number of colonies and in the number of antibody-producing cells was observed (9, 18). This corresponded with the findings in B cells, that Ig secretion is associated with clonal expansion (10).

B cells are generally grown in the presence of FCS. We used a variety of bovine sera; one of them, Hy-clone calf serum, proved to be very active in combination with LPS, DxS, and/or RCL. HcCS alone was a very poor growth promoter, but in combination with the three mitogens used simultaneously, it proved to have far more growth promoting activity than any other serum or conditioned medium investigated so far. If LPS acts together with some component(s) in the sera, it is not yet clear. Anyhow, the kind of serum seems to be important for the the success of hybridoma growth. Further investigations will be done in this field and the results will be discussed more extensively in a future publication.

We also tested Hy-clone fetal bovine serum. This proved to be as good as HUCS, but was inferior to HcCS enriched with the three mitogens. The improved growth promoting activity of Hy-clone serum could be due to the way of collecting it. Rupture of leukocytes and the consequent release of lysosomal contents from the cells was claimed by the manufacturers to be minimal. It is possible that in the original sera there is an inhibitory factor deriving from lysed leukocytes.

The active factor in red cell lysate is still unknown. It is most likely to be hemoglobin. We are actively investigating this possibility.

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CHAPTER VII

GROWTH PROMOTING ACTIVITY OF BOVINE SERA FROM DIFFERENT AGES IN COMBINATION WITH DEXTRAN SULFATE, LIPOPOLYSACCHARIDE AND HEMOGLOBIN

Regine J.J.M. Westerwoudt, Joke Blom and C.M.H. Harrisson

Abbreviations used: Cs, calf serum; DxS, dextran sulfate; Hb, hemoglobin; HbC, crystallized hemoglobin; LPS, lipopolysaccharide; RCLa, adult red cell lysate; RCLf, fetal red cell lysate.

To be published

SUMMARY

Fetal, newborn and young calf sera (CS) were tested as growth promotors in culture media for hybridoma B cells. For each of these three types of sera, two different qualities were used: an ordinary type and Hyclone or defined type. The latter are high quality sera, with very low endotoxin and hemoglobin levels. Proliferation of hybridoma B cells was more rapid in the presence of Hyclone CS than with ordinary CS.

When a combination of dextran sulfate (DxS) and lipopolysaccharide (LPS) was added, the growth promoting activity of ordinary fetal CS improved, but not that of ordinary newborn CS. Hardly any proliferative activity was observed when ordinary young CS was used, both in the presence and in the absence of DxS and LPS. With regard to the Hyclone sera a somewhat different pattern of activity was observed. Addition of DxS plus LPS to Hyclone fetal CS did not improve proliferation, while addition to Hyclone newborn or young CS did.

The influence of red cell lysate (RCL) was also tested. It enhanced the proliferative activity of the hybridoma cells cultured in medium supplemented with ordinary young CS as well as in medium supplemented with Hyclone young CS to a marked extent. With fetal or newborn CS such an improvement was not seen. Hemoglobin (Hb) was found to be the active moiety of the RCL, with hemin as the stimulatory component.

INTRODUCTION

In a previous study we have shown that hybridoma proliferation in vitro is enhanced by the presence of erythrocyte lysate or a combination of DxS and LPS (1). Erythrocyte lysate (RCL) was found to increase the growth promoting activity of Hyclone young calf serum (CS), but not of fetal CS. On the other hand, a combination of DxS and LPS stimulated the proliferative activity in the presence of Hyclone young CS and ordinary fetal CS (1).

Hemin has shown to enhance the colony formation by Friend erythroleukemic cells, mastocytoma cells, myeloid leukemic cells, chick embryo cells and fibroblasts (2-7), and to participate in two major metabolic intracellular activities:

energy production via cytochromes and initiation of protein synthesis (8,9).

The purpose of the present study is to compare the growth promoting activity of fetal CS, newborn CS and young CS to which either DxS and LPS or RCL is added. Furthermore, the active factor in RCL is determined.

MATERIALS AND METHODS

Sera

The following sera were used: ordinary fetal calf serum (batch no. K512010N), ordinary newborn calf serum (batch no. K710101) and ordinary young (special bobby) calf serum (batch no. K113201) (Gibco, Glasgow, Scotland). Hyclone defined fetal calf serum (batch no. 100355), defined newborn calf serum (batch no. 150318) and Hyclone defined young calf serum (batch no. 200269) (Greiner, Sterile System, Inc. Logan, Utah).

Culture medium and cells

Six different B cell hybridomas, three producing monoclonal antibodies against mononuclear cells (Bl_1, Bl_2 and Bl_3) (10,11), to measure the effect of DxS plus LPS on the different calf sera and two producing monoclonal antibodies against monocytes (RW1 and RW2) (1), to measure the effect of red cell lysate, hemin, α-globin or β-globin, on the different calf sera and a non-antibody producing B cell hybridoma, to measure the effect of DxS plus LPS on fetal calf serum, were cultured in RPMI 1640 medium, supplemented with 10% serum, 1% sodium pyruvate (100x), 1% non-essential amino acid solution (100x), 3% sodium bicarbonate (100x 7.5%), 1% HEPES (100x 1M; Gibco, New York), 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 x 10^{-5}M 2-mercaptoethanol.

Feeder systems

Dextran sulfate (Gibco, NY) and Escherichia coli 055:B5 lipopolysaccharide, prepared according to the Westphal technique (Difco Laboratories, Detroit, Mi), were used at concentrations of 20 μl/ml and 50 μl/ml, respectively, as described earlier (1,11). Red cell lysate was prepared from human adult (RCLa) and fetal (RCLf, collected from the fetus at the moment

of delivery) erythrocytes from heparinized blood. These blood cells were first centrifuged for 10 min at 500 g. The supernatant, heavily contaminated with platelets and leukocytes, was removed. The remaining erythrocytes were washed twice in phosphate-buffered saline. After removing the supernatant, 1 vol distilled water was added to 1 vol packed cells, and after 5 min the cell debris was spun down at 700 g. The lysate was diluted about 20 times and filtered through a Millipore filter (0.45 μm pore size). The final Hb concentration of this stock solution was 42.0 μmol/l, as measured by converting hemoglobin into cyanmethemoglobin, and reading the extinction of 540 nm in a Zeiss PM 6 spectrophotometer.

