STUDIES ON THE PRODUCTION OF HUMAN MONOCLONAL ANTIBODIES

by

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DECLARATION

I declare that I have composed and written this thesis and that the work described in this thesis was entirely my own and performed by me unless otherwise acknowledged.

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ABSTRACT

In view of the potential therapeutic and diagnostic applications of human monoclonal antibodies a series of experiments were undertaken to optimise the production of human monoclonal antibodies by existing cell lines and to develop other human monoclonal antibodies.

Initial studies were performed to improve the stability, antibody secreting capacity and growth characteristics of an anti-tetanus antibody secreting line (ES12) previously produced in the department. The studies involved forming hybridomas of ES12 and various existing partner lines. The partners used were KR4 and Hmy2 (human lymphoblastoid cell lines) and NS0 (murine myeloma cell line). Three hybrids were chosen and their growth characteristics and antibody secretion were studied. It was found that GF5/9.13 (ES12/KR4 hybrid) secreted the largest amount of antibody but that GF4/1.1 (ES12/NS0 hybrid) was the most convenient line to grow.

Later experiments attempted to produce new cell lines secreting antibodies specific for Hepatitis B surface antigen (HBs). The methods used included transformation and direct fusion of peripheral blood lymphocytes as well as a combination of the two. The fusion partners used in this case were Hmy2 and NS0. Despite the fact that many antibody secreting lines were isolated, only one proved to be semi-stable. This line was produced by fusing an EBV transformed line with Hmy2.

The isolation of appropriately immune lymphocytes can

be a problem in human monoclonal antibody production. In an attempt to improve the yield of immune lymphocytes a series of in vitro stimulation experiments were undertaken. The stimulants used included PWM, PHA, human AB serum and recombinant HBs vaccine. From these experiments an antibody secreting line arose spontaneously. It is assumed that the line is EBV transformed as its DNA profile appears normal.

Preliminary experiments were under taken to show that lymphocytes obtained from sources other than blood (initially spleen) could be used to form hybridomas. Donors for these experiments had high anti-HIV and anti-blood group antibodies. despite the high yield of hybrids, no specific lines were isolated.

As a follow up to initial experiments carried out in the department which show that cell lines produced from a normal healthy donor bind structures in human brain, samples from transformation experiments were screened against brain sections. The results here show how transformation experiments can be used to dissect the immune response even when permanent cell lines are not produced.

Finally it is obvious that the production of human monoclonal antibodies is far from easy. Improvements are necessary in several areas which include: the isolation of immune lymphocytes, immortalisation techniques, cloning procedures and an under standing of the growth factor requirements of B cells.

CHAPTER 1

INTRODUCTION

1.1 HISTORY

Monoclonal antibodies are antibodies of a single specificity produced by a cell line derived from the progeny of a single B cell. The whole procedure is entirely dependent on the fact that a single antibody producing cell secretes only one specific antibody as demonstrated by Nossal and Lederberg (1958). During the fifties cell culture in vitro was developed as a routine process, Barski et al (1960) observed spontaneous fusion of cultured mouse cells and it was also noted that the frequency of fusion could be enhanced using certain viruses (Okada and Tadoka, 1962).

The selection of fused cells from the resultant mixture was a problem until Littlefield (1964) produced a cell line deficient in enzymes, which could be selected in medium. It is this same basic method which is still used today. Because the hybrids produced by this mechanism tended to be genetically unstable and lose chromosomes, the method was widely used to map genes to their chromosomes (Croce et al., 1980, Erikson et al., 1981)

At around the same time research workers discovered that myelomas were a neoplasm of antibody secreting cells and that each tumour was derived from a single clone (Cohn 1967). The artificial induction of myelomas in mice using mineral oil (Potter and Boyce, 1962), coupled with in vitro cell culture techniques led to the development of many well characterised immunoglobulin secreting lines, although the epitopes recognised by the antibodies were undefined (Cohn, 1967; Potter et al.,

1977)

Several viruses were used in alternative immortalisation procedures to transform lymphocytes. Strasberg (1974) used Simian virus 40 (SV40) to transform cells from a rabbit immunised with pneumococcal type III polysaccharide and isolated a line secreting small amounts of specific antibody. Baumal et al (1971) used Epstein-Barr virus to transform human lymphocytes from an immune donor; this approach led to the production of several lymphoblastoid cell lines (LCL) secreting large amounts of antibody, although none was specific for the immunising antigen. This technique has been greatly improved in recent years and has been used successfully to produce many cell lines secreting specific antibody (see later).

In the meantime progress was being made in attempts to fuse myeloma cells, these studies being undertaken mainly to analyse the expression of immunoglobulin genes. In these experiments Sendai virus induced fusion was largely unsuccessful, probably because the majority of murine cells lack a viral receptor.

The low frequency of hybrid formation continued to be a problem until Davidson and Gerald (1976) reported that polyethelyne glycol (PEG) promoted fusion of adherent cells. This method was rapidly adapted for fusing myeloma and lymphocytes in suspension (Kohler and Milstein, 1975; Marguilies et al., 1976) and has been the fusion method of choice ever since.

Cotton and Milstein (1973) successfully fused a murine and rat line which continued to secrete both murine heavy and light chains and rat light chains. At the same time Schwaber and Cohn (1973) formed hybrids between human lymphocytes and murine myelomas that continued to secrete both human and mouse immunoglobulin. These and further studies led to the establishment of basic principles concerning the expression of immunoglobulin in hybrid cells. Hybrids continue to secrete all of the immunoglobulin chains secreted by both parent lines (Marguilies et al., 1976; Milstein et al., 1976) unless the chromosomes are lost due to genetic instability. No new immunoglobulins are synthesised (Milstein and Kohler 1977) and fusion of an antibody producing cell line with a non-producing cell does not prevent antibody production. Detailed studies of the immunoglobulin produced in these experiments indicated that many were mixed molecules produced by assembling heavy and light chains from both parents (Marguilies et al ., 1976)

All of the above developments acquired greater significance when Kohler and Milstein (1975) reported the production of cell lines secreting antibodies specific for sheep red blood cells. These lines were produced by fusing spleen cells from a previously immunised mouse with a HAT sensitive MOP-21 myeloma cell line. The hybridomas as they were known were cloned and were the first monoclonal antibodies to be produced by deliberate prior immunisation.

Since then the technology has mushroomed into the vast

field which exists today. Monoclonal antibodies are currently being produced against a wide range of epitopes including viral, bacterial, hormones, enzymes and many others. The applications and potential applications are almost as varied, from highly specific assays to immunoaffinity depletion and purification techniques and as human and animal therapeutics.

The great majority of monoclonal antibodies reported to date are of rodent, mainly murine origin. This reflects the fact that murine tissue culture has been extensively developed since the very earliest in vitro studies were undertaken. As a result, murine myelomas which secrete no antibody are readily available for use as fusion partners. Because the majority of tissue culture development has been carried out using murine cell lines, the culture media etc. are designed to give maximum growth of murine cells. The myelomas of other species may require slightly altered conditions for optimal growth.

Advances in murine tissue culture, in particular in relation to monoclonal antibody production, have not always been readily transferred to the human system. The reasons for this are not entirely clear, but it is obvious with even the briefest glimpse through the literature that production of human monoclonal antibodies has not proceeded as quickly as would have been expected. The first human monoclonal antibodies produced by cell fusion were reported almost simultaneously by Croce et al

(1980) and Olsson and Kaplan (1980) although Steinitz et al (1977) had previously reported the production of specific antibodies by Epstein-Barr virus transformed cells. Since this time relatively few antibodies have been reported, indicating that severe technical problems are encountered when attempting to produce human monoclonal antibodies.

There are several key areas where problems arise, these will be discussed in detail later in the text. Briefly the major problems are: 1) identifying a donor who is immune to a particular antigen of interest; because of ethical considerations donors must have become immune via a natural infection or an approved vaccine 2) obtaining sufficient immune lymphocytes; here it is the choice of tissue which is important, 3) immortalising the appropriate cells, and 4) cloning cells to obtain stable cell lines.

1.2 SOURCE OF IMMUNE LYMPHOCYTES

In the murine system, which has by now become extremely well developed, the source of immune lymphocytes is almost exclusively the spleen of hyperimmunised mice. Generally speaking provided a substance is non-toxic and immunogenic then an immunisation schedule can be optimised to ensure sufficient immune cells are available for fusion. Unfortunately this path cannot be followed in the development of human monoclonal antibodies. The main problems are ethical considerations involved when immunising humans with potentially harmful substances. For this reason the range of human monoclonal antibodies

developed to date is limited to antigens for which an approved vaccine is already available or to those which have appeared via a natural infection or disease state.

The source of the lymphocytes is usually restricted for practical purposes to peripheral blood. Spleen, tonsil, lymph node, tumour infiltrate and bone marrow have all been used as lymphocyte sources but in most cases peripheral blood is the most convenient source. The suitability or otherwise of peripheral blood as lymphocyte source will be discussed below.

Despite the problems, lymphocytes immune to a wide range of antigens have been isolated from a variety of sources. These include donors who have been deliberately or accidentally exposed to antigen as well as those from donors with no previous record of exposure to the immunogen and some with ongoing active disease.

Not surprisingly antibodies to bacteria and viruses comprise the majority of antibodies produced following active immunisation. However antibodies to other antigens have also been obtained using lymphocytes from actively immunised individuals. These include monoclonals to colorectal cancer (Haspel et al., 1985) Rhesus D (Boylston et al.,1980; Crawford et al.,1983; Doyle et al.,1985; Paire et al.,1986 and Thompson et al.,1986), HLA antigens (Hulette et al.,1985; Effros et al.,1986) and haptens (Olsson and Kaplan, 1980). The great majority of antibodies generated from the lymphocytes of donors with active disease are directed

against tumour cell antigens (Wunderlich et al.,1981; Sikora et al.,1982; Cote et al.,1983; Glassy et al.,1985; Kan-Mitchell et al.,1986; Strelkauskes et al.,1987) and various autoantigens (Schoenfield et al.,1982; Massicote et al.,1984; Saski et al.,1984). Finally lymphocytes obtained from non-immunised apparently normal donors have given rise to autoantibodies reacting with a variety of cell surface and internal antigens (Simpson et al.,1986).

It is assumed that the state of differentiation and activation of lymphocytes will affect their ability to be immortalised. The time interval between boosting donors and harvesting lymphocytes will therefore be a critical step in successful monoclonal antibody production. For practical reasons there have been very few studies to determine the optimum time for harvesting human lymphocytes for fusion. Where studies have been performed it would appear that the optimum time for harvesting peripheral blood is 6-7 days (Bogard et al.,1985), while for spleen it is 3 days post boosting, as in the mouse (Schwaber et al.,1984). These schedules have been successfully employed to produce monoclonal antibodies against bacterial, red cell and tumour antigens. It should be noted however that monoclonals have been produced using lymphocytes isolated 1-3 months post boosting (Boyd et al.,1984; Tiebout et al.,1984, 1985; Thompson et al.,1986).

The immune state of the donor is obviously of vital importance in the production of human monoclonal antibodies; however successful immortalisation is also

influenced by the state of activation, differentiation and proliferation of the immune lymphocyte. Although these processes are beginning to be understood for Epstein-Barr virus induced immortalisation (see below) their relevance to fusion still remains to be established. As stated by Schwaber et al (1984) B cells fuse at some unidentified stage of differentiation and activation.

1.3 IN VITRO IMMUNISATION

In an attempt to bypass the problems of obtaining immune lymphocytes from donors and thereby increase the potential number of antigens which may be used, increasing attention is being focused on in vitro methods of sensitising and amplifying specific B cells. This approach has already been well studied in the murine system (Reading, 1982; Borrebaeck, 1986). The studies carried out on the human system have yielded less successful results than those previously achieved in mice. The reason for this is not clear. Borrebaeck (1986) suggests it could be due in part to the fact that human studies have used mainly peripheral blood where there is an unfavourable ratio of T suppressor cells to B cells. Another possibility is that the circulating B cells are arrested in a phase in which additional stimuli are required to induce antigen-specific clonal activation.

Developments in this area have been slow but progress is now being made. In vitro immunisation procedures have

been successfully employed with lymphocytes from peripheral blood, spleen and tonsils and antibodies have been produced which react with bacteria, viral, red cell and other antigens. The few comparative studies carried out suggest that spleen and tonsil are superior to peripheral blood as the lymphocyte source (Strike et al.,1984; Teng et al.,1985).

One interesting point which should be made here is that all of the successful stimulations reported so far have used lymphocytes obtained from seropositive donors. Attempts by Matsumoto et al (1986) to sensitise cells from seronegative donors being unsuccessful. So it seems that these in vitro "immunisations" are in fact "re-activations" of memory cells.

In an attempt to improve the performance of peripheral blood lymphocytes, some studies have employed various fractionation techniques. The majority of in vitro immunisations are carried out on lymphocytes obtained by density centrifugation. However several techniques have been developed to deplete the preparation of T cells or more specifically T suppressor cells. These include SRBC rosetting (Teng et al.,1983), separation on nylon wool (Ho et al.,1985; Garzielli et al.,1984; Yamaura et al.,1985) or sephadex G10 columns (Hoffman and Hirst, 1985a,b), specific lysis with an anti-suppressor cell (OKT8) monoclonal (Lagace et al.,1985; Brodeur et al.,1987) or panning on cimetidine coated plates (Ho et al.,1985). In some cases the adherent and non-adherent fractions have been reconstituted to give more favourable

ratios of T_H cells (Ho et al.,1985). The removal of T_S and the enrichment of T_H is not universally practised and more comparative studies will be required before it can be decided whether or not this is a desirable practice (Borrebaeck, 1986). It should be noted however that depletion or inactivation of T cytotoxic cells is advisable especially if subsequent immortalisation is to be by EBV transformation (Crawford et al.,1983,1985; Larrick et al.,1985; Yamaura et al.,1985) Cyclosporin A has proved extremely useful as a T_C inhibitor and has been shown to promote spontaneous outgrowth of EBV induced cell lines (Bird et al.,1981)

Most studies have employed mitogen of some form or another. Pokeweed mitogen has been most extensively used while others include LPS, PHA and Staphylococcus aureus Cowan 1 (a B cell stimulant). The mitogens have been used either alone or in conjunction with an antigen. In some cases the mitogen alone has been found to increase the production of antibody secreting hybrids and to exert a synergistic effect with antigen (Ichimori et al.,1985).

Growth and differentiation of B cells is obviously dependent on growth factors in the culture medium. In most cases these have been elicited in the media by the mitogens, however, some studies have used external sources of growth factors. These include unfractionated lymphocyte conditioned media (Strike et al.,1984; Ho et al.,1985), T cell growth factor (Brodeur, 1987) and B cell growth factor ((Matsumoto et al.,1986).It would

appear that the impure products are of doubtful value (Ho et al.,1985). The choice of serum used in the culture medium will also play an important role in the provision of growth factors. Some authors claim that human serum is essential, at least in the early stages of in vitro cultivation (Strike et al.,1984; Ho et al.,1985; Teng et al 1985b). However in most reports foetal calf serum has been employed without any apparent disadvantages.

The optimum time in culture varies from author to author but generally falls around three days (Crawford et al.,1983; Ichimori et al.,1985; Larrick et al.,1985) while in others seven days or more are used (Yamaura et al.,1985; Brodeur et al.,1987)

1.4 IMMORTALISATION PROCEDURES

There are at present two main techniques being used to immortalise human lymphocytes namely fusion with myeloma (or lymphoblastoid) partners or transformation with Epstein-Barr virus. It is also feasible to combine the two techniques and fuse transformed cells with an immortal partner. Some other techniques involving genetic manipulation have also been attempted but are as yet uncommon.

1. Cell Fusion

The first reports of the production of human monoclonal antibody secreting cell lines produced by fusing splenic lymphocytes with myeloma cells came from Olsson and Kaplan (1980). Since then many papers have reported success using cell fusion as the method of immortalisation. There are however several problems

associated with hybrid formation.

As previously stated the choice of lymphoid tissue plays a major role in determining the success or otherwise of a fusion. Several studies have indicated that spleen or lymph node are superior to peripheral blood for hybrid formation (Chiorazzi et al.,1982; Glassy et al.,1983; Cote et al.,1984; Teng et al.,1985).

2.Fusion Partner

A second major factor to be considered is the fusion partner itself. When murine monoclonals were first reported the myeloma fusion partners being used gave relatively satisfactory results, especially when non-secreting lines became available. This meant that a few partner lines were rapidly available to most laboratories and the development of new lines was not necessary. Good human partner lines have not appeared rapidly and vigorous attempts to develop the same has led to the generation of a plethora of fusion partners. In theory the ideal partner would be a myeloma as these lines have a phenotype with many polyribosomes, well developed golgi apparatus and are thus able to secrete large amounts of antibody (Kozbor et al.,1983, 1985). In reality very few myeloma lines are in existence (see table 1) and those which have been successfully cultured have very poor growth characteristics and perform poorly in fusion experiments (Kozbor et al.,1983). The vast majority of fusion partners used to date have been lymphoblastoid cell lines produced from EBV transformation of

TABLE NO: 1. SOME CELL LINES CURRENTLY USED AS FUSION PARTNERS IN HUMAN MONOCLONAL ANTIBODY PRODUCTION

Fusion Partner	Original Line	Cell Type	EBNA Positive	Secreted Ig	Drug Markers	Reference
SKO-007	U-266	Myeloma	-	IgE(λ)	8-AG	Olsson and Kaplan 1980
RPMI-8226		Myeloma	-		8-AG	Abrams et al. 1983
HFB 1		Myeloma	-	Non-secretor	6-TG	Hunter et al. 1982
KMMI		Myeloma	-	G		Togawa et al. 1982
U-2030		Myeloma	-	Non-secretor		Jernberg et al. 1987
RH-L4		Lymphoma	NI	Non-secretor	8-AG	Olsson et al. 1983
NAT-30	Nawalma	Lymphoma	NI		6-TG	Murakami et al. 1985
GM 1500-6TG-AL	GM 1500	LCL	+	IgG_2 (K)	6-TG	Croce et al. 1980
GM 1500-6TG-OA	GM 1500	LCL	+			Hulette et al. 1985
KR4	GM 1500-6TG-A11	LCL	+	IgG_2 (K)	6-TG,OUA	Kozbor et al. 1982
GM 467						Sato et al. 1972
GM 4672	GM 1500	LCL	+	IgG_2 (K)	6-TG	Croce et al. 1980
ARH-77		LCL	+	IgG (K)	8-AG	Burk et al. 1978
LICR-LON-Hmy 2	ARH 77	LCL	+	IgG_1 (K)	8-AG	Edwards et al. 1982

Table No. 1 (Cont'd.)

Fusion Partner	Original Line	Cell Type	EBNA Positive	Secreted Ig	Drug Markers	Reference
W1 L2		LCL				Emamuel et al. 1984
H35-1-1	W1-L2	LCL	+	IgM (K)	8-AG	Chiorazzi et al. 1982
TAW-925	W1-L2	LCL	NI	NI	6-TG,OUA	Ichimori et al. 1987
W1-L2-729 HF_2	W1-L2	LCL	+	IgM (K)		Strike et al. 1984
HOA.1	W1-L2-829HF2	LCL	+	NI	6-TG,OUA	McDonald et al. 1987
W1-L2-727	W1-L2	LCL	+	IgG (K)		Emanuel et al. 1984
UC 729-6	W1-L2	LCL	+	IgM (K)	6-TG	Glassy et al. 1983
MC/CAR		LCL	+	None		Ritts et al. 1983
MC/MNS-2	MC/CAR	LCL	+	IgG_1 (K)		Ritts et al. 1983
LTR 228		LCL	+	IgM (K)	6-TG	Larrick et al. 1983
LSM 2-7	CRL 1484	LCL	+		6-TG	Schwaber et al. 1984
HS Sultan		LCL	+	None		Lazarus et al. 1982
GK-5		LCL	+	K	6-TG	Satoh et al. 1983
HO-323		LCL	NI			Ohashi et al. 1986

Table No. 1 (Cont'd.)

Fusion Partner	Original Line	Cell Type	EBNA Positive	Secreted Ig	Drug Markers	Reference
KR-12	KR4 x RPMI 8226	h/h hybrid myeloma	+	IgG_2 (K + λ)	6-TG,OUA	Kozbor et al. 1984b
SHM-D3	U266 x 63Ag 8.653	m/h hybrid myeloma	NI	Non-secretor	6-TG,OUA G418	Teng et al. 1983
SBC-H20	B cell x SP2/08 A2	m/h hybrid myeloma	NI	Non-secretor		Foung et al. 1985a
3 HL	SHM-D3 x B lymphoma	m/h/h hybrid myeloma	NI	IgM (λ)	6-TG,OUA G418	Teng et al. 1985a
K6Hb/B5	B lymphoma NS1	m/h hybrid	NI	Non-secretor	8-AG	Carrol et al. 1986

h/h - human hybrids used as fusion partners.

m/h and m/h/h - mouse human hybrids used as fusion partners.

- EBNA - negative

+ EBNA - positive

NI - No information

lymphocytes. These cells are generally easier to handle in culture than myelomas and perform somewhat better in fusion experiments. They have fewer polyribosomes, sparse golgi apparatus and as a result secrete relatively low amounts of immunoglobulin (Kozbor et al.,1983).

Recently several groups have attempted to improve the antibody production and growth characteristics of their lymphoblastoid lines by fusing them with either human or murine myelomas. The rationale behind this approach is that hybrids of these two cell types may retain much of the phenotype of the myeloma while still retaining the division time, ease of handling and rate of hybrid formation of its lymphoblastoid parent. It is claimed that the heterohybrids do in fact perform better than their parents in fusion experiments. Kozbor et al (1984) have fused the human myeloma RPMI 8226 with their lymphoblastoid cell line KR4 to produce a heterohybrid KR12. This line exhibits a myeloma like phenotype and shows improved fusion rates over its parents. By fusing lymphoblastoid lines with murine myelomas several useful partner lines have been produced (Teng et al.,1983, 1985; Foung et al.,1985).

Fusion of human lymphocytes directly with murine myelomas has been successfully attempted by several groups (Wunderlich et al.,1981; Astaldi et al.,1982; Cote et al.,1984; Haspel et al.,1985; Hirohashi et al.,1986; Thompson et al.,1986). It has been known for many years that heterohybrids preferentially eject human chromosomes and indeed this fact has been used to assign genes to

particular chromosomes (Croce et al.,1980; Erikson et al.,1981). It was therefore naturally assumed that mouse/human hybrids would expel human chromosomes. Despite this it would appear that loss of antibody secretion due to genetic instability is no worse in heterohybrids than that encountered in murine hybrids and that these lines can be stabilised by early cloning (Thompson et al.,1986).

The fusion rates achieved using mouse myelomas as fusion partners are much higher than those achieved with human partners in comparative experiments (Cote et al.,1983, 1984, 1986). By using a fusion partner which is itself a mouse/human hybrid the expulsion of human chromosomes can be minimised (Kozbor et al.,1984). It has been shown that hybrids formed when a heteromyeloma is the fusion partner are more stable than when a murine myeloma is used (Teng et al.,1985).

Few comparative experiments have been performed which use more than two available partner lines, however, Abrams et al (1983) suggest that LINC-LON Hmy2, UC 7296 and HF2 are worth further investigation, although this study used only seven of the available partner lines. More recently there have been preliminary reports of a detailed study with a variety of cell lines including mouse myelomas, heteromyelomas etc. (Usagawa et al.,1986). Among the various lines tested KR12, SHM-D33, 3HLA-6 and 3HL3-27 (all heteromyelomas) proved the best. It is obvious that many more time consuming studies are

required before any hard and fast conclusions on the best fusion partners can be reached.

3.Fusion proceedures

Polyethelyne glycol remains the major promoter of cell fusion although the actual technique used varies from one group to another. There are many factors which affect the formation of hybrids by PEG but due to the wide variations encountered in fusions even when carried out by the same experimenter, very few comparative experiments have been undertaken (Kadish and Wenc, 1983; Lane et al.,1984; Dorfman, 1985; Westerwoudt, 1985). The PEG itself plays a major role in determining the success of hybrid formation and any new batch of PEG should be tested in fusions before it is routinely used. The studies recommend PEGs of different molecular weight and source, however, it would appear that if a PEG is performing satisfactorily in fusion experiments then there is no reason to change.

The pH of the PEG solution is important in the formation of hybrids (Lane et al.,1984) and should be optimised for the particular solution being used. The method of sterilising the PEG has also been reported to affect hybridoma formation (Kadish and Wenc, 1983). Filter sterilisation of the PEG solution is suggested to produce larger percentages of hybrids than autoclaving. Time of cell incubation in the PEG is also crucial (Davidson and Gerald, 1976) and again an optimum should be sought.

The entire procedure of fusing cells with PEG solution

is inherently inefficient and dependent to a large extent on chance contacts between appropriate cells. More efficient mechanisms of cell fusion are naturally being sought and these will be discussed later in the text.

1.5 VIRAL TRANSFORMATION

The second method of immortalisation, which chronologically was the first to be used on human lymphocytes (Steinitz et al., 1977), is Epstein-Barr virus induced transformation. The virus used in this technique is Epstein-Barr virus as described by (Epstein and Barr, 1964). It is a herpes virus which preferentially infects the B lymphocytes of humans and some other primates. The normal source of the virus for experimental laboratory work is the culture supernatant of the marmoset cell line B95-8 (Millar and Lipman, 1973). Although the virus has been detected in the cells of patients with Burkitts lymphoma and is known to be the causative agent in infectious mononucleosis these are not used as routine sources of virus. The viral receptor on B cells has been shown to be the C3d (CR2) complement receptor which is a 140KDa glycoprotein (Frade et al.,1985).

There is at present some debate as to which cells become infected and which are transformed, with several different theories being given. It would seem that EBV can bind to and penetrate all B cells which posses the appropriate receptor yet only a small proportion of these become transformed. Binding of heat killed EBV induces

cells to become activated as does antibody directed against the C3 receptor, suggesting that activation and transformation are related but separate events (Aman et al., 1985). Aman et al (1985) suggest that the responsive population is a small high density resting population, while Chan et al (1986) claim that the susceptible population is an activated large cell population.

The activated population are mainly $sIgM^+$ and $sIgD^+$ and are destined to secrete IgM. The small resting population on the other hand gives rise to IgG and IgA secreting populations, the precursors of these being $sIgG^+$ or $sIgA^+$ and $sIgD^-$.

It is also interesting to note at this point that Chan et al (1986) suggest that the susceptibility to transformation may be more dependent on entry into the cell cycle than the density of viral receptors. This suggestion by Chan et al would explain the the bias found by many workers towards IgM secretion while still allowing for IgG and IgA secretion by such lines.

A recent report by Roome and Reading (1987) however tends to support the theory of Aman et al. By fractionating B lymphocytes on percoll and incubating the fractions with EBV they showed that higher rates of transformation were produced in the small resting population. Further experiments on the fractions showed that if the resting population was activated by mitogens or anti-immunoglobulin then the susceptibility to viral transformation declined. Which ever theory is true the

transformation of B lymphocytes can be used to produce antibody secreting cell lines using lymphocytes from most lymphoid tissues. If the work of Roome and Reading is substantiated then peripheral blood would seem an ideal source of lymphocytes as the majority of circulating B cell are in a resting state. This in turn would suggest that EBV could become the immortalisation method of choice because of the ease with which peripheral blood can be obtained.

As mentioned above EBV not only transforms B lymphocytes but also acts as a polyclonal activator, giving transient activation without subsequent transformation. This phenomenon can be a major problem when attempting to produce human monoclonal antibodies. Moreover it appears that there are other problems with transformed lines, particularly relating to the stability of antibody secretion. It is not uncommon for cell lines to grow well secreting specific antibody for 1-2 months before their antibody titre suddenly declines. This fall in titre which is often accompanied by a phenotypic change, may be due at least in part to a defect in the regulatory, secretory or genetic functions of the cell. These possibilities will be discussed at a later stage.

Cloning of any cell line is important if monoclonality is to be established, with EBV transformed lines it is absolutely critical. The fact that some cells stop secreting antibody implies that there may be an overgrowth of secreting cells by non-secretors. Cloning

cell lines at an early stage is the only way to avoid this problem. Although early cloning has in some cases rescued antibody secreting lines (Crawford et al.,1983; Crawford and Callard, 1983; Doyle et al.,1985) many potentially useful lines have been lost due to the extremely poor cloning efficiencies of lymphoblastoid lines (Evans et al.,1984). Attempts to improve cell growth at low densities have not proved successful despite using many feeder cells and supernatants. Tiebout et al (1987) say that the poor cloning is an inherent feature of these cell lines.

It should be noted that viral transformations have occasionally occurred "accidentally" while fusion with an EBV^+ partner cell was being attempted (Boyd et al.,1984; Stricker et al.,1985). These cell driven transformations appear uncommon but do give rise to stable lines despite the fact that the mechanisms underlying this phenomenon have never been investigated.

1.6 LYMPHOCYTE TRANSFORMATION FOLLOWED BY BACK FUSION

Kozbor et al (1982) successfully combined the techniques of fusion and transformation to produce a hybridoma secreting anti-tetanus toxoid antibody. Since then the method has been widely adopted being used to produce antibodies against bacterial (Kozbor et al.,1984; Larrick et al.,1985), viral (Emanuel et al.,1984; Van Meel et al.,1985), red cell (Foung et al.,1986; Thompson et al.,1986) and tumour antigens (Cole et al.,1984; Campbell et al.,1986) among many others (see table 2). By combining the two techniques it was hoped to profit from

TABLE NO: 2. HUMAN MONOCLONAL ANTIBODIES PRODUCED BY EPSTEIN-BARR VIRUS TRANSFORMATION OF HUMAN LYMPHOCYTES FOLLOWED BY FUSION WITH IMMORTAL PARTNER LINES

ANTIGEN	LYMPHOID TISSUE	FUSION PARTNER SPECIES	FUSION PARTNER LINE	IMMUNOGLOBULIN ISOTYPE SECRETED	STABILITY MONTHS	REFERENCE
Tetanus toxoid	PBL	Hu	KR4	M(K)	> 7	Kozbor et al. 1982
Tetanus toxoid	PBL	Hu	KR12	M	>10	Kozbor et al. 1984b
Pseudomonas aeruginosa	SP	Hu	LTR228	M	—	Siadak and Lostrom 1985
Pseudomonas aeruginosa	SP	Hu/Mo	F386	M	—	Siadak and Lostrom 1985
Exotoxin	PBL	Mo	SP2/0	M	—	Larrick et al. 1985
Mycobacterium leprae	PBL	Hu	KR4	M	>12	Atlaw et al. 1985
Gram negative	SP	Hu/Mo	SHM-D33	M		Teng et al. 1985a
Cytomegalovirus	PBL	Hu	W1-L2	G (λ)	—	Emanuel et al. 1984
Rubella Rabies Hepatitis B	PBL (human + chimp)	Hu	Org MHHI	M + G	2.5-6	Van Meel et al. 1985
Rhesus D	PBL	Hu/Mo	SHM-D33	G3(λ)	> 8	Bron et al. 1984
Rhesus D	PBL	Mo	x63-Ag 8.653	M + G3	>14	Thompson et al. 1986
Rhesus G	PBL	Hu/Mo	SBC-H20	G1	—	Foung et al. 1985
Blood group A	PBL	Hu/Mo	SBC-H20		—	Foung et al. 1985a
Blood group A	PBL	Mo	SP2/0	M	—	Larrick et al. 1985
HLA	PBL	Hu	GM1500-6TG-08	M	—	Hulette et al. 1985
Lung carcinoma	PBL, LN, ITL	Hu	KR4	M	>5	Cole et al. 1984
Breast carcinoma	PBL	Hu	KR4		>4	Campbell et al. 1986
Prostatic Acid Phosphatase	SP	Hu/Mo	SHM-D33	M+G	>6	Yamaura et al. 1985
		Mo	SP2/0			
Rheumatoid factor	PBL	Hu	KR4	M, G+A	—	Haskard et al. 1984

Footnotes for Table No. (transformation + fusion).

PBL = peripheral blood lymphocytes
SP = splenic lymphocytes
TON = tonsular lymphocytes
LN = lymph node lymphocytes
ITL = intra-tumoral lymphocytes
Hu = human cell lines
Mo = mouse cell lines

the advantages of the two methods while avoiding some of the problems and to a large extent this has succeeded.

The major advantage of this technique becomes apparent when peripheral blood is the lymphocyte source. When PBL are fused directly hybrids are formed with a frequency of around 20×10^{-7}, however fusion with EBV transformed cells increases this to 100×10^{-7} (Kozbor and Roder, 1984). It has been suggested that the reason why peripheral blood fails to fuse well is that the cells are not actively dividing and therefore their nuclei do not fuse with that of the myeloma partner during mitosis (Burnett et al.,1985). Since EBV acts as a polyclonal activator of B lymphocytes, it allows lymphocytes to fuse more efficiently resulting in a higher yield of hybrids.

Hybrids produced by this method have exactly the same instability problems as those formed by direct fusion, but these can be overcome by early and repeated cloning. Hybrids undoubtedly clone more easily than EBV transformed cells and so lines which may have been lost can potentially be rescued. Antibody secretion by hybrids is generally greater than transformed cells and lines have been reported which are stable for greater than 14 months (Thompson et al.,1986). The antibodies secreted are representative of those being secreted by the transformed lines at the time of fusion and as a result IgM is the most common isotype. IgG's are not uncommon however especially IgG_1, so that potentially useful antibodies could be produced by this method.

1.7 POTENTIAL USES OF HUMAN MONOCLONAL ANTIBODIES

Despite the many problems associated with the production of human monoclonal antibodies, many workers remain optimistic and suggest many potential uses for the reagents they are hoping to produce (see table 3). It is hardly surprising that the main thrust of research has been aimed at producing antibodies which could be used as prophylactic and therapeutic reagents. Infectious diseases and cancers have recieved the lions share of attention in this area. Administration of protective antibodies is extremely important where the organisms reponsible are gradually developing resistance to the drugs currently in use against them. Obvious examples here are the gram negative bacteremias (Sasawada et al.,1985; Teng et al.,1985) and Malaria falciparum (Schmidt-Ullrich et al.,1986)

By producing antibodies which are protective against infection in vivo the potential exists to use these to isolate antigen (Schmidt -Ullrich et al.,1986) and hence develop protective vaccines from either a recombinant or a natural source. The monoclonals themselves may even prove useful as anti-idiotype vaccines (Atlaw et al.,1985; Matsushita et al.,1986; Schmidt-Ullrich et al.,1986). More recently there has been interest in using monoclonals to target drugs to microrganisms.

Many of the above applications also apply to tumour specific antibodies, especialy as regards to drug targeting either drug conjugates (Glassy et al.,1983; Sikora et al.,1983; Cole et al.,1984; Haspel et al.,1985;

TABLE NO: 3. PROPOSED USES OF HUMAN MONOCLONAL ANTIBODIES

Example	Reference
A - DIAGNOSTIC AND MONITORING:	
1. Viral infections e.g. cytomegalovirus, HTLV-1	Emanuel et al. 1986
2. Other infections e.g. chlamydia	Rosen et al. 1983
3. Malignancies e.g. leukaemia, colorectal carcinoma, melanoma, etc.	Glassy et al. 1983; Cole et al. 1984 Haspel et al. 1985a,b; Matsushita et 1985 ; Andreasen et al. 1986; Olsson et al. 1984; Kan-Mitchell et al. 1986; Smith et al. 1987; Strelkauskas 1987a
4. Red cell typing e.g. Rhesus incompatibility	Crawford et al. 1983a; Bron et al. 1984
5. Tissue typing e.g. HLA antigens	Steinitz et al. 1977; Hulette et al. 1985; Effros et al. 1986
6. Immunopathology e.g. antibodies to pathological and normal cell components	Haspel et al. 1985a,b; Imam et al. 1986; Simpson et al. 1986; Kan Mitchell et al. 1986
B. PROPHYLACTIC AND THERAPEUTIC:	
1. General comment on value as prophylactic and therapeutic	Gigliotti and Insel 1982; Glassy et al. 1983; Ichimori et al. 1985; Koizumi et al. 1986; Larrick and Bourla 1986
2. Treatment of infectious diseases:	
a) identifying immunogenic epitopes for use in vaccines	Atlaw et al. 1985; Schmidt-Ullrich et al. 1986; Matsushita et al. 1986
b) purifying immunogenic peptides for use in vaccines	Schmidt-Ullrich et al. 1986
c) investigating regions of genome coding immunogenic peptides	Schmidt-Ullrich et al. 1986
d) as anti-idiotype vaccines	Atlaw et al. 1985; Schmidt Ullrich et al. 1986; Matsushita et al. 1986
e) passive therapy of infections e.g. Gm-ve CMV,HTLV-1,haemophilus influenzae	Hunter et al. 1982; Emanuel et al. 1984
f) passives enhancement of response	Atlaw et al. 1985

Table No. 3 (Cont'd.)

Example	Reference
3. Treatment of malignancies:	
a) identifying immunogenic peptides for vaccines	Kan-Mitchell et al. 1986
b) as anti-idiotype vaccines	Gaffar et al. 1986a
c) as therapeutic agents (either alone or drug conjugates)	Glassy et al. 1983; Sikora et al. 1983; Cole et al. 1984; Haspel et al. 1985a,b; Matsushita et al. 1985; Andreasen et al. 1987; Borup-Christensen et al. 1986; Sikora et al. 11987; Smith and Teng 1987; Strelkauskas 1987a
d) imaging or localisation of tumours	Sikora 1983; Glassy et al. 1985; Borup-Christensen et al. 1986; Burnett et al. 1987; Sikora et al. 1987; Strelkauskas et al. 1987a
4. General application as immunomodulators e.g. antibodies to suppressor cells, interleukins, anti-idiotypes	Dorfman et al. 1985 Sasaki et al. 1984
5. Therapy of autoimmune diseases	Croce et al. 1980; Sasaki et al. 1984; Chiorazzi 1986
6. Management of transplant patients e.g. antibodies to histocompatibility antigens	Hulette et al. 1985; Effros et al. 1986
7. Prevention of Rhesus incompatibility	Crawford et al. 1983a; Bron et al. 1984
8. In contraception:	
a) as contraceptive per se	
b) in identifying immunogenic peptides for use in contraceptive vaccines	Kyurkchiev et al. 1986
C. INVESTIGATING THE IMMUNE SYSTEM IN HEALTH AND DISEASE:	
1. Studying the normal B cell repertoire in health and disease	Winger et al. 1983; Glassy et al. 1983; Cote et al. 1985,1986
2. Examining the specificity/ activity of B cells in micro environments e.g. tumour, synovial cavity	Winger et al. 1983; Watson et al. 1983; Glassy et al. 1983

Table No. 3 (Cont'd.)

Example	Reference
3. Analysing the relevance of humoral immunity in situations of clinical importance:	
a) infection	Matsushita et al. 1986: Redmond et al. 1986
b) malignancy	Paulie et al. 1984; Cote et al. 1986; Kan-Mitchell et al. 1986; Andreasen et al. 1986; Glassy et al. 1985,1987; Watson et al. 1983; Sikora et al. 1982; Smith and Teng 1987
c) autoimmunity and tolerance	Eisenbarth et al. 1982; Shoenfield et al. 1982; Glassy et al. 1983; Satoh et al. 1983; Sasaki et al. 1984; Chiorazzi 1986
d) allergic disorders	Chiorazzi 1986
4. Characterising and isolating antigens of clinical relevance:	
a) antigens for use in vaccines - see above	
b) tumour antigens	Smith and Teng 1987
c) autoantigens	Satoh et al. 1983
d) red cell antigens	Bron et al. 1984
5. Studying the molecular biology of the immunoglobulin locus	Carrol et al. 1986

Matsushita et al.,1986; Borup-Christensen et al.,1986; Andreasen and Ollson, 1987; Sikora et al.,1987; Smith and Teng, 1987; Strelkauskas 1987) or direct imaging of tumours (Sikora, 1983; Glassy et al.,1985; Borup-Christensen et al.,1986; Burnett et al.,1987; Sikora et al.,1987; Strelkauskas et al.,1987).

Another exremely important therapeutic application is the use of monoclonal antibodies as modulators of the immune reponse (Sasaki et al.,1984). Of particular interest in this context are antibodies against human lymphocyte subsets, antigens of the major histocompatability complex and any soluble mediators of the immune reponse such as the interleukins which might be used to reverse graft rejection (Hulette et al.,1985; Effros et al.,1986) or treat autoimmune diseases (Croce et al.,1980; Sasaki et al.,1984; Chiorazzi, 1986).