The concentrations of stock solutions of crystallized human hemoglobin (HbC, 42.0 μmol/l) 2,3-diphosphoglyceric acid (DPG, 42.0 μmol/l) (12), bovine hemin (270 mg/l) Sigma Chemical Co., St Louis, Mo), and the concentration of α-globin and β-globin (each at 30 mg/l) (kindly provided by Prof. Dr. L.F. Bernini) were made up to correspond with the concentration of the relevant component in the RCLa.

^{3}H-thymidine uptake

Hybridoma cells were cultured in microtiter tissue-culture plates at a concentration of 50 cells/well in media supplemented with different sera. The cultures were radioactively labeled after 4,6,8 and 10 days with 0.5 μCi of ^{3}H-thymidine (TRA 310, spec. act. 2.0 mCi/mmol; Radiochemical Centre, Amersham, UK) and harvested 16 h later using a multisample collector. Radioactivity was counted in a liquid scintillation spectrometer. The data were analysed using the Wilcoxon method for statistical significance. Results are expressed as the mean c.p.m. of 6 determinations (± S.D.).

RESULTS

The influence of DxS and LPS on the growth promoting activity of different bovine sera

The influence of the addition of DxS plus LPS on the growth promoting activity of different bovine sera was investigated. The growth promoting activity of ordinary fetal CS

improved when DxS plus LPS were added (Fig. 1A). DxS plus LPS did not cause an increased proliferation when the medium was used supplemented with Hyclone fetal CS (Fig. 1B).

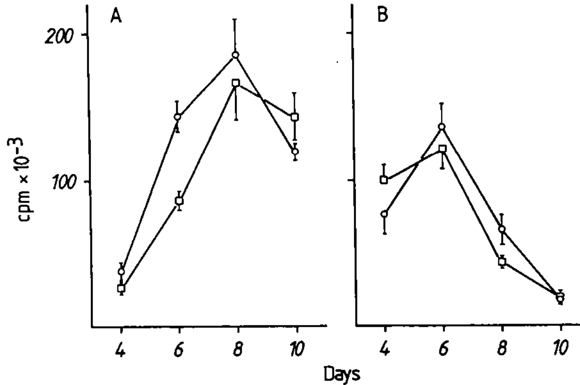

Fig.1. The effect of the addition of DxS and LPS on the growth of non-antibody producing hybridoma cells and Bl_1 cultured in media supplemented with different fetal calf sera. (A) Ordinary fetal CS. There are significant differences between the serum only and the serum supplemented with DxS and LPS on days 4 and 6 (p=0.01). (B) Hyclone fetal CS. There are no significant differences. □ 10% serum, ○ 10% serum + DxS + LPS. Bars represent S.D.

A different pattern was observed when newborn CS was used; DxS plus LPS in combination with ordinary newborn CS did not improve the proliferative activity (Fig. 2A), whereas the proliferative activity was increased and sustained when Hyclone newborn CS was used (Fig. 2B).

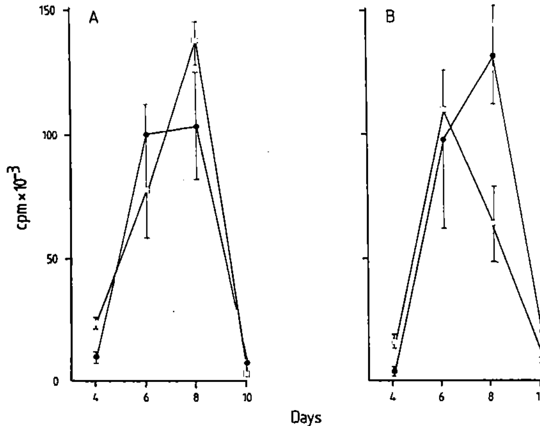

Fig.2. The effect of the addition of DxS and LPS on the growth of hybridoma cells Bl_2, cultured in media supplemented with different newborn calf sera. (A) Ordinary newborn CS. There are no significant differences between the serum only and the serum supplemented with DxS and LPS. (B) Hyclone newborn CS. There is a significant difference between the serum only and the serum supplemented with DxS and LPS on day 8 (p=0.01). □ 10% serum, ● 10% serum + DxS + LPS. Bars represent S.D.

The hybridoma cells hardly proliferated in medium supplemented with ordinary young CS, and the addition of DxS and LPS hardly improved this (Fig. 3A). When Hyclone young CS was used in combination with DxS plus LPS, the proliferative activity was at least 10 times increased (Fig. 3B). Table I represents a summary of the effects of addition of DxS and LPS on the growth promoting activities of the different sera.

Table I. Growth promoting activity of DxS plus LPS and RCL, compared to sera without additions

Sera		DxS + LPS	RCL
Ordinary fetal	CS	+ *	o
Hyclone "	CS	o	o
Ordinary newborn	CS	o	o
Hyclone "	CS	+	o
Ordinary young	CS	o	+
Hyclone "	CS	+	+

* + means: significant difference between serum with and without additions

o means: no significant difference between serum with and without additions

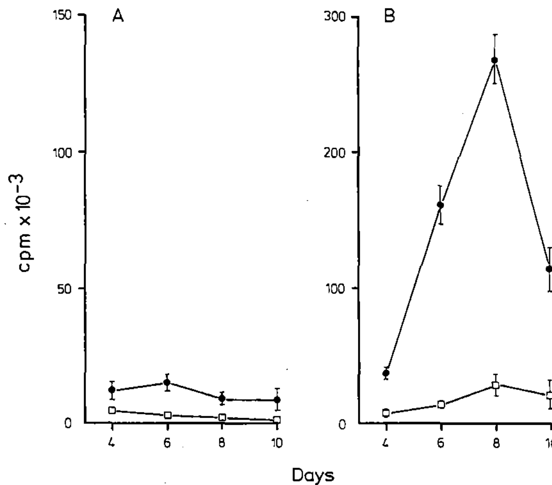

Fig.3. The effect of the addition of DxS and LPS on the growth of hybridoma cells Bl_3, cultured in media supplemented with different young calf sera. (A) Ordinary young CS. Both in the presence and the absence of DxS plus LPS a weak proliferative activity was found. (B) Hyclone young CS. There are significant differences between the serum only and the serum supplemented with DxS and LPS on days 4, 6, 8 and 10 (p=0.01). □ 10% serum, ● 10% serum + DxS + LPS. Bars represent S.D.

The influence of RCL on the growth promoting activity of different bovine sera

In a previous publication (1) we have shown that addition of RCL to Hyclone young CS improved the growth of hybridoma B cells. Here we describe the effect of addition of RCL to other bovine sera. When RCL was added to fetal CS or newborn CS there was no increase in proliferative activity, neither when added to sera of ordinary quality, nor in combination with sera of the Hyclone quality. In contrast, in combination with ordinary young CS (Fig. 4A) or Hyclone young CS (Fig. 4B), RCL improved the growth of the hybridoma cells 20-40 times. Table I summarizes the growth promoting activity of the addition of RCL to the various calf sera tested.

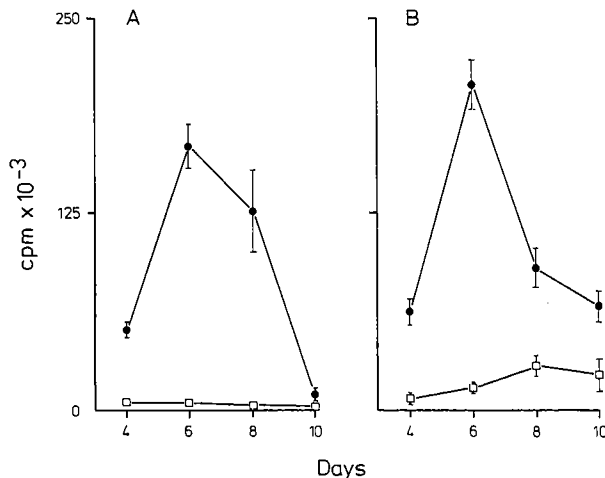

Fig.4. The effect of the addition of RCL on the growth of hybridoma cells RW1, cultured in media supplemented with different young calf sera. (A) There are significant differences between ordinary young CS and ordinary young CS plus RCL on days 4, 6 and 8 (p=0.01). (B) There are significant differences between Hyclone young CS and Hyclone young CS plus RCL on days 4, 6, 8 and 10 (p=0.01). □ 10% serum, ● 10% serum + 10% RCL. Bars represent S.D.

The stimulating factor in red cell lysate

In order to identify the component in RCL that accounts for the growth promoting effect on hybridoma cells cultured in medium supplemented with ordinary young CS or Hyclone young CS, we compared lysates from adult erythrocytes (RCLa) and fetal erythrocytes (RCLf), and crystallized hemoglobin (HbC). It

appeared that all preparations improved the cell proliferation to the same extent (Table II). Diphosphoglyceric acid, an organic phosphate which occurs in the red cell and facilitates the unloading of oxygen from hemoglobin, did not improve the growth of the hybridoma cells.

Table II. The growth promoting activity of RCL and the different polypeptide chains of hemoglobin

Additions to Hyclone young CS	cpm × 10^{-3}
none	28 ± 8
RCLa	93 ± 15
RCLf	102 ± 12
HbC	86 ± 9
α-globin	27 ± 10
β-globin	33 ± 6
α- and β-globin	24 ± 6

Figures represent the mean and standard deviation of six determinations. The hybridoma cells, RW2, were cultured for 8 days.

Subsequently we investigated the growth promoting activity of the different polypoptide chains of hemoglobin. It appeared that α-globin and β-globin in combination with Hyclone young CS did not improve proliferation (Table II). However, hemin did improve proliferation, but to a smaller extent than RCL (Fig. 5).

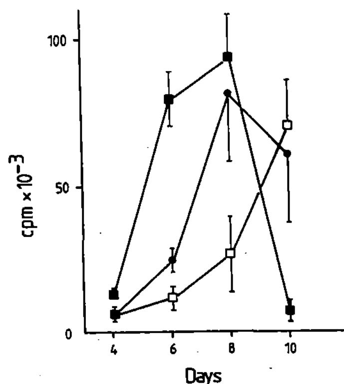

Fig.5. The effect of the addition of hemin on the growth of hybridoma cells RW1, cultured in media supplemented with Hyclone young calf serum. There are significant differences between the serum only (□) and the serum supplemented with RCL (■) or hemin (●) on days 6 and 8 (p=0.01). Bars represent S.D.

DISCUSSION

The data presented in this paper show that hybridoma cells grow equally well in media supplemented with either fetal CS or newborn CS, at least with the batches CS and hybridomas used in this study. Young CS, however, promoted hardly any cell proliferation during the test period. Remarkably, the growth promoting activity of high quality sera (Hyclone sera, with very low endotoxin and hemoglobin levels) was not better than the growth promoting activity of the equivalent ordinary sera, at least not with the batches CS and hybridomas used.

Commercially available lots of fetal CS are usually collected without special aseptic procedures and stored several days before the pools are sterilized. Shiigi et al. (13,14) therefore screened serum samples sterilly taken from single fetuses by cardiac puncture. Such samples proved to be very deficient in growth promoting activity when compared with sera collected non-aseptically. They showed that when serum was preconditioned by the growth of gram-negative, gliding bacteria, a strong growth promoting effect was obtained. Hyclone sera are supposed to be collected aseptically. In contrast to the findings of Shiigi they proved to have a better growth promoting activity than sera known to have been collected non-aseptically (data not shown).