While the main interest in research so far has been to produce potential therapeutic reagents perhaps the largest benefits will arise from the information gained about the immune system itself. Our understanding of the B cell repertoire in health and disease has been advanced considerably by immortalising human B cells (Glassy et al.,1983, 1987; Winger et al.,1983; Cote and Houghton, 1985; Cote et al.,1986). While the investigations carried out into the growth requirements and control events on B cells has led to detailed knowledge of the humoral immune response and provided further insight into autoimmunity and tolerance (Eisenbarth et al.,1982;

Shoenfield et al.,1982; Glassy et al.,1983; Satoh et al.,1983; Sasaki et al.,1984; Chiorazzi et al.,1986). Although the studies performed to date have resulted in considerable advances in knowledge, it is readily apparent that further studies will both increase our under standing of the basic components of the immune system and provide valuable therapeutic reagents.

1.8 OUTLINE OF THE PROJECT

This project was commenced at a point where relatively few human monoclonal antibodies had been reported. Kozbor et al (1983) had recently published their data on back fusing EBV transformed cells with the lymphoblastoid cell line KR4. Within the Department of Surgery an anti-tetanus toxoid secreting cell line ES12 had been produced by Dr. J Boyd. This cell line had been fairly well characterised and its growth characteristics studied. It was decided to use ES12 as a model line to investigate the possibilities of fusion with several partner lines available in the laboratory.

Hybrids produced in the initial experiments were studied extensively for their growth characteristics, antibody secretion and general stability. All three of the hybrids chosen proved to be stable and showed increased immunoglobulin secretion over the parent line ES12.

The methods learnt in handling the anti-tetanus lines were applied in attempts to produce an antibody which bound Hepatitis B surface antigen (HBs). It was assumed that the production of a cell line by EBV transformation

would be a relatively simple process, however this proved not to be the case. Problems were encountered with the stability of antibody production and providing antibody secreting cells in sufficient numbers for fusion.

After many attempts using various combinations of in vitro stimulation, EBV transformation and cell fusion, two cell lines were produced which secreted specific antibody. The first (GF41/27) was derived from the fusion of an EBV transformed line with the human partner Hmy2. The second HB1 arose from a spontaneous transformation during an in vitro immunisation experiment. Both of these lines have proved difficult to clone effectively especially the transformed line. The hybrid even though it can be cloned does not produce stable clones. The parent hybrid is also unstable with antibody secretion stopping at around 12 weeks after fusion. As both of these lines appeared relatively late in the course of experimental work neither have been particularly well characterised.

The success achieved with the anti-tetanus was not repeated in the anti-Hepatitis experiments. Why this should be will be discussed in more detail within the text. The line produced most recently (HB1) has characteristics very similar to ES12 and may react in a similar manner in fusions. If this is the case then it should be possible to develop a panel of hybrids from the various fusion partners and so compare them with the anti-tetanus lines.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL LINES

During the course of this project several cell lines have been either used or produced. The lines were supplied by various laboratories as stated in table (4). The precise details of their pedigree and growth requirements can be found by referring to the papers quoted in the table. Lymphoblastoid cell lines and myelomas were used as fusion partners in experiments with either Epstein-Barr virus transformed or untreated B-lymphocytes. The marmoset cell line B95-8 (Miller and Lippman, 1973) was used as the routine source of Epstein-Barr virus for transformation of lymphocytes. Other lines were produced by fusing various partner lines with antibody secreting cells.

2.2 MEDIA

The basic complete media used in most experiments, was RPMI 1640 containing 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100IU/ml penicillin, 100ug/ml streptomycin, (Gibco Biocult, Paisley) 100 ug/ml kanamycin and 5 x 10^{-5} M 2-mercaptoethanol. Preselected foetal calf serum (Flow Labs , batch number 29015111) was added at either 10% or 20% (volume/volume). Complete media was prepared and sterility tested by adding 3ml of media to 3ml of nutrient broth and incubating for 1 week at $37^{o}C$. Media was stored at $-20^{o}C$ until required; once thawed media was stored at $4^{o}C$ and any media which had not been used after 4 weeks at $4^{o}C$ was discarded.

Selective media was prepared by adding HAT solution (Gibco Biocult) to give a final concentration of 10^{-4}M

TABLE NO: 4. CELL LINES USED IN EXPERIMENTS

Line	Cell Type	Drug Markers	Ig Secreted	Reference
NSO	Murine myeloma	8-AG	None	
KR4	Human/LCL	6-TG,OUA	IgG_2 (K)	Kozbor et al. 1982
KR12	Human LCL/Humam myeloma	6-TG,OUA	IgG_2 (K+)	Kozbor et al. 1984
Hmy2	Human LCL	8-AG,OUA	IgG_1 (K)	Edwards et al. 1982
UC7296	Human LCL	6-TG	IgM (K)	Glassy et al. 1983
B95-8	Marmoset	None	None	Miller & Lipman 1973
ES12	Human LCL	None	IgG_3 (K)	Boyd et al. 1984

TABLE NO: 4. CELL LINES PRODUCED DURING EXPERIMENTS

Line	Cell Type	Antibody Affinity
GF4/1.1	Human/mouse hybrid	Tetanus toxoid
GF5/9.13	Human/human hybrid	Tetanus toxoid
GF7/3.2	Human/human hybrid	Tetanus toxoid
GF41/27	Human/human hybrid	Hepatitis B surface AG.
HB1	Human LCL	Hepatitis B surface AG.

hypoxanthine, 10^{-5}M aminopterin, 1.6 x 10^{-5}M thymidine. When Epstein-Barr virus transformed lymphocytes were being fused, ouabain was also included at a concentration of 5 x 10^{-6}M.

2.3 SERUM FREE MEDIA

When trying to adapt cells to growth in serum free media, four different media supplements were used. The source and description of each supplement is given in table (5). Complete serum free media was prepared and sterility tested as normal complete media. Aliquots of 3ml were incubated in 3ml of nutrient broth for 1 week at 37°C. In these particular experiments media was always used within 4 weeks therefore it was not necessary to freeze media.

2.4 FREEZING MEDIA

In initial experiments, freezing medium was prepared with 50% foetal calf serum, 10% dimethylsulphoxide (DMSO) and 40% RPMI 1640. This preparation was later modified to 90% FCS + 10%DMSO as this was shown to give higher viability on recovery from cryopreservation. After preparation freezing media was sterility tested by incubating 3ml media with 3ml nutrient broth for 1 week at 37°C. The medium was stored frozen at -20°C in aliquots of 20ml until required.

2.5 FOETAL CALF SERUM

Six batches of foetal calf serum from different suppliers were tested for their ability to support the growth of human cells at cloning dilutions. The serum which gave

TABLE 5

SERUM FREE MEDIA USED IN EXPERIMENTS

Product name	Supplier	
SF-1	N.B.	Supplied as a complete liquid medium requiring only antibiotics and L-glutamine.
HB 104	NEN	Supplied as liquid medium plus liquid supplement of defined composition.
NU-SER	C.R.	Supplied as in serum like batches, reputed to have batch to batch consistancy. Added to RPMI 1640 instead of serum.
Ultroser-G	LKB	Supplied as lyophilised powder. Reconstituted with water and added to RPMI 1640 instead of serum

N.B. Northumbria Biologicals Ltd.

NEN NEN Research Products

C.R. Collaborative Research Inc.

LKB LKB Instruments Ltd.

the best growth rates (see experiments in section 3.6) was ordered and used exclusively in all experiments requiring foetal calf serum. The serum which was supplied sterile and heat inactivated was stored at -20°C until required.

2.6 CRYOPRESERVATION OF CELL LINES

Cells taken either from culture, from fresh tissue or from blood samples were prepared for freezing by washing 3 times in Dulbecco A solution. After washing, the pellet with supernatant, was placed on ice for several minutes to cool. The supernatant was decanted and the pellet loosened by tapping gently. Freezing medium was added such that the final cell concentration was 10^7 cells/ml and 0.5ml aliquots were dispensed into NUNC freezing vials. The vials were placed inside polystyrene containers in a -70°C freezer overnight before transfer to the vapour phase of a liquid nitrogen vessel.

2.7 RECOVERY FROM CRYOPRESERVATION

Vials removed from liquid nitrogen were warmed in a 37°C water bath as quickly as possible. As soon as the ice in the vial had completely melted the cell suspension was removed to a sterile universal and 10ml of prewarmed complete medium was added. Cells were allowed to stand for 5-10 min at room temperature before being centrifuged for 10 min at 1500rpm. The pellet was then resuspended in fresh, complete medium and the cells were incubated in a humidified incubator with 5% CO_2 at 37°C.

2.8 PREPARATION OF VIRUS SUPERNATANT

Epstein-Barr virus was routinely used to transform human

lymphocytes. The normal source of virus was the supernatant of the marmoset cell line B95-8 (Miller and Lippman, 1973). Cells were grown for 3-4 days to allow complete recovery from cryopreservation then seeded at 3×10^5 cells/ml in complete RPMI 1640 with 10% FCS. Cultures were incubated for 7 days before cells were removed by centrifugation for 10 min at 1500rpm. Cell debris was removed by filtering the supernatant through 0.45um filter (Gelman Sciences). Viral supernatant was aliquoted to 1ml vials and stored at -196°C in liquid nitrogen.

2.9 PREPARATION OF FEEDER SUPERNATANTS

(1). Mixed Thymocyte Medium (MTM)

Thymus from two 4-5 week old rats, one Sprague-Dawley, the other Wistar, were homogenized and the cells washed 3 times in Dulbecco A solution. Cells were seeded at a density of 5×10^6 per ml in complete RPMI 1640 with 10% FCS. Cultures were incubated for 48h, incubation for periods longer than this can result in the production of inhibitory factors in the supernatant (Dr L Micklem, personal communication). The cells were removed by centrifugation for 10 min at 1500rpm and the supernatants were filter sterilised. Aliquots of 15ml were stored frozen at -20°C until required.

(2). Human Mixed Thymocyte Medium (HMTM)

Human thymus or frozen thymus cells were kindly supplied by Dr E. Dewar, Department of Pathology, Edinburgh University. To prepare medium, thymus cells were isolated

by homogenising tissue and washing 3 times in Dulbecco A solution. Cells were incubated at 5 x 10^6 per ml in complete RPMI 1640 containing 10% FCS for 48h. Cells were removed by centrifugation for 10 min at 1500rpm and the supernatant was filter sterilised. HMTM was stored frozen at -20°C in 15ml aliquots until required.

(3). Human Endothelial Cell Supernatant (HECS).

HECS was prepared by Mr. N. Hunter at Scottish National Blood Transfusion Service Headquarters Laboratory,Forrest Road, Edinburgh. A subconfluent layer (5-7x10^6) of the endothelial cell line H40PS was fed fresh growth medium (Medium 199, 20% pooled human serum, 2.5ug/ml fungizone, 150ug/ml endothelial cell growth supplement) and incubated for 3 days at 37°C in a humidified 5% CO_2 incubator. The supernatant was filter sterilised and stored at -20°C until required.

2.10 EBV NEGATIVE LEUCOCYTES USED AS FEEDER CELLS

Leucocytes were obtained previously by Dr. J. Boyd from a leocopheresis donor at Edinburgh and Southeast Scotland Blood Transfusion Service Donor Suite, Lauriston Place Edinburgh. The cells were supplied as the leukocyte fraction from a blood separation. Cells were irradiated (4000 rads) before cryopreservation in aliquots of 10^7 cells.

2.11 ISOLATION OF MURINE PERITONEAL MACROPHAGES

Balb/c mice were killed by exposure to ether fumes and then their bodies soaked in Savlon solution for 2 min. The skin was removed around the abdomen and 3-4 ml of Dulbecco A solution injected into the peritoneal cavity;

the abdomen was then gently massaged and the fluid withdrawn, this process being repeated several times. The cell suspension obtained was placed in a siliconised glass universal and the cells were washed 3 times with Dulbecco A solution. After the washes cells were resuspended in complete RPMI 1640 + 10% FCS and incubated overnight to ensure that the cells had remained sterile. The macrophages were counted by staining with nigrosin and cells were plated with PEG treated cells at a final concentration of 10^3 cells per well.

2.12 ISOLATION OF LYMPHOCYTES FROM BLOOD

Whole blood samples (50ml) were collected fresh from informed, consenting plasmapheresis donors at Edinburgh and Southeast Scotland Blood Transfusion Service donor suit, Lauriston Place, Edinburgh. EDTA (0.03% final concentration) was added as anti-coaggulant. Blood was layered onto Ficoll (Flow Labs) and spun at 1800 rpm for 25 min. Lymphocytes were collected from the interface and washed 3 times in Ca and Mg free Hanks balanced salt solution (HBSS). Cells were resuspended in RPMI 1640 and viability was estimated by counting in nigrosin dye. cell concentration was adjusted and lymphocytes were used according to which experimental procedure was being followed.

2.13 PREPARATION OF LYMPHOCYTES FROM SPLEEN

Spleen samples were kindly supplied by Dr. E. Dewar, Department of Pathology, Edinburgh University and Edinburgh Royal Infirmary. In all three spleens were

used, 2 from patients with lymphoma the third from an HIV positive patient. Because of the extremely high numbers of lymphocytes present in the spleen only a small piece of tissue approximately 8 cm^3 was used from any single spleen. The tissue was supplied in a sterile container and always maintained under sterile conditions. Small pieces of tissue were macerated either in a glass homogeniser or by pushing through a sterile tea strainer. The cells were suspended at this point in a small volume of Hanks BSS. The homogenising was continued until approximately 10-20 ml of cell suspension was collected, any connective tissue or larger pieces of undisrupted tissue were allowed to settle out. The cell suspension obtained was layered onto Ficoll and after centrifugation for 25 min at 1800 rpm the cells were washed three times in Hanks BSS before counting. If at all possible spleen cells were used fresh as recovery from cryopreservation was extremely poor. The viability of recovered cells could be greatly improved by adding 0.03% EDTA to the freezing medium. The EDTA seemed to prevent the cells from clumping as they did in its absence, presumably as a result of fibrin formation.

2.14 AET-TREATMENT OF RED BLOOD CELLS FOR E ROSETTES

Sheep red blood cells in 50% Alsevers solution obtained from the Scottish Antibody Production Unit (SAPU), Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke, were washed three times in Dulbecco A and spun at 2000 rpm for 10 min. A solution was prepared by dissolving 402 mg of 2-aminoethylisothio-

uronium bromide hydrobromide (AET) in 10ml of distilled water and adjusting the pH to 9.0 with 4N NaOH. The solution was filter sterilised through a 0.2um filter and 4ml of AET solution was added to 1ml packed sheep red cells. After incubation for 30 min at 37°C with gentle mixing, the treated cells were washed 3 times in ice cold Dulbecco A and once in RPMI 1640 before finally being resuspended in 9ml of cold RPMI 1640 + 10% FCS.

2.15 REMOVAL OF E^+ ROSETTING LYMPHOCYTES

The lymphocyte preparation from the Ficoll spin was made up to approximately 100 x 10^6 cells in 2.5ml and 2.5ml of AET-SRBC added. Cells were incubated for 15 min at 37°C with gentle mixing every 5 min, then spun to a loose pellet (500 rpm for 5 min) and further incubated on ice for 1h. The pellet was then disrupted by shaking gently and the cells were layered onto cold Ficoll, a small sample (10-20µl) being retained for counting rosettes. After spinning for 25 min at 1800 rpm the E^- cells were removed from the interface and washed three times in Hanks BSS. The cells were then used as a B cell population.

2.16 VIRAL TRANSFORMATION

The E^- rosetting population (B cells) was washed three times in Hanks BSS. After the final wash the supernatant was decanted and the cell pellet resuspended in the residual volume by tapping the container firmly on the bench. Supernatant from B95-8 cells was added such that the concentration was approximately 10^7 cells/ml

with a minimum volume of 0.5ml of supernatant. The lymphocytes were incubated at 37°C for 1h before the suspension was topped up with RPMI 1640 and the cells spun to a pellet. Supernatant containing live virus was soaked in a Savlon solution overnight before being discarded. The cell pellet was disrupted and resuspended in RPMI 1640 containing 20% FCS. The concentration was adjusted and cells were plated at either 10^4 cells/well in 96 well, round bottomed microtitre plates (100µl/well) or 10^5 cells/well in 24 well plates (1ml/well) or 10^5 cell/ml in 25 cm^2 tissue culture flasks. Peripheral blood leucocytes obtained previously by Dr. J. Boyd from an EBV negative donor were added as feeders at a concentration of 10^4 cells per microtitre well or equivalent. Wells were fed by adding 150µl of RPMI 1640 + 20% FCS after 7 days incubation. Growth was normally visible by day 14 and the cultures were assayed for specific antibody between days 14 and 28 depending on the size of the colonies growing. Any positive wells were expanded into 1ml of RPMI 1640 + 20% FCS in 24 well plates then via 2x1ml wells to a 25 cm^2 tissue culture flask. It was important at this stage to keep the cell density fairly high, as diluting the culture with too much fresh medium tended to cause cells to stop growing. If cultures remained positive at this stage several vials of cells were cryopreserved.

2.17 CELL FUSION

The fusion technique used was based on that of Cole et al 1984. Cells to be fused were washed three times in serum

free RPMI 1640 and counted. The two cell types were then mixed together in a 1:1 ratio and spun for 10 min. The supernatant was completely removed and using a 1.0ml pipette, 0.5ml of warm (37°C) PEG solution was added over a 1 min period with the pipette tip resting on the bottom of the tube and stirring gently. After the addition of PEG, mixing was continued for 1 min then 10 ml of prewarmed RPMI 1640 was added over the next 7 min. To ensure consistent technique this was done at a set rate of 1 drop/5 sec for 2 min, 2 drops/5 sec for 2 min, 3 drops/ 5 sec for 2 min, and the remainder over a further 1 min. The suspension was then placed in a 37°C water bath for 40-60 min, after which cells were centrifuged at 1800 rpm for 10 min and washed once in RPMI 1640.

The PEG treated cells were finally resuspended in the appropriate selective media (ie. RPMI 1640 + 20% FCS + HAT for lymphocytes or RPMI 1640 + 20% FCS + HAT + OUA for transformed lymphocytes) at 10^6 cells/ml and plated into the centre wells of 96 well microtitre plates (100µl/well). MTM diluted 1 in 3 in the selective media was added to each well (100µl). In later experiments the selective media was added 24h after fusion and for some partner lines this seemed to improve the yield of hybrids.

Wells were fed 3-4 days after fusion with selective media, then fed as required every 3-5 days, the selective media being stopped after 2-3 weeks. Hybrids could be observed microscopically within 14 days and wells

were assayed when cells covered approximately 25% of the base of the well. Positive cultures were expanded via 1ml wells to 25cm^2 tissue culture flasks, and cloned as early as possible. Any positive hybrids were cryopreserved for later study.

2.18 CLONING OF CELL LINES

Hybrid cells and EBV transformed cells were cloned by limiting dilution. Cells were counted using the vital stain nigrosin, dilution prepared in RPMI 1640 +20% FCS and 100µl aliquots plated into 96 well u-bottomed plates at 1 and 10 cells per well. Occasionally if cells were extremely difficult to grow, initial densities would be raised to 25 and 50 cells per well. The cultures were then incubated at 37^{o}C in a humidified CO_2 incubator until growth was visible. When growing colonies were 1-2mm in diameter, the supernatants were assayed for specific antibody production. If no growth was visible after 4 weeks cultures were discarded.

2.19 ELISA TO MEASURE HUMAN IMMUNOGLOBULINS

Goat anti-human immunoglobulins (Sigma) were diluted to 10µg/ml protein in coating buffer (see appendix B) and 100µl of this solution was dispensed into each well of Titertek 96 well microtitre plates. The plates were incubated overnight at 4^{o}C to allow wells to become completely coated. The wells were washed 3 times using ELISA wash buffer and a Titertek plate washer. Non-specific binding was blocked by incubation for 1h at 37^{o}C with 150µl/well of a 1% Bovine serum albumin solution (PBS-BSA). Standard (Hoescht, IgM and IgG) and test

solutions (100µl/well) were added to appropriate wells and incubated at 37°C for 1h. Plates were washed 3 times as before, then 100µl of alkaline phosphatase conjugated anti-human IgG (or IgM as appropriate) was added to test wells and the plates again incubated for 1h at 37°C.

Conjugate solution was washed off and 100µl of substrate was added to each well (p-nitrophenyl phosphate, Sigma, dissolved at a concentration of 1mg/ml in substrate buffer as described in appendix B). Plates were incubated at 37°C for 30 min to allow colour development. Plates were read at 410 nm on a Titertek plate reader. The standard solutions used were prepared by diluting the neat solutions in PBS-1%BSA to concentrations of 10µg/ml in doubling dilutions to 0.3µg/ml human immunoglobulin.

2.20 ELISA TO MEASURE ANTI-TETANUS TOXOID

Wellcome Tetanus vaccine (containing 40Lf/ml) was dialysed against PBS overnight at 4°C. The toxoid was then diluted to 17 µg/ml in 0.9% NaCl and 100ul aliquots of the diluted solution were placed in the centre wells of 96 well microtitre plates (Dynatech). After incubation for 3h at 37°C then overnight at 4°C, the wells were emptied and blotted dry. Plates were sealed with clingfilm and stored at 4°C until required (plates have been shown to be stable for up to 6 months Farzad et al (1986).

Plates were removed from storage and washed 3 times with washing buffer using the Titertek plate washer.

Unbound sites in the wells were reblocked by incubation with 150µl of PBS-1%BSA for 1h at 37°C. Plates were washed 3 times before the addition of 100µl of sample or standard solution to appropriate wells; the plates were then incubated for 1h at 37°C. After washing 3 times, 100µl of alkaline phosphatase conjugated goat anti-human IgG (Sigma, diluted 1/1000 in PBS-1%BSA) was added and incubation continued for a further 1h at 37°C. Plates were again washed 3 times before the addition of 100µl of p-nitrophenyl phosphate (1mg/ml) dissolved in substrate buffer (see appendix B) to each well. After a final incubation period of 1h at 37°C plates were read at 410nm on a Titertek multiscan plate reader.

The standard anti-tetanus toxoid used in this case was a preparation obtained from Protein Fractionation Centre (Scottish National Blood Transfusion Service, Edinburgh). In the assay doubling dilutions from 1/3200 to 1/102400 were prepared in PBS-1%BSA, these being equivalent to 0.0768-0.0024 IU/ml of anti-tetanus toxoid activity.

2.21 ANTI-HEPATITIS B SURFACE ANTIGEN DETECTION

To detect anti-Hepatitis B surface antigen activity in the culture supernatant a commercial hemagglutination assay was used (Serodia-anti-HBs, Diamed Diagnostics LTD). This kit consisted of four major parts, positive control serum, diluting fluid (also used as a negative control) and two sets of lyophilised chicken red blood cells, the first antigen sensitised, the second control cells.

The assay was performed with slight variations from

the manufacturers recommendations. Briefly each vial of lyophilised red cells was reconstituted in 1500µl of diluting fluid. Sample supernatants (25µl) were placed into duplicate wells in a 96 well U-bottomed microtitre plate and 25µl of sensitised or control chiken red cells were added; positive and negative controls were included on every plate. After 1 min agitation plates were allowed to stand at room temperature for 30-60 min before agglutination was assessed.

2.22 IMMUNOFLUORESCENT STAINING OF CELLS FOR FLOWCYTOMETRY

Lymphocytes or hybrid cells were washed three times in Dulbecco A solution, counted and aliquots of 0.5-1x10^6 cells added to test tubes. Cells were pelleted by spinning for 10 min at 1800rpm, the supernatant decanted and the pellet disrupted by tapping the tube firmly. The first antibody was added to the residual volume (normally a mouse monoclonal directed against a specific lymphocyte subset) diluted 1:1 in human serum albumin and incubated for 30 min on ice. Cells were washed once in Dulbecco A then second antibody (5µl anti-mouse FITC conjugated FAB_2) was added and incubated for a further 30 min on ice. After two washes in Dulbecco A solution cells were analysed on the EPICS C cell sorter.

2.23 KARYOTYPING OF CELLS

Cells were karyotyped with the help of Dr S Szymaniec. Hybrid cells were harvested after 2-3 days incubation in RPMI 1640 + 10% FCS, washed three times and incubated in

fresh RPMI 1640 + 10% FCS containing 0.4ug/ml colchicine for 4h. Cells were spun to a pellet at 1500 rpm, 0.8% sodium citrate was added dropwise to a volume of 5-10ml, the cells being maintained at 37°C in a water bath. The the cells were spun gently again (700 rpm) and the supernatant was removed. Cold fresh fixative (3:1 methanol:acetic acid) was added dropwise to a volume of 5ml. The cells were spun again and the fixation process repeated. Fixed cells were dropped onto clean cold slides from a height of 5-10cm and allowed to dry.

2.24 STAINING OF CELLS FOR CELL CYCLE ANALYSIS

An aliqot of 10^6 cells was pelleted and washed three times in Dulbecco A solution to remove any serum. Cells were then resuspended in 1ml of 10^{-2}M Tris pH 7.0 containing 5mM $MgCL_2$ and 50 µg/ml propidium iodide. After incubation for 10 min at room temperature cells were washed again and resuspended in Dulbecco A solution and analysed on the EPICS C.

When stimulated with laser light of wavelength 488nm propidium iodide stained DNA fluoresces red, with the intensity of fluorescence directly proportional to the amount of DNA present within the cell (Larsen et al., 1986). By examining the profiles of various cell lines and comparing them to normal peripheral blood lymphocytes it is possible to determine if cells have a normal diploid karyotype or if they are carrying extra chromosomes and are therefore hybrids (Larsen et al., 1986).

In an actively dividing population cells will be

present in all stages of the cell cycle. Cells on the G_2-M boundary will appear as tetraploid and so will appear as a small secondary peak on the screen. The particular program used to analyse the data obtained uses the ratio of the two peaks to calculate the number of cells at any particular stage in the cell cycle. It is possible therefore to measure how actively dividing a cell population is at any point in time.

2.25 IMMUNOCYTOCHEMISTRY

(1). Preparation of slides

Cells which had been maintained in culture were centrifuged and washed once with Dulbecco A solution. Cells were counted and resuspended at a concentration of 5×10^5 cells/ml in Dulbecco A. The cell suspension was loaded onto a Shandon-Elliot cytospin with 200µl of cells being spun onto each slide. After spinning for 15 min at 1500rpm the slides were air dried under a hairdrier. Cytospins were fixed by immersion for 10 min in acetone and air dried again. Slides were stored wrapped in tinfoil at -20°C until required.

(2). Staining of slides

Slides were removed from -20°C and allowed to stand at room temperature for a few minutes to defrost. An indirect immunoperoxidase stain was used as follows. The cytospun cells were overlaid with normal rabbit serum diluted 1 in 4 in tris buffered saline (see appendix B) for 10 min to block non-specific binding of protein. After incubation the slides were washed in tris buffered

saline for 5 min. The cells were then overlaid with mouse anti-human immunoglobulin M or mouse anti-human immunoglobulin G (Unipath). After incubation for 30 minutes in a moist chamber, the antibody solution was washed off by submersion in 2 changes of tris buffered saline for 5 min each.

The sections were overlaid with peroxidase conjugated rabbit anti-mouse antibody (Dako) containing normal human serum diluted 1:50 in tris buffered saline. Slides were incubated for 30 minutes followed again by washing in 2 changes of tris buffered saline.

The final colour reaction was achieved by incubating the smears for 5-8 min in 0.01% H_2O_2 and 0.05% diaminobenzidine (BDH) freshly prepared in tris buffered saline. The reaction was terminated by rinsing the slides in tap water. To enhance and increase the intensity of staining, the slides were incubated in 2% $CuSO_4$ in distilled water for 4 min and washed in tap water. The smears were counter stained in Mayer's haematoxylin for 30-60 seconds and mounted in Apathy's aqueous mounting medium.

(3). Control slides

The object of these experiments was to determine if human immunoglobulin was present in any of the test cells. To ensure that the procedure was working properly, positive and negative controls were always used. Negative control slides were prepared using the murine myeloma NSO. It was assumed that this line did not secrete any human immunoglobulin. For a positive control the anti-tetanus

toxoid secreting line GF4/1.1 was chosen as this line appeared stable for antibody secretion.

CHAPTER 3

ANTI-TETANUS TOXOID ANTIBODIES

3.1 INTRODUCTION

All of the experiments carried out in this section were based on the cell line ES12 (Boyd et al.,1984). This line is an EBV transformed line produced in the Department of Surgery (Edinburgh University) by Dr J. Boyd. ES12 is somewhat unusual in that it was created by a "cell driven" transformation. During a fusion between peripheral blood from an anti-tetanus toxoid producing donor and an EBV^+ partner cell, the lymphocytes became infected with virus and were transformed.

Initially it had been assumed that the line was a hybrid as it had grown in the selective medium. Subsequent karyotyping proved the line was in fact an EBV transformed line. ES12 behaves in a manner typical of lymphoblastoid cell lines, in that it produces relatively low levels of immunoglobulin and is extremely difficult to clone. Growth at larger volumes is however relatively simple and the line appears to be quite stable, so large numbers of cells were available for experimental purposes.

ES12 seemed a good model to attempt fusions with both human and mouse partners. Large numbers of cells were readily available as was a good reliable assay. At the time of these experiments there had been very few reports on back fusions published in the literature, especially using murine myelomas as the fusion partner. It was known that human/murine hybridomas expelled human chromosomes, therefore it was assumed that the heterohybrids would be unstable. The fact that the

murine cells were much easier to handle in culture and secreted greater amounts of immunoglobulin made a human/mouse fusion a desirable experiment. As the results below indicate the experiments were very successful and have subsequently been supported by other work published since the experiments were carried out.

As well as fusing ES12 with murine cells it was successfully fused with two human partner lines KR4 and Hmy2. The reason for fusing with human lines as well as murine was to ensure that any immunoglobulin produced was processed entirely by human cells and so any glycosylation etc. would be in the correct position. It was also thought at the time that the human/mouse hybrids would be unstable.

The results described below are for fusion experiments where ES12 is the antibody producing parent. A flow chart (fig 1) shows at a glance how each of the lines described in this section was derived.

3.2 FUSION OF ES12 WITH MURINE PARTNERS

In the first experiments ES12 was fused with the murine myeloma line NSO in four separate fusion experiments. Only two of these experiments were fully cloned (GF3 and GF4) and the resulting lines studied in any detail. The other experiment GF6 was expanded through 1ml wells and the cells cryopreserved as uncloned lines for later study. Results of the NSO fusions are shown on table 6. When hybrids were screened by ELISA, very few gave no anti-tetanus activity and in fact only 17 hybrids from

TABLE 6

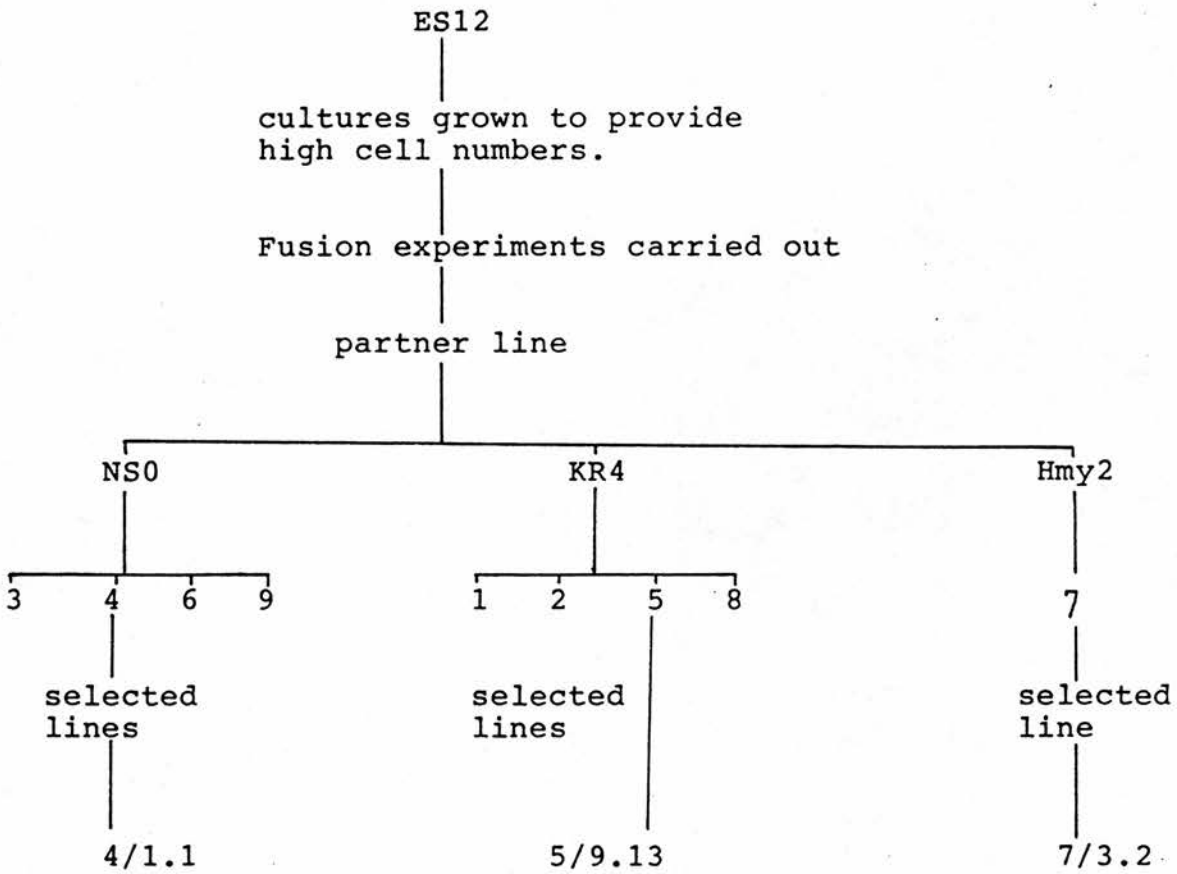
RESULTS OF FUSIONS OF ES12 WITH NS0

Fusion number	wells seeded	wells with growing hybrids	no of specific hybrids	(%)
GF3	60	60	57	(95)
GF4	75	16	10	(63)
GF6	360	360	357	(99)
GF9	360	54	7	(13)

(%) is calculated as percentage of hybrids present which secrete specific antibody

FIGURE 1

FLOW CHART SHOWING FUSIONS CARRIED OUT WITH ES12 CELL LINE



436 tested were negative. This result may at first seem to be very high, but it should be noted that the EBV transformed cells are already a relatively pure, actively dividing and antibody secreting cell population. All of these factors are thought to be an advantage when fusions are being attempted.

In experiment GF3, 57 of 60 wells seeded on a microtitre plate contained actively growing cells between 2 and 3 weeks after fusion. The supernatants from these wells were tested by ELISA for activity against tetanus toxoid and all gave positive results (ie. $OD_{405} > 0.1$, negative control was $OD_{405} < 0.01$). Three of the wells containing the highest antibody titres were cloned by limiting dilution at 10, 5 and 3 cells per well. Of the three lines cloned only GF3/8 produced clones giving positive readings in the ELISA.

The second NSO/ES12 fusion GF4 produced only 16 hybrids, 5 of which showed anti-tetanus toxoid activity. The well with the highest antibody titres GF4/1 was cloned immediately at 10, 5 and 1 cell per well while other hybrids were expanded and cryopreserved for possible future study.

Two clones GF3/8.4 and GF4/1.1 were chosen for further investigation because of their high antibody titre and good growth characteristics.

3.3 FUSION OF ES12 WITH HUMAN PARTNERS

Fusion GF5 involved hybridising ES12 with the human lymphoblastoid line KR4. It was known from previous experiments by Dr J. Boyd that the fusion efficiency of

KR4 was much lower than that achieved by NSO. A large number of wells (360) were seeded for this fusion. As expected the fusion rate proved to be much lower with fewer than 5% of the wells seeded containing growing hybrids. These hybrids also grew at a much slower rate than those formed by NSO, taking 3-4 weeks to grow large enough for testing. The hybrid giving the highest titres of anti-tetanus activity was GF5/9. This line was cloned immediately at 10, 5 and 1 cell per well. From the cloning 15 wells gave growth , 9 were positive for antibody secretion. The best line GF5/9.13 was selected for further study, others were expanded and cryopreserved.

The lymphoblastoid cell line LINC-LON-Hmy2 (Hmy2) was used as the fusion partner in experiment GF7. From 360 wells seeded at 10^5 cells/well 264 contained growing hybrids, all of which were positive for anti-tetanus when assayed by ELISA. Five hybrids were chosen for their high anti-tetanus activity and cloned by limiting dilution at 10, 5 and 1 cell per well. Three positive clones from each plate were cryopreserved and GF7/3.2 was chosen for further study.

3.4 THE EFFECT OF FEEDER CELLS ON HYBRID PRODUCTION

Balb/c peritoneal exudate cells were used as feeders in the three fusions which produced anti-tetanus secreting lines. It is not clear if the feeders made any significant difference to the number of hybrids produced. Several mechanisms have been described which would

suggest that this could be the case. It is possible that the PEC's produce a growth factor which is released into the supernatant (see section 6.4) or that cell contact is important. When the hybrids were growing in the wells it was not clear whether they normally grew beside PEC's, but this could have been the case. The only visible difference when PEC's were used as feeders as opposed to supernatants was that the wells contained much less debris. This suggests that the macrophages "cleaned up" any cells which were killed in the selective medium, so leaving growing hybrids with more room.

In other fusions where MTM has been used as the feeder, hybrids have been formed very successfully especially when the fusion partner was Hmy2 (see section 4.6). Various feeder systems have been claimed to increase hybridoma formation (table 7) but the results obtained here failed to show any increase in either hybrid growth or cloning efficiency with any of the systems tested.

3.5 CHOICE OF POLYETHYLENE GLYCOL FOR FUSION

Several different polyethelyne glycol (PEG) preparations were available as fusogens. In order to decide which PEG would give the best results they were compared for their ability to fuse ES12 and KR4. Three different PEG solutions were prepared, each at pH 7.0 and pH 9.0 and used in fusion experiments with ES12 and KR4. In each case 60 wells were plated with 10^5 cells per well and the results are shown on table 8. These results demonstrate the low fusion rates achieved with KR4 as the fusion

TABLE NO: 7. FEEDER LAYERS USED IN HUMAN MONOCLONAL ANTIBODY PRODUCTION

Feeder Used	References
Cord blood lymphocytes	Sasaki et al. 1984; Melamed et al. 1985
Peripheral blood T cells	Winger et al. 1983; Garzelli et al. 1984
Peripheral blood monocytes	Bischoff et al. 1982; Olsson et al. 1983 Melamed et al. 1985; Andreasen & Olsson 1986
Allogeneic peripheral blood leukocytes	Kozbor & Roder 1981; Astaldi et al. 1982; Irie et al. 1982; Crawford et al. 1983; Paulie et al. 1984; Tiebout et al. 1984; Ho et al. 1985; Garzelli et al. 1986; Schmidt-Ullrich et al. 1986
Lymphoblastoid cell lines	Irie et al. 1982; Doyle et al. 1985
Foreskin fibroblasts	Zurawskiet al. 1978; Emanuel 1984; Siadak & Lostrom 1985
Thymus fibroblasts	Strike et al. 1984
Fibroblasts not specified	Raubitchek et al. 1985; Van Meel et al. 1985
Embryonic lung fibroblasts	Steinitz et al. 1979
Embryonic fibroblasts not specified	Kozbor & Roder 1981; Satoh et al. 1983; Watson et al. 1983; Rosen et al. 1983
Embryonic kidney	Zurawski et al. 1978
Embryonic lung	Evans et al. 1984
Embryonic amnion	Zurawski et al. 1978
Endothelial cells	Astaldi et al. 1982
Melanoma cells	Watson et al. 1983
Mouse thymocytes	Nowinski 1980; Olsson et al. 1983; Teng et al. 1985a
Mouse spleen	Kozbor et al. 1981,1982,1984; Cole et al. 1984; Atlaw et al. 1985
Mouse peritoneal exudate	Kozbor et al. 1982; Satoh et al. 1983; Cote et al. 1983,1985; Cole et al. 1984; Gigliotti et al. 1982,1984; Haskard et al 1984; Doyle et al.1985; Melamed et al. 1985; Borup-Christensen 1986; Carroll et al. 1986; Matsuho et al. 1986; Thompson et al. 1986; Brodeur et al. 1987

TABLE NO: 8. SHOWING NUMBER OF POSITIVE HYBRIDS FORMED WHEN FUSING ES12 to KR4 USING VARIOUS PEG's UNDER VARYING CONDITIONS

PEG	Molec. Wt. of PEG	pH	Wells with Hybrids(a)	%	Positive Wells
Sigma	1500	7.0	0	0	0
Sigma	1500	9.0	3	5	1
BDH (biochem grade)	4000	7.0	5	8	3
BDH (biochem grade)	4000	9.0	1	2	1
BDH	4000	7.0	3	5	1
BDH	4000	9.0	4	7	4

(a) In each case 60 wells were seeded.

partner. The rates were never significantly increased no matter what fusion conditions were used.