We have argued previously that B cell hybridomas and normal B cells can be stimulated to growth and/or maturation by the same mitogens and that cells in different stages of development can respond to different stimuli (15). Davis et al. (16) described an enhanced proliferation following the addition of LPS to hybridoma cultures. The data presented here show that a combination of DxS and LPS, when added to ordinary fetal CS, can increase hybridoma cell proliferation. On the other hand, such an enhancing effect was not observed with Hyclone fetal CS. Similar observations were made by Melchers et al. (17). Just the opposite happened when newborn CS was used. DxS plus LPS did not improve the growth supporting activity of ordinary newborn CS but did improve that of Hyclone newborn CS. When ordinary young CS was used, the cells hardly proliferated, both in cultures supplemented with DxS and LPS, and in cultures that were not supplemented

with mitogens. The growth promoting activity of the Hyclone CS, however, was greatly improved.

Hybridoma cells usually multiply poorly in medium supplemented with young CS only, but the addition of a low concentration of hemin can greatly improve the growth-promoting activity. Similar observations have been made by others with different cell types such as chick embryo cells, fibroblasts, T cells and erythroid cells, when bovine calf serum (3,7), bovine plasma (18,19), horse serum, mouse serum or cat serum (2,5-7,20) were used.

While hybridoma growth is markedly increased by addition of small doses of hemoglobin to young CS, it is reduced at higher doses (1). A similar inhibitory effect by increasing concentrations of hemin has been observed on colony growth on semi-solid media supplemented with horse serum, fetal CS and rabbit serum (2,21).

Hemin can interact with the cell surface (22) and is bound in a close proximity to the lipid layer of the cell surface membrane. It can regulate enzymatic pathways and later protein function (23-25). Hemin was described as a stimulator of guanylate cyclase, an enzyme which catalyzes the generation of cGMP from GTP. cGMP serves as a positive signal to cell growth (26).

Hemin can be cytotoxic in the absence of serum (22, 23) and this is in agreement with some additional experiments we performed which show a reduced proliferation of hybridoma cells. Experiments of Malik and Djaletti (22) suggested that fetal CS could inhibit the cytotoxic effect of hemin. Addition of hemin to fetal or newborn CS had no or very poor effect on the growth of hybridoma cells. This is in agreement with the findings of others, who showed no effect on the growth of cell types like chick embryo cells, granulocytes and neuroblastoma cells (4,5,27). Verger et al. (20), on the other hand, reported that the growth of fibroblasts is improved by addition of hemin to the fetal CS to be used in the medium. This is consistent with our own data, since we also sometimes found some growth promoting effect when the fetal CS was supplemented with hemin. This, however, was dependent on the batch of fetal CS used (data not shown).

In conclusion, the data presented indicate that hemin is

highly effective in enhancing the hybridoma growth supporting activity of young calf sera, but not of fetal or newborn calf serum. Possibly, young calf sera contain a component which, in combination with hemin, can stimulate the proliferative activity of the cells. This is in contrast to DxS and LPS which do not seem to enhance the activity of a particular serum component.

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CHAPTER VIII

GENERAL DISCUSSION

The fusion technique has been used extensively to study a variety of basic cell biological and genetic questions. About ten years ago the production of monoclonal antibodies was started by using the same technique. Nowadays, hybridoma formation by fusion of murine plasmacytoma and antibody-forming cells is prosperous, but hybridoma formation by cells from other species is often less successful. The reason for this discrepancy is not yet clear. One explanation could be that the mouse plasmacytoma cell line is cultured since many years and that a well-growing and well-fusing subline has been selected, in contrast to cell lines from other species. Another explanation is that the usually applied fusion agent polyethylene glycol(PEG) is suitable for mouse B lineage cells, but less for B lineage cells of other species. According to Roos et al. (1985a, b) the fusion frequency in PEG would depend on unsaturated fatty acids on the cell membrane. So far, however, comparative studies of the unsaturated fatty acids in the cell membranes of different cell types have not been done.

One of the purposes of the studies reported in this thesis is to attempt to answer the question whether antibody-producing hybridomas are outgrown by non-antibody producing hybridomas and whether limiting dilution experiments soon after hybridization increase the chance to obtain stable antibody-producing clones without contamination of non-antibody producing clones. According to our findings, the hypothesis that cells producing antibody are in a growth disadvantage compared to non-antibody producing cells (Lemke et al., 1979; Goding, 1980) proved to be incorrect. If the initial cell concentration per well is not too high, antibody producing clones can outgrow non-antibody producing clones soon after fusion (Chapter IV).

The data presented in this thesis show that the number of clones after hybridization probably depends on the number of spleen cells and not so much on the number of plasmacytoma cells (Chapter IV). A possible explanation is that spleen cells which are in the resting state do not form viable hybridomas.

Miyahara et al. (1984) incubated plasmacytoma cells in colcemid prior to fusion and showed that this treatment increased the fusion frequency and the number of antibody-producing hybridomas. This suggests that many spleen cells are in the M-phase of the cell cycle at the moment of hybridization. Fusion of cells in different phases of the cell cycle brings about premature chromosome condensation (Johnson and Rao, 1970) and most or all chromosomes from one parent will be eliminated (Rao and Johnson, 1972). This would mean that the fusion frequency is not only dependent on the number of spleen cells, but also on the cell cycle distribution of the antibody-forming spleen cells and plasmacytoma cells.

The aim of the second part of this thesis was to get more insight into the factors that influence the proliferation and antibody-secretion of hybridomas. A number of polyclonal B-cell activators proved to stimulate the growth of hybridomas. The effects of these factors are discussed below.

Dextran sulfate (DxS) and lipopolysaccharide (LPS)

DxS and anti-IgM have been shown to activate immature B cells (Kettman and Wetzel, 1980; Howard and Paul, 1983). Activation of undifferentiated B cells by DxS leads to an increased responsiveness of these cells to LPS, that preferentially activates more mature B lymphocytes. Low concentrations of macrophage-depleted spleen cells do not proliferate in the presence of DxS, either with or without thymus filler cells (Persson et al., 1977b; Melchers et al., 1983; Corbel and Melchers, 1984). The addition of LPS to DxS, however, appears to impose a uniformity on B cell activation to growth. The magnitude of the synergy observed between DxS and LPS increases as the initial cell density decreases. Cell interactions are required for LPS-induced proliferation (Melchers et al., 1983). The addition of DxS decreases the requirement for cell interaction in induction of B lymphocyte proliferation (Kettman and Wetzel, 1980; Wetzel and Kettman, 1981a, b). A possible explanation might be that the negative charge of DxS changes the electrostatic barrier permitting growth stimulators to enter the cells.

LPS is required during the first 24 hours of culture (Lipsky et.al., 1983). It triggers B cells cultured at high cell density directly in the absence of helper factors (Persson et al., 1977a).

The B cells respond well to LPS by DNA synthesis but less by Ig secretion (Janossy et al., 1973; Kishimoto et al., 1975; Hoffmann et al., 1979; Jaworski et al., 1982; Fernandez and Severinson, 1983).

Macrophage supernatant

Peritoneal macrophages that are activated in vivo by thioglycollate release considerably more B cell-activating factor than unactivated peritoneal macrophages. Human monocytes release a B cell-activating factor comparable to that obtained with activated mouse macrophages (Wood and Gaul, 1974). The active factor is secreted for the first 24 to 48 hours of an in vitro incubation (Wood and Cameron, 1975; Rosenberg and Lipsky, 1981). Addition of LPS to the monocyte culture markedly increases the B cell activating factor concentration (Wood and Cameron, 1976). The monokine is present in culture supernatant of adherent cells of both thioglycollate-induced peritoneal exudates and non-induced peritoneal macrophages, but not in media conditioned by the non-adherent components of the mouse peritoneal cell population. The factor is termed interleukin-1 (IL-1) (Aarden et al., 1979). Antibody against leukocytic pyrogen (anti-LP) has been shown to bind IL-1 and to remove the activity in monocyte supernatants that augment stimulated B cell proliferation and differentiation. The degree of inhibition increases with prolongation of the culture (Lipsky et al., 1983). These results suggest that IL-1 plays an essential role in B cell proliferation and differentiation.

Some authors have identified IL-1 mediated augmentation of B cell responsiveness but have generally demonstrated effects on differentiation of antibody-forming cells rather than on B cell proliferation (Schrader, 1973; Wood and Gaul, 1974; Calderon et al., 1975; Wood et al., 1976; Wood, 1979; Finelt and Hoffmann, 1979; Koopman et al., 1978). In complete absence of monocytes, however, IL-1 containing monocyte supernatant by itself has no effect on the proliferation or differentiation of unstimulated B cells or on proliferation of in vivo activated B cells (Smith and Hammerström, 1978; Hoffmann et al., 1979; Rosenberg and Lipsky, 1981; Vaux et al., 1981; Jaworsky et al., 1982; Thiele and Lipsky, 1982; Falkoff et al., 1983; Howard et al., 1983; Corbel and Melchers, 1984). There is no detectable increase in mean B cell volume, in RNA synthesis, or in mean

Ia expression of resting B cells cultured with various concentrations of IL-1 (Kehrl et al., 1984). It seems likely that the stimulating activity of IL-1 is correlated with expression of IL-1 receptors on the cell surface. However, no direct evidence for receptors for IL-1 on activated B cells has yet been obtained.

IL-1 is weakly or not mitogenic compared to LPS (Schrader, 1973; Wood and Gaul, 1974; Wood et al., 1976; Wood and Cameron, 1978; Hoffmann et al., 1979; Vaux et al., 1981). Proliferation and differentiation of B cells are improved when LPS is added to supernatant of macrophages stimulated with agar extract (Corbel and Melchers, 1983). When LPS stimulated macrophage supernatant is used, the mitotic response can be attributed to residual LPS. Hoffmann et al. (1979) showed that even very low concentrations of LPS induce B cell proliferation.

In conclusion, IL-1 by itself can stimulate B cells in interphase but not resting B cells to proliferate. The cell density in culture affects the proliferation of normal B cells. In the presence of LPS and a B cell concentration of over 10^4 cells per 0.2 ml well or in the presence of DxS and a higher B cell concentration, proliferation and differentiation are improved.

Human endothelial culture supernatant and human umbilical cord serum

A soluble factor(s) produced by human endothelial cells, HECS, supports growth of hybridomas. When the cells have been in contact with HECS for 24 hours, they continue to proliferate, even when the supernatant is removed (Astaldi et al., 1981). We therefore added HECS only once at the beginning of the procedure. When HECS is added to cultures containing various numbers of hybridoma cells, proliferation is increased at all cell densities (Chapter V).

Cultured human endothelial cells produce more colony-stimulating activity than human monocytes for non-adherent human and murine bone marrow cells (Quesenberry and Gimborne, 1980) and hybridomas (Chapter V). Little is known about the nature of the stimulating factor in HECS. However, it seems that this factor shares characteristics with aortic endothelial cell-derived growth factor from monkey or from ox (Gadjusek et al., 1980), an acidic fibroblast and endothelial growth factor from bovine brain (Maciag et al., 1979, 1982; D'Amore and Klagsbrun, 1984; Thomas et al., 1984), a macropha-

ge-derived growth factor that promotes fibroblast proliferation (Leibovich and Ross, 1976), a WEHI-3 derived growth factor stimulating Lyb-5^+ B cells (Booth et al., 1983), and a human monocyte-derived growth factor inducing proliferation in B cell hybridomas (Aarden et al., 1984; DeGroot et al., 1984). The growth factors exhibit three major biochemical similarities including isoelectric point (pI 5.0), elution at high salt concentrations (0.9 - 1.1 M NaCl) and molecular weight (16,000 - 22,000). To determine the degree of similarity of the growth factors requires comparison of their amino acid sequences and a spectrum of target cells.