The Sigma PEG gave very poor hybrid production in this experiment, although it had been used successfully in murine fusions on many previous occasions (personal communication Dr. J. Boyd). Of the two PEGs obtained from BDH, the biochemical grade when used at pH 7.0 gave the best yield of hybrids. When the number of positive hybrids was measured then the ordinary grade BDH PEG used at pH 9.0 gave the best results. This PEG was used in all subsequent fusions. It should be noted that the rate of hybrid formation achieved when Hmy2 or NSO are the fusion partners is very much higher than the rates achieved by KR4.

3.6 CHOICE OF FOETAL CALF SERUM

The foetal calf serum used for culturing cells obviously has a great influence on the condition and growth of any hybrids produced. In order to ensure continuity throughout all of the experiments described, only one batch of foetal calf serum was used throughout the entire project. To decide which foetal calf serum should be used, several batches were obtained from commercial sources and used to clone four different cell lines.

The cell lines chosen represented the four major cell types grown in the Department of Surgery, a human EBV transformed line, a fusion of the EBV line and NSO and a fusion of the EBV line to KR4 as well as a murine hybridoma line. This group covered all of the cell types

used in this study ie. an EBV transformed line, a human/human hybrid and a human/murine hybrid. The murine hybrid was included for a comparison of the relative cloning efficiencies of the cell types.

In each case the four cell lines were cloned by limiting dilution to 1 cell/well in medium containing 20% of the appropriate foetal calf serum. The results are shown on table 9, the most immediate and striking observation is the vast difference in the cloning ability between the human and mouse cells. Fusion of human cells to mouse cells greatly enhances the number of clones growing to the extent that they perform as well as murine hybrids. Overall the Flowlabs sera was by far the best with over 95% growth in GF4/1.1 and J15/123. It also achieved almost 10% growth of the human/human hybrid GF5/9.13 this being almost twice as great as the next best serum.

Growth of cells in 95% of the wells may appear high, indicating that the counting of viable cells was not as accurate as it should have been. The relative growth within a given cell line should not have been affected by this as cells were counted in serum free medium and dispensed to the various complete media from a single stock. As as result of this experiment the Flow labs serum batch number 29015111 was reserved and used in the preparation of all subsequent media.

TABLE NO: 9. NUMBER OF WELLS WITH GROWING COLONIES OF EACH CELL TYPE IN COMPLETE MEDIA MADE USING DIFFERENT FOETAL CALF SERA

FCS Source \ Cell Line	ES12 (%)	GF5/9 (%)	GF4/1.1 (%)	J15/123 (%)
Flow 29015111	0 (0)	28 (9.7)	275 (95.5)	283 (98.3)
Northumbria Biologicals S1052	3 (1)	15 (5.2)	219 (76)	250 (86.8)
Northumbria Biologicals 32414	2 (0.7)	1 (0.3)	169 (58.7)	136 (47.2)
Gibco 20Q0940R	1 (0.3)	0 (0)	163 (56.6)	100 (34.7)

288 wells of each cell type were plated in each serum.

3.7 IMPROVING THE CLONING OF CELL LINES

Various supplements have been suggested to improve the growth of human cells in vitro (Astaldi et al.,1981; Mao and France, 1984; Sugasawara et al.,1985). In an attempt to improve the cloning of human hybrids, some of these supplements were used in two separate experiments. In the first experiment cells were cloned in the presence of each supplement and the results tabulated as the number of wells containing growing colonies (table 10). This measure was thought to be subject to too much variation due to inaccurate counting and other chance factors. To obtain a more accurate and objective measure of cell growth in these supplements, the uptake of ^{3}H-thymidine was measured. In order to do this cells were seeded at a density of 10^{4} cells per well in the appropriate media and incubated for 3 days. The cultures were pulsed overnight with ^{3}H-thymidine and the radioactive uptake of each group of cells measured. The results of the experiments are tabulated (table 11); they show that none of the feeder supernatants gave a significant increase in growth as measured by either ^{3}H-thymidine uptake or cloning. The only supernatant which gave any boost in ^{3}H uptake was a control ie. the medium used to culture the human endothelial cells. Normal human serum which was a pooled AB serum supplied by the Edinburgh and Southeast of Scotland Blood Transfusion Service gave an increase in cloning in one experiment, but this could not be repeated in further experiments.

TABLE NO: 10. COMPARISON OF GROWING CLONES of GF5/9.13 IN DIFFERENT FEEDER SUPERNATANTS IN TWO SEPARATE EXPERIMENTS

Supplement[a]	Experiment 1			Experiment 2		
	Wells Seeded	% with[b] Growth	% with[c] Antibody	Wells Seeded	% with Growth	% with Antibody
R20	480	1.9	1.5	480	1.9	1.9
EC-1	444	7.2	7.0	480	2.3	2.3
HECS	468	1.3	1.3	480	1.7	1.7
MTM	456	8.1	7.9	480	2.3	2.3
HMTM	ND[d]	ND	ND	480	2.7	2.7
NHS	444	8.8	8.8	480	1.9	1.9

(a) The supplements used were :-

EC1 - 10% endothelial cell supernatant.
HEC - 10% endothelial cell supernatant.
MTM - 10% mixed thymocyte medium.
HMTM - 10% human mixed thymocyte medium.
NHS - 2% pooled normal human AB serum.

(b) Plates scored for growth after 14-16 days.

(c) Wells with growth were assayed.

(d) Not done.

TABLE NO: 11. COUNTS PER MINUTE FOR CELLS AFTER OVERNIGHT INCUBATION WITH ^{3}H-THYMIDINE CULTURED IN VARIOUS FEEDER SUPERNATANTS

	Experiment 1		Experiment 2	
	10%	50%	10%	50%
R20	ND	ND	36,172	ND
R10	ND	31,549	ND	30,637
MTM	ND	27,140	44,708	33,609
HMTM	ND	42,813	38,993	28,098
HECS	ND	1,096	762	283
HEC-cont	ND	66,086	54,176	42,961
NHS	ND	32,344	49,115	27,453

ND - Not done
MTM - Mixed thymocyte medium (rat thymus)
HMTM - Human mixed thymocyte medium
HECS - Human endothelial cell supernatant
HEC-cont - The medium used for culturing the endothelial cells
NHS - Normal human serum

From these experiments no firm conclusion are possible. It appears that the cells derive no significant advantage from any of the feeder supernatants used. It must be stressed that in these experiments only supernatants were used in order to reduce any background uptake of ^{3}H-thymidine. It is possible that using feeder cells such as irradiated peripheral blood leukocytes or PEC may have had a greater effect, but these were not tested.

3.8 GROWTH CHARACTERISTICS OF THE CELL LINES

The hybrids produced in these experiments all have different growth characteristics. GF4/1.1 which is a human/mouse hybrid, tends to grow in a manner very similar to NSO (plate 1). The cells are large, round and tend to adhere to the sides of the plastic culture vessels. The adherence is firm but cells can be removed easily by washing the cell monolayer with a 0.02% EDTA solution, stronger measures such as trypsin are not required. In static culture cells remain almost 100% viable until the cell layer becomes confluent; at this point some cells begin to detach from the plastic and grow in suspension. Cell viability declines steadily as the culture becomes more acidic (as indicated by the supernatant turning yellow).

The KR4 hybrid GF5/9.13 tends to grow in tightly packed clumps (plate 2). On microscopic examination cells of this hybrid seem to have a definite polarity, pointed at one end and rounded at the other. The clumps form with the rounded ends inwards and the points

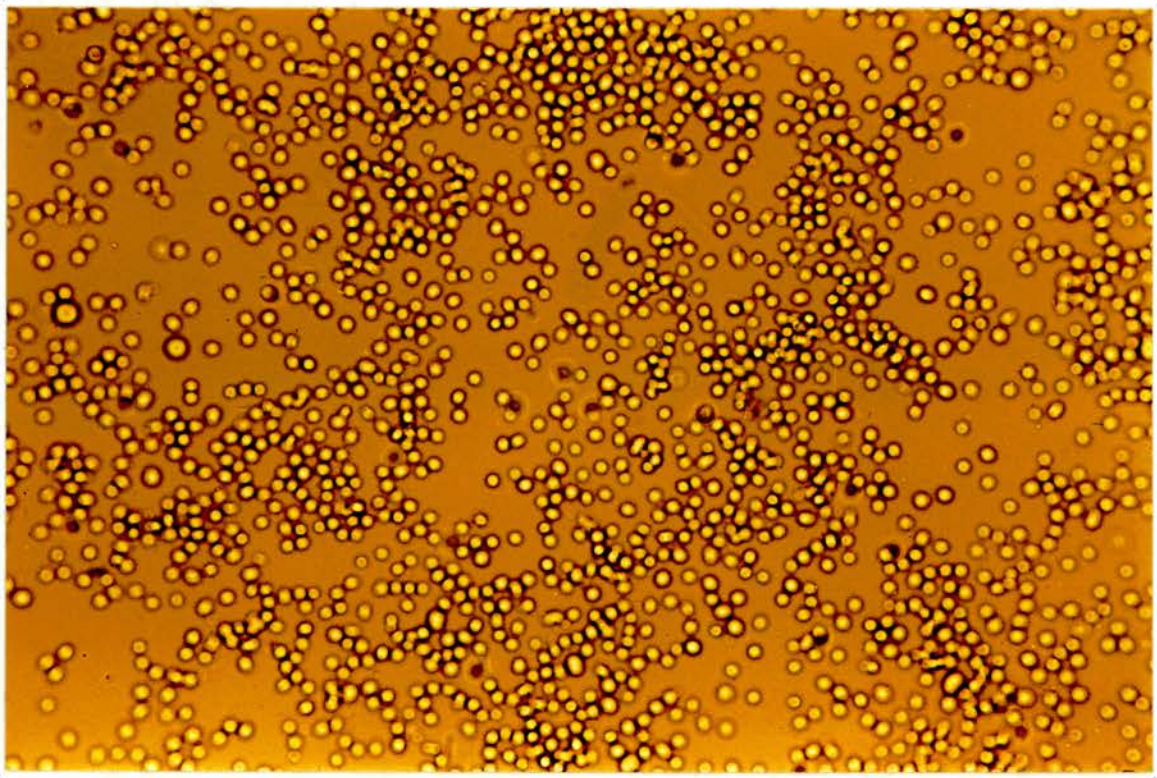


PLATE 1: Wet film preparation of GF4/1.1 cells in culture. Phase contrast microscope, note the cells are uniform in size and shape. cells in culture grow as an adherent monolayer.

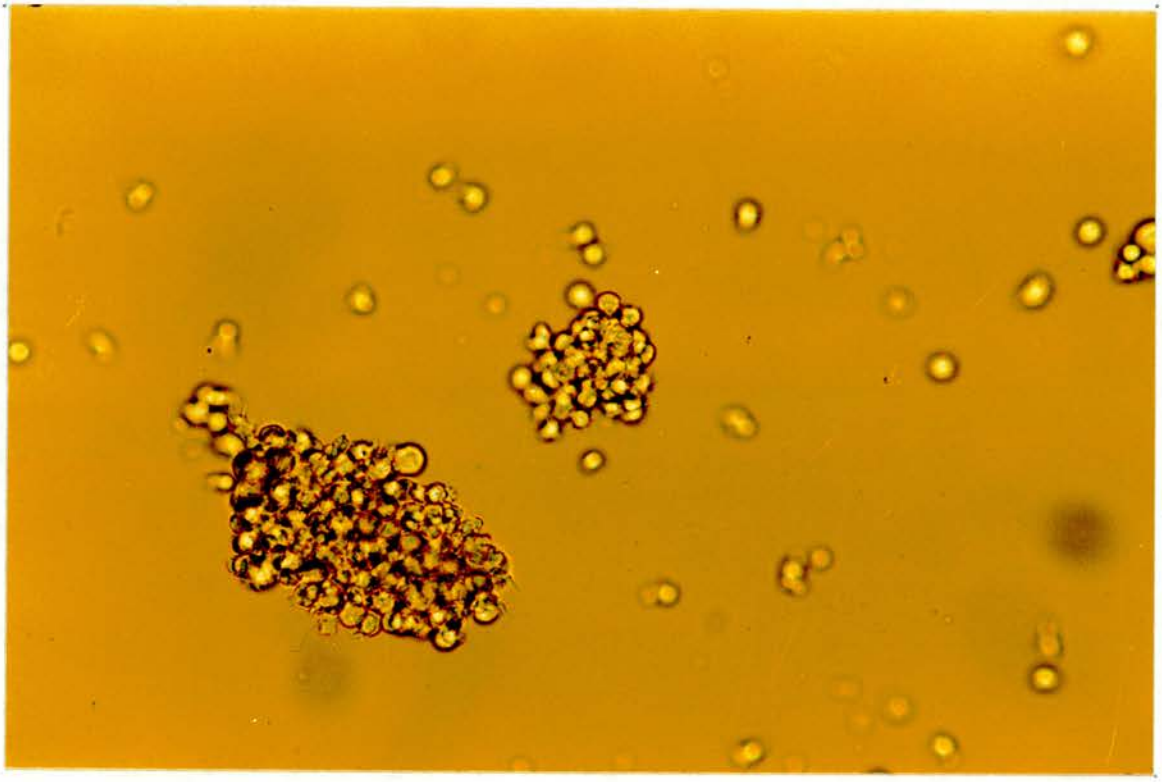


PLATE 2: Wet film preparation of GF5/9.13 in culture. Phase contrast microscopy. Note the clumps are tightly packed and the cells have "villi" like processes.

sticking out, giving the colonies a star shaped appearance with many small spikes on the surface (similar to ES12, plate4). The colonies tend to form within 24 hours of cells being seeded into fresh flasks and in order that the cells grow to a reasonable density they must be regularly disrupted. If the colonies are not disrupted they grow to around 1mm in diameter then growth stops. The viability of a culture will remain high (>90%) for several days until the pH begins to fall at which point the viability also begins to decline.

GF7/3.2 is a hybrid of ES12 and Hmy2 and grows in suspension as large, mainly single cells (plate 3). Occasionally the cells will form clumps but these are much looser aggregations than those formed by GF5/9.13. This hybrid grows to much higher densities than either of the others, because it's growth is not inhibited by a lack of surface area or by the formation of clumps. Even in populations with cell densities of greater than 10^6 cells/ml (which is greater than those found in the other hybrids) viability remains relatively high (>95%).

In order to compare the antibody secretion of the various lines, clones of each line were grown by a standard technique. GF4/1.1 and the GF7 clones were seeded at a cell density of 5×10^4 cells/ml, GF4/1.1 was seeded in 10 ml and GF7 was seeded in 20 ml. The other hybrid GF5/9 was seeded at 10^5 cells/ml as this proved to give better initial growth. The cultures were incubated until they had reached the point where the

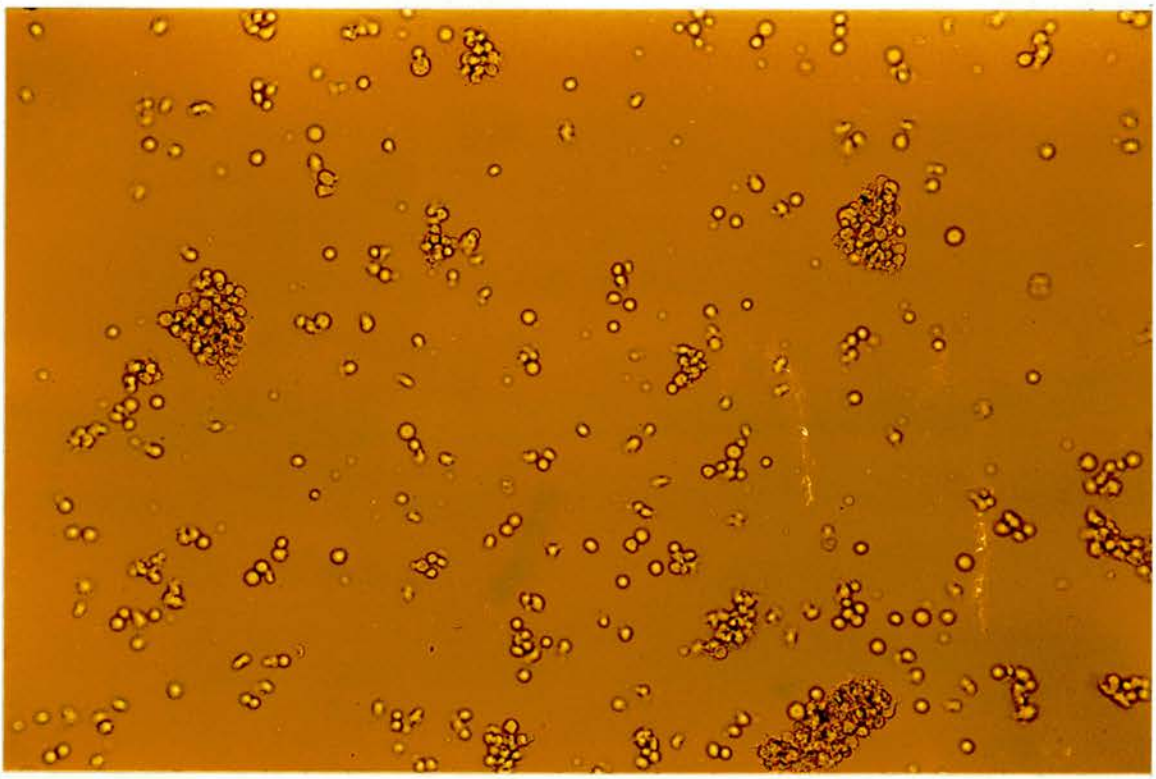


PLATE 3: Wet film preparation of GF7/3.2 cells in culture. Phase contrast microscopy. Note although the cells are growing in clumps they are not so tightly packed, also many more cells are growing free in the medium.

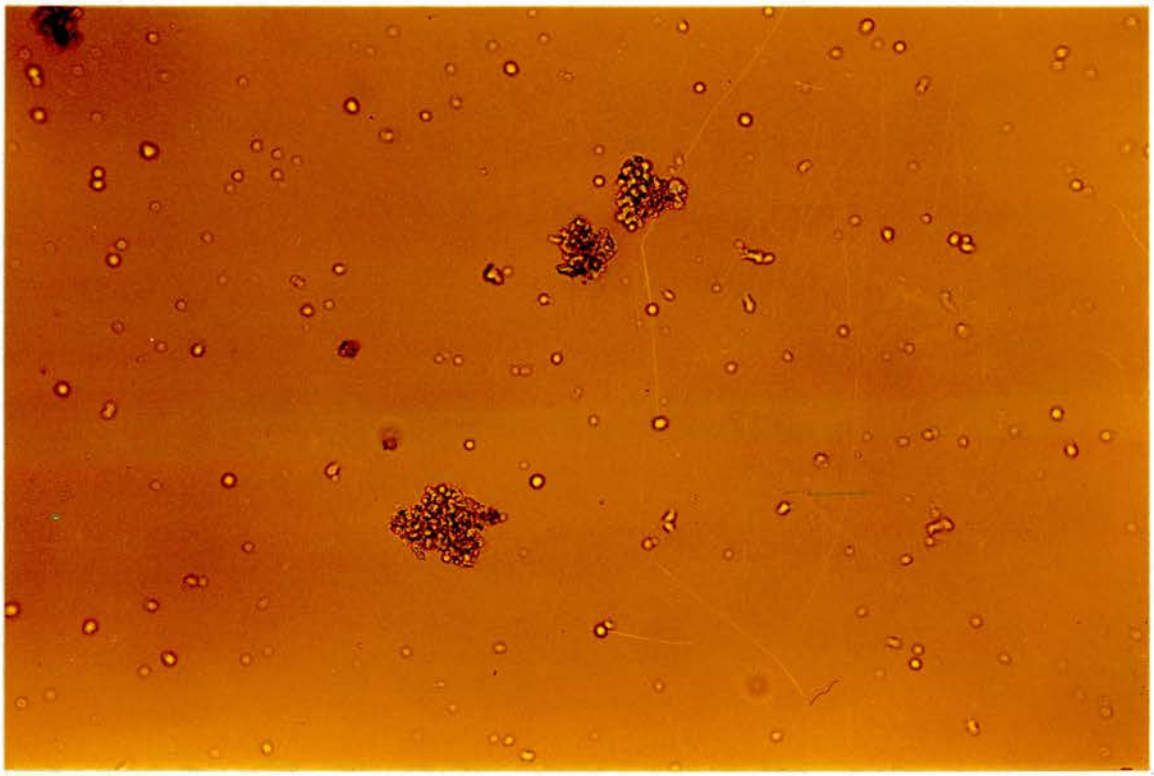


PLATE 4: Wet film preparation of ES12 cells in culture. Phase contrast microscopy. Note here the cells are again tightly clumped (the free cells here were mainly a result of disruption during preparation of the slide).

viability was about to decline. In the case of GF4/1.1 this was 3 days, for GF5/9 it was seven days and for GF7 clones it was 4 days. At the appropriate time the cultures were harvested, the cells counted and the supernatants assayed for anti-tetanus toxoid activity. The results are shown on table 12. Cultures were harvested while the viability was high so that the amount of antibody being produced per cell per day could be calculated.

The results show some interesting features of each cell line. The amount of antibody being produced per cell per day for an individual line remains fairly constant, especially within the GF4/1.1 subclones. The GF7 hybrids show more variation, but that is not surprising as they are derived from different wells in a single fusion experiment rather than being derived from a single hybrid.

As calculated by this method GF5/9.13 and the other GF5/9 clones produce the most antibody per cell. The best line produces almost four times as much antibody as the parent line ES12. The GF4/1.1 clones produce twice as much antibody as ES12 while the GF7 hybrids range from less than ES12 to almost two times as much.

Although the antibody production by GF5/9.13 is much higher than that of GF4/1.1 the growth characteristics make GF4/1.1 a much easier line to handle. Cloning is possible and relatively simple, whereas with GF5/9.13 it is extremely difficult. For these reasons most of the detailed studies and growth of cells at larger volumes

TABLE NO: 12. ANTIBODY PRODUCTION AND CELL GROWTH OF SELECTED ANTI-TETANUS TOXOID PRODUCING LINES

Line	Cells[e] Seeded	Volume Seeded	Final[e] Cell No.	Total[f] Antibody	Antibody/ Cell	No. of days Incubation	Ab/cell/day x 10^{-7}
ES12(a)	2.0	20	8.75	25.6	2.9	7	4.2
4/1.1.3(b)	0.5	10	3.25	7.8	2.4	3	8.0
4/1.1.38	0.5	10	4.35	10.8	2.5	3	8.3
4/1.1.39	0.5	10	5.70	13.6	2.4	3	8.0
4/1.1.43	0.5	10	4.18	8.8	2.1	3	7.0
5/9.8 (c)	2.0	20	2.75	21.2	7.7	7	11.0
5/9.10	2.0	20	8.00	78.0	9.7	7	13.9
5/9.11	2.0	20	4.00	34.6	8.6	7	12.3
5/9.13	2.0	20	4.75	52.4	11.0	7	15.8
5/9.14	2.0	20	7.75	12.8	1.6	7	2.4
5/9.15	2.0	20	9.63	11.2	1.2	7	1.6
7/1.14(d)	1.0	20	11.88	13.8	1.2	4	2.9
7/1.15	1.0	20	6.88	3.6	0.5	4	1.3
7/1.16	1.0	20	11.63	12.4	1.1	4	2.7
7/3.1	1.0	20	6.25	13.8	2.2	4	5.5
7/3.2	1.0	20	4.00	11.2	2.8	4	7.0
7/4.2	1.0	20	4.00	7.6	1.9	4	4.8

(a) EBV transformed line
(b) NSO-ES12 hybrids
(c) KR4-ES12 hybrids
(d) Hmy2-ES12 hybrids
(e) Cells x 10^6
(f) Antibody expressed as international units of antitetanus toxoid activity.

have been carried out with GF4/1.1.

3.9 GROWTH OF CELL LINES IN SERUM FREE MEDIA

Attempts were made to adapt ES12, GF4/1.1, GF5/9.13 and GF7/3.2 to growth in serum free medium. Four different defined media or media supplements were used (see section 2.3). The media chosen were HB104 and SF-1, both formulated media which did not require serum and UltroserG and NU-SER which were used as supplements instead of serum.

In initial experiments cells were transferred directly from their normal media to the serum free media. In all cases cells failed to adapt to the new conditions indicating that a gradual removal of serum would be required. Cells were seeded into 25 cm^2 flasks at 5 x 10^4 cells/ml in media consisting of 25% serum free media and 75% (RPMI 1640 + 10% FCS). After 2 passages in this medium (approximately one week) the cells were reseeded at 5 x 10^4 cells/ml in media consisting of a 50:50 mixture of serum free and normal media. Cells were again passed twice before increasing the percentage of serum free media to 60%. In this way cells were grown in continually decreasing percentages of serum (10% reductions from a ratio of 50:50 to 90:10). None of the cell lines would grow in completely serum free medium even after several passages at 90% serum free medium to 10% RPMI 1640 + 10% FCS (a final concentration of FCS of 1%)

The cells showed phenotypic changes as the percentage

of FCS was reduced. GF5/9.13 which normally grows as tight clumps began to grow as larger more refractile cells which although they still tended to form clumps did so in much looser aggregations. GF7/3.2 stopped forming clumps and grew as single cells spread evenly across the surface of the flask. GF4/1.1 which grows as single round adherent cells began to change so that the cells were different sizes where previously they were more uniform. Some cells were almost twice as large as others.

The doubling time of all of the lines was affected as the serum concentration was reduced. The doubling times were not actually measured but were deduced from the time taken from seeding to reach confluence; this was generally longer in cultures which contained lower concentrations of FCS. When cells were transferred to completely serum free medium they survived for several days without undergoing any appreciable increase in cell numbers, then the viability began to decline. Cells which were growing in 90% serum free media were cryopreserved and may be used in future studies on cell growth. The cells growing in 90% serum free media produced measurable amounts of anti-tetanus toxoid antibody as measured by ELISA. No comparisons were made between serum free and complete medium because the incubation time and the cell numbers varied between cultures.

3.10 GROWTH AT HIGHER VOLUMES

If human cell lines are to be of any commercial value then they must have the ability to be grown easily at higher volumes. In order to show that GF4/1.1 could adapt to growth in larger volumes several different culture techniques were employed.

Cells were grown in static culture in RPMI 1640 + 10% FCS simply by passing confluent logarithmically growing cultures into increasing volumes in larger culture flasks until a volume of 200 ml was reached in a 150 cm^2 flask. At this point the cultures were left to grow until most of the cells appeared dead on microscopic examination. Cultures were harvested and assayed for anti-tetanus toxoid activity.

GF4/1.1 cells were also grown on a roller bottle system to a final volume of 300 ml. Cells were seeded from confluent static 75 cm^2 flasks into 100 ml of RPMI 1640 + 10% FCS in a roller bottle. The roller system was housed in a 37^{o}C room with no gas control. As the cells were seeded at a relatively high density (5 x 10^4) in medium containing 20mM HEPES, the flasks did not require gassing.

The cells grew as a monolayer surrounding the inside of the roller bottle. After 7-8 days the layer began to disintegrate and on sampling most of the cells were found to be dead. The culture was harvested and the supernatant assayed for anti-tetanus toxoid activity. A static and roller culture were seeded from the same flask

and grown to death. The supernatants from each culture were then assayed for anti-tetanus activity and the results which are shown on table 13 are directly comparable.

The Celligen bioreactor (New Brunswick Scientific LTD) was available in the department and was used as a third bulk culture method. The apparatus consists of a 1l culture vessel with entry and exit ports, dissolved oxygen (dO_2), pH and temperature probes (see fig 2). The probes are connected to a control unit which adjusts pH and dO_2 by sparging the medium with a varying mixture of O_2, N_2, CO_2 and air. Gasses are passed into the medium behind a mesh which excludes cells, this prevents cell damage by bubbles and also helps to avoid foaming. The temperature is maintained by an electrically heated jacket surrounding the vessel and the medium is mixed by a constantly rotating central rotor.

The entire vessel complete with pH and dO_2 probes was sterilised by autoclaving. Medium was poured into the vessel by means of a sterile glass funnel inserted into the entry port, the entire procedure being carried out in a laminar flow hood. No sterility problems were encountered using this method of loading.

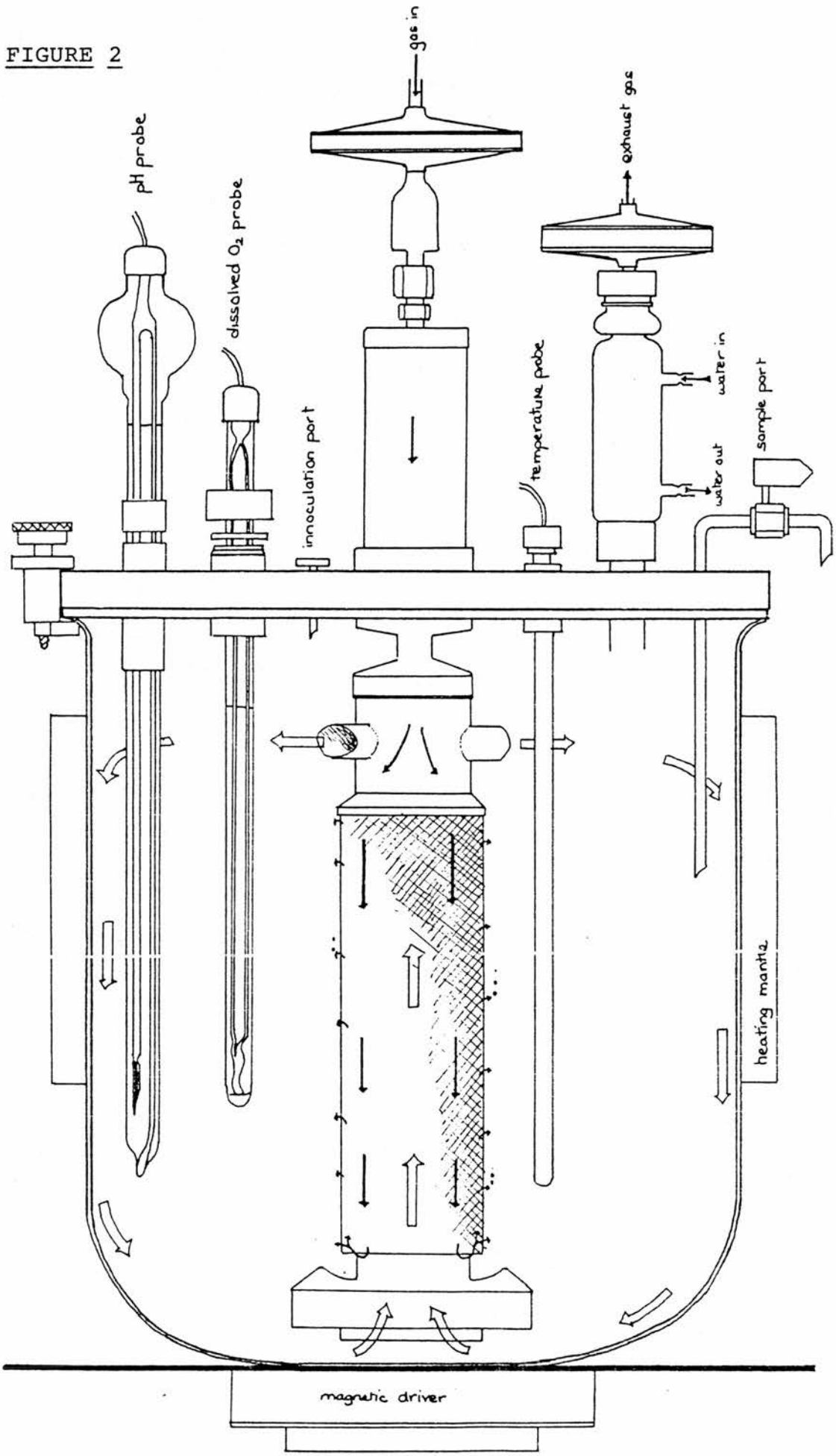
The O_2 concentration was increased until the medium was saturated, the control unit was set as 100% and the dO_2 was subsequently controlled to 30% of this level. The pH was controlled to 7.2 while the medium was stirred at 30 rpm and the temperature maintained at 37^{o}C. Once the reactor had reached equilibrium, it was left overnight to

TABLE 13

Antibody Titres In GF4/1.1 Supernatant Produced By Three Bulk culture Methods

Method of culture	Final [Ab] (IU/ml)	Total time in culture (Days)	Total volume produced (ml)
Static flask	1.84	14	200
Roller culture	1.52	8	300
Celligen Bioreactor	1.42	12	1000

FIGURE 2



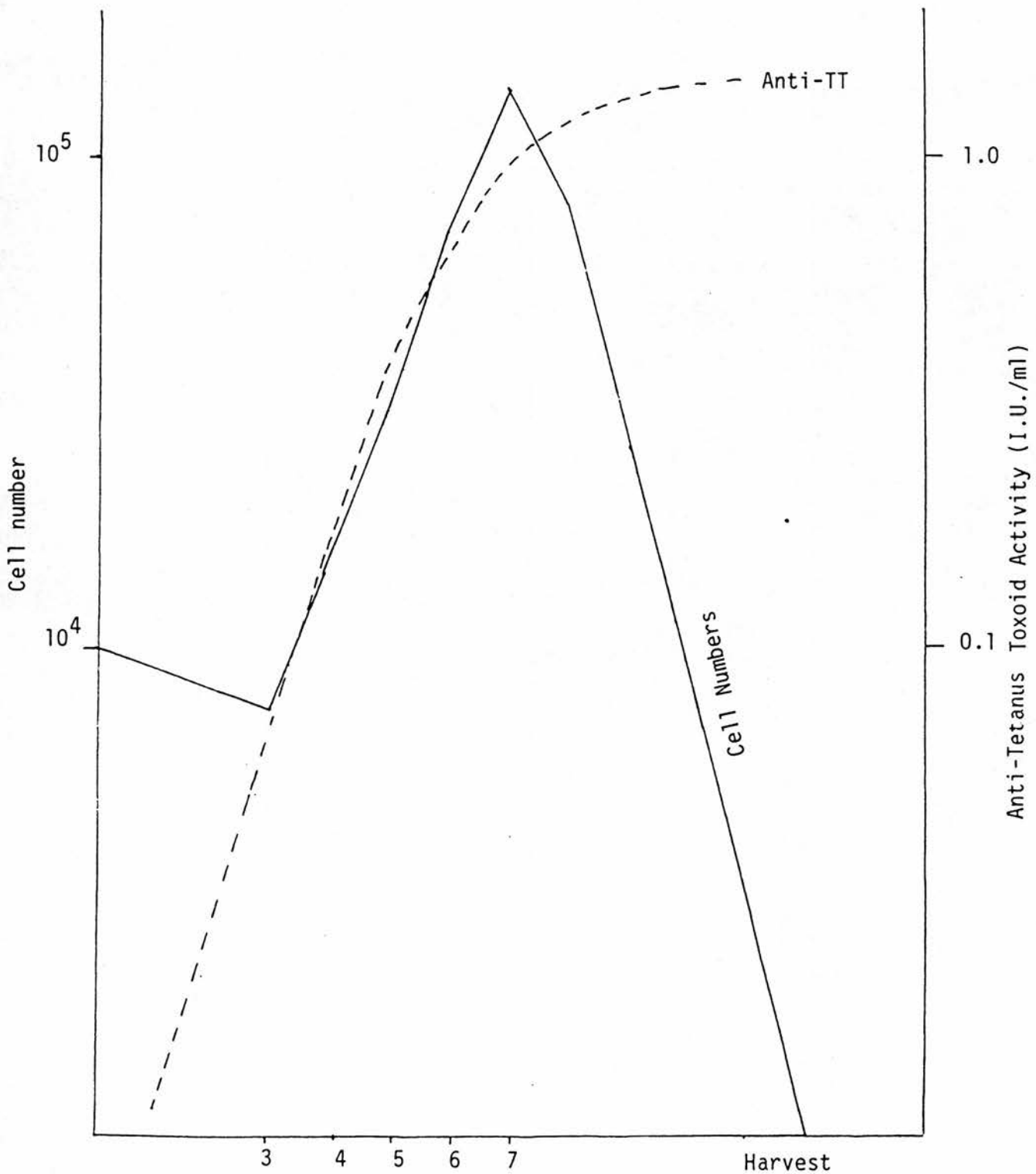
CELLIGEN BIOREACTOR

ensure that the medium was sterile. Cells were seeded at a final density of 10^4 cells/ml by injection through a septum in one of the ports. Daily samples of 5-10 ml were withdrawn and the viability and cell concentration determined. The supernatants were stored and assayed for anti-tetanus activity in one assay at the end of the run. Results of growth and antibody production are shown on graph 1.

Despite the fact that these cells normally grow as a monolayer, they adapt well to growth in a stirred reactor. The viability remained high until the cell numbers reached 1.6×10^5 cells/ml on day 7 then declined sharply. The controlled factors ie. pH, dO_2, and temperature did not alter appreciably, therefore it must be assumed that at this point some nutrient in the medium became limiting and this lead to cell death.

Assay of the daily samples shows that the antibody titre rises along with the cell numbers during the log phase of growth, then plateaus as the cell number begin to decline. It is interesting to note that antibody titres in the bioreactor did not rise as high as those in a static culture seeded at the same time from the same stock. There are several possible explanations for this observation, the most likely is that the cells were growing more quickly in the bioreactor and that the nutrient limit was reached before the antibody titre had a chance to reach those in the static flask.

Graph 1: Cell Concentration and Antibody Concentration with Time for GF4/1.1 Growing in Celligen Bioreactor



3.11 DNA ANALYSIS AND KARYOTYPING OF CELL LINES

Cultures of the cell lines ES12, GF4/1.1, GF5/9.13, GF7/3.2, NSO, KR4 and Hmy2 were grown in RPMI 1640 + 10% FCS. Aliquots containing 10^6 cells of each cell line were centrifuged in 10 ml centrifuge tubes and washed three times in Dulbecco A solution. To the cell pellet in each tube, 1 ml of 50µg/ml propidium iodide in 10^{-2}M tris pH 7.0 containing 5mM $MgCL_2$ was added. After 10 min incubation at room temperature, cells were centrifuged and resuspended in Dulbecco A solution. Analysis was carried out on an EPICS C cell sorter and the positions of the peaks on the red fluorescence noted. Propidium iodide stains DNA and causes it to fluoresce in the red wavelengths of the spectrum. Since the amount of DNA present within a cell is directly proportional to it's fluorescence, the position of the peak on the EPIC's can be used to compare the amount of DNA present in the cell line tested with that of normal peripheral blood lymphocytes.

The results not shown here indicated that ES12, Hmy2 and KR4 all have profiles similar to normal lymphocytes. The profiles show that the cells are dividing much more than lymphocytes as would be expected but appear to have the same amount of DNA ie. they are diploid. Cell lines GF5/9.13 and GF7/3.2 contain twice as much DNA as peripheral blood lymphocytes confirming that they are in fact hybridomas. The mouse line NSO contains more DNA than the human cell and the mouse human hybrid GF4/1.1

also contains more than a normal human cell. GF4/1.1 does not however contain enough DNA to accommodate a complete set of chromosomes from both the parent cells. This is consistent with the idea that some chromosomes are lost during the period immediately after fusion.

The hybrid cell lines GF4/1.1, GF5/9.13 and GF7/3.2 were all karyotyped by Dr Szymaniec in the Department of Surgery. The results showed that GF5/9.13 had 86 chromosomes, GF7/3.2 had 88 chromosomes and GF4/1.1 had 138. The human/mouse hybrid contained a mixture of human and murine chromosomes indicating that it is a hybrid. The karyotypes obtained were not clear enough for Dr Szymaniec to say exactly how many chromosomes were human and how many murine. The general impression gained was that more murine than human chromosomes were present, this would correspond with the theory that huamn chromosomes are expelled.

3.12 DISCUSSION

The results achieved in this section were extremely encouraging. The cell line ES12 originally secreted small amounts of immunoglobulin and grew quite slowly in culture. By fusing ES12 with each of the three available partner lines as described in section 3.2 the properties of the cell line were improved. The use of a murine fusion partner had previously been described (Wunderlich et al., 1981, Astaldi et al., 1982, Gigliotti et al., 1982). In all of these reports human lymphocytes were fused directly to murine myelomas without prior viral

stimulation. By adapting the method of Kozbor et al (1983) and fusing EBV transformed lymphocytes with murine myelomas, large numbers of hybrids could be easily produced.

When the human partner lines KR4 or Hmy2 were used, although fewer hybrids were produced, specific antibody secreting lines were derived extremely easily. This fact is reflected in the number of fusions described in this section. From only 9 fusions 3 high secreting lines were chosen and many others were cryopreserved as reserves. It is possible that the lines chosen were not in fact the highest secreting hybrids. With the relatively high number of positive hybrids produced, especially with the murine partners, positive lines were chosen almost at random. It was only at the cloning stage that titrations were carried out to determine which clones secreted the largest amounts of antibody.

It has been suggested that murine/human hybrids preferentially eject human chromosomes and are thus unstable (Croce et al., 1980, Erikson et al., 1981). Work carried out with the two human/murine hybrids chosen in these experiments does not confirm these findings. Since the two hybrids were chosen at random from 63 positive wells in 2 fusion experiments there seems no reason to suppose that they are not representative of the hybrid population in general.

After the initial cloning procedures GF4/1.1 was maintained in culture continuously for 6 months without any apparent decline in antibody titre. During this time

flasks were split 1-2 times per week by detaching the cell monolayer with a 0.02% EDTA solution and the cells poured off. Complete RPMI 1640 + 10% FCS was added to the residual volume and incubation at 37^{o}C resumed. Every 2-4 weeks a flask was allowed to grow until nearly all of the cells were dead. Supernatants from these flasks were assayed for anti-tetanus toxoid activity by ELISA. In the six month period there was very little variation in the titres of antibody produced indicating that GF4/1.1 is stable.

The two human hybrids produced were treated in a similar manner, except that stability studies extended for only 4-5 months. Again there was no noticable fall in antibody titres up to this point. This must imply that all of the hybrids investigated are genetically stable in long term culture.

Since the human/mouse hybrid GF4/1.1 has proved to be the easiest of the cell lines to work with, most of the studies have been carried out on this line. This fact alone suggests that heterohybrids are desirable lines to produce.

GF4/1.1 has been sent to Damon Biotech who have attempted to clone the immunoglobulin genes from this line. Briefly the approach taken was to wash GF4/1.1 cells in PBS before extraction with guanidine isothiocyanate. The RNA was pelleted through a CsCl cushion and poly(A)+ RNA was then selected on an oligo (dT)-cellulose column. The purity and integrity of the

mRNA was verified by agarose gel electrophoresis.

Poly(A)+ RNA was then used as a template for first-strand cDNA synthesis using reverse transcriptase and an oligo (dT) primer. The second strand was then copied using RNAse H and E coli DNA polymerase. By filtering over a Sephadex G-50 column the cDNA was purified and the ends were linkered. After electropheresis in a native acrylamide gel, two fractions were recovered: 850-1200bp (kappa) and 1400-2200bp (gamma). The size fractionated cDNAs were then ligated into bacteriophage lambda gt10 and these were in turn packaged with lambda proteins to form infective particles.

Bacteriophage were plated onto lawns of E coli host cells and plaque lifts were taken onto nitrocellulose paper. By hybridising specific probes (for kappa or gamma) onto the filter paper it was possible to isolate phage colonies expressing the appropriate gene. At the time of writing the positive phage are being plaque purified and readied for further analysis. Details of experimental procedures and results were kindly supplied by Dr. Reilly, Damon Biotech, Boston, USA. The techniques described above open up a whole new area for future study, this will be discussed in more detail later in the text.

In conclusion this section has shown that when starting with an EBV transformed line it is possible to produce a series of hybrids with different fusion partners. The antibody secreted by these hybrids is the same as that produced by ES12. Levels of antibody secretion measured

in an ELISA are higher in hybrids than in the parent line. Growth characteristics of these hybrids are as favourable if not better than ES12 and the hybrids are stable in long term culture. If experiments along these lines could be repeated for other antigens then human monoclonal antibody production would be a relatively easy procedure.

CHAPTER 4

ANTI-HEPATITIS B ANTIBODIES

4.1 INTRODUCTION

Following the success in producing anti-tetanus toxoid secreting cell lines, it was decided that experiments should be carried out with another antigen. The antigen chosen was Hepatitis B surface antigen (HBs). This target was chosen for several reasons; a source of highly immune donors was easily available, a convenient assay system was present, the antigen was already well defined and finally any antibodies produced would be potentially useful as a therapeutic reagent.

In the previous chapter dealing with anti-tetanus, an EBV transformed line was already present in the department. In the case of anti-HBs the first step was to attempt to produce an antibody secreting line. Initial experiments were aimed at EBV transforming peripheral blood lymphocytes of immune donors. When these failed to give satisfactory results, other methods were attempted. Eventually two anti-HBs secreting cell lines were derived. Very little biochemical analysis has been carried out on these lines because they were produced very late in the project.

4.2 DONORS

Plasmapheresis donors at Edinburgh Blood Transfusion Service, who were donating plasma for anti-HBs were used as the lymphocyte source. All of these donors had high titres of antibody as measured by the technique used by the regional transfusion centre. Donors normally donated two 450ml packs for plasma separation and between these packs samples were withdrawn for routine screening. It

was at this point that an additional 50ml of whole blood was withdrawn for lymphocyte isolation. Alternatively the first blood donation was spun at 500 rpm for 10 min and a buffy coat removed into a separate pack. Lymphocytes were isolated according to the materials and methods section 2.12.

4.3 TRANSFORMATION OF PERIPHERAL BLOOD LYMPHOCYTES

Lymphocytes isolated either from whole blood or a buffy coat preparation were passed through lymphocyte separation medium (Flow) to remove red cells and platelets followed by three washes in Hanks BSS. Rosettes were formed with AET-treated sheep red cells and non-rosetting cells were separated by a further pass through lymphocyte separation medium. The non rosetting cells were assumed to be mainly B cells (Dr. J Boyd personal communication) and were used as the B cell population in subsequent experiments.

The B cell population was incubated for 1h at 37°C with B95-8 supernatant and the cells were plated at 10^4 cells per well with irradiated EBV^- leukocytes as feeders. In general growth was visible within 14 days and supernatants were ready for testing between 21 and 28 after viral infection. Colonies were tested when they were clearly visible to the naked eye ie. approximately 1-2mm in diameter.

Cells which were positive on the first test were transferred to 1ml culture wells and fed with 0.5ml of fresh RPMI 1640 + 20% FCS. The cells were examined

daily and when cell growth had covered most of the well floor the cells were tested again. After this time cell supernatants were tested regularly every 3-4 days to ensure that cells were still secreting antibody. Cultures were split into 2 x 1 ml culture wells and subsequently into small 25cm^2 flasks. If the lines were still positive at this stage they were either cryopreserved or hybridised with one of the available fusion partners.

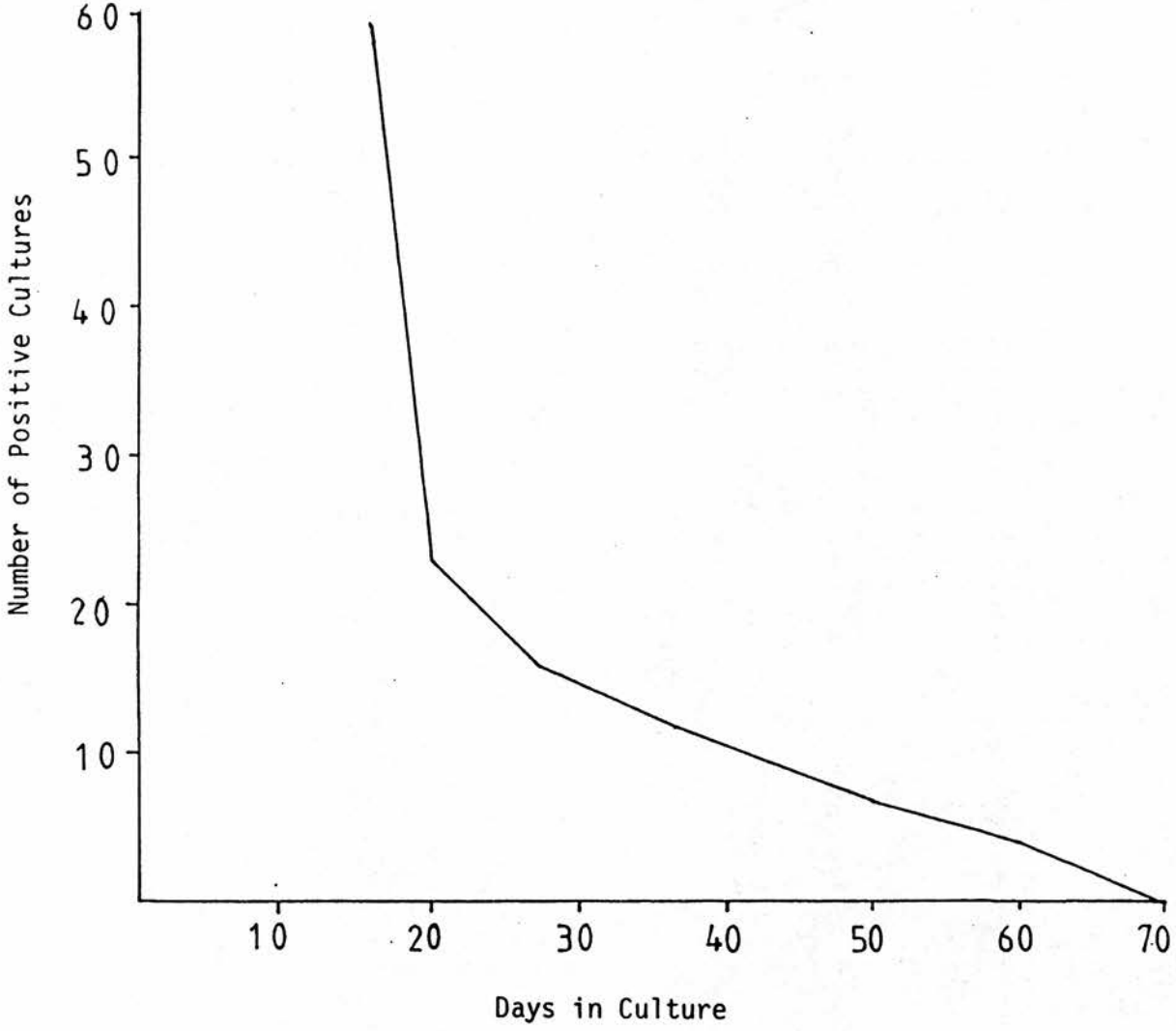
In all cases 100% of the wells seeded at 10^4 cells per well gave growth by 4 weeks. When these were assayed by a haemagglutination assay all experiments gave some positive wells, but the actual number varied considerably. Table 14 shows a summary of all of the results from transformation experiments. The number of wells which gave positive results always began to decline as soon as the cultures were expanded to 1 ml wells. When a graph is plotted of the number of positive cultures versus time as in graph 2 the fall off in antibody secretion is most noticable.

The disappearance of specific antibody from a culture was often accompanied by a phenotypic change in the cells. Lymphoblastoid cells normally have a tightly clumped appearance with the cells being irregularly shaped however when antibody secretion ceases the cells tend to become rounder and appear dissociated in culture. The fall off in antibody secreting lines usually begins around 20 days after exposure to the virus when cells are fed fresh medium. The process continues gradually until

TABLE NO: 14. TRANSFORMATION EXPERIMENTS ASSAYED FOR ANTI-HBs

Experiment	Wells Seeded	% Growth	Wells Positive %	Lines Cryopreserved
GT4	672	100	35 (5.2)	16
GF32	768	100	59 (7.6)	3
GF38	768	100	75 (9.7)	17
GF54	470	100	17 (3.6)	0
GF56	384	100	7 (1.8)	0
GF59	768	100	9 (1.1)	0

Graph 2: Number of EBV Transformed Lines Secreting Specific Antibody versus Time for Transformation GF32.



at 70 days no positive cultures remain. The lines which have ceased antibody secretion normally continue to divide and grow. Occasionally growth stops completely and cells simply remain viable in culture without dividing.

4.4 CLONING OF EBV TRANSFORMED CELL LINES

It was known from previous experiments on anti-tetanus toxoid lines that cloning EBV transformed lines was extremely difficult. However as it was obvious that by seeding wells with 10^4 cells, growth was almost certainly polyclonal, then the lines had to be cloned. Cells were cloned initially at 25 and 10 cells per well into 96 well, U-well plates each containing 200ul of RPMI 1640 + 20% FCS. To each well 10^4 EBV^- leukocytes were added as feeders. Plates were incubated at 37°C in a humidified 5% CO_2 incubator for up to 4 weeks. Growth was normally visible microscopically within 14 days and the colonies were tested when they reached 1-2mm in diameter.

Table 15 shows the results of the cloning of the 35 HBs positive lines obtained from transformation experiment GT4. As can be seen the outgrowth of colonies from both 25 and 10 cells per well was extremely poor, resulting in 11% and 4.5% respectively of the wells seeded. the positive wells were expanded into 1ml wells and assayed after 3-4 days, however at this point all of the wells had ceased antibody production and were abandoned.

In subsequent experiments similar results were obtained, with any positive clones produced being as

TABLE 15

NUMBER OF WELLS CONTAINING GROWTH FOR EBV TRANSFORMED CELLS SEEDED AT TWO DENSITIES

Cell line	wells growing at 25 cells/well	wells growing at 10 cells/well
GT4/1	6	0
GT4/2	6	2
GT4/3	3	2
GT4/4	11	1
GT4/5	5	1
GT4/6	0	0
GT4/7	0	0
GT4/8	3	0
GT4/9	2	2
GT4/10	1	1
GT4/11	15	2
GT4/12	1	2
GT4/13	3	3
GT4/14	3	0
GT4/15	11 (1+)	1
GT4/16	11	6
GT4/17	5	3
GT4/18	10 (1+)	3
GT4/19	3	1
GT4/20	12	5
GT4/21	3 (1+)	6
GT4/22	11	1
GT4/23	0	0
GT4/24	0	0
GT4/25	11	3
GT4/26	5	5
GT4/27	0	0
GT4/28	10 (1+)	3
GT4/29	6	4
GT4/30	10 (2+)	4
GT4/31	4	2
GT4/32	2	3
GT4/33	4	2
GT4/34	1	2
GT4/35	7	6

Total wells seeded	1680	
total number of clones at 25 cell/well	185	(11%)
total number of clones at 10 cell/well	76	(4.5%)
total number of positive clones	6	(0.4%)

unstable as the parent line. Clonings have also been carried out in medium containing mixed thymocyte medium (MTM). No increase in the number of clones produced has been observed using this medium instead of feeder cells.

4.5 DIRECT FUSION OF PERIPHERAL BLOOD LYMPHOCYTES TO FUSION PARTNERS.

A total of 9 fusions were carried out where peripheral blood lymphocytes (PBL) were fused directly to lymphoblastoid or murine fusion partners. Table 16 shows the results obtained from these fusions and it is immediately obvious that the rate of hybrid formation is extremely low. Although not all of the fusions can be compared directly it appears that none of the variables noted had any great effect on hybrid formation. Fusion numbers GF50 and GF51 used lymphocytes from the same donor treated in an identical fashion except that in fusion GF50 the cells were prestimulated. The AET^- population was incubated for 72h prior to fusion with 100 µg/ml of PHA and 100 ng/ml of HBs. This preincubation led to the production of hybrids in 12.2% of wells as opposed to 1.7% in GF50 where no incubation occurred. These results are highly suggestive that prestimulation of lymphocytes leads to an increase in hybrid formation. None of the hybrids produced were secreting specific antibody so that the possibility exists that an inappropriate cell population was stimulated by mitogen. It should be noted that so far no firm evidence has been published to support this theory.

By comparing fusions 51 and 53 or 63 it can be seen

TABLE NO: 16. FUSION OF PERIPHERAL BLOOD LYMPHOCYTES DIRECTLY TO PARTNER LINES

Fusion	Lymph. Pop.	Partner	Pre-stimulation	Wells Seeded	Hybs.	%
13	Whole	UC7296	-	300	0	0
21	Whole	Hmy2	-	720	9	1.3
29	Whole	UC7296	-	300	0	0
50	AET^-	NSO	-	240	4	1.7
51	AET^-	NSO	+	107	13	12.2
53	Whole	NSO	+	86	3	3.5
55	Whole	NSO	+	720	0	0
63	Whole	NSO	+	600	20	3.5
70	AET^-	UC7296	+	120	0	0
58	Whole	NSO	+	600	64	10.6

that by fractionating whole blood and using the AET^- population for fusion, again the rate of hybridisation is markedly increased. Although these fusions are not directly comparable as the lymphocytes were donated by different individuals, the results obtained in fusions 53 and 63 are sufficiently similar to suggest that the effect observed is real.

4.6 FUSION OF EBV TRANSFORMED LYMPHOCYTES

Because of the instability problems encountered with the EBV transformed lines, it was decided to attempt to hybridise antibody secreting lines with lymphoblastoid or myeloma partners. The particular fusion partner chosen were Hmy2 and NSO as these had produced satisfactory numbers of hybrids in previous experiments (see previous chapter).

As described above the EBV transformed lines secreted specific immunoglobulin only up to a maximum of 70 days after exposure to virus. Cells had to be subject to fusion before this end point was reached.

As large a number of cells as possible are required for fusion as this increases the number of hybrids formed. By increasing the total number of hybrids formed it is assumed that the chances of isolating a positive hybrid will also be increased. However, this had to be balanced against the fact that frequently antibody production stopped abruptly and therefore a compromise had to be reached. The optimum time would be where the maximum number of cells were available and the antibody

production is still at its peak.

In early experiments the EBV transformed cells were expanded from 96 well plates through 1ml wells to 10ml in a $25cm^2$ tissue culture flask. Lines still producing specific antibody at this stage were fused with one of the partner lines. This point normally came between 4 and 6 weeks after exposure to the virus, a point midway down the decay curve on graph 2. Many lines became negative before they reached this point and so could not be used in fusions.

In later experiments lines were fused when cell numbers were much lower, with the result that the total number of hybrids produced in these experiments was lower. Fusions were carried out at an earlier stage because it was reasoned that if overgrowth by non-secreting lines was responsible for the loss of antibody secretion then at an earlier stage the proportion of antibody secreting cells would be greater. The chances of obtaining a specific hybrid would be correspondingly increased. The results obtained did not lend support to this theory as very few specific hybrids were produced in any of the experiments carried out.

Positive hybrids were treated as described in materials and methods section 2.17, being expanded through 1ml wells to $25cm^2$ flasks. Instability of antibody secretion was a problem encountered in hybrids as well as with transformed cells All of the hybrids produced except one stopped secreting antibody while growing in 1ml wells. The cause of loss of antibody

secretion in hybrids is generally accepted to be genetic instability immediately after fusion. Early cloning however failed to produce any stable lines. The results from all fusions are contained in appendix C, the results of one transformation experiment and its subsequent fusions will be described in detail. The procedures described and the results obtained are generally similar to those of other transformation experiments.

4.7 RESULTS OF TRANSFORMATION GF32 AND SUBSEQUENT FUSIONS

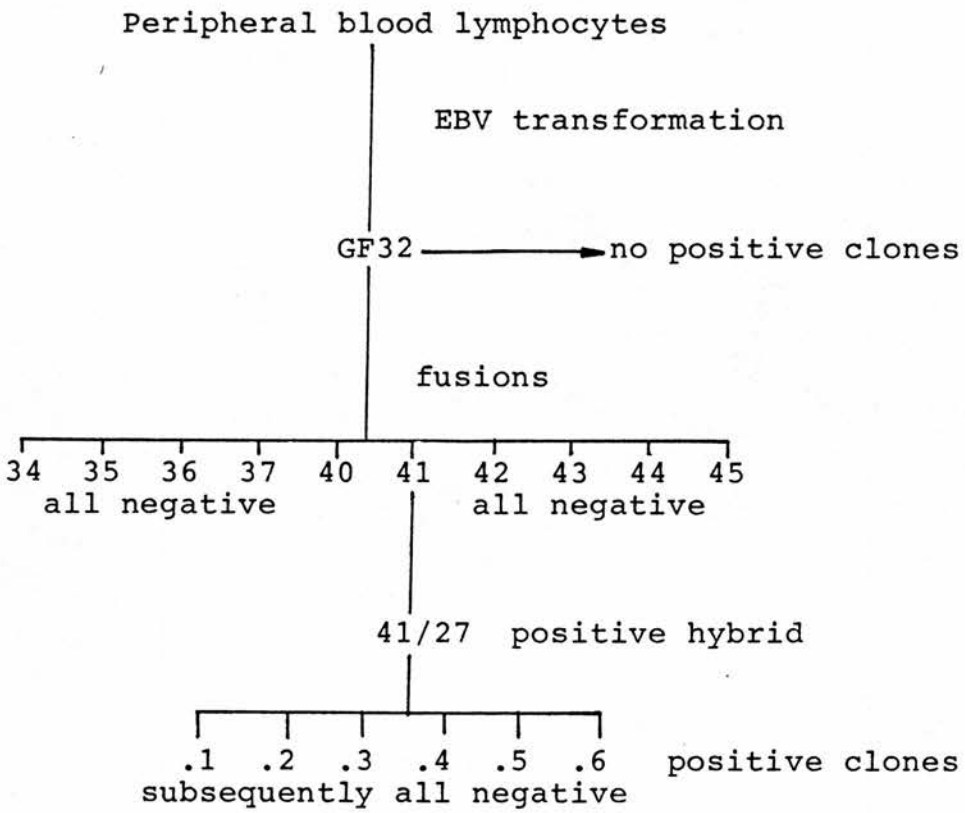
Cells were obtained from a female donor who had high levels of anti-HBs in her plasma. She had not received vaccine in the previous six months. Lymphocytes were isolated, AET^+ rosetting cells were removed and the remaining population was infected with EBV and plated at 10^4 cells/well in 200µl of RPMI 1640 + 20% FCS in 96 well plates (see fig. 3).

When growth was visible after approximately 21 days, all wells were tested and those positive for anti-HBs were transferred to 1ml wells. Cells were maintained in 1ml wells until growth was confluent, at which point they were transferred to flasks and then fused.

On initial assay 59 of 768 wells contained anti-HBs activity and were transferred to 1ml wells. Six of these lines were cloned immediately at 25 and 10 cells/well and three other lines were cloned at a later stage. No positive lines were derived from these clones. When the 1ml wells were assayed four days after transfer from the 96 well plates, 35 of the original 59 lines had stopped

FIGURE 3

FLOW DIAGRAM OF TRANSFORMATION GF32 AND SUBSEQUENT BACK FUSION



secreting specific antibody.

Lines were expended as required and fused when confluence was reached in 10ml of medium. By the time cells reached this stage only 10 lines remained positive and were subsequently fused. The results from these fusions (numbers 34-45) are shown on table 17. In these particular fusions the fusion partner was the human cell line Hmy2, however, in other experiments the murine myeloma NSO has been used quite successfully to produce hybrids (see appendix C).

From all of these fusions only one well GF41/27 assayed positive for anti-HBs by hemagglutination. This line was expanded through 1ml wells to 25 cm^2 flasks and aliquots were stored in liquid nitrogen. The line was immediately cloned at 25 and 10 cells per well, but no positive clones grew from this experiment. GF41/27 was expanded in volume up to 200ml so that sufficient supernatant to fully characterise the antibody would be available. However the titre of the antibody in the supernatant was almost negligible as the lined had ceased antibody production.

Subsequent experiments with frozen samples showed that the cells continued to secrete antibody until eight to ten weeks after fusion at which point the titre declined. After several further cloning experiments positive clones were produced at 25 and 10 cells/well. These were immediately recloned and the original clones expanded. The clone GF41/27.1-GF41/27.6 stopped secreting specific antibody at around the same time that the parent lines

TABLE NO: 17. RESULTS OF FUSIONS USING GF32 TRANSFORMED CELLS AS PARENTS

Fusion	Parents	Partner	Wells Seeded	Hybs.	%	No. Positive
34	32/6	Hmy2	60	44	73	0
35	32/18	Hmy2	60	41	68	0
36	32/51	Hmy2	60	10	16	0
37	32/4	Hmy2	60	37	61	0
40	32/1	Hmy2	24	18	75	0
41	32/16	Hmy2	35	35	100	1
42	32/45	Hmy2	40	36	90	0
43	32/28	Hmy2	43	33	77	0
44	32/36	Hmy2	20	1	5	0
45	32/43	Hmy2	43	27	82	0

from which they were cloned did. So far, the line has never been successfully stabilised as regards antibody secretion.

When assayed by ELISA the supernatant contains both IgG and IgM confirming that the line is not monoclonal. It is not known which isotype is specific for HBs, but as Hmy2 is known to secrete IgG_1 and the majority of EBV transformed lines secrete IgM the chances are high that the IgM is specific for HBs.

4.8 IN VITRO IMMUNISATION

After carrying out six transformation experiments it had become apparent that there was a very real problem with stability which was not going to be easily solved. Fusion of transformed lines had produced only one semi-stable hybrid from some 1200 hybrids tested and so a different approach was tried.

Previous experiments (section 4.5) had shown that by stimulating peripheral blood lymphocytes with mitogen prior to fusion, the number of hybrid obtained could be increased. It was decided to stimulate so that anti-HBs cells would be specifically stimulated. To accomplish this a series of experiments were designed which used various combinations of antigen, mitogen and adjuvant peptide. Lymphocytes for these experiments were isolated from buffy coat preparations (see section 2.12) obtained from informed, consenting plasmapheresis donors. In several cases donors who had been used in transformation experiments were also used in the in vitro

stimulation studies.

The mitogen chosen was pokeweed mitogen (PWM) as this was routinely used in the laboratory by other workers. PWM also has the advantage that it stimulates T cells as well as B cells , so that any essential T cell derived growth factors will also be produced in the culture. The concentration of PWM was fixed at 2.5µg/ml final strength as this had been shown to cause blast formation in other experiments (results not shown). MDP was used at a concentration of 20µg/ml as recommended by Boss (1984). Cells were plated into 1ml wells in a final volume of 500µl. Three different concentrations of antigen plus a control were used in each experiment. In all cases the final concentration of antigen was 10 ng/ml, 100 ng/ml and 1000 ng/ml.

Fig 4 shows the typical layout of an in vitro immunisation experiment. This design covered as many variables as possible while still allowing each set of results to be present in triplicate. In all six experiments were carried out and table 18 shows the combinations of antigen and mitogen used in each case.

In all of the experiments wells were tested after 3-4 days then again after 14 days. The medium was replaced as much as possible after the first test to remove any free antigen which may have been absorbing antibody from the medium. Only one well from experiment 6 gave any anti-HBs activity. The conditions in this well were 100 ng/ml of HBs with 10% pooled human AB serum as supplement. The lymphocytes had been fractionated by

TABLE NO: 18. CONDITIONS FOR IN VITRO IMMUNISATION

No.	Donor	Sex	Cell Conc./ml.	Time since[a] last boost	Cell Population	Positive Assays
1	A.C.		2×10^6	4	Whole lymph.	0
2	D.P.		2×10^6	3	Whole lymph.	0
3	E.D.		2×10^6	7	Whole lymph.	0
4	I.G.		4×10^6	4	Whole lymph.	0
5	E.L.		2×10^6	4	AET^-	0
6	M.M.		2×10^6	3	AET^-	2

TABLE NO: 18. CONDITIONS FOR IN VITRO IMMUNISATION

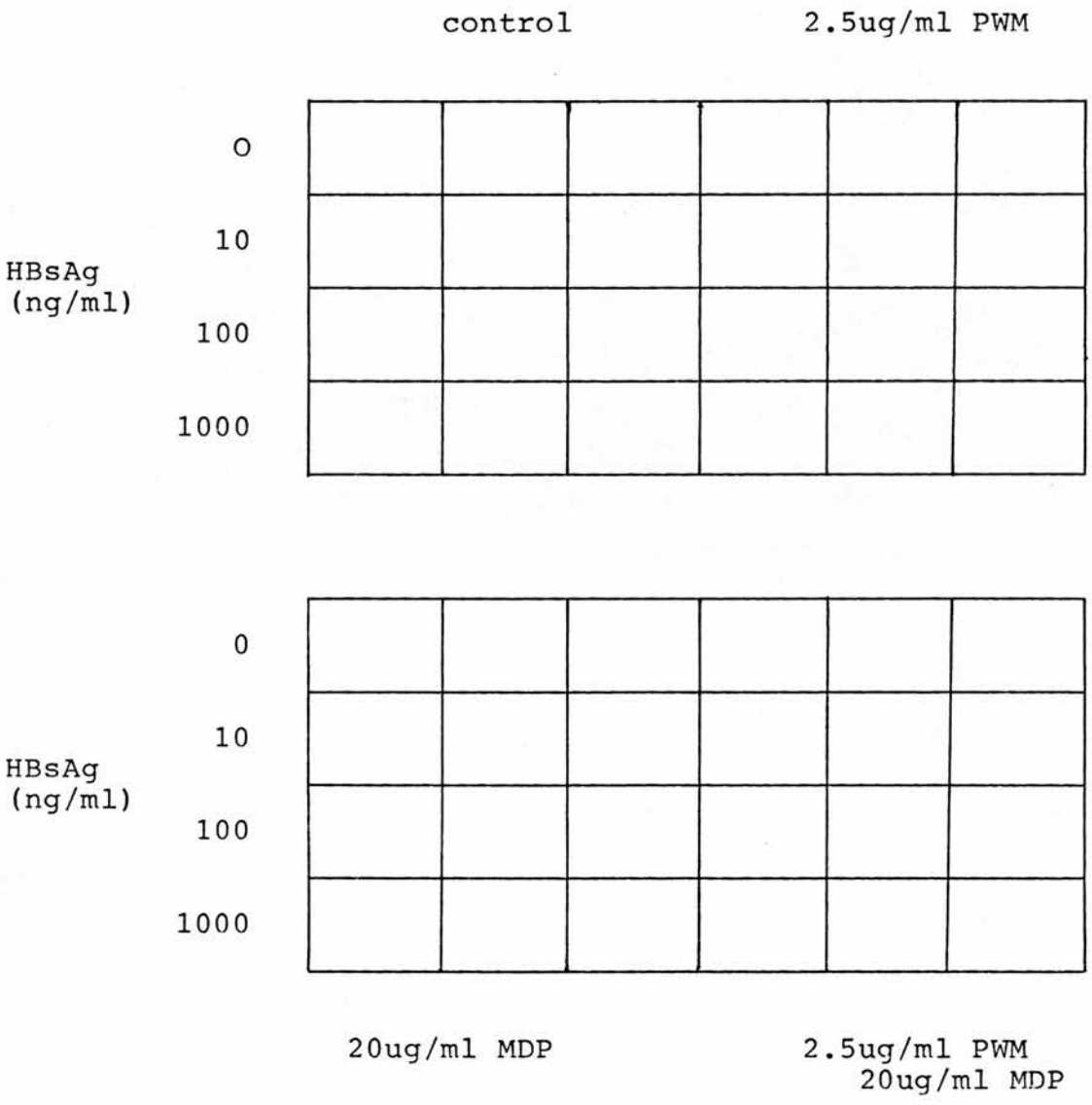
No.	MDP[b]	PWM[c]	PHA[d]	AB Serum[e]
1	+	+	-	-
2	+	+	-	-
3	+	+	-	-
4	-	+	+	-
5	+	+	-	-
6	-	+	-	+

Combinations of two factors used as in Fig.

a Time in months
b MDP - 20 ug/ml.
c PWM - 2.5 ug/ml.
d PHA - dilution
e AB Serum - 10% v/v

FIGURE 4

TYPICAL LAYOUT OF IN VITRO IMMUNISATION EXPERIMENT



Cells plated at 10^6 cells/well

Final well volume 500ul

Basic medium is complete RMPI 1640 + 10% FCS

AET-sheep red cell rosetting and wells were plated. Unfortunately in this particular experiment only single wells were plated because there were relatively few cells available. It is unclear whether the observations were real or merely due to a chance event.

As only one well had been observed positive in all of the experiments carried out, it was decided that the conditions in this well should be replicated on a larger scale and immortalisation procedures attempted. A buffy coat preparation from the same donor was obtained on her next visit to the donor suite. Lymphocytes were isolated and fractionated by the AET-SRBC rosetting technique. The AET^- population was incubated for 48h in RPMI 1640 + 10% FCS with 100 ng/ml HBs vaccine (Wellcome) and 10% pooled AB serum. After 48h the cells were washed twice and divided into 3 parts; the first part was fused with NSO, the second was transformed with EBV, and the third was returned to culture with fresh RPMI 1640 + 10% FCS. The fusion and transformation were treated as normal (see materials and methods section 2.16 + 2.17) The cultured stimulated lymphocytes were kept to determine if any anti-HBs activity could be detected.

The fusion yielded very disappointing results with few hybrids being formed (10 hybrids in 240 wells). The hybrids formed grew very slowly and none secreted specific antibody. The transformation produced 6 positive wells from 600 wells plated, again very low compared to other transformation experiments. The

positive wells were expanded but became negative approximately 5 weeks after initial infection with virus.

The flask with untreated lymphocytes was maintained at 37^{o}C in a humidified 5% CO_2 incubator for 2 weeks. When examined microscopicaly at this point it was noticed that although most of the lymphocytes had died a small proportion remained viable. The culture was fed fresh RPMI 1640 + 10% FCS and incubated for a further 2 weeks. At this point it was obvious that a cell line had developed from the lymphocytes.

The line (HB1) was maintained at first merely out of interest, however when supernatant was assayed it was positive for anti-HBs at a titre of 1:16. Cells were grown up and cryopreserved. Fourteen weeks after the initial experiment began supernatant from a confluent flask had a titre of 1:1024. This titre was stronger than any measured in previous experiments.

The cell line HB1 was fused with NSO in a single fusion experiment. PEG treated cells were plated at 10^5 cells/well in 96 well plates. The fusion efficiency achieved in this experiment was satisfactory with 130 wells from 180 seeded containing growing hybrids. All of the hybrids tested showed anti-HBs activity, 12 were transferred to 1ml wells for further study. Of the 12, six were cloned immediately at 10, 5 and 0.5 cells/well. All of the lines produced growing clones but none showed any anti-HBs activity. The 1ml wells were negative for anti-HBs activity when they were confluent around 1 week after transfer from 96 well plates. In retrospect it

seems likely that the positive results obtained with the hybrids were due to antibody secretion by unfused HB1 cells before they were killed in the selective medium. No further fusions were carried out with this line as time did not permit, however, it is anticipated that if more fusions were undertaken then HB1 would behave in a manner very similar to ES12.

4.9 DISCUSSION

The second part of this project proved to be much more difficult than the first. The initial attempts to produce an anti-HBs secreting cell line by EBV transformation were quite successful. Around 10% of the wells tested were producing anti-HBs antibodies and initially it was thought that at least one cell line would be derived quickly from these experiments. The stability problems which were encountered, however, were extremely severe.

In the first experiment all of the lines were lost in a short space of time and as this was completely unexpected very few lines had been cryopreserved. The large number of cloning experiments carried out on transformation GT4 were undertaken as a counter measure against what at the time was suspected to be overgrowth by non-secreting lines. The number of clones growing was low, but this was not entirely unexpected, what was surprising was that so few of the clones were actively secreting antibody. Positive clones switched off antibody production almost as soon as they were

transferred from small wells to larger 1ml wells. The instability of antibody production has been one of the few predictable results in all of the transformation experiments carried out.

The mechanism and cause of the loss of antibody secretion has not as yet been elucidated however there are several possibilities. The most obvious cause is overgrowth by non-secreting cells which gradually dilute out specific cells. This problem is particularly relevant in the experiments described here, as the lines in question were almost certainly derived from wells containing several EBV activated precursors and are consequently polyclonal. Early cloning would provide a simple solution but as stated in section 4.4 cloning in these experiments was not successful.

A second possible cause of the loss of antibody secretion is the loss of the heavy and/or light chain genes. This is more relevant to hybrids than transformed lines but the possibility of gene loss in these lines must be considered. Other areas which should be investigated are a shortage of exogenous or endogenous growth factors or equally important growth factor receptors. Further possibilities include defects in translation/transcription of DNA via mRNA to protein or even faults in the secretory mechanisms of the cells.

It seems unlikely that a loss of genetic material is responsible for the inability to produce immunoglobulin. This statement is based on the the results of two fusion experiments between EBV transformed cells and normal

peripheral blood lymphocytes. Cells from an anti-HBs$^+$ donor were transformed with virus and expanded as described previously. When one particular line under investigation stopped producing antibody, it was maintained in culture until sufficient cells were available for a fusion

The cells were fused with peripheral blood lymphocytes isolated from a donor showing no anti-HBs activity in their plasma. The cells were plated into 96 well plates as described in methods section 2.12. In this case no selective mechanism was available so hybrids could not be differentiated from unfused lymphoblastoid cells. Cells grew quickly in all wells as would be expected and so wells were assayed at 14 days instead of 21-28 days as would be the case in a normal fusion. Of the 384 wells assayed, 4 gave anti-HBs activity. No attempts were made to clone or expand these wells as it was considered at that time that the chances of success were too low. These results clearly indicate that even although the EBV transformed lines stopped secreting antibody they retain the genetic material which allows re-expression of antibody under appropriate conditions.

It was obvious from very early in these experiments that a line such as ES12 was not going to be easily derived from EBV transformed lymphocytes. Fusion of an antibody secreting population seemed to be the obvious next step. Satisfactory numbers of hybrids were produced in most of the experiments carried out, but again the number of positives was disappointingly low.

Hybrids were being produced regularly and reproducibly with both Hmy2 and NSO but relatively few hybrids secreting anti-HBs were detected.

In experiments where anti-HBs was found, the hybrids were both cloned and transferred to 1ml wells. Unfortunately no positive clones were produced and of all of the positive hybrids only 1 maintained antibody secretion for any significant time. This hybrid, derived from a fusion with Hmy2, also proved to be ultimately unstable in culture and eventually stopped secreting antibody.

Since fusion of EBV transformed lines had not produced satisfactory results it was decided to change the approach once again. From previous experiments it was known that prestimulation of peripheral blood lymphocytes with mitogen increased the number of hybrids produced when this population was subsequently fused with one of the partner lines. In order to produce a specific stimulation and so hopefully produce specific hybrids, cells were incubated with mitogens and antigen in various combinations. Only one combination gave positive results for anti-HBs secretion in these particular experiments. In previous stimulations, for example fusion GF51, antibody could be detected in the medium after 3 days, no explanation can be given for these results. The only common factor between fusion GF51 and in vitro immunisation 6 was that in both cases the cells were AET-rosetted prior to incubation with antigen. It is possible that the removal of some AET^{+} rosetting cells is

important in the stimulation of B-lymphocytes to secrete antibody.

The transformation and fusion experiments subsequently carried out on lymphocytes from the same donor as in IVI.6 and incubated in a similar manner did not give many positive lines. In the transformation experiment only 6 positive wells were detected from 600 which were growing and these stopped secreting antibody within 1 month. No positive hybrids appeared and overall the number of hybrids produced was low.

The cell line which appeared in the flask stimulated by HBs and 10% human AB serum was completely unexpected. The fact that specific antibody was being produced was a bonus, but with the previous experience of EBV transformed lines was not expected to last very long. However at the time of writing, the line is still secreting antibody 3 months after the start of the experiment and the titre is still rising.

The only reasonable explanation for the appearance of this line was that it was produced by a spontaneous transformation of B lymphocytes. Such spontaneous transformations have been previously reported (Bird et al., 1981) when cyclosporin A is added to the culture medium. Since cyclosporin A acts by inhibiting T cells it is not unreasonable to assume that by removing T cells a similar phenomena could be produced. When the supernatant was assayed for immunoglobulin content it was found to contain both IgM and IgG in roughly equal amounts. This indicates that the transformation seems to

have been polyclonal thus producing a mixed population. So far cloning experiments have yielded very few clones none of which have been positive.