Human umbilical cord serum (HUCS) increases the proliferation of bone marrow cells (Odavic and Beck, 1976) and hybridomas (Chapter V). The growth promoting effect of HUCS is superior to that of the growth-promoting factor(s) produced by human endothelial cells in culture (HECS). Probably, the growth-promoting factor(s) of HECS is also present in HUCS. The concentration of amino acids in fetal plasma is much higher than in the maternal plasma. Most amino acids are actively transported from the maternal plasma and travel down a concentration gradient to the fetus resulting in a higher amino acid concentration in the fetus than in the mother (Lindblad and Baldesten, 1967; Young, 1971). Probably because of such nutritional elements, HUCS is superior to HECS.

In this thesis special attention is paid to the effects of polyclonal B cell stimulators on the growth of hybridomas immediately after cell fusion and at culturing as single, isolated hybridomas. These two different stages of the procedure are important to obtain monoclonal antibody-producing clones. In the first stage, only spleen cells that fuse with plasmacytoma cells in the same phase of the cell cycle form viable hybridomas (Chapters II and III).

Fusion is performed at a ratio of 10 : 1 of spleen cells to plasmacytoma cells and a concentration of 1.1×10^5 cells per well. At this concentration an average of one hybridoma per well will grow and form a clone (Chapter IV). The remaining plasmacytoma and spleen cells have a "filler" function rather than a hybridization function. In this phase of the procedure the newly formed hybridomas, but not their companion unfused cells, have to be stimulated to become proliferating and antibody-producing clones. Addition of growth promoters increases the yield of antibody producing clones compared to

the number of input spleen cells but does not increase the number of antibody-producing clones compared to the number of hybrid clones formed (Chapter V). This was found for all growth promoters tested, except for macrophage supernatant supplemented with LPS (end concentration of 5 μg/ml). In the presence of the latter, the number of antibody producing clones is 1.5 times that of the other stimulators (Table I).

TABLE I

EFFECT OF DIFFERENT FEEDER SYSTEMS ON RECOVERY AND YIELD OF ANTIBODY PRODUCTION BY HYBRIDOMA CELLS AFTER FUSION

Stimulators	Wells plated[a]	Wells with clones		Antibody producing clones		Ratio clones/ Ab producing clones[d]
		% (A)	Frequency of spleen cells forming clones[c] x 10^5 (B)	% (C)	Frequency of spleen cells forming clones[c] x 10^6 (D)	
FCS	384(4)[b]	65	1.05	12	1.3	8.1
LPS + DxS	384(4)	99	4.61	33	4.0	11.5
Mø sup.	384(4)	98	3.91	42	5.4	7.2
HECS	288(3)	99	4.61	34	4.2	11.0
HUCS	384(4)	99	4.61	33	4.0	11.5

a. Concentrations of 10^5 spleen cells per well

b. Number of fusions in parentheses

c. Calculated with the formula: average clones/well ($-\ln F_o$) / 10^5 spleen cells

d. Calculated from B/D

The second stage in the procedure is the culture of hybridomas as single cells. The circumstances here are quite different from those immediately after fusion. Hybridomas are grown separately to select antibody producing hybridomas out of non-producers. Essential in this stage is that isolated hybridoma cells move from the resting G_0 phase into the activated G_1 phase and through this phase into the mitotic cycle. There is no cell-cell interaction in this stage of the procedure.

Feeder cells are known to stimulate proliferation of B cells. Aged monocytes, which no longer secrete active factors, fully reconstitute the proliferative response of monocyte-depleted lymphocytes (Rosenstreich et al., 1976; DeVries et al., 1979; Rosenberg and Lipsky, 1981). This function cannot be accomplished by monocyte membranes, nor by heat-killed monocytes (Rosenberg and Lipsky, 1981).

When highly enriched B cells or normal spleen cells, both activated by LPS, are cultured at suboptimal concentrations, the relation between cell density and magnitude of the proliferative response is non-linear and rapidly declines to background proliferation levels at cell numbers below 10^4 cells per well. The dilution curve is sigmoid and not linear (Andersson et al., 1977a; Sieckmann et al., 1978; Ulmer and Maurer, 1978; Corbel and Melchers, 1984). When hybridomas are cultured at decreasing cell concentrations, the response to LPS is rapidly lost below a cell density of 25 cells per well. Addition of thymus or spleen filler cells to normal B cells or hybridomas, however, will increase the overall response and the dilution curves become linear (Anderson et al., 1977b; Fernandez and Severinson, 1983). Also the addition of thymus filler cells to X63-AG8 myeloma cells grown at low cell densities increases the efficiency of growth, whereas no increase is obtained when the myeloma cells are cultured at elevated cell densities (Lernhardt et al., 1978). Spieker-Polet et al. (1985) demonstrated that in the early phase of the cell cycle contact between B cells is important for the activation of the cells. These observations suggest that B cells, hybridomas and plasmacytomas require cell-cell interaction in order to proliferate, either between the cells mutually or between these cells and the feeder cells. This could explain the different results with macrophage supernatant stimulated hybridomas directly after cell fusion, where about 1.1×10^5 cells are cultured per well, and with macrophage supernatant stimulated hybridomas when dispensed at one cell per well.

When the mitogens DxS and LPS were added to the culture medium, single B cells are stimulated and form clones. Both mitogens are able to deliver electronegative charges and cause membrane depolarization. Low levels of anti-IgM induce also membrane depolarization (Cambier and Monroe, 1984). The membrane depolarization is followed by increased Ia antigen expression, suggesting that these events may be linked (Cambier et al., 1985). In this phase the cells are in an activated state and are able to proliferate in response to B cell-activating factors (Proust et al., 1985). These studies indicate that cell-cell contact is not always required for B cell activation. This is in agreement with our own observations concerning the growth of hybridomas, since DxS plus LPS were found to stimulate

the growth of single hybridoma cells (Chapter V).

A new stimulator, red cell lysate (RCL), proved to stimulate the hybridoma growth rate more than any other B cell stimulator tested when added to Hyclone young calf serum. When used immediately after fusion, there was no difference in either the frequency of wells that contained proliferating clones and of wells with antibody-producing clones when compared to HUCS. The addition of DxS and LPS to RCL and Hyclone young calf serum did not improve the hybridoma growth rate significantly. When starting from one cell per well, there was no difference in the number of clones when the hybridoma cells were grown in medium supplemented with HUCS or with Hyclone young calf serum together with DxS, LPS and RCL (Chapter VI). The active factor in RCL proved to be hemin (Chapter VII). Hemin is not a specific hybridoma stimulator, because it has a variety of effects. It can affect the proliferation of different cell types such as chick embryo cells (Verger, 1979), T cells in the presence of macrophages (Stenzel et al., 1981), Friend erythroleukemic cells and mastocytoma cells (Rothmann et al., 1983). It also promotes erythroid cell differentiation (Porter et al., 1979; Bonanou-Tzedali et al., 1981; Monette and Holden, 1982; Holden et al., 1983). Furthermore it promotes the differentiation of fibroblasts to adipocytes (Chen and London, 1981) and the formation of neurites by mouse neuroblastoma cells (Ishii and Maniatis, 1978).

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SUMMARY

Specific antibodies can be generated by immunization of animals with an antigen. This antigen binds to receptors on the cell membrane of B lymphocytes, which results in proliferation and differentiation into antibody-secreting plasma cells. A disadvantage of this method is that always antisera containing heterogeneous populations of antibodies are obtained. To overcome this problem Köhler and Milstein developed in 1975 a method by which antibody production by a single clone of antibody-forming cells was made "immortal". Therefore antibody-producing plasma cells are fused with plasmacytoma cells which have an unlimited proliferative capacity. The hybrid cells (hybridomas) produced in this procedure retained these important properties of both parental cells. From a population of hybrid cells individual clones can be selected that continuously proliferate and produce unlimited amounts of physico-chemically homogeneous, specific antibodies.

The aim of the study presented in this thesis was to get more insight into the fusion process, to simplify the time consuming manipulations and to improve the hybridization frequency and growth of hybridomas.

A major problem concerning the fusion technique is the loss of antibody production after hybridization. Much information about cell fusion concerning non-lymphoid cells is available in the literature. Because this information might be applied for improving the fusion technique for the production of hybridomas, the literature about this subject is reviewed. An important aspect is that only cells that are in the same phase of the cell cycle can be successfully fused to form hybrid clones. Besides, the fusion frequency may be improved by increasing the pH during the fusion process and by addition of certain drugs like colchicine.

From our investigations it appeared that the number of hybridomas formed after fusion depends on the number of spleen cells and not so much on the number of myeloma cells. Furthermore, the suggestion in the literature that non-antibody producing clones outgrow antibody producing clones after fusion appeared to be incorrect. Many hybridomas rather loose spontaneously their antibody production soon after hybridization. This means that selection of the hybrid cells by limiting dilution shortly after fusion can be ommitted.

To improve the fusion frequency a number of B-cell stimulators were tested. It appeared that human umbilical cord serum induced a rapid growth of hybridomas. Somewhat slower growth was induced by human endothelial culture supernatant, macrophage supernatant and a combination of dextran sulfate and lipopolysaccharide. The total number of clones and the number of antibody producing clones obtained after fusion increased to almost the same extent with all stimulators. The only exception concerns macrophage supernatant, which caused an increase of the number of antibody producing clones when cultured at high density only. When starting from one cell per well, however, macrophage supernatant did not promote growth.

In this thesis also a new hybridoma growth stimulator is introduced, namely hemoglobin in combination with young calf serum. The α and β chain of hemoglobin did not improve growth, but the hemin part did, indicating that the latter is the active growth promoting component in hemoglobin.

When the results of our investigations about hybridoma growth factors are compared to those presented in the literature about B-cell stimulation and proliferation, it appears that there is hardly any difference between the growth of normal B cells and the growth of hybridomas.

Based on the data obtained we propose that the best growth of hybridomas immediately after fusion is obtained in medium supplemented with macrophage supernatant, whereas established hybridomas cultured as single cells grow best in Hyclone young calf serum supplemented with hemoglobin.

SAMENVATTING

Door proefdieren te immuniseren met een antigen kunnen specifieke antilichamen worden geproduceerd. Na immunisatie bindt het antigeen zich aan receptoren op het oppervlak van B lymphocyten, waarna deze cellen gaan prolifereren en differentiëren tot antilichaam-secernerende plasmacellen. Een nadeel van deze methode is dat de antisera die op deze manier worden verkregen altijd heterogeen zijn, dwz. verschillende antilichaam-populaties bevatten. Om aan dit bezwaar tegemoet te komen is door Köhler en Milstein in 1975 een methode ontwikkeld waardoor van afzonderlijke klonen de antilichaam produktie "onsterfelijk" gemaakt kan worden. Hierbij worden antilichaam vormende plasmacellen gefuseerd met plasmaceltumor cellen. De aldus ontstane hybride cellen (hybridomen) behouden de belangrijkste eigenschappen van de beide oudercellen. Zo wordt een in principe continu prolifererende tumorcel gevormd die onbeperkte hoeveelheden van fysisch-chemisch homogene, specifieke antilichamen produceert.

Het doel van het onderzoek dat in dit proefschrift wordt beschreven was om meer inzicht te krijgen in het fusieproces zelf, om de vele tijdrovende manipulaties iets te kunnen beperken en om de hybridisatie frequentie en de groei van hybridomen te verbeteren.