This line has been fused with NSO in one fusion experiment and the rate of hybrid formation achieved was high (133 wells with hybrids from 180 wells seeded at 10^5 cells/well). Initially it seemed that positive hybrids were present in the fusion but later testing showed that they had most likely been due to carry over of unfused EBV transformed cells.

The spontaneous appearance of HB1 has meant that in one sense the project has come full circle. A stable antibody producing cell line has been produced by an unknown mechanism. It seems likely that antibody secreting hybrids will be derived from this line as they were from ES12. Between times much has been learned about the characteristics and production of both hybrids and transformed cell lines. It must be considered extremely doubtful whether a similar experiment would produce another cell line.

CHAPTER 5

MISCELLANEOUS EXPERIMENTS

5.1 INTRODUCTION

The experiments which will be described in this section do not fall readily into a single catagory. They do however provide additional information essential to this thesis as a whole. Many of the statements made in the discussion later in the text are based on a combination of results from the previous two chapters and this one. The Chapter is divided into several sections which stand on their own and do not neccessarily bear any relation to each other.

5.2 FUSION OF SPLEEN CELLS WITH MURINE MYELOMA

Reports in the literature had indicated that the fusion rates achieved using splenic or lymph node lymphocytes were much higher than those achieved with peripheral blood lymphocytes (Chiorazzi et al.,1982; Schwaber et al.,1984; Teng et al.,1985; Koizumi et al.,1986; Bibier and Teng, 1987). These conclusions were reached on the basis of our own study (James and Bell, 1987) and table 19 shows the results published in this paper. It was decided therefore that some fusion experiments should be carried out using splenic lymphocytes to determine whether or not this was true.

Two spleens were supplied by Dr E Dewar, Department of Pathology, Edinburgh University. Lymphocytes were isolated from tissue samples as described in section 2.13 and the cells were prepared for fusion. In both cases NSO was chosen as the fusion partner because in most experiments this had yielded higher numbers of hybrids than any of the other available partners. In experiment

TABLE NO: 19. THE CHOICE OF LYMPHOID TISSUES FOR HUMAN MONOCLONAL ANTIBODY PRODUCTION

Lymphoid Tissue Examined	Immunogen	Immunisation		Immortalisation			Best Tissue	Reference
		In Vivo	In Vitro	Fusion	Trans	Both		
Blood,spleen,tonsil	Tetanus toxoid		+	+			Spleen/ tonsil	Chiorazzi et al. 1982
Blood,nodes[a] intra-tumour lymphocytes	Lung carcinoma				+	+	Nodes	Cole et al. 1984
Blood,nodes,spleen intra-tumour lymphocytes	Miscellaneous tumours	+		+			Nodes[b]	Cote et al. 1984, 1985
Blood,spleen	Pneumococcus capsular poly-saccharide	+		+			Spleen	Schwaber et al. 1984
Blood,tonsil	Sheep erythrocytes		+	+			Tonsil	Strike et al. 1984
Blood,node, spleen	Miscellaneous tumours	+		+			Node	Glassy et al. 1983
Blood,node, spleen	Tetanus toxoid	+		+			Node/ Spleen	Burnett et al. 1985
Blood,spleen, tonsil	Tetanus toxoid Bombesin		+	+			Spleen/ tonsil	Ho et al. 1985
Blood,spleen	Miscellaneous antigens		+	+			Spleen	Teng et al. 1985b
Blood,node,spleen, tonsil		+			+		Spleen	Koizumi et al. 1986
Blood,spleen	Miscellaneous antigens		+	+			Spleen	Bieber & Teng 1987

(a) Refers to tumour draining lymph nodes. (b) Depends on cell line ued.
Note the superior performance of spleen, nodes and tonsil was observed with a wide variety of fusion partners both human and mouse.

GF65 spleen cells from donor W.B. were fused with another murine myeloma P653. This experiment was carried out to discover if the fusion rate would be similar in both of these murine lines.

Cells were fused with a 50% PEG Mwt 4000 solution containing 10% dimethylsulphoxide, the solvent for this mixture being RPMI 1640 containing 20mM HEPES buffer. PEG treated cells were incubated in 96 well microtitre plates and fed as described in section 2.17. When growing hybrids covered 25% of the well surface, 150ul samples were removed for assay.

In all, three experiments were carried out in this section (numbers GF64, GF65 and GF68). The first two experiments used lymphocytes derived from donor W.B. This donor was found to possess an allo anti-kell antibody in his serum. This antibody was of interest to the Blood Transfusion Service and it was decided that any hybrids should be screened by Mr C. Green at the Edinburgh and South East of Scotland Blood Transfusion Service. Samples were passed to Mr Green and the results returned within 24h enabling any positive samples to be identified rapidly and cloned.

The donor in experiment GF68 (D.R.) was female and was shown to have serum antibody reactive with cytomegalovirus. This antibody was of interest to Dr M. McCann of the Protein Fractionation Centre, Edinburgh and consequently supernatants from this experiment were passed on for assay against CMV by ELISA.

The results from these experiments are shown in table 20 and as can be seen, none of the hybrids tested secreted specific antibody. No conclusions can be drawn either as to whether or not P653 forms as many hybrids as NSO. The range produced in these experiments was much too wide and shows again the vast variation in hybrid formation encountered when carrying out fusions.

When the results from this section are compared to those where fusion was direct to unstimulated peripheral blood lymphocytes, it can be seen that more hybrids are produced when splenic lymphocytes are used. The reasons for this are not clear. It is possible that lymphocytes fuse more easily when they are activated. To prove this it should be possible to measure the state of activation in relationship to fusion frequency. The number of cells in any particular stage of the cell cycle can be assessed using the EPICS C cell sorter and correlation between cell state and fusion efficiency could be measured.

The spleen cells in question were derived from a patients with B cell lymphoma. Although it was not measured at the time it, it could be assumed that a higher than normal proportion of the cells was actively cycling at the time of fusion. A better comparison of the relative efficiencies of peripheral blood and splenic lymphocytes would undoubtedly have been obtained with matched fusions of both types of lymphocytes from the same donor. Unfortunately the opportunity to carry out such an experiment never arose.

TABLE NO: 20. RESULTS OF FUSIONS USING SPLEEN AS LYMPHOCYTE SOURCE

Fusion	Donor	Partner	Wells Seeded	No. Hybrids	Assay	No. Positive
64	W.B.	NSO	130	104	anti-Kell	0
65	W.B.	P653	94	24	anti-Kell	0
68	D.R.	NSO	300	43	anti CMV	0

5.3 ATTEMPTS TO PRODUCE ANTI-HIV MONOCLONAL ANTIBODIES

A patient at the Royal Infirmary Edinburgh presented with thrombocytopenia which was not being controlled by conventional therapies. It was decided that the patient should be splenectomised in an attempt to improve his condition. Previous blood tests had shown that the patient was seropositive for anti-HIV. Since it was known that splenic lymphocytes fused better than peripheral blood lymphocytes and anti-HIV would be a particularly interesting antibody to produce the decision was taken to attempt a fusion with the spleen cells. In order to maximise the chances of isolating a positive line transformation experiments were also undertaken.

The spleen in question was assumed to have a highly unfavourable ratio of T_H:T_S, reflecting the counts carried out on peripheral blood. Since it is well documented that T_S have a deleterious effect on transformed cells, T-cells were removed prior to viral transformation. Since no such effects have been shown on hybrids and in the murine system T-cells do not seem to have any effect, the whole population was used in fusion experiments.

Initially fusion was favoured for several reasons. Removal of T-cells requires extra incubation and washing steps and since all of this work had to be carried out in a class 1 hood in the Department of Bacteriology, it was considered that fewer steps and less time occupying the hood was to be preferred. The first experiment carried out was a fusion and the second a transformation.

Finally a second fusion was carried out. The methods followed and the results produced are summarised in fig 5 and table 21.

Briefly for the fusion, cells were removed from liquid nitrogen and cultured overnight before fusing with Hmy2 and NSO. Cells were plated into 96 well microtitre plates at 10^5 cells per well. For transformation lymphocytes were cultured for 2h on flasks coated with goat anti-human Ig (SAPU). Non-adherent cells were washed off and viral supernatant was placed directly onto the adherent population in the flask. Cells were incubated in this supernatant for 48h after which they were washed and placed in fresh RPMI 1640 + 10% FCS. After 2 weeks growth was apparent and cells were plated into 16 x 1ml wells which were then assayed when growth became confluent. In all experiments supernatants were tested for anti-HIV activity in the Department of Bacteriology using the DU-PONT direct binding ELISA.

The first two fusion experiments carried out were unfortunately lost due to the incubator in the containment laboratory being allowed to run dry. Half of the wells seeded were lost before the condition could be rectified and no hybrids grew in any of the other wells. The volume of liquid present in each well each well had been reduced by half so increasing the salt concentration correspondingly. It is not surprising that hybrids did not grow after being stressed in this manner.

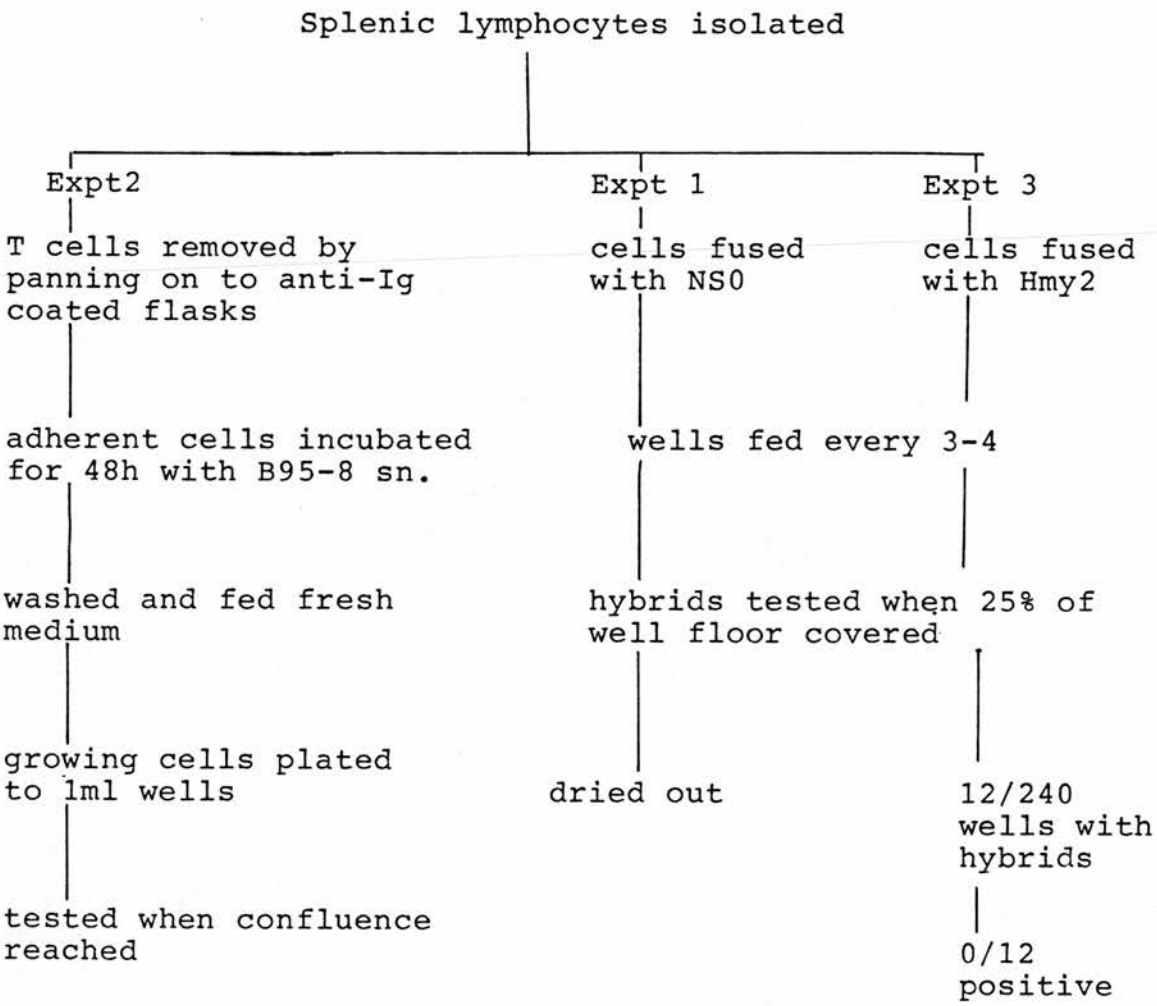
In the final fusion experiment HIV 3, the fusion

TABLE NO: 21. RESULTS OF EXPERIMENTS TRYING TO PRODUCE HUMAN ANTI-HIV MONOCLONAL ANTIBODIES

Expt.	Method of Immortalisation	Wells Seeded	Wells Growing	Wells with Anti-HIV
1	Fusion	480	Dried out	
2	Transformation	16	16	0
3	Fusion	240	12	0

FIGURE 5

FLOW DIAGRAM OF EXPERIMENTS CARRIED OUT ON HIV^{+} SPLEEN



partner was Hmy2. In all 240 wells were seeded at 10^5 cells per well. Growing colonies were present in 12 wells but when assayed they contained no anti-HIV activity. Several of the wells in this experiment contained colonies which grew as tightly packed clumps, more like EBV transformed cells than typical Hmy2 hybrids. The cells in these wells also grew much more slowly than Hmy2 hybrids produced in previous experiments. It may be that the cells observed in fact been transformed in a manner similar to that previously reported by Boyd et al (1984). It is also possible that unusual state of the hybrids produced simply reflected the poor state of the immune system as a whole.

The transformation experiment, although initially slow to grow, soon took on the growth characteristics of other EBV transformed cell lines ie. growing in tight clumps and doubling every 24h or so. When the 1ml wells were assayed after approximately 4 weeks in culture none of showed any anti-HIV activity and were discarded.

Disappointingly no specific antibody activity was detected in any of the assays carried out on supernatants. However these experiments have shown the feasibility of carrying out fusions and transformations under the strict containment conditions required for growing HIV infected lymphocytes. More lymphocytes are stored in liquid nitrogen and are available for future experiments if the facilities are available. Because of the potential uses for a human anti-HIV monoclonal antibody, these experiments are well worth repeating.

5.4 PRODUCTION OF ANTI-BRAIN ANTIBODIES

Previous work in this department (Simpson et al 1986) has shown that patients with Alzheimers disease have antibodies which bind to structures in brain sections. Antibodies were produced which bind to plaques and tangles, diagnostic features of the disease. Age matched controls were also shown to possess antibodies which could bind these features.

In order to investigate whether these antibodies are present in younger donors or are characteristic of older patients, a transformation experiment was carried out. The donor was a 30 year old female who donated 50ml of peripheral blood. The method transformation is described previously (section 2.16). Samples were taken from the 96 well plates 14-21 days after seeding and assayed by Dr J. Simpson (MRC Brain Metabolism Unit, Department of Pharmacology, Edinburgh University). The assay consisted of layering the test supernatant onto frozen sections of brain from Alzheimers patients or normal control. After incubation and washing, a conjugated secondary antibody was added, followed by substrate. Slides were examined for evidence of staining and scored for intensity and specificity.

In all more than 80 samples were tested, 37 of which bound to one or more structures in both Alzheimers and control brain. Table 22 shows the specificity of the strongest samples ie. >3 on the intensity scale (the scale was judged from 1-5). The staining patterns of

TABLE 22

REACTIVITIES OF SOME EBV TRANSFORMED CELL SUPERNATANTS WITH NORMAL AND ALZHEIMER HUMAN BRAIN

Sample no.	Tangle	Astrocyte	Neuron	Nuclei
2	3-4*		1*	
5	4*	3*		
7			2*	4-3*
12		4*		
18	5*			
20		3-4*		
30				4*
34		3-4*		
35	3-4*			

Samples stained by the immunoperoxidase technique.

Intensity of staining scored from 0-5* by Dr. J. Simpson MRC Brain Metabolism Unit

some of the transformed lines are shown in plates 5-10. It was surprising to find that a donor with no history of disease and who was young possessed these antibodies.

It is possible that these antibodies play some role in the mechanism of clearing cell debris from the blood stream, much work remains to be done to fully characterise and explain the presence of such antibodies. Since these experiments were carried out, brain binding murine antibodies have been raised from unimmunised mice of various ages from neonates to 12 months (Simpson et al 1987). These monoclonals which are at present being fully characterised show that the reaction is not species specific and is likely to be against some internal or membrane structure exposed by cell damage. The monoclonals have not as yet been used to stain other tissues although this may prove an extremely interesting experiment.

The human cell lines were not expanded or cloned as the lines had shown signs of dropping titres and it was felt that the chances of success were too low compared to the time involved. The experiment was however considered a success as it has shown that antibodies of the required specificity were present in the blood. Transformation experiments of this type can be used to investigate in detail the immune response of an individual to any antigen, indeed Logtenberg et al (1987) used a similar approach with fusions to investigate the production of auto-antibodies. Even where no permanent cell lines are produced in the end, valuable information can be gained

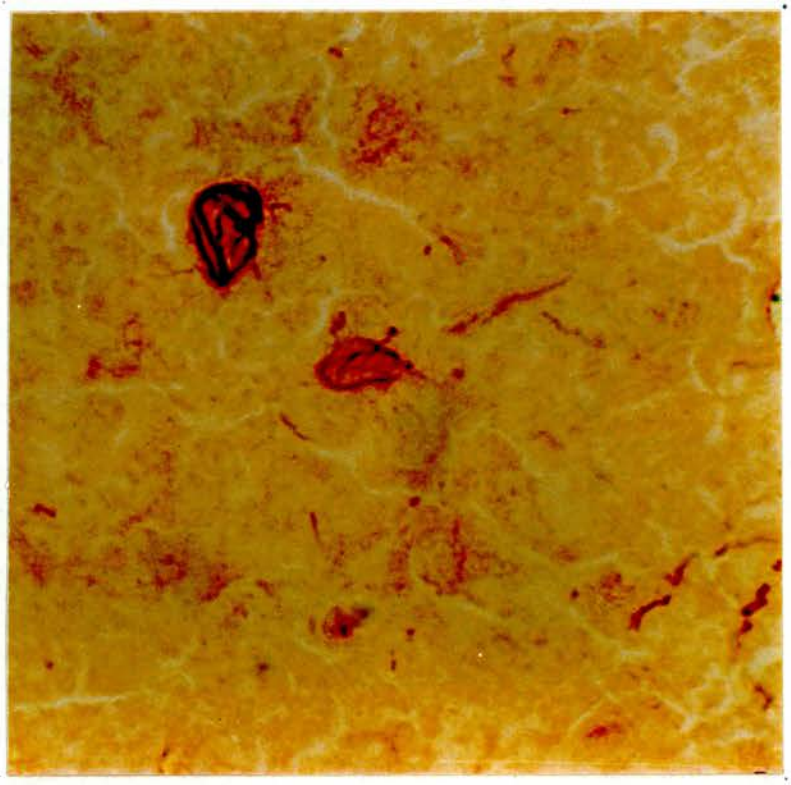


PLATE 5: Immunoperoxidase staining of Alzheimer brain section with human antibody as primary antibody. Low power (X50) field showing typical Alzheimers tangle.

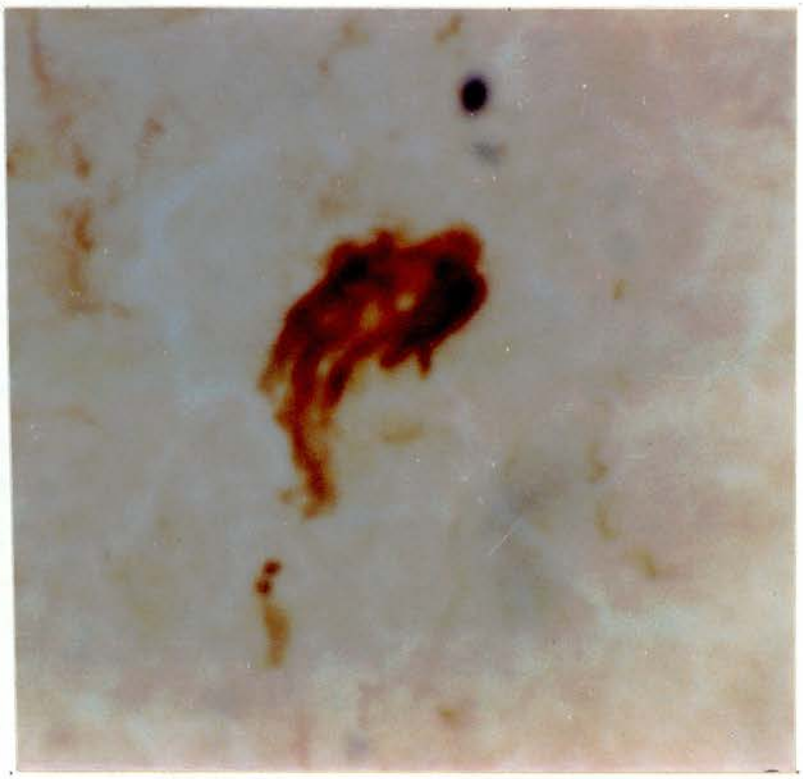


PLATE 6: Immunoperoxidase staining of Alzheimers brain section with human antibody as primary antibody. High power (x100) oil immersion field showing typical Alzheimers tangle.

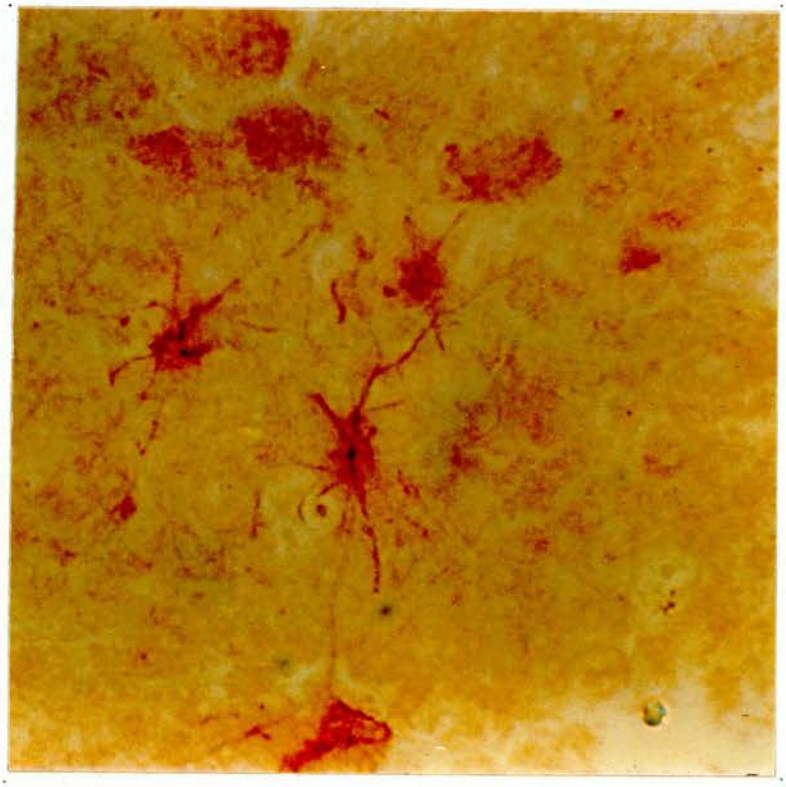


PLATE 7: Immunoperoxidase staining of Alzheimers brain section using human antibody as first antibody. Low power (X50) field showing stained astrocytes.

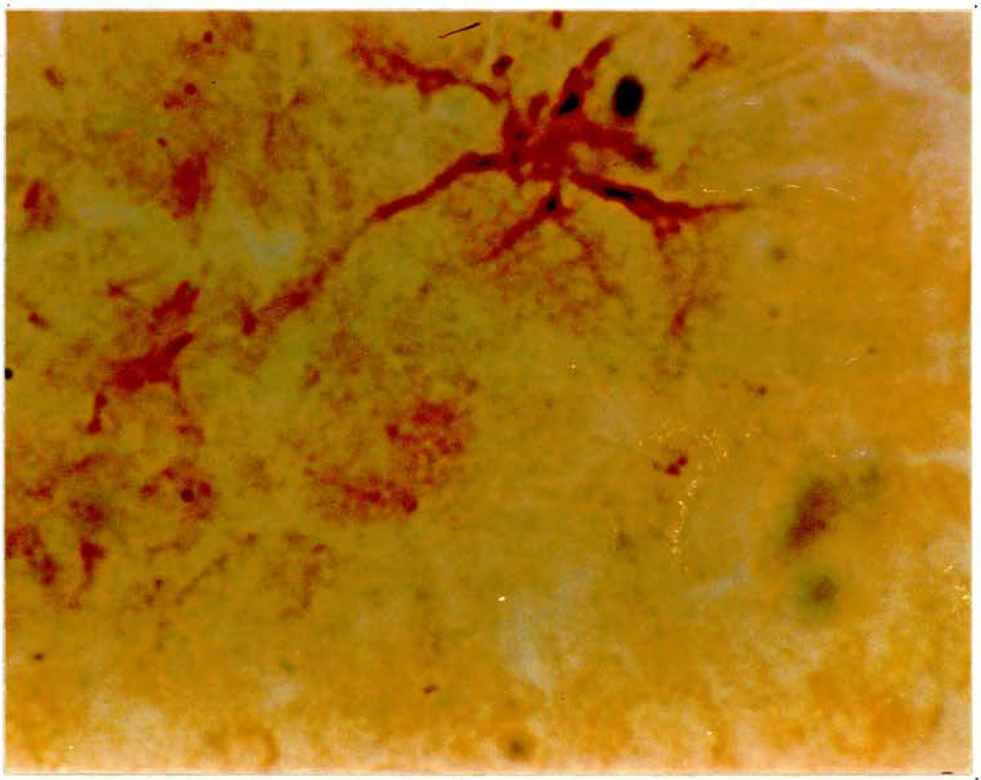


PLATE 8: Immunoperoxidase staining of Alzheimers brain section using human antibody as primary antibody. High power (x100) oil immersion field showing astrocytes.

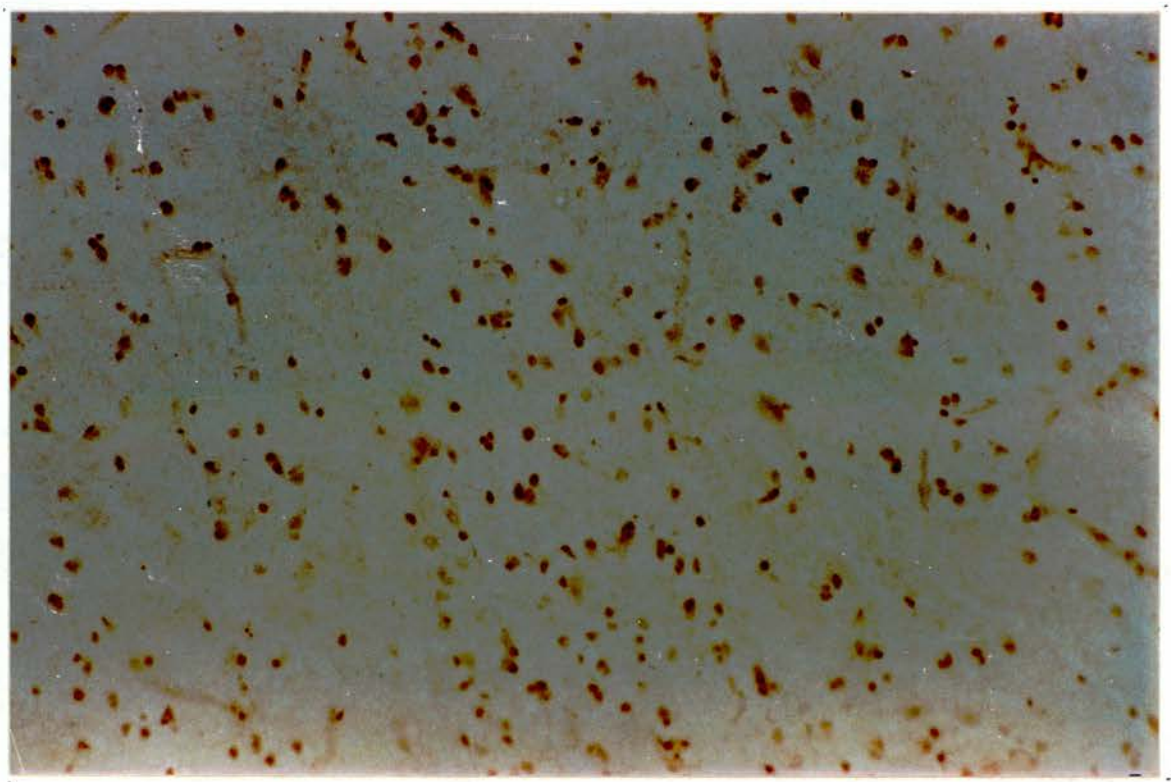


PLATE 9: Immunoperoxidase staining of normal brain section using human antibody as primary antibody. Low power field (X25) showing staining of nuclei.

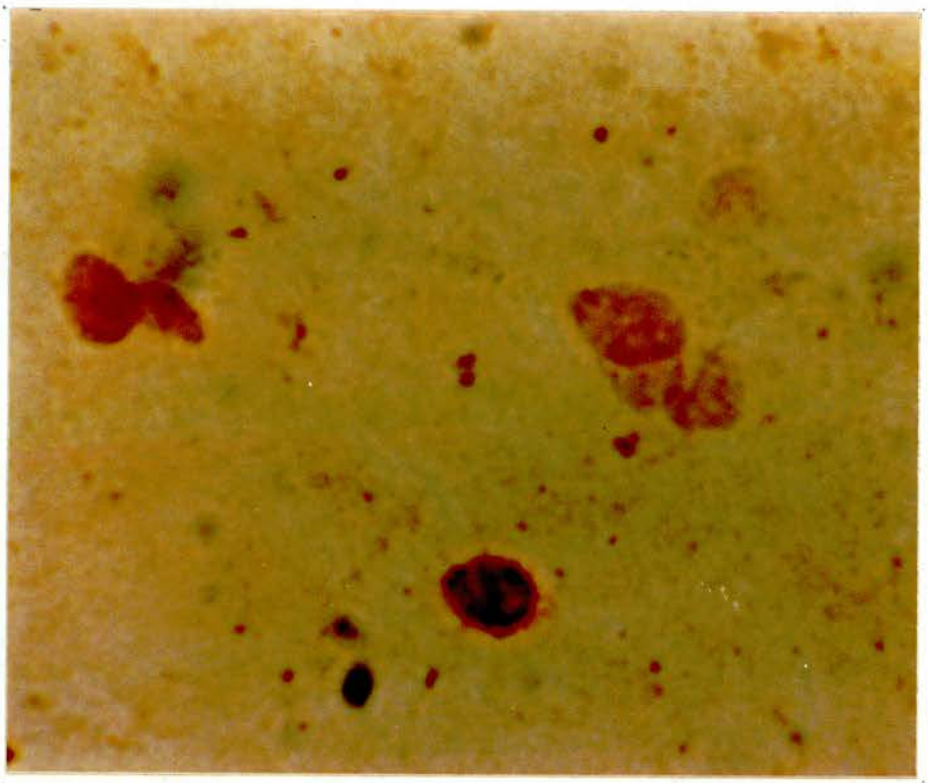


PLATE 10: Immunoperoxidase staining of normal brain section using human antibody as primary antibody. High power (X100) oil immersion field showing staining of nuclei

about the immune system.

5.5 IMMUNOCYTOCHEMICAL STAINING OF CELLS FOR IMMUNOGLOBULIN CONTENT

As stated in previous sections there was an extreme problem with stability of antibody secretion. There are many possible causes for this (table 23) one of which is that cells have a defect in their secretory mechanism. In order to test if cells produced immunoglobulin but were unable to secrete it, cells were stained for IgG and IgM as described in section 2.25.

The studies described in this section were carried out on the hybrid line GF41/27. This hybrid had not been cloned and secreted both IGM and IgG. Cells stopped secreting antibody some 12-14 weeks after the initial fusion. Aliquots of cells frozen in liquid nitrogen were recovered and grown in RPMI 1640 + 10% FCS. At regular intervals cells were removed and spun onto washed microscope slides. The cells were air dried, fixed and stored at -20^{o}C until required.

Slides from sequential samples were stained for IgG and IgM by the method previously described (section 2.25) The plates (11 to 17) show the staining patterns which were produced. It is obvious that the percentage of cells which stain for IgG falls off at the same time as the titre of specific antibody (see table 24). The number of cells staining for IgM does not alter dramatically.

In order to show that the IgG is the active

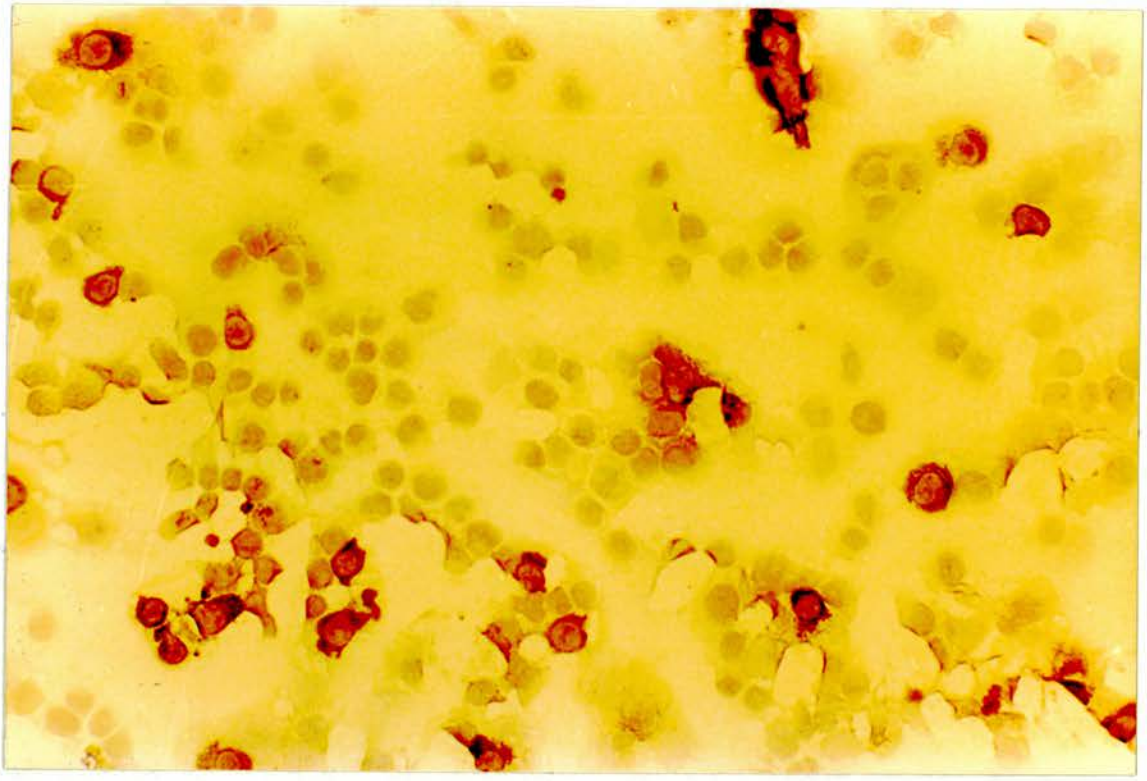


PLATE 11: Immunoperoxidase staining of cytocentrifuge smear of GF41/27 cells harvested on 30.4.87. Cells stained with anti-human IgG.

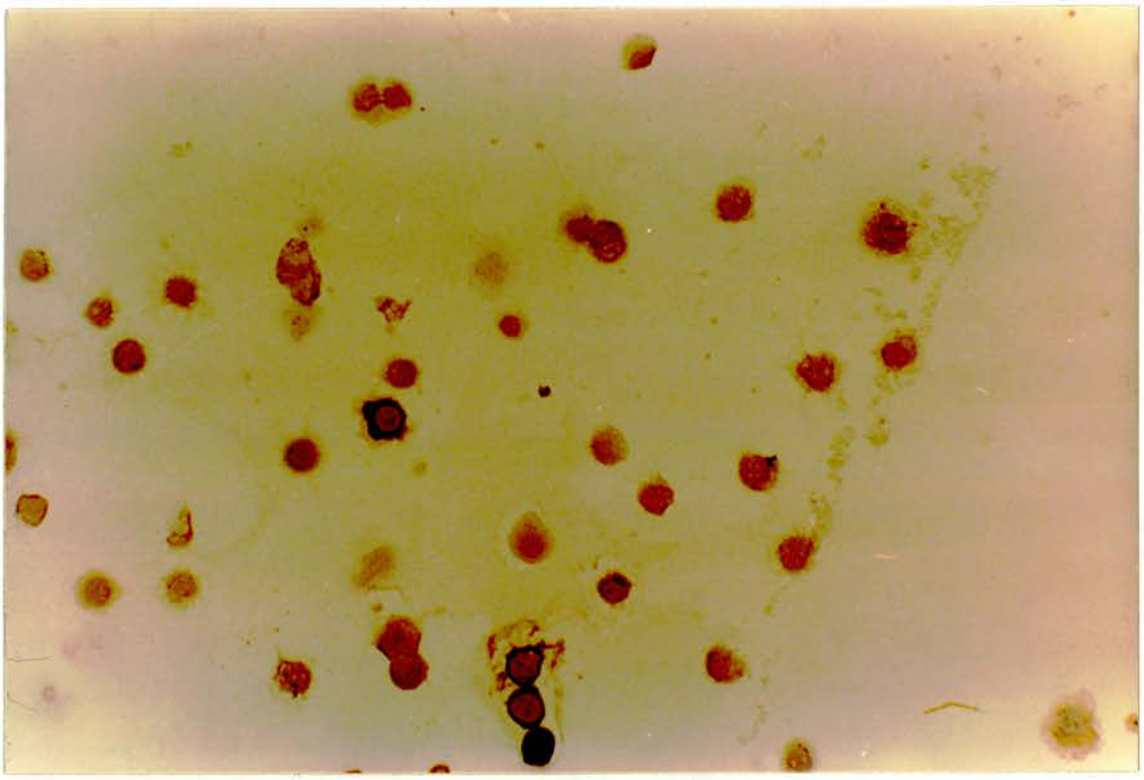


PLATE 12: Immunoperoxidase staining of cytocentrifuge smear of GF41/27 cells harvested on 1.5.87. Cells stained with anti-human IgG.

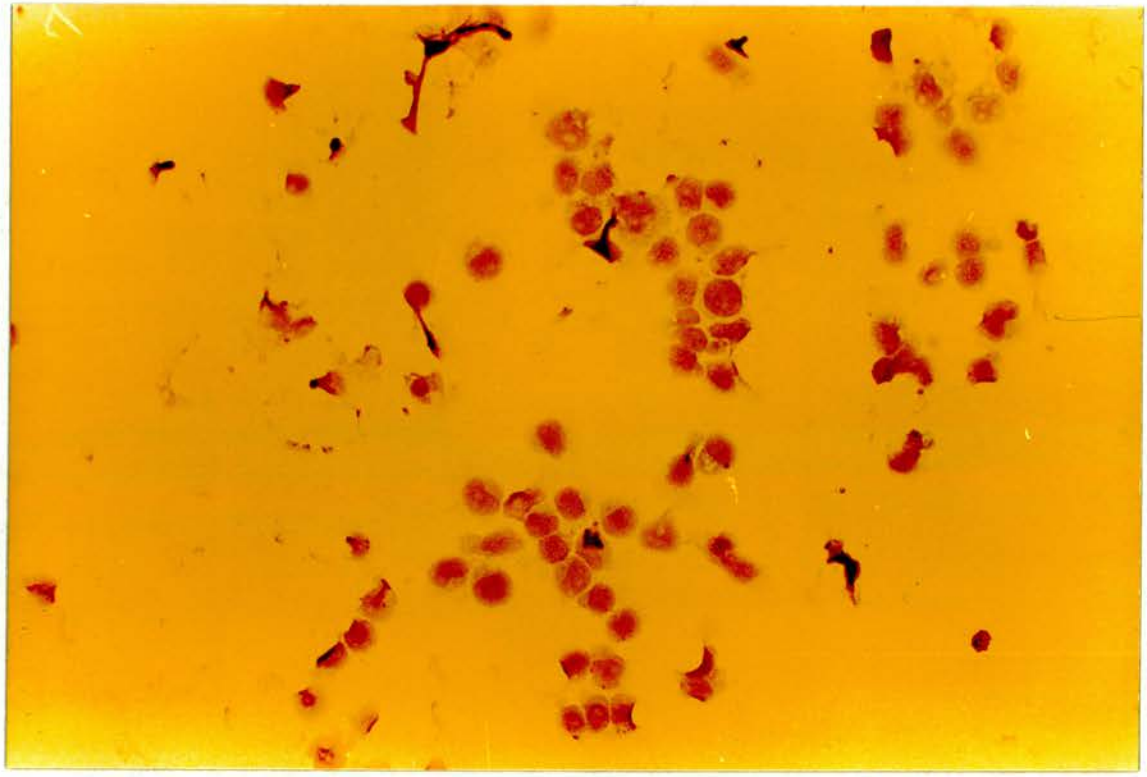


PLATE 13: Immunoperoxidase staining of cytocentrifuge smear of GF41/27 cells harvested on 21.5.87. Cells stained with anti-human IgG.

PLATE 14: Immunoperoxidase staining of cytocentrifuge smear of GF41/27 cells harvested on 1.5.87. Cells stained with anti-human IgM.

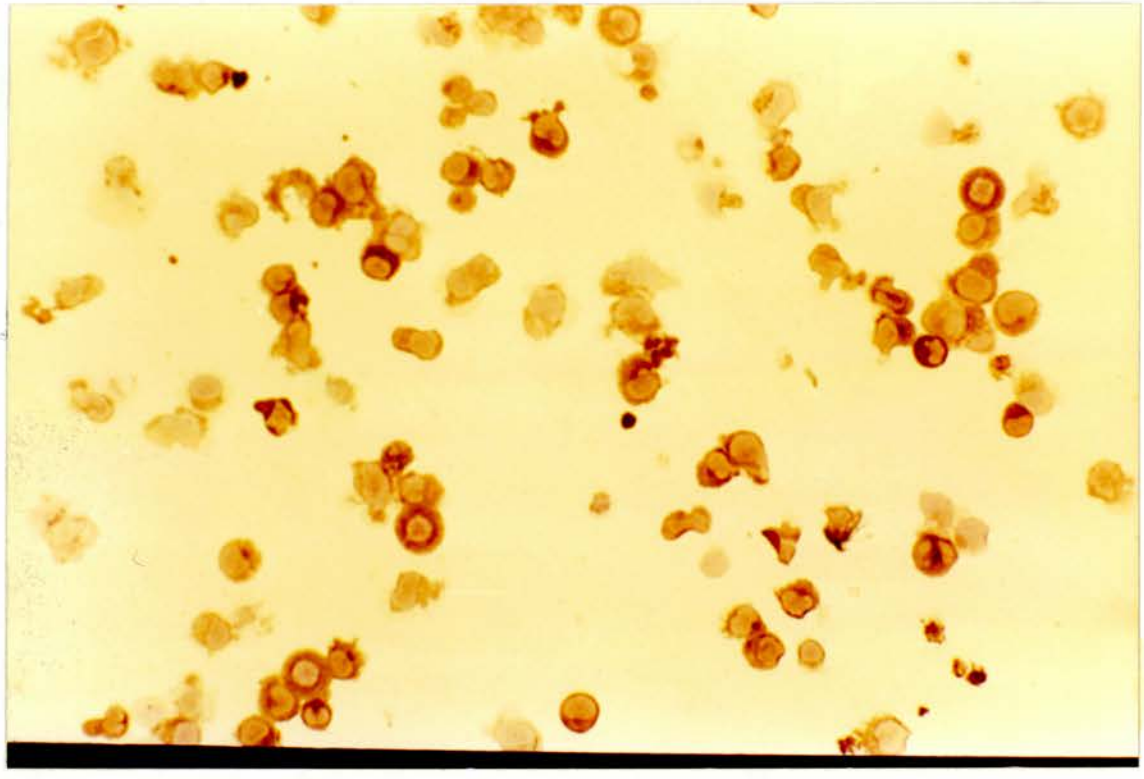


PLATE 15: Immunoperoxidase staining of cytocentrifuge smear of GF41/27 cells harvested on 21.5.87. Cells stained with anti-human IgM.

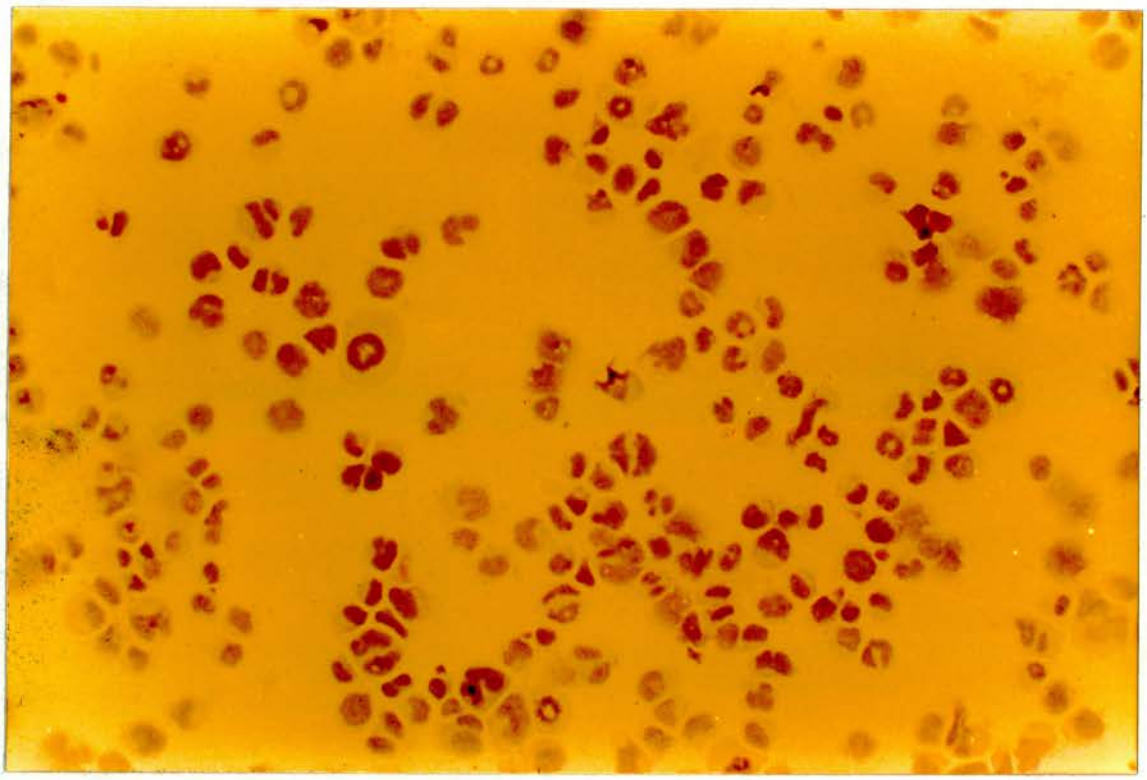


PLATE 16: Immunoperoxidase staining of NS0 cells stained with anti-human IgG. Note this is a negative control and no non-specific staining occurs.

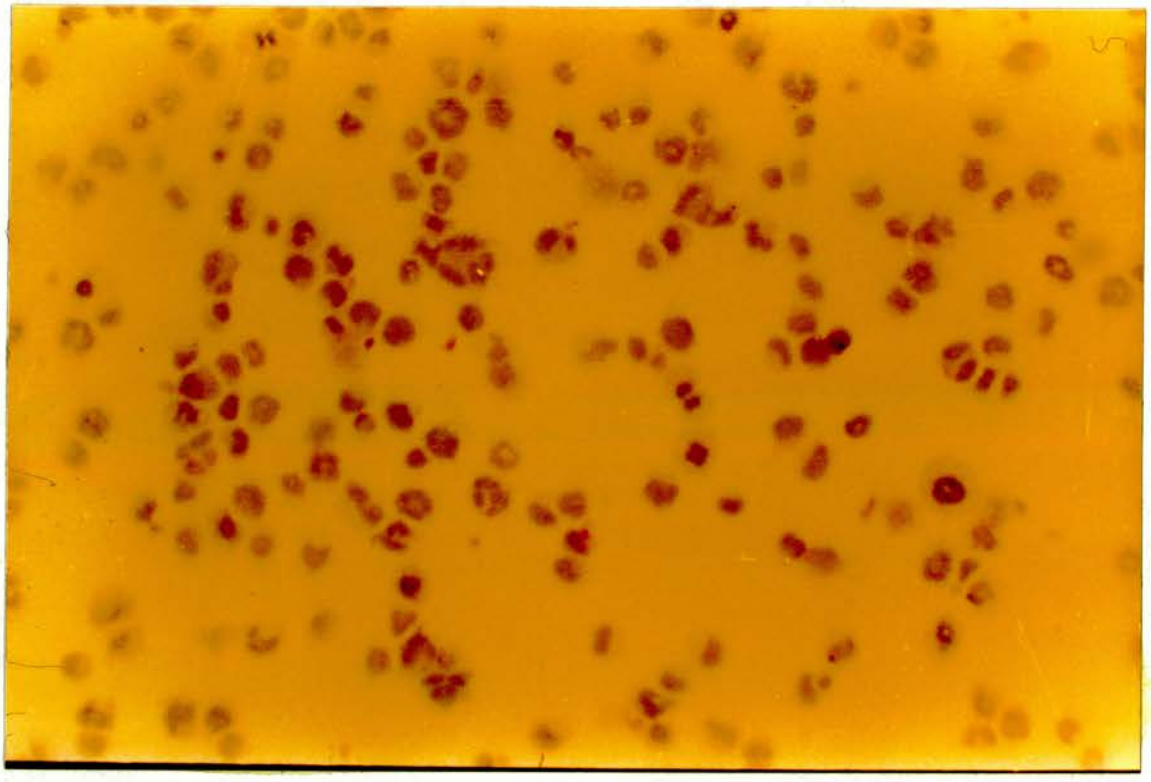


PLATE 17: Immunoperoxidase staining of NS0 cells stained with anti-human IgM. Note this is a negative control and no non-specific staining occurs.

TABLE 23

POSSIBLE REASONS FOR LOW OR ABSENT IMMUNOGLOBULIN SYNTHESIS BY HUMAN MONOCLONAL CELL LINES.

SUGGESTED DEFECT	REFERENCES
• Inhibitory effect of mycoplasma or other microorganisms	Van Meel et al. 1985
• Overgrowth of secreting cells by non secretors	Zurawski et al. 1978
• Shortage of relevant growth and differentiation factors	Winger et al. 1983
• Absence of appropriate receptors for growth and differentiation factors	Melamed et al. 1985
• Loss or inappropriate incorporation of structural genes for immunoglobulin H and L chain	Schwaber et al. 1984; Teng et al. 1985b
• Insufficient copies of structural (and regulatory genes)	Schwaber et al. 1984; Sahagan et al. 1986
• Inappropriate H and L chain combination resulting in hybrid molecules	
• Failure/Loss of relevant regulatory genes or other defects in regulation	Raison et al. 1982; Kozbor and Croce 1985a; Schwaber et al. 1984
• Defects in the synthetic machinery resulting in impaired transcription, translation or assembly	Sikora et al. 1983; Kozbor and Croce 1985a; Teng et al. 1985b
• Defects in the secretory machinery of the cell	Kozbor and Croce 1985a; Teng et al. 1985; Gaffar et al. 1986b

TABLE 24

Percentage of GF41/27 Cells Staining With Anti-IgG or Anti-IgM in Immunocytochemistry

Date	IgM^+	IgG^+	Negative (a)	Specific Ab. (b)
30.4	NA	11.9 (c)	NA	+
1.5	62.2	10.7	27.1	+
21.5	76.1	0.7	23.3	-

(a) negative cells were deduced by totaling IgM and IgG positive cells and subtracting the total from 100.

(b) as assayed by hemagglutination.

(c) In each case 1000 cells were counted.

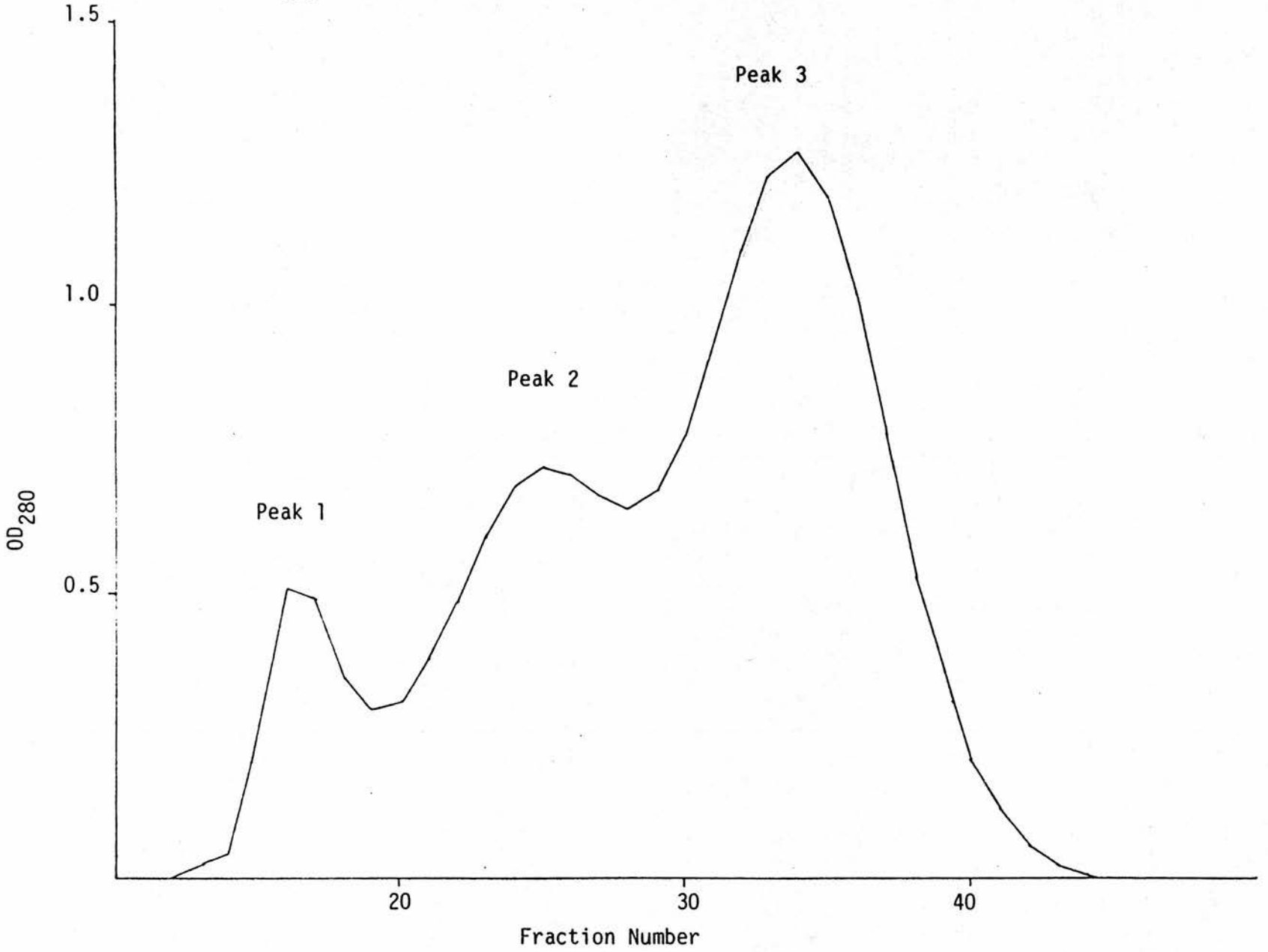
NA no result available.

immunoglobulin species, supernatant from a GF41/27 culture was concentrated by a factor of 10 and mixed with a human serum known to be negative for anti-HBs. This mixture was passed through a sephadex G200 column and the samples were collected. The profile shown in figure 6 was obtained by measuring the absorption of individual samples at 280nm. The pattern obtained is typical of serum and the contents of each peak are well known. Peak 1 contains IgM and 2 macroglobulins, peak 2 is mainly IgG with IgA and peak 3 consists of a mixture of albumin and other small molecules.

When the peak samples for peaks 1-3 were assayed for anti-HBs, only peak 2 contained specific antibody. When the number of IgG containing cells declined then the specific antibody titre also declined. It seems fair therefore to assume that the IgG is the active immunoglobulin species in this particular case.

The cause of decline in antibody titre cannot be concluded directly from this experiment, however, it is apparent that in this case the secretory mechanism of the cell is not at fault. Since the experiments carried out here were performed on an uncloned cell line, a strong argument could be put forward that in this case the specific cells are being overgrown. The results achieved in other experiments would dispute this. For example in cloning experiments, positive clones were produced by this line, but when they were expanded they stopped secreting antibody. This would indicate that even in a homogeneous cell population the switch off event is

FIGURE 6: OD_{280} Versus Fraction Number for GF41/27 Supernatant run through Sephadex G200 Column



occurring.

The mechanism of loss of antibody secretion by these hybrids may not be the same as that in transformed cells. Preliminary experiments are underway where EBV transformed lines are treated in a similar manner, sampled and stained at regular intervals. The time taken for EBV transformed cells to switch off seems to be very short. That is when cultures are fed fresh medium every 2-3 days and reseeded at the same cell density each time, specific antibody titre will decline within one three day culture. The speed with which this occurs would tend to argue against overgrowth by non-secreting cells. The supernatant from these cell lines however still contain non-specific immunoglobulin indicating that some cell populations retain the ability to secrete antibody.

The whole picture is further confused in virus transformed lines because all of the lines tested are oligoclonal. In the case of hybrid GF41/27 it was apparent that only three and at most four separate hybrids were present in the original culture well, with the transformed lines however this could have been as high as ten or eleven. This higher number makes it more difficult to interpret the data from the staining experiments. However it would appear that in most cases the number of IgG secreting cells is falling at around the same point that specific antibody titre drops.

It would be interesting to label some HBs and stain the cells directly for anti-HBs activity. This would

show conclusively whether or not the specific cells declined slowly (as in overgrowth) or quickly (due to active switch off or genetically controlled events).

Many more experiments will be required to show that in other lines IgG antibodies are specific and that there is in fact some correlation between loss of specific antibody and the observed reduction of IgG secreting cells. It seems strange that some populations, mainly IgM, retain their antibody secretion while others lose theirs. It is possible that IgM and IgG secreting lines are derived from different areas of the B cell activation pathway and therefore have different growth factor requirements. This could explain why one species of immunoglobulin is lost while the other is retained.

CHAPTER 6

DISCUSSION

6.1 GENERAL DISCUSSION

The results produced in the preparation of this thesis are generally encouraging. Despite the problems encountered with stability of antibody secretion, several specific antibody producing cell lines have been produced. Investigations were carried out into the characteristics of cell fusion and of hybrid growth, and also into the growth of EBV transformed cell lines. Finally preliminary attempts were made to expand the antibody specific B cell population by in vitro immunisation.

The most successful technique in the experiments described in this thesis was the fusion of EBV transformed lymphocytes to murine myeloma cells. The technique was first advocated by Kozbor et al (1982) and has since been employed by many other workers with varying degrees of success (table 2). The original work was carried out with a human rather than a murine fusion partner, but since this time various workers have successfully used murine partners (Cote et al., 1983, 1984, 1985, 1986; Thompson et al., 1986; Larrick et al., 1985; Yamaura et al., 1985; Macdonald et al.,1987).

It would appear that the instability problems anticipated in human/mouse hybrids have not arisen. It is untrue to suggest that all human/mouse hybrids are stable and it is likely that human chromosomes are preferentialy segregated from hybrids (Kozbor and Croce, 1985; Teng et al., 1985) however the vast problems which were anticipated do not seem to have arisen. Published

work (Cote et al., 1983; Burnett et al., 1985; Sugano et al., 1987; Masuho et al., 1987) supports the results obtained here using murine cells as fusion partners. Thompson et al (1986) state that loss of antibody secretion due to chromosomal instability is no more significant in heterohybrids than in human/human or indeed entirely murine hybrids.

Murine myelomas undoubtedly give higher fusion rates than any of the currently available human fusion partners when comparative experiments are carried out (Abrams et al., 1983; Cote et al., 1983, 1984, 1986; Usagawa et al., 1986). There is also the advantage that lines are available which do not secrete any immunoglobulin, until recently this was not the case for human myelomas (Jernberg et al., 1987). Therefore by using non secreting murine partners the possibility of producing mixed antibody molecules is negated. It is possible that for various reasons the products of human/mouse hybrids will not be acceptable as therapeutic grade reagents, the reasons for this will be discussed in more detail below.

The production of antibody secreting lines by EBV transformation was not particularly successful in experiments described here. Despite the fact that many transformation experiments were carried out, only one cell line was derived and that was of doubtful origin. The difficulty faced was not one of detecting or isolating specific antibody secreting cells, but rather with the stability of antibody secretion. The pattern of

secretion was almost identical in each experiment with initially quite large numbers of positive cells being detected. Over a period of several weeks the number of positive wells slowly declined until at 8 weeks none remained. Cloning of these cells failed to isolate any useful lines.

There are many possible causes for the loss of antibody secretion and these have been discussed previously. A great deal of work is required into the basic cell biology of EBV transformed lines before any firm conclusions can be drawn as to the mechanism of loss of antibody secretion. The preliminary experiments described in section 5.5 tend to suggest that the secretory mechanisms of the cells are not at fault and that the switch off event is occurring at the gene level. This event could be due to an active "switch off" message or lack of a "switch on" message. further experiments will hopefully identify which if either of these hypotheses are correct.

The second major problem encountered in these experiments was the poor performance of the human lines under cloning conditions. This statement refers especially to EBV transformed cell lines. Even when the lines were stable and well established such as ES12 or latterly HB1, cloning was extremely difficult. In the experiments described in section 3.7 the effects of various feeder supernatants on clone growth were examined. From these particular experiments no firm conclusions can be drawn. The problem of cloning EBV

cells has also been encountered by others (Tiebout et al., 1987). The impression gained by these investigators is that EBV transformed cells are inherently difficult to clone.

This fact brings up another interesting point and that is the lack of soluble factors. Because of the difficulty in cloning mentioned above, many types of feeder cells and supernatants have been employed. These fall into two distinct groups, cells of the lymphocyte and mononuclear phagocyte lineage of human or murine origin and human cells of embryonic origin or fibroblasts (see table 7). Some groups believe they greatly enhance the isolation of antibody producing lines (Crawford et al., 1983, 1985; Rosen et al., 1983; Tiebout et al., 1984; Teng et al.,1985a). While others feel they are of limited value (Cote et al., 1983, 1985; Strike et al., 1984; Schmidt-Ullrich et al., 1986).Experiments performed here support the later view. It must be pointed out that in these experiments, it was the ability of the feeder supernatants to support growth at lower cell densities which was assessed and not the ability to increase the outgrowth of specific antibody producing cells.

It has been suggested that the abnormal growth of some cancer cells may result from the self perpetuating mitogenic response to autogenous growth factor (Kaplan et al., 1982). The activation of this autocrine loop has also been implicated in the continued growth of other cell lines such as T-lymphomas and EBV transformed lines

(Gootenberg et al.,1981; Gorden et al., 1984). Scala et al (1987) have recently shown that an EBV transformed line ROHA-9 produces an Il-1 like substance which acts as an autocrine growth factor. Other EBV transformed lines have also been shown to produce similar growth factors (Oppenheim et al., 1984). Since the major source of Il-1 is normally monocytes, it may be considered that cells of this lineage are the most appropriate feeder cells.

It is possible that the EBV transformed cell lines produced here are dependent on an autocrine growth factor. When cells were passaged into fresh medium it was normal practise to transfer 2-3ml of spent medium. This practise was performed due to an empirical observation that cells tended to grow better when this was done. When actively growing cells split into fresh medium but still maintained at a fairly high density (say 5×10^4 cells/ml) it could be imagined that the concentration of the autocrine factor would begin to rise fairly rapidly. While this may result in a lag phase before exponential growth begins, it would never leave the cells completely without growth factor. On the other hand when cells were seeded at cloning densities (1-10 cells/100µl), even if cells were secreting growth factor immediately after cloning the concentration is not likely to rise quickly. The lack of growth factor could then in turn cause its' own production to cease and so cells would not grow at all.

One way to avoid this would be to clone cells using supernatant from their own culture as a feeder. This

would then raise problems when assaying for specific antibody at a later stage. If all of the EBV transformed lines were dependent on the same growth factor then it should be possible to clone using supernatants from well established lines as feeder. Unfortunately these experiments were not carried out and so the discussion is purely speculative; it would however be interesting to try. It is also possible that growth factors other than IL-1 or indeed a series of factors is involved, if this is the case cloning in media conditioned by other EBV transformed lines is unlikely to have much effect.

6.2 LIMITATIONS OF THE EXPERIMENTS CARRIED OUT

It is not always easy to know in advance what is going to happen in any particular experiment. Hindsight however reveals many faults and limitations in the design of almost all scientific experiments. In most cases where a completely unforeseen result is obtained the problem is that the appropriate controls have not been used. Thus extrapolations made from any one experiment must then be considered speculative rather than being based on sound scientific evidence.

Many of the experiments carried out here suffer from such faults. Experiments tended to overlap due to the period of time taken from isolating lymphocytes from a donor through to testing cloned EBV transformed lines or their hybrids. for example transformation GF38 was started while transformation GF32 was being cloned and fused. This overlap of experiments meant that any

alteration suggested by the results of one particular experiment took time to filter through the system.

Again because of the time involved changes had to be made to the system constantly in order to test as many variables as possible. All of these changes meant that when the results are reviewed as a complete work, very few can be compared directly. The final decision as to when changes should be made and the reasoning behind them was taken with the ultimate aim of trying to produce a stable cell line rather than on systematically testing all of the variables. In reviewing the results it would seem that this was perhaps not the best approach to adopt. In many experiments the control systems are not as stringent as would be liked, but this lack again only became apparent with hindsight.

The system itself is basically a biological one which is inherently variable. The donors chosen for anti-HBs, had varying antibody titres and had been boosted at various times prior to bleeding. From this point it is likely that each donor would react differently to EBV transformation. Perhaps it would have been advisable to to carryout some identical test on all of of the donors to establish a base line or starting point in each case. Possible parameters to measure include the state of activation of the B cells, AET rosettes, antigen specific cells and antibody titre. In each case any variation in positive cultures could then be ascribed to a poorly reactive individual or a suboptimal step in the experimental procedure.

The antibody titre in the plasma are known for each donor. These titres however indicate only that actively antibody secreting cells are present at some point within the individual and not necessarily circulating in the blood. Since only peripheral blood lymphocytes were used in these experiments, high circulating antibody level is not necessarily the best indicator of an appropriate donor, it was however the only marker available.

The binding of EBV is basically a chance event. This can however be optimised by varying the concentration of cells, virus etc. In the transformation experiments carried out here, growth always appeared in 100% of the wells seeded. Experiments were performed previously where wells were seeded with fewer cells. Results indicated that the number of wells with growing colonies quickly dropped as the seeding density was reduced. Not only did the number of colonies decline but so did the total number of wells secreting specific antibody. It was decided to sacrifice the possibilities of isolating the progeny of a single clone in the initial culture well in order to achieve an as high as possible number of positive cultures. This was done in the knowledge that cloning would then be vital, but it was decided that producing more lines would increase the chances of finally isolating a positive line.

This approach may again have been the wrong choice as it leaves open the question of whether or not the loss of antibody secretion was due to overgrowth by non-specific

lines. Here the failure to produce reasonable numbers of clones has had a particularly bad effect on the project as a whole. The failure to clone effectively reduces the chances of producing a monoclonal line by this method to zero.

Fusion of EBV transformed lines was carried out for two reasons. Firstly it was known that transformed cells had a tendency to stop antibody secretion. Secondly it had been shown that hybrids would clone where transformed cells would not. By fusing transformed cells it was hoped that antibody secretion would be "stabilised" and that from these hybrids the specific antibody secreting cells could be cloned.

The fusion technique used was standarised so that the actual technique varied as little as possible. Incubations and the addition of media were carefully timed so that they were reproducible. The polyethylene glycol used was always from the same source (the same container), and it had been shown in early experiments to produce reasonable rates of hybridisation (see for example GF3 and GF4).

Despite all of these steps the numbers of hybrids produced during any particular fusion varied considerably. For example, if a single EBV transformed line and a fusion partner were prepared for fusion and then divided into two aliquots, the number of hybrids produced in the two aliquots varies tremendously. This large variation makes direct comparison between fusions almost pointless. It is possible however to distinguish

trends; for example in a fusion experiment EBV transformed cells will almost certainly produce more hybrids than an identical fusion using non-transformed peripheral blood lymphocytes. However even such general statements do not always hold firm and there are some EBV transformed lines which fuse very poorly.

In order to avoid these problems a great deal more research is required into the physiology and mechanism of cellular hybridisation. It has been stated that cells fuse at some unknown point in their differentiation activation pathway (Schwaber et al.,1984) and the definition of this point should take a high priority in future research. The point in the cell cycle may also prove important and experiments could easily be designed to see if any correlation exists between these parameters and the efficiency of hybrid production.

6.3 THE FUTURE OF HUMAN MONOCLONAL ANTIBODY PRODUCTION

From the previous discussion it should be obvious that the production of human monoclonals is still very much a chance affair. There are several key areas which require further investigation if the efforts spent so far are not to be wasted. These areas are listed in table 25 and will be discussed in more detail below.

6.3a SUPPLY OF IMMUNE LYMPHOCYTES

The isolation of immune lymphocytes in an appropriate state of differentiation remains a major problem in human monoclonal antibody production. Improvements in this area could be affected by optimising immunisation

TABLE NO: 25. KEY AREAS FOR FUTURE RESEARCH IN HUMAN MONOCLONAL ANTIBODY PRODUCTION

Improved Immunisation strategies	In vivo In vitro
Selection of immune B cells	Tissue source Specificity Differentiation status Proliferative capacity
Immortalisation procedures	Optimisaion of existing technologies Improved B cell lines More efficient fusion procedures e.g. electrofusion Alternative immortalisation strategies e.g. transfection
Lymphocyte cloning techniques	Alternative cloning techniques e.g. micromanipulation, fluorescence activated cell sorter Use of recombinant growth and differentiation factors
Regulation of B cell growth	Growth factor effects Growth factor receptor expression
Regulation of immunoglobulin synthesis and secretion	Expression of immunoglobulin structural genes Role of regulatory genes
Production by recombinant techniques	Human antibody molecules Hybrid antibody molecules Mixed antibody protein molecules Site-directed antibody mutants

techniques either in vivo where appropriate or more especially in vitro. Selection procedures also require more research in order to maximise the isolation of specific B cells.

In situations where active immunisation is feasible we need to optimise injection and bleeding protocols. This will undoubtedly require a better understanding of the factors influencing the activation, proliferation, differentiation and circulation of committed B cell precursors. Where possible it would be interesting to discover whether adjuvants such as BCG can be used to increase the circulating pool of committed B cell precursors or their activated progeny as has been previously suggested (James and Bell, 1987).

No matter how much in vivo immunisation advances it is clear that ethics allow a limited number of antigens to be introduced in this manner. The future of human monoclonal antibody production will therefore depend to a large extent on the use of in vitro immunisation (Dorfmann, 1985; Borrebaeck, 1986). Initial advances in this field have shown that this approach permits the specific expansion of B cell precursors and the development of antigens against immunogens which cannot be safely administered in vivo (see for example Wasserman et al.,1986; Ho et al., 1987).

Of particular importance in the out come of both in vitro immunisation and immortalisation steps is the cellular composition of the lymphocyte suspension used. This in turn depends on the choice of tissue. As

previously described, lymph node and spleen have been shown to provide a better starting material than peripheral blood. The non B cell content is of particular importance for in vitro immunisation, with close cell-cell interactions having a major role in B cell activation. Viral transformation is also impaired by cytotoxic T cells and NK cells (James and Ritchie 1984; Kuwano et al.,1986). Further studies on the effects of removal or enrichment of specific cell phenotypes (such as T_C and NK) should lead to improvements in both in vitro immunisation and immortalisation techniques.

The use of cell sorters will greatly enhance the isolation of B cells of defined phenotype and may also be used to remove T_C and NK cells. Several methods have already been suggested (Casal et al.,1986, 1987) these involve mainly staining specific phenotypes with fluorescent monoclonal antibodies and then sorting the positively stained cells either to be used or to be discarded.

6.3b IMMORTALISATION PROCEDURES

The most widely used techniques of cellular immortalisation are EBV transformation an PEG facilitated cell fusion. In the immediate future this is almost certain to continue. Many refinements are possible in both techniques but much basic research is still required. There is still some debate as to which subset of B cells is immortalised by EBV. According to Chan et

al (1984) it is the large cycling population while Aman et al (1984) claim it is the small high density resting population, Roome and Reading (1987) provide evidence in support of Aman et al. It is vitally important that this question be resolved quickly as the optimal choice of tissue may vary depending on which cell population transforms most effectively.

The cell driven viral transformation described by Siadak and Lostrom (1985) also requires further evaluation. This technique which involves co-cultivation of normal peripheral blood lymphocytes with a HAT sensitive EBV transformed B cell line is reputed to be simple, reproducible and more efficient than conventional transformation and fusion and has been used by others to produce cell lines (Nugent et al., 1987). It was this type of reaction which was believed to have been responsible for the production of ES12 (Boyd et al, 1984).

The anti-HBs secreting cell line HB1 appears to have arisen through a similar type of spontaneous transformation. In previous experiments workers have shown that removal or inhibition of the T cells with cyclosporin A leads to the production of transformed lines. To establish if HB1 is indeed a transformed line it would be necessary to show that the EBV associated antigen was present within the cell. This could be done by a simple staining technique. It would also be preferable to produce a karyotype to assess which chromosomes were present.

As far as the fusion process is concerned, the most obvious limitation is the lack of a suitable non-secreting partner line. While much effort has been spent in developing new partners, none have so far been produced which can compare with the murine lines. What is encouraging is the success achieved so far with the mouse and particularly with the human/mouse fusion partners. As stressed earlier a number of investigators find that these lines fuse more efficiently than human partners. The resultant hybrids also clone more easily, secrete more antibody and have proved to be relatively stable.

One exciting prospect which has not been fully exploited as yet is the use of electrofusion as described by Zimmerman and Vienkin (1982) and Bischoff et al (1982). This technique is reputed to be 100,000 times more efficient than the PEG method (Dorfmann,1985) and has been used for the production of T-cell hybridomas (Gravekamp et al.,1985) as well as B-cell hybridomas. The possibility exists of using this technique for the specific fusion of extremely small numbers of cells.

A further enhancement of this technique is possible with the development of the avidin-biotin techniques for linking specific antibody forming B cells and myeloma cells prior to electrofusion (Lo et al.,1984; Wojchowski and Sytkowski 1986). The avidin-biotin linking has also been used to increase the yield of specific hybrids from a PEG induced fusion (Reason et al., 1987). It is

conceivable that in the near future procedures will be developed involving the fusion of single cells, followed by the expansion of the hybrid in carefully formulated media containing growth and differentiation factors produced by recombinant techniques. This procedure could take advantage of the sophisticated single cell procedures developed by Nossal and his colleagues (Nossal and Pike 1983; Pike et al.,1984).

With current immortalisation procedures cloning will remain a major problem. Transformed cells undoubtedly clone less well than hybrid cells and human hybrids less efficiently than their murine counterparts. If as suspected growth factors are limiting, then new supplies of highly purified growth factors and knowledge of their mode of action should be of enormous benefit.

In the meantime alternative cloning techniques other than limiting dilution and soft agar cloning are available. These include the micromanipulation of single cells (Thompson et al.,1986) and the use of fluorescent activated cell sorter based techniques (Shay 1985). These of course will remain of limited value so long as adequate growth media are lacking.

6.3c IMPROVING STABILITY AND ANTIBODY SECRETION

At the present time one of the major limiting factors in human monoclonal antibody production is the stability of antibody secretion by cell lines. The problems arise in transformed cells, hybrids and heterohybrids and are generally attributed to either overgrowth by non-secreting lymphocyte contaminants or the loss of

structural genes coding for immunoglobulin. Overgrowth is perhaps the most appropriate explanation in the case of transformed lines where lymphocytes are polyclonaly activated and cloning in most hands is difficult. Experiences here however, tend to support the view that the explanation is more complex than simple overgrowth.

Loss of regulatory genes is believed to be particularly relevant to heterohybrids which are regarded as being chromosomally unstable, preferentially segregating human chromosomes (Kozbor and Croce, 1985; Teng et al.,1985). In most cases the reason for the decline in antibody titre has not been investigated but merely noted and the lines abandoned. The synthesis of immunoglobulin is nearly always measured by assaying the supernatant for either specific or total immunoglobulin. These techniques totally ignore the possibility that there is a defect in the secretory mechanism of the cell.

The literature suggests that the apparent loss of immunoglobulin secretion observed with most cell lines together with the low levels secreted initially, can be attributed to a number of factors (see table 27). These include the chromosomal deletions mentioned above, shortages of growth factors or their receptors as well as faults in the synthetic and secretory machinery of the cell. Possible growth factor problems suggested include a lack of endogenous (Scala et al.,1987) or exogenous growth and differentiation factors (Winger et al.,1983) or their receptors (Melamed et al.,1985).

It should be noted that the problem with many human cell lines lies with antibody secretion and not with cell proliferation, so that some of the suggested growth factor problems may not be relevant. Furthermore attempts to improve antibody secretion by co-cultivation with HLA-DR matched specific T helper cells or antibody to HLA-DR, which is believed to substitute for T cell help, have not been successful (Crawford, 1985).

The problems of cell secretion and synthetic capability are believed to be largely related to cell phenotype. In particular changes within the cell such that they are deficient in rough endoplasmic reticulum, polyribosomes and golgi apparatus (Kozbor et al.,1983; Sikora et al.,1983; Kozbor and Croce, 1985).

Considerable efforts will be required to improve the levels of antibody secreted by human cell lines and to ensure that these levels are maintained over long periods. Improvements in immunisation, selection and immortalisation procedures may help to some extent, but it is likely that new methods will have to be developed. The most obvious approach is to frequently enrich specific antibody secreting lines and to pursue a rigorous cloning policy. The former has been successfully applied to lines secreting anti-red cell antibodies (Crawford et al.,1985; Doyle et al.,1985).

In view of the observations by Schwaber et al (1984) that cell lines which have stopped secreting immunoglobulin can be reactivated by fusion with normal peripheral blood lymphocytes or by stimulation with LPS

(Raison et al.,1982), it may be that other approaches are more appropriate. These observations which have been largely overlooked suggest that the failure of immunoglobulin secretion may be a consequence of defective regulatory genes or mechanisms concerned with synthesis and secretion of immunoglobulin.

Techniques which can enhance gene expression may now warrant further investigation. This may involve back fusion with peripheral blood lymphocytes as suggested above, stimulation with LPS or treatment with phorbol esters. The last approach has been shown to enhance receptor expression and immunoglobulin secretion by B cells (Polke et al.,1986) and is of considerable importance in view of recent suggestions that senescence in human antibody-secreting cell lines might be associated with a decline in receptor expression (Melamed et al.,1985). More sophisticated approaches could involve transfecting cells with specific enhancer sequences (Teng et al.,1985) or gene silencing techniques involving dimethylation of the genome (Ollson and Brams, 1985).

Whichever approach is adopted, a better understanding of the basic cause of senescence of antibody secretion is required. Once the cause has been identified, the appropriate corrective measures can more easily be adopted.

6.4 B CELL ACTIVATION

As previously implied, it is thought that the state of activation of B cells is a crucial factor in the success of immortalisation procedures. It is known for example that in the case of EBV transformation, small resting B cells are infected at a higher rate than large activated B cells (Roome and Reading, 1987). It seems that a better understanding of the B cell pathway is required before human monoclonal antibody production can progress much further.

A massive amount of research has been carried out into the control systems of B cell activation, differentiation and proliferation. This area has been reviewed by Hamaoka and Ono (1986), Melchers and Andersson (1986) and Paul and O'Hara (1987). The basis of the humoral (or antibody producing) immune response to antigen is the activation, proliferation and maturation of antigen specific B lymphocytes (Burnet, 1957). Basically antigen selects specific B cells from a pool of resting B cells. Once stimulated either in vitro or in vivo, the cells begin to divide and mature. It is the cell contacts and the soluble factors controlling this process which are vital.