Na celfusie doet zich het probleem voor dat de hybridomen vaak stoppen met de productie van antilichamen. Uit de celbiologie is veel meer bekend over de processen die plaatsvinden wanneer cellen met elkaar fuseren. Omdat dit van belang zou kunnen zijn bij het verbeteren van de fusietechniek, is hiernaar een literatuurstudie gedaan. Hieruit bleek o.a. dat vrijwel alleen cellen die tijdens de fusie in dezelfde fase van de celcyclus verkeren, hybride klonen kunnen vormen. De fusiefrequentie zou verder verbeterd kunnen worden door tijdens het fusieproces de pH te verhogen of door het toevoegen van bepaalde chemicaliën zoals colchicine.

Uit het onderzoek bleek dat het aantal hybridomen dat gevormd wordt, vooral bepaald wordt door het aantal miltcellen in het fusiemengsel en niet zozeer door het aantal myeloomcellen. In tegenstelling tot wat er in de literatuur gesuggereerd wordt, bleek dat antilichaam producerende klonen niet overgroeid worden door niet-antilichaam producerende klonen. Het verlies aan antilichaam produktie van een groot aantal cellen vindt spontaan plaats. Dit betekent dat de selectie van de hybride cellen door kweken onder "limiting dilu-

tion" kweekcondities kort na fusie overbodig is.

Om een verbetering van de groei resultaten te verkrijgen zijn een aantal B-cel stimulatoren getest. Het bleek dat menselijk navelstreng serum in de onderzochte gevallen in hoge mate de hybridoomgroei bevorderde. Iets minder, maar toch ook erg goed bleken de supernatanten van kweken van menselijke endotheelcellen en van macrofagen en een combinatie van dextraansulfaat en lipopolysaccharide te zijn. Het totaal aantal klonen en het aantal antilichaam producerende klonen dat na fusie werd verkregen was vrijwel gelijk voor alle geteste B-cel stimulatoren. Een uitzondering daarop vormde macrofaag supernatant. Wanneer dit wordt toegevoegd, worden er in kweken met een relatief hoge celdichtheid meer antilichaam-producerende klonen verkregen dan bij de andere stimulatoren. Echter, bij limiting dilution experimenten waarbij wordt uitgegaan van een cel per kweekputje, gaf het macrofaag supernatant geen vergroting van het aantal klonen te zien.

In dit proefschrift wordt voorts een nieuwe hybridoom-stimulator, hemoglobine in combinatie met kalverserum, beschreven. De α en β ketens van het hemoglobine droegen nauwelijks bij tot de stimulerende aktiviteit, in tegenstelling tot hemine, waaruit blijkt dat dit gedeelte van het hemoglobine molecuul de stimulerende faktor is.

Uit het voorafgaande kan de konklusie worden getrokken dat hybridomen direkt na fusie het beste kunnen worden gekweekt in medium verrijkt met macrofaag supernatant. Voor het opzuiveren van een kloon worden de beste resultaten bereikt met medium met Hyclone kalverserum en hemoglobine.

ABBREVIATIONS

AG	8-azaguanine
Anti-LP	antibody against leukocytic pyrogen
APRT	adenine phosphoribosyltransferase
BAF	B-cell activation factor
CS	calf serum
DMSO	dimethyl sulfoxide
DPG	2,3-diphosphoglyceric acid
DxS	dextran sulfate
EBV	Epstein-Barr virus
HAT	hypoxanthine, aminopterin, thymidine
Hb	hemoglobin
HbC	cristallized hemoglobin
HcCS	Hy-clone calf serum
HECS	human endothelial culture supernatant
HPRT	hypoxanthine phosphoribosyltransferase
HUCS	human umbilical cord serum
IL-1	interleukin-1
LPS	lipopolysaccharide
mtDNA	mitochondrial DNA
NOR	nucleolus organizer regions
PBL	peripheral blood lymphocytes
PCC	premature chromosome condensation
PEG	polyethylene glycol
PGE	prostaglandin E
RCLa	red cell lysate from adult erythrocytes
RCLf	red cell lysate from fetal erythrocytes
Rb(8.12)	Robertsonian 8.12
rRNA	ribosomal RNA
TG	6-thioguanine
TT	tetanus toxoid

CURRICULUM VITAE

De schrijfster van dit proefschrift werd in 1938 in Bentveld geboren. Op 19-jarige leeftijd behaalde zij het diploma van de Middelbare Meisjes School in Vught. In de daaropvolgende jaren volgde zij een analisten opleiding, enkele talen cursussen en werkte zeven jaar bij het Nederlands Centraal Instituut voor Hersenonderzoek in Amsterdam, waar zij uiteindelijk ontslag nam vanwege haar universitaire studie. In 1967 begon zij de avond M.O. opleiding biologie aan het Nutsseminarium voor Pedagogiek in Amsterdam. In 1972 slaagde zij voor het colloquium doctum en in datzelfde jaar behaalde zij het candidaatsexamen aan de Universiteit van Amsterdam. Het doctoraalexamen met als hoofdvak neurofysiologie en de bijvakken farmacologie en electronenmicroscopie legde zij af in 1974 aan de Vrije Universiteit in Amsterdam. In 1975 werkte zij een jaar op een tijdelijke arbeidsplaats aan een gecombineerd onderzoek van electrofysiologie en gedrag aan het Dierfysiologisch Laboratorium van de Universiteit van Amsterdam. Van 1978 tot 1979 werkte zij op vrijwillige basis aan de afdeling Gastro-Enterologie van het Academisch Ziekenhuis in Leiden; van mei 1980 tot november 1982 werkte zij op de afdeling Immunohaematologie van het Academisch Ziekenhuis Leiden, waar het onderzoek voor dit proefschrift werd verricht. De bewerking van de resultaten daarvan tot dit proefschrift vond plaats onder leiding van Prof. Dr. R. Benner van de afdeling Immunologie van de Erasmus Universiteit Rotterdam en Prof. Dr. J.J. van Rood van de afdeling Immunohaematologie van het Academisch Ziekenhuis Leiden. Van 1982 tot november 1986 was zij werkzaam op het Laboratorium voor Electronenmicroscopie van de Rijksuniversiteit Leiden.