Three cell types are involved in the production of a B cell response, these being Accessory cells (A cells), T cells and of course B cells. One of the early events after antigen is added to the system is its uptake by cells of the reticulo-endothelial system. These cells endocytose, digest and present antigen on their cell

surface in association with MHC class II antigens. The antigen MHC complex is recognised by T cells of the helper/enducer subset. Many cell types can act as A cells for T cells, all of which express MHC class II on their surface and have the ability to "process" antigen. Macrophage process and present any antigen to T_H cells while B cells use their antigen receptors to present "specific" antigen to T_H cells. As a result of this, antigen specific B cells require much lower (up to 10^4 times) concentrations of antigen for presentation (Rock et al.,1984; Lanzavecchia 1985; Tony and Parker, 1985).

One result of the interaction of A cells and T cells is the production of lymphokines that act on T and B cells to stimulate proliferation and maturation. Media conditioned by these interactions can serve to stimulate antigen specific B cell responses in vitro. Since this occurs even in T cell depleted B cell populations the factors have been termed T cell replacing factors (Dutton et al.,1971; Schimpl and Wecker, 1972). Considerable confusion exists as to which lymphokines are active on B cells and which cells produce which lymphokines.

It appears that the lymphokines can be broken into several groups, α factors, β factors and maturation factors (Melchers and Lernhardt, 1985). The factors are generally detected using one of three assay systems; the proliferation of J558 plasmacytoma cells that require α factors (Corbel and Melchers, 1984), the proliferation of A cell depleted B cell blasts (Melchers and Lernhardt,

1985) and the activation of A cells depleted resting B cells into the cell cycle (Corbel and Melchers, 1983). When B cells are activated by α factors, polyclonal activators such as LPS are required to complete the cell cycle.

It has been shown that α factors control the entry onto S phase at a restriction point some 4-6h after mitosis in the G_1 phase within the 20h cell cycle (Melchers and Lernhardt, 1985). The effect of α factors can be inhibited by the soluble complement component C3d (Melchers et al.,1985). However, C3b or C3d either aggregated or sepharose bound, replaces the effects of factors in the B cell cycle (Erdei et al.,1985; Melchers et al.,1985).

The finding that soluble C3d is inhibitory while aggregated C3, C3b or C3d is stimulatory raises the question why soluble factors are stimulatory if they are in fact complement components. It has been suggested (Melchers and Andersson, 1986) that the microaggregation of CR2 receptors could be achieved by C3b interacting with other complement components such as C4b and C2a thus providing a positive signal for B cells.

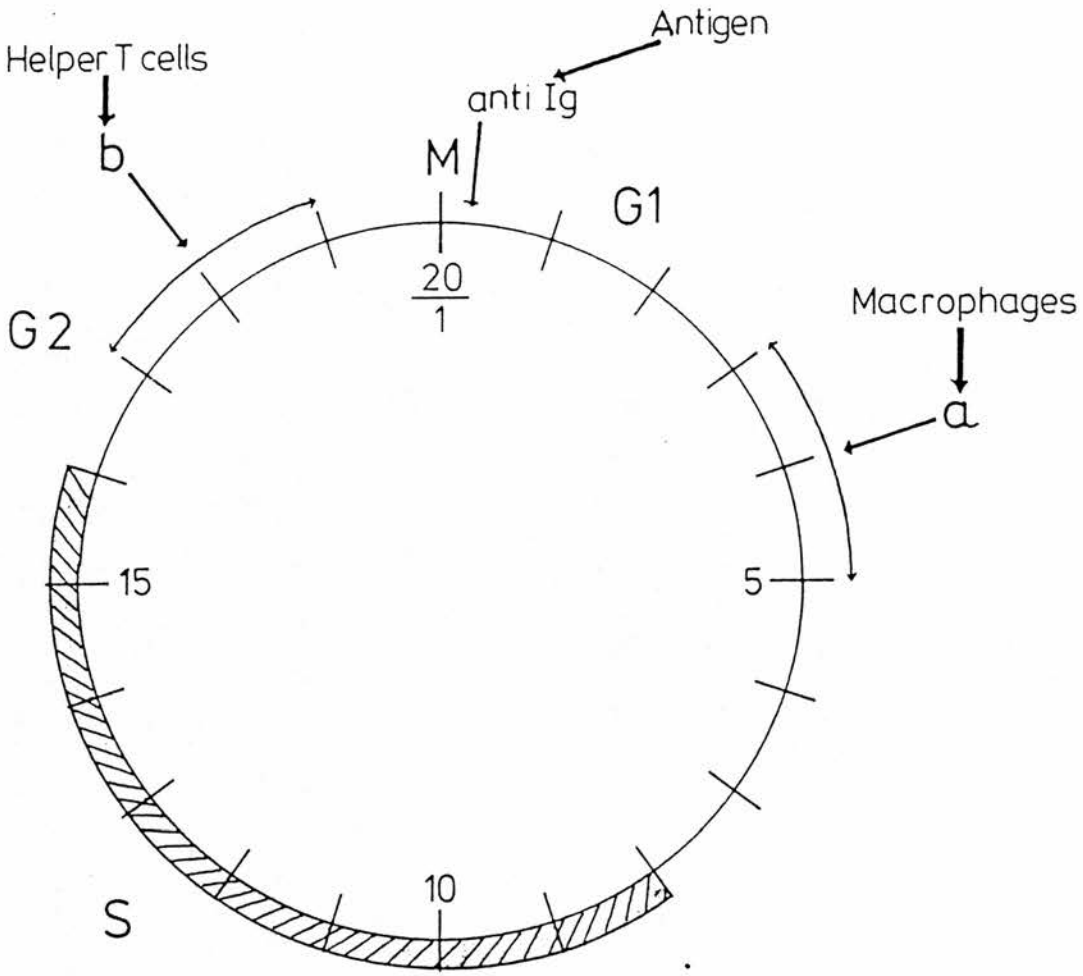
It is interesting to note at this point that EBV, which stimulates resting but not activated B cells (Roome and Reading, 1987) enters the human B cell via the CR2 receptor (Aman et al.,1984; Nadler et al.,1985). This route of entry may provide a clue as to how B cells are activated by EBV. The factor stimulated entry of the B cells into S phase provides the DNA synthetic activity

which would be required for the incorporation of viral DNA into the genome. It is not known however whether this is also a requirement for subsequent immortalisation.

Many growth factors are produced by T cells in response to stimulation by antigen in association with MHC. One well characterised factor is BSF-1 (Howard et al.,1982) recently designated IL-4. This growth factor has been shown to be different from IL-1, IL-2 and IL-3 (O'Hara et al.,1985; Takatou et al.,1985). Many other T cell derived growth factors have been described which stimulate proliferation and/or maturation of B cells. These include BCGF-II, TRF, BCDF and IL-2 (Melchers and Lernhardt, 1985). These factors appear to act on B cells at a point late in the cell cycle in the G_2 phase at a restriction point 2-4h before mitosis (Melchers and Lernhardt, 1985).

It seems therefore that several stimuli are required for any particular round of cell division in the normal B cell. Fig 7 shows the points at which the various factors have their effect. There are three restriction points controlled by Ig specific antibodies; α factors (from macrophages) and β factors (from T_H cells). It is likely that cells immortalised either by viral transformation or cell fusion will still require some growth factors. Much work is still required to define which factors are required by hybrids and transformed cells and at what point they can most usefully be

FIGURE 7



The 20h cell cycle of activated murine B lymphocytes, divided into 8h of G1-phase, followed by 8h of S-phase, then by 4h of G2-phase and finally by mitosis (M). In this cycle are placed the three restriction points controlled by Ig-specific antibodies (anti-Ig); α factors (a); and β factors (b). α Indicates the point of influence of macrophages, β that of helper T lymphocytes and anti-Ig that of antigen in immune responses of B cells. Reproduced from Melchers and Andersson, 1986.

employed. It is hoped that in the near future pure growth factors will be made available from recombinant sources. These ultra pure reagents should greatly aid research in this particular area of cell biology.

6.5 THE EXPANSION OF HUMAN MONOCLONAL CELL LINES

If human monoclonal antibodies are to be used on a commercial basis, then large volumes must be grown. The approaches used have generally followed those used in murine monoclonals. They include a variety of in vitro culture procedures together with growth in immunodeficient mice or rats. Where relatively small amounts of antibody are required then growth in static culture is perfectly adequate and routinely used. Cell lines have been successfully expanded using spinner flasks (Hunter et al.,1982; Thompson et al.,1986) and roller bottles (Wunderlich et al.,1981; Sikora et al.,1983; Thompson et al.,1986). Others have grown human cell lines in immobilised form (Haspel et al.,1985a,b). In most cases if larger volumes are required then air lift reactors and immobilised cell techniques are the methods of choice. These procedures have been reviewed recently (Birch et al.,1985; Duff, 1985; Altschuler et al.,1986).

The production of large volumes of cell supernatant inevitably involves the investment of large amounts of cash, especially if FCS is to be used as a supplement. To try to avoid this cost several defined serum-free media have been developed specifically for the culture of human and mouse cell lines. While experience with these

media are as yet limited, it is interesting to note that there are reports which suggest that cell lines will produce at least as much antibody when grown in serum free medium as they do in high concentrations of FCS (Cole et al.,1985b; Glassy et al.,1985; Teng et al.,1985; Shacter et al., 1987). This has lead to suggestions that FCS may contain factors which impair immunoglobulin production (Glassy et al.,1985). It should be noted at this point that none of the cell lines produced during the course of this project would adapt to growth in serum free medium. Growth was apparent at 1-2% FCS but complete removal effectively stopped cell division.

There are several reports of attempts to grow human monoclonals in nude mice. From these it is apparent that human/human hybridomas do not grow readily in nude mice in spite of their T cell deficiency. This is perhaps not surprising in view of the fact that such mice are capable of reacting xenogeneic cells by NK and macrophage mediated mechanisms. A higher success rate of ascites production can be obtained by irradiating (350-400 rad) the nude mice prior to innoculation (Bogard et al.,1985; Kozbor et al.,1985), by adapting the cells to in vivo growth (Kozbor et al.,1985) or by mixing the cells with human skin fibroblasts prior to innoculation (Ollson and Brams, 1985). Even with these procedures the take may be as low as 50% (Bogard et al.,1985; Kozbor et al.,1985) or depend on the route of injection (Ollson and Brams,

1985).

Heterohybrids appear to grow more easily in mice, several reports suggest such cells will grow without prior adaptation (Bron et al.,1984; Insel et al.,1984; Burnet et al., 1985; Tiebout et al., 1985; Yamoura et al.,1985). This observation is perhaps not surprising in the light of previous observations that human/mouse B cell hybrids express the H-2 haplotype of the murine parent but fail to express the human HLA antigen which would evoke an immune rejection (Raison et al.,1982).

There have been conflicting reports on the amount of antibody produced in the ascites. According to Kozbor et al (1985) cells which were adapted for growth in nude mice by in vivo passage showed an increase in both tumorigenicity and immunoglobulin production. These properties were associated with a change in cell morphology. Return to in vitro culture showed that this change was transient and reversible. The differentiation observed was attributed to growth factors released during the inflammatory process induced by pristane treatment. In contrast others have observed that hybridomas adapted for growth in ascites do not secrete large amounts of immunoglobulin (Ollson and Brams, 1985).

When the monoclonal antibodies in question are required for prophylactic, therapeutic and diagnostic purposes (such as imaging), it is highly unlikely that production via ascitic fluid will be considered appropriate. Since the mice used may harbour unknown viral infections it would seem prudent not to use ascitic

fluid (or antibodies derived from it) for in vivo therapies. It is almost inevitable therefore that the majority of human monoclonal antibody production will depend on bulk culture either in air lift fermenters or immobilised cell systems.

6.6 MOLECULAR BIOLOGY AND MONOCLONAL ANTIBODY PRODUCTION

So far all of the discussion has concentrated on conventional methods of monoclonal antibody production. There are however other approaches which are being investigated, namely those involving genetic engineering techniques. It should be stressed that these techniques have already been successfully applied to the cloning of the immunoglobulin genes themselves. Although there are still some problems to be overcome, the achievements to date are extremely encouraging and worthy of discussion for they open up a the way to establishing stable cell lines secreting antibodies with unique properties (Aguila et al.,1986).

To date complete immunoglobulin molecules have been produced by expressing mouse H and L chains in E coli (Boss et al.,1984), yeast (Wood et al.,1985) and myeloma lines (Neuberger, 1985) using conventional recombinant procedures.

A less sophisticated approach has been to either transfect antibody secreting cells with calcium-precipitated DNA from a permanent cell line (Jonak et al.,1984) or alternatively, permanent cell lines with similar preparations from antibody producing cell lines

(Srelkauskas et al.,1987).

There are also reports on the cloning and expression of genes coding for hybrid molecules containing mouse variable and human constant region domains. This approach, which has recently been reviewed by Oi and Morrison (1986), enables the production of "humanised" monoclonals against immunogens which cannot be injected into humans or which fail to produce a good immune response either in vitro or in vivo.

The genes for these hybrid immunoglobulins have been introduced into myeloma cells by both spheroplast fusion (Jones et al.,1986; Sharon et al.,1986) and electroporation techniques (Sahagon et al.,1986). The immunoglobulin produced by these recombinants retains the specificity of the original mouse antibody. With the human heavy chain domains however, it will be intrinsically less immunogenic in man than the original mouse antibody. Using this approach chimeric antibodies have been produced against haptens (Boulianne et al.,1984), tumour antigens (Sahagan et al.,1986) and other antigens (Takeda et al.,1985).

More recently the potential of the technology has been further advanced with Jones et al (1986) replacing the genes coding for the hypervariable region of the the H chain with genes coding for the hypervariable region of a murine monoclonal heavy chain. The product of this procedure is an almost entirely human immunoglobulin molecule containing the specific binding regions of a murine antibody. Exploitation of this type of approach

should lead to hybrid molecules of even weaker immunogenicity in humans.

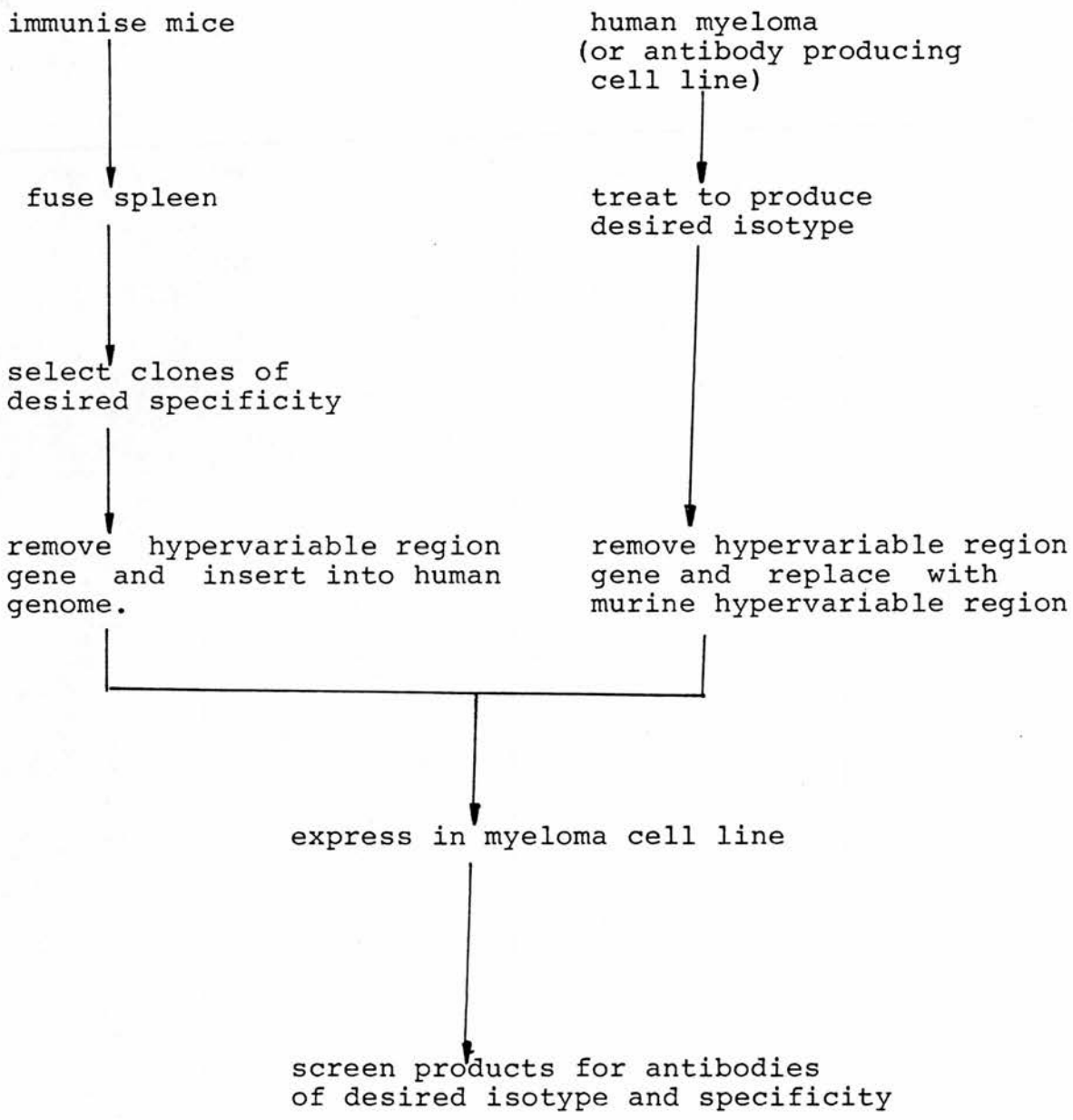
Sharon et al (1986) have shown that site directed mutagenesis can be used to alter the specificity and idiotype expression of murine monoclonals. This approach involves the insertion of specific oligonucleotide sequences into heavy chain variable region genes and could enable the development of antibodies with novel specificities.

The potential of this technique for the production of antibodies with novel effector functions should not be overlooked. Thus Neuberger and his colleagues (Neuberger et al.,1984; Neuberger, 1985) have expressed in myeloma cells genes coding for the $F(ab)_2$ region of mouse immunoglobulin linked to genes for either enzymes or c-myc antigenic determinants. Human constructs of this type could prove extremely useful in diagnosis, targeting and imaging.

Fig 8 shows a flow chart of how a molecular biology approach could be used to produce a human monoclonal antibody of desired specificity. This approach combines the conventional murine monoclonal production with genetic manipulation. Murine monoclonals are relatively easy to produce so several clones could be tested and one with appropriate properties selected. Human cell lines secreting non-specific immunoglobulin are also relatively simple to produce and so would be available as the source of the "human" part of the molecule.

FIGURE 8

FLOW DIAGRAM SHOWING POSSIBLE MOLECULAR BIOLOGICAL APPROACH TO MONOCLONAL ANTIBODY PRODUCTION



6.7 REGULATORY CONSIDERATIONS FOR HUMAN MONOCLONAL ANTIBODY USE IN THERAPY AND DIAGNOSTICS

Work is proceeding at a great pace developing both human and murine monoclonal antibodies and novel suggestions as to how they may be used are regularly proposed. In the rush to produce new therapeutic and diagnostic tools however, many workers fail to mention the strict regulations controlling the purity and specificity of any new pharmaceutical reagent. Some of these conditions will be discussed briefly below.

Monoclonal antibodies are derived from growing cells which are in turn derived from immune individuals. They can therefore be regarded as "natural" products, which might imply some degree of safety from side effects.

One of the major arguments for producing human monoclonal antibodies as opposed to murine is that when used for prophylactic purposes they would be less likely to be antigenic and so would avoid problems of sensitisation. This fact has yet to be shown in vivo and as humans are not in bred in the same way as the murine models used to establish these theories, there remains the slight chance that sensitisation may occur. Serum products however, are already widely used and it seems unlikely that monoclonals would fail any tests on these grounds.

In most cases in vivo use is envisaged mainly for life threatening diseases. These patients are normally already immunocompromised to some extent and are therefore highly susceptible to any viral particles which

may be present within the final antibody preparation. It should be remembered that all human monoclonal antibodies from either transformed lines or hybridomas are produced by cells which are in fact tumours. There is a possibility that a tumorigenic agent may be present in the culture supernatant. It would be necessary to show that the final product was free of any genetic material. Other possible viral contaminants which are already defined such as HTLV-1, HIV and HB would also be required to be removed during processing.

Even for in vitro diagnostic use where the requirements of safety are not as harsh, the efficacy would need to be shown. These evaluation tests are based on considerations of specificity, sensitivity, stability, reproducability and clinical correlation. It is important to determine the role of clinical factors such as age, sex, genetics, nutrition, geography and concomitant medications (Schauf, 1987).

When a new product is licensed, it is not only the final product which is assessed, but the entire process. The cell line producing the antibody and any fusion partners and possibly the lymphocyte donor may require testing for harmful viruses. The purification process would be required to show its efficiency by removing a standard amount of virus deliberately added to the raw material. The final packaged material would need to show stability and continued sterility.

All of the above tests take a great deal of time and

are extremely expensive. It seems most likely therefore that only large companies with the resources and experience of running clinical trials will be able to produce an end product for the market. It can only be hoped that they will adopt antibodies which will have beneficial effects clinically and not only those which will provide huge profits.

6.8 HUMAN MONOCLONAL ANTIBODIES PRODUCED

As stated previously many workers have attempted to produce human monoclonal antibodies against a wide range of antigens. Despite all of the problems described here antibodies have been produced against a wide range of antigens. Appendix D gives a list of some of the antibodies produced so far and although by it is by no means a complete list, it gives a fair indication of which types of antigen are recieving the most attention.

6.9 CONCLUDING DISCUSSION

Research into the production of human monoclonal antibodies continues apace. It should be obvious that there are many problem areas which still require much basic research. However despite these problems there remains an overall optimism that human monoclonals will soon be routinely produced. The potential uses of human monoclonals are many and varied and the knowledge which will be gained from this research is immense.

It seems likely that if in a few years the problems of genetic instability and low levels of antibody secretion are not overcome by improvements in tissue culture, then the molecular biologists will take over. Feeder systems

and growth factors will require close investigation and this can only improve our understanding of the immune system as a whole. It is almost certain that the area of in vitro immunisation will produce information leading to a greater understanding of how immune cells regulate their functions and which cells are important in the initiation of an immune response and the production of immunological memory.

Viral transformation experiments will enable us to dissect the immune response and perhaps shed more light on the mechanisms involved in immune tolerance in general and autoimmunity in particular. Studies on transformed cells which lose antibody secretion may further elucidate the control mechanisms for secretion. Antibodies binding to "tumour specific" antigen may help us to understand the mechanisms underlying neoplasias. Finally any human monoclonal antibodies produced will have immense clinical or diagnostic potential.

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APPENDICES

APPENDIX A

SUPPLIERS OF EQUIPMENT

BDH CHEMICALS LTD,
Burnfield Avenue,
Thornlebank,
Glasgow.

DAKO LTD,
22 The Arcade,
The Octagon,
High Wycombe,
Bucks.

DIAMED DIAGNOSTICS LTD,
Mast House,
Derby Road,
Bootle.

DYNATECH LABORATORIES LTD,
Daux Road,
Billingshurst,
Sussex.

EDINURGH AND SOUTH EAST SCOTLAND,
BLOOD TRANSFUSION SERVICE
Lauriston Road,
Edinburgh.

FLOW LABS LTD,
Woodcock hill,
Harefield road,
Rickmansworth,
Herts.

GELMAN SCIENCES LTD,
10 Harrowden Road,
Brackmills,
Northampton.

GIBCO LIFE SCIENCES LTD,
PO 35 Abbotsinch Ind. Estate,
Paisley.

HOESCHT (UK) LTD,
50 Salisbury Road,
Hounslow,
Middlesex.

NORTHUMBRIA BIOLOGICALS LTD,
South Nelson Ind. Estate,
Camlington.

SIGMA CHEMICAL CO.
Fancy Road,
Poole,
Dorset.

SCOTTISH ANTIBODY PRODUCTION UNIT,
Law Hospital,
Carluke,
Scotland.

UNIPATH
co OXOID
Wade Road
Basingstoke

WELLCOME FOUNDATION,
Langley Court,
Beckenham,
Kent.

APPENDIX B

BUFFER SOLUTIONS

1. Antibody Diluting Buffer
   (Phosphate buffered saline + 1% BSA)

   0.145M NaCl,
   0.018M K_2HPO_4
   0.01M KH_2PO_4
   1% (v/v BSA)
   pH7.4

2. Plate Coating Buffer

   0.05M $NaHCO_3$
   pH 9.6

3. ELISA Wash Buffer

   0.9% (w/v) NaCl
   0.05% Tween 20
   pH 7.4

4. ELISA Substrate Buffer

   0.05M Na_2CO_3
   0.001M $MgCl_2$
   0.1M NaN_3
   pH 9.8

5. Tris Buffered Saline

   Was prepared using 0.85M NaCl buffered with Tris-HCl
   pH 7.6, 0.01M

   working solution:

   dissolve 17g NaCl in

   100ml 0.2M Tris
   157ml 0.1N HCl

   Then add distilled water to a final volume of 2l

APPENDIX C

RESULTS OF FUSION AND TRANSFORMATION EXPERIMENTS

Expt. No.	partner	antigen	wells seeded	wells growing	wells +
1	KR4	TT	30	0	0
2	KR4	TT	30	0	0
3	NS0	TT	60	60	57
4	NS0	TT	75	16	5
5	KR4	TT	360	16	10
6	NSO	TT	360	360	357
7	Hmy2	TT	360	264	264
8	KR4	TT	360	0	0
9	NS0	TT	360	54	7
10	Hmy2	HBs	288	63	0
11	NS0	HBS	315	48	0
12	Hmy2	HBs	96	87	0
13	UC-7296	HBs	300	0	0
14	Hmy2	HBs	96	54	1
15	Hmy2	HBs	96	1	0
16	Hmy2	HBs	96	2	0
17	Hmy2	HBs	96	2	0
18	Hmy2	HBs	96	0	0
19	Hmy2	HBs	96	0	0
20	Hmy2	HBs	300	59	0
21	Hmy2	HBs	720	9	0
22	Hmy2	HBs	240	20	0
23	Hmy2	HBs	120	28	1
24	NS0	HBs	120	81	1
25	NS0	HBs	240	235	0
26	NS0	HBs	120	102	0
27	Hmy2	HBs	120	53	0
28	Hmy2	HBs	120	30	0
29	UC-7296	none	300	0	-
30	KR4	HBs	380	0	0
31	KR4	HBs	20	0	0
32	Transf	HBs	600	600	59
33	Hmy2	HBs	210	130	0
34	Hmy2	HBs	60	44	0
35	Hmy2	HBs	60	41	0
36	Hmy2	HBs	60	10	0
37	Hmy2	HBs	60	37	0
38	Transf	HBs	768	768	75
39	Transf	HBs	flask ⟶	fusions	46+47
40	Hmy2	HBs	24	18	0
41	Hmy2	HBs	34	34	1
42	Hmy2	HBs	40	36	0
43	Hmy2	HBs	43	33	0
44	Hmy2	HBs	60	1	0
45	Hmy2	HBs	33	27	0
46	Hmy2	HBs	230	119	0
47	NSO	HBs	120	0	0

Expt.No.	Partner	antigen	Wells seeded	wells growing	wells +
48	PBL	HBs	240	?	
49	PBL	HBs	240	?	4
50	NS0	HBs	180	4	0
51	NS0	HBs	107	13	0
52	NS0	HBs	215	133	27
53	NS0	HBs	86	3	0
54	Transf	HBs	470	470	17
54a	NS0	HBs	108	3	0
55	NS0	HBs	60	Contaminated	
56	Transf	HBs	420	420	0
57	mouse spleen	HBs	720	?	0
58	NS0	HBs	600	60	Contam
59	Transf	HBs	480	480	9
60	NS0	HBs	40	37	0
61	NS0	HBs	85	23	Contam
62	KR12	HBs	60	51	Contam
63	NS0	HBs	600	20	0
63a	KR12	HBs	60	0	0
64	NS0	kell	130	103	0
65	P653	kell	120	23	Contam
66	NS0	kell	240	Contaminated	
67	P653	kell	240	Contaminated	
68	NS0	Brain	300	48	20

APPENDIX D.

A SURVEY OF HUMAN MONOCLONAL ANTIBODIES

TARGET	REFERENCES
Bacterial Antigens	
Diptheria toxin	Gigliotti et al.
Gm-ve endotoxin	Teng et al. 1983; Teng et al. 1985a, Feely et al. 1987
Endotoxin A	Larrick et al. 1985
Haemophilus influenzae	Hunter et al. 1982; Gigliotti et al. 1984; Brodeur et al. 1987
-Lipid A-core	Pollack et al. 1987
Mycobacterium leprae	Atlaw et al. 1985; Foung et al. 1985a
Neisseria meningitides	Brodeur et al. 1987
Pneunomoccus poly-saccharide	Schwaber et al. 1984
Pseudomonas auruginosa	Siadak and Lostrom 1985; Sawada et al. 1985
Tetanus toxoid	Zurawski et al. 1978; Kozbor and Roder 1981, 1982; Chiorazzi et al. 1982; Teng et al. 1983; Larrick et al. 1983; Tiebout et al. 1984, 1985; Boyd et al. 1984b; Ho et al. 1985; Ichimori et al. 1985; Mirata and Sugawara, 1987
Tetanus toxin	Gigliotti and Insel 1982
Chlamydia	Rosen et al. 1983
Viral Antigens	
Cytomegalovirus	Emanuel et al. 1984; Matsumoto et al. 1986; Redmund et al. 1986; Masuho et al. 1987
Epstein-Barr	Koizumi et al. 1986
Hepatitis A	Van Meel et al. 1985

Appendix D (cont'd)

Hepatitis Bs	Burnett et al. 1985; Ichimori et al. 1985, 1987; Van Meel et al. 1985; Stricker et al. 1985
Hepatitis Bc	Furuya et al.1982
Herpes simplex	Seigneurin et al. 1983; Evans et al. 1984; Masuho et al. 1986
HTLV-1	Matsushita et al. 1986
Influenzae virus nucleoprotein	Crawford et al. 1983b; Ostberg and Pursch 1983
Rubella	Croce et al. 1980; Ritts et al. 1983; Van Meel et al. 1985; Hilfenhaus et al. 1986
Rabies	Van Meel et al. 1985
T cell leukaemia	Matsushita et al. 1986
Varicella zoster	Foung et al. 1985a and b, Sugano et al. 1987

Other Infectious Agents

Plasmodium falciparum	Schmidt-Ullrich 1986; Udomsangpetch et al. 1986

Red Cell and White Cell Antigens

Blood group A	Foung et al. 1985a; Larrick et al. 1985; Raubitschek et al. 1985; Goosens et al. 1987
Blood group i	Hirohashi et al. 1986
Rhesus D	Boylston et al. 1980; Astaldi et al. 1982; Crawford et al. 1983a; Bron et al. 1984; Doyle et al. 1985; Melamed et al. 1985; Paire et al. 1986; Thompson et al. 1986; Boyd et al. 1987; Goosens et al. 1987; MacDonald et al. 1987
Rhesus G	Foung et al. 1986
Forsmann	Nowinski et al. 1980
Sheep red blood cells	Strike et al. 1984; Terashima et al. 1987
Chicken red blood cells	Winger et al. 1983

Appendix D (cont'd)

HLA	Hulette et al. 1985; Effross et al. 1986
T cell	Alpert et al. 1987

Tumour Antigens

Acute lymphocytic leukaemia	Nishimura et al. 1987
Bladder	Paulie et al. 1984
Brain glioma	Sikora et al. 1982,1983
Breast	Wunderlich et al. 1981; Cote et al. 1983,1984, 1985,1986; Campbell et al. 1986; Strelkauskas et al. 1987a; Schoenfield et al. 1987
Chronic lymphocytic leukaemia	Abrams et al. 1984
Colorectal carcinoma	Hirohashi et al. 1982b; Haspel et al. 1985a,b; Borup-Christensen et al. 1986; Shaw et al. 1987
Gastric carcinoma	Hirohashi et al. 1982b; Sugiyama et al. 1986
Lung carcinoma	Hirohashi et al. 1982a; Cote et al. 1983,1984, 1985,1986; Cole et al. 1984,1985a; Murakami et al. 1985
Lymphoma	Carroll et al. 1986
Melanoma	Irie et al. 1982; Warenius et al. 1983; Watson et al. 1983; Kan-Mitchell et al. 1986; Yamaguchi et al. 1987.
Myloid leukaemia	Andreasen and Olsson 1986
Ovarian carcinoma	Al-Azzawi et al. 1987
Renal carcinoma	Cote et al. 1983, 1984, 1985, 1986

Autoantigens

Astrocytes	Simpson et al. 1986
Cytoskeletal proteins (actin, myosin, tubulin)	Cote et al. 1983,1986; Cote and Houghton 1985

Appendix D (cont'd)

DNA (ss and ds)	Shonfield et al. 1982; Teng et al. 1983; Massicote et al. 1984; Sasaki et al. 1984, 1985; Casali et al. 1987
Endothelial cells	Hamburger et al. 1985
Factor Xa	Annamalai et al. 1987
Glial fibrilary acid protein	Cote and Houghton 1985
Golgi	Cote et al. 1986
Myelin associated glycoprotein	Murray et al. 1984, Spatz et al. 1987
Nuclear antigens	Satoh et al. 1983; Someya and Kondo 1986; Simpson et al. 1986; Cote et al. 1986
Nucleolar antigens	Simpson et al. 1986; Cote et al. 1986; Chiorazzi and Reeves 1987
Nerve axons	Garzelli et al. 1984
Neurones	Simpson et al. 1986
Neurofibrillary plaques	Simpson et al. 1986
Neurofibrillary tangles	Simpson et al. 1986
Pancreatic islet cells	Eisenbarth et al. 1982; Satoh et al. 1983; Garzelli et al. 1984
Pancreatic duct cells	Satoh et al. 1983; Someya and Kondo 1986
Parathyroid gland	Garzelli et al. 1984
Pituitary	Satoh et al. 1983
Platelets	Shonfield et al. 1982; Nugent et al. 1987
Polynucleotide	Hashizuma et al. 1987
Prostatic acid phosphatase	Yamoura et al. 1985
Ribosomal RNA	Teng et al. 1983
Sperm	Winger et al. 1983; Isojima et al. 1987
Sperm coating protein	Kyurchiev et al. 1986

Appendix D (cont'd)

Stratified squamous epithelium	Garzelli *et al.* 1984
Thyroglobulin	Dighiero *et al.* 1983
Thyroid antigen	Tan *et al.* 1987
Thyroid follicle	Satoh *et al.* 1983
Vimentin	Cote and Houghton 1985
Rheumatoid factor	Haskard and Archer 1984

Others

Bombesin	Ho *et al.* 1985
2-4 dinitrochloro-benzene	Olsson and Haplan 1980
2-4 dinitrophenol	Teng *et al.* 1983; Bieber *et al.* 1987
4 hydroxy 3-5 dinitro-phenacetic acid	Steinitz *et al.* 1977,1979
Keyhold limpet haemocyanin	Lane *et al.* 1982; Bieber and Teng 1987
Phosphorylcholine	Yoshie and Ono 1980
Sperm whale myoglobin	Bieber and Teng 1987

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Review article

Human monoclonal antibody production

Current status and future prospects

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Introduction

Since the first description of monoclonal antibody secreting cell lines by Köhler and Milstein in 1975, rodent monoclonal antibody technology has been rapidly and successfully applied to a wide variety of biological problems of both theoretical and practical importance. In the health care field for example, this has resulted both in the development of new and improved diagnostic and therapeutic procedures and an increase in our understanding of human biology. Unfortunately progress in the human monoclonal antibody field has been far less spectacular. Although immortalised human cell lines secreting specific antibody were described as long ago as 1977 and their potential immediately recognised (Steinitz et al., 1977) the development of human monoclonal antibody technology has been a slow, laborious and often unrewarding exercise (see later). Nevertheless in spite of the many problems encountered to date work has continued unabated, sustained by the widely held belief that this technology would result in improved diagnostics and therapeutics and would increase our knowledge of the B cell repertoire in health and disease.

While many technical problems still remain to be solved, sufficient progress has been made to encourage further efforts in this field. The aim of the present review is to evaluate the main approaches which have been used to date in human monoclonal antibody production with the view to establishing if there are any general guidelines suggesting possible ways in which the technology might be improved in the future. Throughout this discussion the emphasis will be on general technical and theoretical principles rather than on precise technical details which can be obtained from the publications cited and a number of recent reviews (Kozbor and Roder, 1983; Boyd et al. 1984a). For those intending to venture into this field the recent book edited by Engleman et al. (1985) is highly recommended as it contains a detailed methodological appendix.

The principal areas to be covered in this review will be the procurement of sensitised lymphocytes for immortalisation, the immortalisation strategies themselves, procedures for cloning and expanding antibody-secreting cell lines and future technologies. In addition, the potential of human monoclonal antibody technology will be illustrated by a survey of the range of human monoclonal antibodies currently available together with a summary

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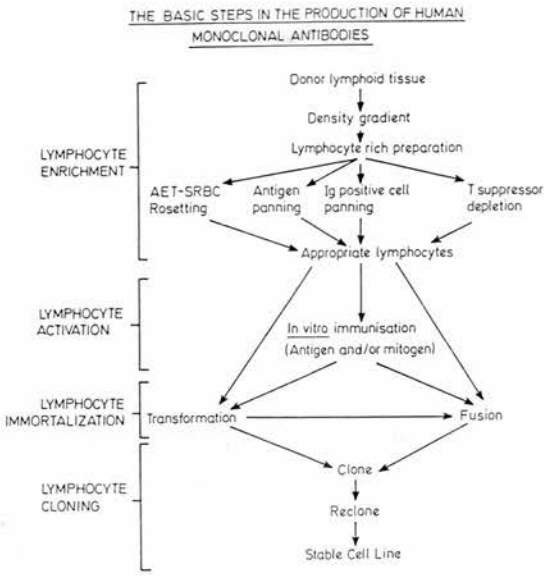

Fig. 1. This figure outlines the key steps which can be involved in the production of human monoclonal antibodies. It should be noted that some of the steps, though desirable, are optional. These include the enrichment of specific antibody-forming cell precursors and in vitro stimulation with antigen and/or mitogen.

of their possible applications. However, in order to set the background to this review the basic steps currently employed in human monoclonal antibody production have been summarised in Fig. 1.

Sources of immune lymphocytes for immortalisation

In vivo immunisation

Generally speaking the successful development of murine monoclonals has not been limited by the availability of immune lymphocytes. Providing the substance to be injected was available in adequate amounts, was non-toxic and immunogenic, immunisation schedules could be optimised to ensure sufficient immune cells for fusion. Unfortunately this is not the case with human monoclonal antibody production where the range of immunogens which can be injected, together with the immunisation schedules which can be employed is limited by ethical and other considerations. Furthermore, the source of immune lymphocytes is usually restricted to peripheral blood, which, as will be discussed later, is not the ideal choice. These shortages, together with our lack of knowledge on what is the ideal differentiation stage for fusion (Schwaber et al., 1984) are believed by some to have been neglected in a frantic search to obtain improved fusion partners (Olsson et al., 1983; Gigliotti et al., 1984).

Although these practical and theoretical problems have hampered human monoclonal antibody development lymphocytes specifically immune to a wide range of target antigens can, and have been, readily obtained from a variety of sources. These include donors who have been immunised following approved schedules or as a result of disease processes or inadvertent exposure to immunogens. In addition monoclonal antibodies with a wide range of interesting specificities have been obtained using lymphocytes from individuals with no previous record of exposure to the immunogen in question or history of disease. These various approaches are summarised in Table I and discussed in greater detail below.

As is to be expected monoclonal antibodies to bacteria and viruses comprise the majority of antibodies which have been produced following active immunisation, though some have been generated using lymphocytes obtained following natural infection. However, monoclonal antibodies to a variety of other immunogens have been obtained using lymphocytes from actively immunised individuals. These include monoclonals to colorectal cancer, Rhesus D, HLA antigens and various haptens (Table I).

It will also come as no surprise that the great majority of antibodies generated using lymphocytes from people with active disease are directed against tumour cell targets and various autoantigens. However, some antibodies against infectious agents have also been produced using lymphoid tissues from individuals with ongoing disease. Finally lymphocytes from 'non-immunised', 'normal' donors have frequently given rise to autoantibodies reacting with a variety of cell surface and internal antigens.

In the light of experience with mouse monoclonal antibody production it is reasonable to assume that the outcome of human immortalisation procedures is influenced by the interval be-

TABLE I

IN VIVO IMMUNISATION PROCEDURES EMPLOYED IN HUMAN MONOCLONAL ANTIBODY PRODUCTION

Immunisation strategy	Immunogen category	Examples	References
Natural infection or inadvertent exposure	Bacterial	Capsular polysaccharide	Gigliotti et al., 1984
		Haemophilus influenzae (type B)	Hunter et al., 1982
		Phosphorylcholine	Yoshie et al., 1980
		Pseudomonas aeruginosa	Siadak and Lostrom, 1985
		Purified protein derivative	Garzelli et al., 1986
		Chlamydia	Rosen et al., 1983
	Viral	Cytomegalovirus	Emanuel et al., 1984; Redmund et al., 1986
		Epstein-Barr virus	Koizumi et al., 1986
		Hepatitis A	Van Meel et al., 1985
		Hepatitis B	Stricker et al., 1985
		HTLV-1	Matsushita et al., 1986
		Influenza	Crawford and Callard, 1983
		Rabies	Van Meel et al., 1985
		Rubella	Van Meel et al., 1985
	Parasite	*Plasmodium falciparum*	Schmidt-Ullrich, 1986; Udomsangpetch et al., 1986
	Other	Blood group A	Raubitschek et al., 1985
		Rhesus D	Bron et al., 1984
		Rhesus G	Foung et al., 1986
		4-Hydroxy-3,5-dinitro-phenacetic acid	Steinitz et al., 1977, 1979
Active immunisation or boosting	Bacterial	Diphtheria toxoid	Gigliotti et al., 1984
		Gram-negative endotoxin	Bogard et al., 1985; Teng et al., 1985a
		Haemophilus influenzae	Gigliotti et al., 1984
		Pneumococcus polysaccharide	Schwaber et al., 1984
		Tetanus toxoid	Zurawski et al., 1978; Kozbor and Roder, 1981; Chiorozzi et al., 1982; Gigliotti and Insel, 1982; Larrick et al., 1983; Gigliotti et al., 1984; Boyd et al., 1984b; Tiebout et al., 1984, 1985; Burnett et al., 1985
	Viral	Hepatitis B	Burnett et al., 1985
		Colorectal cancer	Haspel et al., 1985a,b
		Rhesus D	Boylston et al., 1980; Crawford et al., 1983; Doyle et al., 1985; Paire et al., 1986; Thompson et al., 1986
	Other	HLA	Hulette et al., 1985; Effros et al., 1986
		2,4-DNCB	Olsson and Kaplan, 1980
		Keyhole limpet haemocyanin	Lane et al., 1982
Active diseases	Bacterial	*Mycobacterium leprae*	Atlaw et al., 1985
	Viral	Epstein-Barr virus	Koizumi et al., 1986
		Rubella	Croce et al., 1980
	Tumour	Bladder	Paulie et al., 1984
		Brain glioma	Sikora et al., 1982, 1983
		Breast	Wunderlich et al., 1981; Cote et al., 1983, 1984, 1985, 1986; Campbell et al., 1986; Strelkauskas et al., 1987a

(continued on next page)

(Table I continued)

Immunisation strategy	Immunogen category	Examples	References
		Colorectal	Hirohashi et al., 1982b; Borup-Christensen et al., 1986
		Gastric	Hirohashi et al., 1982b
		Lung	Hirohashi et al., 1982a; Cote et al., 1983, 1984, 1985, 1986; Cole et al., 1984; Murakami et al., 1985
		Lymphoid system	Abrams et al., 1984; Andreasen and Olsson, 1986; Carroll et al., 1986
		Melanoma	Irie et al., 1982; Warenius et al., 1983; Watson et al., 1983; Kan-Mitchell et al., 1986
		Renal	Cote et al., 1983, 1984, 1985, 1986
	Autoantigen	Astrocytes	Simpson et al., 1986
		Cytoskeleton	Cote et al., 1986
		DNA (ss and ds)	Shoenfield et al., 1982; Massicotte et al., 1984; Sasaki et al., 1984
		Erythrocytes	Shoenfield et al., 1982
		Golgi	Cote et al., 1986
		Islet cells	Eisenbarth et al., 1982
		Multiple organ	Satoh et al., 1983; Garzelli et al., 1984
		Neurofibrillary plaques	Simpson et al., 1986
		Neurofibrillary tangles	Simpson et al., 1986
		Nucleus	Cote et al., 1986; Simpson et al., 1986; Someya et al., 1986
		Nucleolus	Simpson et al., 1986; Cote et al., 1986
		Platelets	Shoenfield et al., 1986
		Sperm	Kyurchiev et al., 1986
No obvious immunisation or disease	Autoantigen	Astrocytes	Simpson et al., 1986
		Cytoskeleton	Cote et al., 1983, 1986
		DNA (ss and ds)	Winger et al., 1983
		Erythrocytes	Winger et al., 1983
		Golgi	Cote et al., 1986
		Multiple organ	Garzelli et al., 1986
		Neurons	Simpson et al., 1986
		Neurofibrillary plaques	Simpson et al., 1986
		Neurofibrillary tangles	Simpson et al., 1986
		Nucleus	Cote et al., 1986; Simpson et al., 1986
		Nucleolus	Cote et al., 1986; Simpson et al., 1986

tween boosting and harvesting of the immune lymphocytes which influence their state of differentiation and proliferation and their circulatory properties. Unfortunately for practical reasons there have been very few studies to determine the optimum time for harvesting lymphocytes with a view to immortalisation. Where comparative studies have been performed it would appear that the optimum time for harvesting peripheral blood cells is 6–7 days (Bogard et al., 1985), while for spleen it is 3 days post boosting as in the mouse (Schwaber et al., 1984). These time schedules have been successfully employed by a number of groups in producing antibodies against bacterial, viral, red cell and tumour antigens. It should be noted however that monoclonals have been produced using lymphocytes harvested 1–3 months post boosting (Boyd et al. 1984b; Tiebout et al., 1984, 1985; Thompson et al., 1986).

While it is obvious that the ability to produce specific monoclonal antibodies is dependent upon the immune status of the donor, it is also influenced by the state of differentiation, proliferation and activation of the cells fused or transformed. Although at the present time we have some insight into how these properties influence

TABLE II

MONOCLONAL ANTIBODIES PRODUCED USING LYMPHOCYTES STIMULATED IN VITRO WITH IMMUNOGEN

Immunogen	Example	Lymphoid tissue	Immortalisation procedure	Additional in vitro conditions [a]	Reference
Bacterial	Tetanus toxoid	PBL	Fusion (KR4)	Not specified	Kozbor and Roder, 1984
	Tetanus toxoid	Spleen, tonsil	Fusion (NS1)	Mixture adherent and non-adherent cells, conditioned media, AB serum	Ho et al., 1985; Ho, 1987
	Tetanus toxoid	PBL	Fusion (HM-5)	PWM	Ichimori et al., 1985
	H. influenzae	Tonsil	Fusion (SP2/SP and SP2/HPT)	TCGF	Brodeur et al., 1987
	Neisseria meningitides	Tonsil	Fusion (SP2/SP and SP2/HPT)	TCGF	Brodeur et al., 1987
	Group streptococcus	Spleen, tonsil	Hmy2, HF2	T cell-depleted, PWM and T cell-irradiated feeders	Wasserman et al., 1986
Viral	Influenza	PBL	Transformation	T cell depleted before transformation	Crawford et al., 1983b
	Rubella	PBL	Fusion (MNS-2)	Not specified	Ritts et al., 1983
	Rubella	PBL	Transformation plus fusion	T cells inactivated (cyclosporine) before transformation	Hilfenhaus et al., 1986
	Hepatitis B surface antigen	PBL	Fusion (HM-5)	PWM	Ichimori et al., 1985
	Herpes simplex	Tonsil	Fusion (P3X63-Ag-U1)	PWM	Masuho et al., 1986
	Cytomegalovirus	Spleen	Fusion (P3X63-Ag-U1)	BCGF	Matsumoto et al., 1986
Erythro-cyte	Sheep	PBL, tonsils	Fusion (W1-L2--729 and HF2)	PWM, AB serum	Strike et al., 1984
	Blood GpA	Spleen	Transformation + fusion (SP2/0Ag14)	T cell depleted before trans-formation	Larrick et al., 1985
	Forssman antigen	Spleen	Fusion (NS-1)	Not specified	Nowinski et al., 1980
	Rhesus D	PBL	SP2/0Ag14		Astaldi et al., 1982
Other	DNP	PBL, spleen	Fusion (SHM-D33)	T cell depleted, LPS, MDP, PHA, *S. aureus* Cowan I	Teng et al., 1985b
	DNP, KLH, sperm whale myoglobin	Spleen	Fusion (SHM-D33 and D36)	T cell depleted, LPS, MDP, PHA, *S. aureus* Cowan I	Bieber et al., 1987
	Prostatic acid phosphatase	Spleen	Transformation and fusion (SP2/0Ag14 and SHM-D33)	PWM, then T cell depleted	Yamaura et al., 1985
	Bombesin	Spleen, tonsil	Fusion Ns-1	Mixture adherent and non-adherent cell lines conditioned medium AB serum	Ho et al., 1985; Ho, 1987
	KLH-ABS	Spleen, tonsil	Hmy2 and HF2	T cell-depleted, PWM and T cell-irradiated feeder	Wasserman et al., 1986

[a] Conditions specified usually those which gave optimum results.
Note also that antigen dose and time of culture varied.

Epstein-Barr virus-induced immortalisation procedures (see later) the relevance of these to fusion still remains to be established. As stressed by Schwaber et al. (1984) B cells fuse at some unidentified stage of differentiation and activation.

In vitro immunisation

In an effort to circumvent the problems of obtaining suitable immune lymphocytes from donors, increasing attention is being devoted to in vitro methods of sensitising and amplifying specific B cells, an approach which has already been extensively used in the development of murine monoclonals (Reading, 1982; Borrebaeck, 1986). Unfortunately, however, the in vitro immunisation of human lymphocytes has been more difficult to achieve than that of their murine counterpart. According to Borrebaeck (1986) this could be due to the fact that human studies have usually been performed with peripheral blood where there is an unfavourable ratio of T suppressor cells to B cells or the circulating B cells are arrested in a phase in which additional stimuli are required to induce antigen-specific clonal activation. Whatever the explanation, Dorfmann has stressed (1985) that the lack of a well-established technique for the in vitro immunisation of human blood cells remains the most significant obstacle to human monoclonal antibody development.

While developments in this area have undoubtedly been slow, progress has been made, especially in the last 2 years. Thus there are now reports of a variety of human monoclonal antibody-secreting cell lines which have been obtained by the immortalisation of lymphocytes which have been stimulated in vitro with antigen. These include antibodies to bacterial, viral, red cell and other antigens (Table II). Details of the precise procedures used can be found in the references listed and elsewhere (see for example Reading, 1982; Hoffman and Hirst, 1985b; Borrebaeck, 1986).

Analysing the results to date the following points emerge. The in vitro immunisation procedure has been successfully employed with lymphocytes from peripheral blood, spleen and tonsils. As stressed later the small amount of comparative data available suggests that spleen and tonsils are superior to peripheral blood (Strike et al., 1984; Teng et al., 1985b). It should be noted however that almost all of the studies reported to date have been undertaken with lymphocytes from seropositive donors. Indeed attempts to sensitise cells from seronegative donors with certain antigens have been unsuccessful (Matsumoto et al., 1986). In most cases in vitro immunisations have been performed with lymphocyte preparations obtained by separation on density gradients. However, in a few cases such preparations have also been depleted of T cells or T suppressor cells (T_S) by a variety of techniques. These include SRBC rosetting (Teng et al., 1985a), separation on nylon wool (Garzelli et al., 1984; Ho et al., 1985; Yamaura et al., 1985) or Sephadex G-10 columns (Hoffman and Hirst, 1985a,b), specific lysis with an anti-suppressor cell (OKT8) monoclonal (Lagace et al., 1985; Brodeur et al., 1987) or panning on cimetidine-coated plates (Ho et al., 1985). On other occasions the lymphocyte-rich suspensions have been separated into non-adherent and adherent fractions which have then been reconstituted to give a more favourable ratio of T helper (T_H) cells (Ho et al., 1985). While there are sound theoretical reasons for removing T_S cells and enriching T_H cells the value of this approach with respect to in vitro immunisation is still a matter of debate and will not be resolved until more comparative studies are undertaken (Borrebaeck, 1986). It should be stressed however that depletion or inactivation of cytotoxic T cells is advisable if the stimulated cell lines are to be subsequently immortalised or expanded by EBV transformation (Crawford et al., 1983, 1985; Larrick et al., 1985; Yamaura et al., 1985). Of particular value in this context is the use of cyclosporine A which has been shown to promote the spontaneous outgrowth in vitro of Epstein-Barr virus-induced B cell lines (Bird et al., 1981). It should be noted that other aspects of cell enrichment relating particularly to in vivo immunisation will be considered below.

The success of the in vitro approach is obviously influenced by the dose, and possibly the form, of the immunogen used. While the impure nature of some of the immunogens employed renders precise quantitation difficult it is apparent that high doses of immunogen may inhibit (Matsumoto et al., 1986) and that the optimum

dose may vary from one lymphocyte preparation to another (Brodeur et al., 1987) as well as from immunogen to immunogen. It has also been suggested that the form of the immunogen influences the isotype of the antibody secreted, insoluble antigens favouring the production of IgG antibodies (Ho et al., 1985). Further detailed studies in this area are obviously desirable.

In most in vitro immunisation procedures mitogens have been used. PWM has been most extensively employed but others include LPS, PHA and *Staphylococcus aureus* Cowan I (a B cell stimulant). In some cases mitogen alone has been found to increase the production of antibody-secreting hybrids and to exert a synergistic effect with antigen (Ichimori et al., 1985).

Of particular interest have been recent reports that the inclusion of the adjuvant peptide (muramyl dipeptide) in the culture media greatly enhances the chance of obtaining antibody-specific hybrids (Teng et al., 1985b). This compound has also been shown to enhance the generation of murine monoclonal antibodies following in vitro sensitisation (Boss, 1984). Another possible approach is to use antigen/silica complexes (Olsson et al., 1985), a procedure previously proposed for generating murine monoclonals against weakly immunogenic insoluble proteins (Van Ness et al., 1984). The success of the in vitro approach is obviously dependent upon the presence in the culture of the relevant differentiation and growth factors. In most cases these have been elicited in the culture by the added mitogens. On occasions, however, studies have been performed with exogenously added growth factors. These include unfractionated lymphocyte-conditioned media (Strike et al., 1984; Ho et al., 1985), T cell growth factor (Brodeur et al., 1987) and B cell growth factor (Matsumoto et al., 1986). It would appear that the impure products are of doubtful value (Ho et al., 1985).

There is also debate about the source of serum used. Some authors claim that human serum is essential, at least during the early stages of in vitro cultivation (Strike et al., 1984; Ho et al., 1985; Teng et al., 1985b). However it should be noted that in most reports to date FCS has been successfully employed.

The optimum time for in vitro culture varies from author to author, presumably reflecting differences in the immune status of the donors as well as the nature of the immunogenic stimulus. In some cases 3 days appears to be optimal (Crawford et al., 1983; Ichimori et al., 1985; Larrick et al., 1985) while in others, 7 days or more (Yamaura et al., 1985; Brodeur et al., 1987). It is also interesting to note that following initial culture with immunogen, some investigators undertake a further period of culture in the presence or absence of mitogen prior to immortalisation (Teng et al., 1985b; Masuho et al., 1986).

The choice of lymphoid tissue

Opinions in this area have been derived largely from personal experience and considerations of a theoretical nature rather than detailed, controlled comparative studies. The latter, of course, are extremely difficult to perform for even if the relevant lymphoid tissues were available the variations normally experienced in fusion work, even in experienced laboratories, mean it is difficult to reach hard and fast conclusions. In addition analysis of the data already reported is frequently complicated by the fact that the results are presented as the numbers of hybrids produced rather than specific antibody-secreting cell lines. Nevertheless in spite of these difficulties a body of opinion has emerged to indicate that as in the mouse (see for example Olsson et al., 1983) spleen, tonsils, etc., make better fusion partners than peripheral blood (Table III). There is some suggestion, however, that this may depend on the human fusion partner used and may be less critical where mouse myelomas are employed (Cote and Houghton, 1985). From these limited impressions it seems that peripheral blood lymphocytes are both more difficult to sensitise in vitro than lymphocytes from other tissues (see Lagace and Brodeur, 1985; Borrebaeck, 1986) and give rise to fewer antibody-secreting cell lines following immortalisation. This inferior performance has been attributed to a numer of factors and these are summarised in Table IV. They include inherent deficiencies in the circulating B cells themselves and adverse interactions with other cells of the immune system. In brief, it is believed that peripheral blood does not contain enough antigen-reactive specific B cells in the appropriate state of

TABLE III

THE CHOICE OF LYMPHOID TISSUES FOR HUMAN MONOCLONAL ANTIBODY PRODUCTION

Lymphoid tissue examined	Immunogen	Immunisation		Immortalisation			Best tissue	Reference
		In vivo	In vitro	Fusion	Trans.	Both		
Blood, spleen, tonsil	Tetanus toxoid		+	+			Spleen/ tonsil	Chiorazzi et al., 1982
Blood, nodes [a] intra-tumour lymphocytes	Lung carcinoma				+	+	Nodes	Cole et al., 1984
Blood, nodes, spleen intra-tumour lymphocytes	Miscellaneous tumours	+		+			Nodes [b]	Cote et al., 1984, 1985
Blood, spleen	Pneumococcus capsular poly-saccharide	+		+			Spleen	Schwaber et al., 1984
Blood, tonsil	Sheep erythrocytes		+	+			Tonsil	Strike et al., 1984
Blood, node, spleen	Miscellaneous tumours	+		+			Node	Glassy et al., 1983
Blood, node, spleen	Tetanus toxoid	+		+			Node/ spleen	Burnett et al., 1985
Blood, spleen, tonsil	Tetanus toxoid Bombesin		+	+			Spleen/ tonsil	Ho et al., 1985
Blood, spleen	Miscellaneous antigens		+	+			Spleen	Teng et al., 1985b
Blood, node, spleen, tonsil	Epstein--Barr virus	+			+		Spleen	Koizumi et al., 1986
Blood, spleen	Miscellaneous antigens		+	+			Spleen	Bieber and Teng, 1987

[a] Refers to tumour draining lymph nodes.

[b] Depends on cell line used.

Note the superior performance of spleen, nodes and tonsil was observed with a wide variety of fusion partners both human and mouse.

differentiation and proliferation. Furthermore peripheral blood contains too many suppressor and cytotoxic cells and not enough antigen-presenting cells or T_H cells.

Although it is apparent that peripheral blood is not the best source of lymphocytes for human monoclonal antibody production, for practical and ethical considerations it will continue to be the main source in the foreseeable future. In the meantime, as emphasised elsewhere, methods for improving the supply of antigen-primed lymphocytes from peripheral blood will remain a priority. In this context it is interesting to note that adjuvants such as BCG are believed to improve the efficiency of the immortalisation process by recruiting the appropriate progenitors into the circulation (Haspel et al., 1985a,b). This could be important in view of claims that bone marrow is an excellent source of committed B cells for immortalisation (Seigneurin et al., 1983).

Further comments on cell enrichment

In this section on in vitro immunisation we stressed the importance of removing T_S cells which might inhibit the immune response and T_c cells which could prove deleterious following EBV-induced immortalisation. Of added importance, particularly when in vivo immunised lymphocytes are used, is the specific enrichment of antigen-reactive cells which are usually present in very low num-

TABLE IV

POSSIBLE REASONS WHY PERIPHERAL BLOOD LYMPHOCYTES PERFORM LESS WELL IN HUMAN MONOCLONAL ANTIBODY PRODUCTION THAN CELLS FROM OTHER LYMPHOID TISSUES

Reason advanced	References
(1) Insufficient B cells	Cote and Houghton, 1985; Ho et al., 1986
(2) Insufficient memory cells or antigen specific B cells	Seigneurin et al., 1983; Olsson et al., 1983 Ho et al., 1986
(3) Transient appearance of antigen specific B cells in blood following immunisation	Burnett et al., 1985
(4) Inappropriate state of differentiation of B cells	Schwaber et al., 1984; Cote and Houghton, 1985
(5) Low mitotic activity of B cells	Burnett et al., 1985; Westerwoudt, 1985
(6) Contains fewer Ia positive macrophages	Teng et al., 1985
(7) Contains too many cytotoxic T cells	Cote and Houghton, 1985
(8) Contains suppressor B cells	Cote and Houghton, 1985

bers (1 in 10^6–10^7 lymphocytes). As stressed above this can be achieved partly by incubating the lymphocytes with antigen (Ho et al., 1985; Matsumoto et al., 1986) or mitogen alone (Chiorazzi et al., 1982; Olsson et al., 1983) or both (Astaldi et al., 1982; Strike et al., 1984; Yamaura et al., 1985; Matsumoto et al., 1986). Alternative strategies which are available include rosetting with red cells bearing the relevant antigen (Steinitz et al., 1979; Boylston et al., 1980; Doyle et al., 1985) or panning on antigen-coated wells (Winger et al., 1983; Cole et al., 1985). Another approach is to mix the cells with FITC-labelled antigen followed by fluorescence-activated cell sorting (Casal et al., 1986). The latter technique is probably the most accurate but it is also the most expensive and least widely available. In addition, to obtain adequate numbers of specific B cells for use in current immortalisation strategies involves extremely long runs in the cell sorter.

Any or all of the above techniques could be used in the preparation of lymphocytes for immortalisation but two facts should be borne in mind when choosing which methods to use. Firstly the maximum number of antigen-positive cells should be isolated but possibly more importantly cells which may have a deleterious effect should be removed. In general when large numbers of B cells are available such as from lymph nodes or spleen then specific B cell enrichment methods are worth attempting. When lower cell numbers are available, such as from blood, then the stimulation techniques can play a role by increasing the total number of specific cells present.

Procedures for immortalising human B cells

Lymphocyte fusion

Following the development of murine monoclonal antibodies the next logical step was to apply a similar approach to the production of human monoclonal antibodies. In the first report Olsson and Kaplan (1980) produced human hybridoma-secreting monoclonal antibodies against the 2,4-dinitrophenyl hapten by fusing splenic lymphocytes with a myeloma cell line. Since this time fusion has remained probably the most popular immortalisation strategy.

Several problems have been encountered using the fusion technique. The difficulties relating to the choice of lymphoid tissue have already been discussed but in the majority of cases peripheral blood is the only tissue available. A second problem encountered is the choice of partner for fusion. In murine work a few very satisfactory lines were developed early in the history of the technology. These lines soon became widely available to most laboratories and so at present there are relatively few murine myeloma partners in general use. In the case of human partner lines the opposite is true; no satisfactory lines were developed quickly and as a result many laboratories spent a great deal of time and effort developing their own partners. This fact is reflected when the literature is surveyed with many different partner lines having been used, but none having come to dominate (Table V and Table VI).

The partner lines produced are of several phenotypes; myelomas (which are used so successfully in the murine system) are used very rarely, mainly because they grow very poorly in culture. The majority of human fusion partners are lympho-

TABLE V

SOME CELL LINES CURRENTLY USED AS FUSION PARTNERS IN HUMAN MONOCLONAL ANTIBODY PRODUCTION

Fusion partner	Original line	Cell type [a]	EBNA positive [b]	Secreted Ig	Drug markers	Reference
SKO-007	U-266	Myeloma	−	IgE(λ)	8-AG	Olsson and Kaplan, 1980
RPMI 8226		Myeloma	−	λ	8-AG	Abrams et al., 1983
HFB 1		Myeloma	−	Non-secretor	6-TG	Hunter et al., 1982
KMMI		Myeloma	−	G		Togawa et al., 1982
RH-L4		Lymphoma	NI	Non-secretor	8-AG	Olsson et al., 1983
NAT-30	Nawalma	Lymphoma	NI		6-TG	Murakami et al., 1985
GM 1500-6TG-AL	GM 1500	LCL	+	IgG2(κ)	6-TG	Croce et al., 1980
GM 1500-6TG-OA	GM 1500	LCL	+			Hulette et al., 1985
KR4	GM1500-6TG--A11	LCL	+	IgG2(κ)	6-TG, OUA	Kozbor et al., 1982
GM 467						Sato et al., 1972
GM 4672	GM 1500	LCL	+	IgG2(κ)	6-TG	Croce et al., 1980
ARH-77		LCL	+	IgG(κ)	8-AG	Burk et al., 1978
LICR-LON--Hmy2	ARH 77	LCL	+	IgG1(κ)	8-AG	Edwards et al., 1982
W1-L2		LCL				Emanuel et al., 1984
H35-1-1	W1-L2	LCL	+	IgM(κ)	8-AG	Chiorazzi et al., 1982
W1-L2-729 HF2	W1-L2	LCL	+	IgM(κ)		Strike et al., 1984
W1-L2-727	W1-L2	LCL	+	IgG(κ)		Emanuel et al., 1984
UC 729-6	W1-L2	LCL	+	IgM(κ)	6-TG	Glassy et al., 1983
MC/CAR		LCL	+	None		Ritts et al., 1983
MC/MNS-2	MC/CAR	LCL	+	IgG1(κ)		Ritts et al., 1983
LTR 228		LCL	+	IgM(κ)	6-TG	Larrick et al., 1983
LSM 2-7	CRL 1484	LCL	+		6-TG	Schwaber et al., 1983
HS Sultan		LCL	+	None		Lazarus et al.,
GK-5		LCL	+	κ	6-TG	Satoh et al., 1983
HO-323		LCL	NI			Ohashi et al., 1986
KR-12	KR4 × RPMI 8226	h/h hybrid myeloma	+	IgG2(κ + λ)	6-TG, OUA	Kozbor et al., 1984
SHM-D3	U266 × 63-Ag-8.653	m/h hybrid myeloma	NI	Non-secretor	6-TG, OUA, G418	Teng et al., 1983
SBC-H20	B cell × SP2/08 A2	m/h hybrid myeloma	NI	Non-secretor		Foung et al., 1985a
3 HL	SHM-D3 × B lymphoma	m/h/h hybrid myeloma	NI	IgM(λ)	6-TG, OUA G418	Teng et al., 1985a

[a] h/h = human hybrids used as fusion partners; m/h and m/h/h = mouse human hybrids used as fusion partners.
[b] − EBNA, negative; + EBNA, positive; NI, no information.

blastoid cell lines derived by Epstein-Barr virus transformation of lymphocytes. These lines are easier to handle in culture than myelomas, but they may not be in an appropriate differentiation state for high levels of antibody secretion. Kozbor et al. (1983, 1985) have stressed that while myelomas have many polyribosomes, well developed golgi apparatus and can secrete large amounts of antibodies, lymphoblastoid cell lines have few polyribosomes, sparse golgi apparatus and secrete relatively little immunoglobin. Several groups have attempted to improve the fusion rates and growth characteristics of their existing lymphoblastoid lines by fusing with either mouse or human myelomas. It is claimed that these hetero-myelomas perform better than the original lines in

TABLE VI

USE OF HETEROMYELOMAS IN HUMAN MONOCLONAL ANTIBODY PRODUCTION

Heteromyeloma designation	Derivation		Lymphoid cells used in fusion	Immunogen	Stability (months)	Reference
	Mouse parent	Human parent				
PSV2.Neo^R	P3X63-Ag8.653	FU 266	Peripheral blood and lympho-blastoid cell lines	2,4-Dinitrophenol ds- and ss-DNA Ribosomal RNA J5 endotoxin RhD, tetanus toxoid	> 7	Teng et al., 1983
SHM-D33		FU 266	Peripheral blood Spleen Lymph nodes Spleen	RhD Prostatic acid phosphatase Lymphoma J5 Endotoxin	> 8 > 6 < 2	Bron et al., 1984 Yamaura et al., 1985 Carroll et al., 1986 Teng et al., 1985b
HM/5		LICR-LON Hmy2	Peripheral blood	Tetanus toxoid Hepatitis B surface antigen		Ichimori et al., 1985
Org M HHl		Normal B cell	Peripheral blood	Rubella Rabies Hepatitis A and B	> 2.5 to 6.0	Van Meel et al., 1985
SBC/H20	SP2/0	FU 266	Lymph nodes Peripheral blood and spleen	Lymphoma Blood group A Varicella zoster EBV early antigen *Mycobacterium leprae*	< 2	Carroll et al., 1986 Foung et al., 1985
SP2/SP		Normal spleen	Tonsil	*Haemophilus influenzae*	> 5	Brodeur et al., 1987
SP2/HPT		Normal peripheral blood and tonsils		*Neisseria meningitides*		
K6H6/B5	NS 1	B cell lymphoma	Lymph nodes	Lymphoma	< 2	Carroll et al., 1986
K6H9/G12		B cell lymphoma	Lymph nodes	Lymphoma	< 2	

fusion experiments. Fusion of lymphoblastoid cell lines with mouse myelomas has produced several useful partners (Teng et al., 1983, 1985a; Foung et al., 1985a) which give a greater number of stable hybrids when fused with lymphocytes than do mouse cells alone (Table VI). Kozbor et al. (1984) have used the same principle to improve the growth characteristics of the human myeloma line RPMI 8226, by fusing with their lymphoblastoid line KR4. The resultant hybrid KR12 shows improved fusion rates over either of its parents and also exhibits a phenotype, more like a myeloma than other lines which may increase the amount of antibody secreted.

The problems of fusing lymphocytes with human partners has encouraged several groups to fuse with mouse myelomas instead (Wunderlich et al., 1981; Astaldi et al., 1982; Cote et al., 1984; Haspel et al., 1985; Hirohashi et al., 1986; Thompson et al., 1986) (Table VII). It has been known for many years that heterohybrids preferentially reject human chromosomes and indeed this fact has been used to assign genes to particular chromosomes (see later). Despite this fact it

TABLE VII

HUMAN MONOCLONAL ANTIBODIES PRODUCED BY FUSION OF HUMAN LYMPHOCYTES WITH IMMORTAL PARTNER CELLS

Antigen	Lymphoid tissue	Fusion partner		Immunoglobulin isotype secreted	Stability in months	Reference
		Line	Species			
Tetanus toxoid	PBL [a]	P3X63-Ag8.653	Mo [f]	G1(κ)	> 30	Giolotti et al., 1982
Tetanus toxoid	SP [b], TON [c]	NS-1	Mo		> 10	Ho et al., 1985
Tetanus toxoid	PBL	NS-1	Mo		7–13	Tiebout et al., 1985
Haemophilus influenzae B	SP	HFB-1	Hu [g]	G	–	Hunter et al., 1982
Haemophilus influenzae B	PBL	P3X63-Ag8.653	Mo		7–19	Gigliotti et al., 1984
Pseudomonas aeruginosa	PBL, TON	P3X63-AgU1	Mo		> 6	Sawada et al., 1985
Pneumococcal polysaccharide	SP, PBL	LSM 2-7	Hu		< 2	Schwaber et al., 1984
Hepatitis B surface antigen	PBL	NS-1	Mo		7–13	Tiebout et al., 1985
Cytomegalovirus	SP	P3X63-AgU1	Mo	G1	> 6	Matsumoto et al., 1986
Herpes simplex virus	TON	P3X63-AU1	Mo	G	> 15	Masuho et al., 1986
Hepatitis B surface antigen	PBL	P3/HT	Mo		> 8	Burnett et al., 1985
Plasmodium falciparum	PBL	GM4672	Hu		–	Schmidt-Ullrich et al., 1986
Rhesus D	PBL	SP2/0Ag/4	Mo		< 3	Astaldi et al., 1982
Colorectal tumour	PBL	NS-1	Mo	M	> 12	Haspel et al., 1985a,b
Colorectal tumour	LN [d]	LICR-LON--Hmy2	Hu	M	> 12	Borup-Christensen et al., 1986
Breast tumour	LN	NS-1	Mo		> 6	Wunderlich et al., 1981
Breast tumour	All possible	GM4672, UC729-6 SKO-007, NS-1 Hmy2	Mo + Hu		–	Cote et al., 1983, 1984, 1985, 1986
Lung tumour	LN	Namalwa	Hu	M	–	Murakami et al., 1985
Lung adeno-carcinoma	LN	P3X63-Ag8.653	Mo		> 36	Hirohashi et al., 1986
Leukaemia	PBL	RH-L4	Hu		–	Olsson et al., 1984
Lymphocytic leukaemia	PBL	NS-1	Mo		–	Abrams et al., 1984
Melanoma	LN		Mo	M + G	> 2	Kan-Mitchell et al., 1980
Miscellaneous tumours	LN, SP, PBL	UC729-6	Hu		> 2	Glassy et al., 1985
Glioma	ITL [e]	LICR-LON--Hmy2	Hu		–	Sikora et al., 1982, 1983
Sperm-coating antigen	PBL	P3X63-Ag81653 + SP2/0Ag/4	Mo		> 3	Kyurkchiev et al., 1986

[a] Peripheral blood lymphocytes.
[b] Splenic lymphocytes.
[c] Tonsular lymphocytes.
[d] Lymph node lymphocytes.
[e] Intra-tumoral lymphocytes.
[f] Mouse cell line.
[g] Human cell line.

appears that loss of antibody-secreting lines due to genetic instability is no worse in heterohybrids than in murine hybrids (Thompson et al., 1986) and these lines can be stabilised by early cloning. The fusion rates achieved when using mouse myelomas as partners are significantly higher than those achieved with human partners in comparative experiments (Cote et al., 1983, 1984, 1986).

For the human lines, Abrams et al. (1983) suggest that LICR-LON-Hmy2, UC729-6 and HF2 are worth further investigation, although their study used only seven of the available human partners. More recently there have been preliminary reports of detailed studies with a variety of cell lines including mouse myelomas, heteromyelomas, etc. (Usagawa et al., 1986). Among the various cell lines tested KR-12, SHM-D33, 3HLA-6 and 3HL3-27 proved the best. It is obvious that further time-consuming studies in this area will be necessary before hard and fast conclusions on the best fusion partners can be reached.

The actual fusion procedures used vary widely from group to group and workers all have their own methods of producing hybrids. Due to the variations between fusions even when the same experimenter is involved very few comparative studies are possible (Kadish and Wenc, 1983; Lane et al., 1984; Dorfman, 1985; Westerwoudt, 1985). One conclusion which seems to be agreed upon is that the polyethylene glycol (PEG) preparation used for fusion plays a major role in hybrid formation and any new batch must be tested before it can be used routinely. The studies all recommend PEGs of different molecular weight and sources but it appears that if a batch of PEG produces good yields of hybrids in one experiment then there is no reason why it should not continue to do so. In our department one batch of PEG has produced many successful murine fusions over several years. The pH of the PEG when it is in contact with the cells can influence the rate of fusion (Lane et al., 1984) as can the time the cells remain in contact with the fusogen (Davidson and Gerald, 1976). The choice of foetal calf serum and other supplements used play a major role in the growth of all cells and will be discussed below.

Lymphocyte transformation

Human lymphocytes can be immortalised by a second completely different mechanism, namely viral transformation. The virus used in this technique is the Epstein-Barr virus (EBV) and its normal source is the culture supernatant derived from the marmoset cell line B95-8 (Miller and Lipman, 1973). EBV is a herpes virus which preferentially infects human B lymphocytes, and can on occasion insert into their genome thus causing transformation. There is at present some debate as to which cells are infected with EBV and of these which go on to become transformed. The receptor for EBV on human lymphocytes is the C3d (CR2) complement receptor which is a 140 kDa glycoprotein (Frade et al., 1985). It would appear that EBV can bind to and penetrate all B cells, yet only a small proportion are transformed. Binding of killed EBV induces cells to become activated as does antibody directed against the C3 receptor suggesting activation and transformation are related but separate events. Aman et al. (1985) suggest that the responsive population is a small high density resting population while Chan et al. (1986) claim that the most susceptible population is an activated large cell population. This population is also $sIgM^+$, $sIgD^+$ and is destined to secrete IgM. The small resting population on the other hand gives rise to IgG and IgA secreting populations, the precursors of these cells being $sIgG^+$ or IgA^+ and $sIgD^-$. It is also interesting to note that Chan et al. (1986) suggest that the susceptibility to transformation may be more dependent on entry into the cell cycle than the density of EBV receptor expression. If Chan et al.'s observations are correct, this would explain the bias found by many workers towards IgM secretions, while still explaining IgG and IgA secretion by some lines. It is interesting to note that many of the long-lived antibody-secreting cell lines obtained by EBV transformation secrete IgG antibodies especially IgG1 (Table VIII). As suggested by others this may indicate that cell lines secreting IgG antibodies are more stable in culture or merely reflect a bias in the selection procedures (Redmond et al., 1986).

The transient activation of B lymphocytes by EBV binding without subsequent transformation can be a major problem when attempting to produce monoclonal antibodies by this method. Moreover, it appears that there are other problems

with transformed cell lines; it is not uncommon for cell lines to grow well for 1 or 2 months before their antibody titre suddenly declines. This fall in antibody titre is often accompanied by a visible change in cell phenotype from small highly clumped irregularly shaped cells to larger more uniformly round single cells. These cells do not grow as quickly in culture as the irregular cells.

Although early cloning has in some cases rescued antibody-secreting lines, many potentially useful lines have been lost due to the extremely poor cloning efficiencies of lymphoblastoid lines. Many different strategies have been tried to improve the growth at low cell densities including early repeated cloning but the fact remains that the majority of workers find these cells extremely difficult to clone. It should be noted that viral transformation has occasionally occurred 'acci-

TABLE VIII

HUMAN MONOCLONAL ANTIBODIES PRODUCED BY EPSTEIN-BARR VIRUS TRANSFORMATION OF HUMAN LYMPHOCYTES

Antigen	Lymphoid tissues	Immunoglobulin isotype secreted	Stability in months	References
Tetanus toxoid	PBL [a]	G	> 6	Zurawski et al., 1978
Tetanus toxoid	PBL	G1(κ)	–	Tiebout et al., 1984
Tetanus toxoid	PBL	G3	> 10	Boyd et al., 1984a,b
Pneumococcal polysaccharide	TON [b]	M	> 3	Yoshie and Ono, 1980
Chlamydia salpingitis	PBL	G1(κ + λ)	> 12	Rosen et al., 1983
Herpes Simplex II	PBL	G1 + G2	< 2	Evans et al., 1984
Herpes Simplex glycoprotein D	BM	G1(κ)	> 24	Seigneurin et al., 1983
Hepatitis Be	PBL		–	Furuya et al., 1982
Influenza virus	PBL	G1(κ)	> 12	Crawford and Callard, 1983b
Cytomegalovirus	PBL	G(κ)	> 12	Emanuel et al., 1984
Cytomegalovirus	PBL	G1(κ + λ)	–	Redmond et al., 1986
Epstein-Barr capsid and membrane	PBL, LN, TON, SP [c]	G1(κ)	> 8	Koizumi et al., 1986
Plasmodium falciparum	PBL	M + G	12	Udomsangpetch et al., 1986
Rhesus (D)	PBL	M	1.5	Boylston et al., 1980
Rhesus (D)	PBL	G1(κ)	> 9	Crawford et al., 1983
Rhesus (D)	PBL	G1 and G4	> 10	Doyle et al., 1985
Rhesus (D)	PBL	G1(κ)	–	Melamed et al., 1985
Blood group A	SP	M	–	Raubitschek et al., 1985
Melanoma	ITL [d]	M + G	–	Watson et al., 1983
Transitional cell carcinoma	PBL	M	> 1	Paulie et al., 1984
Lung tumour	LN [e]	M	–	Hirohashi et al., 1982a
Gastric tumour	LN	M(κ)	–	Hirohashi et al., 1982b
OFA-1	PBL	M(κ)	–	Irie et al., 1982
T cell leukaemia	LN	G1	> 2	Matsushita et al., 1986
DNA	PBL	M	> 36	Sasaki et al., 1984
DNA	PBL	M	–	Sasaki et al., 1985
DNA, sperm, erythrocytes	PBL	M + G	> 1	Winger et al., 1983
Thyroid antigens	PBL	M, G + A	> 6	Garzelli et al., 1984
Neurofibrillary plaques + tangles	PBL	M + G	> 4	Simpson et al., 1986
N.N.P.	PBL	M(κ)	–	Steinitz et al., 1979

[a] Peripheral blood lymphocytes.
[b] Tonsular lymphocytes.
[c] Splenic lymphocytes.
[d] Intra-tumoral lymphocytes.
[e] Lymph node lymphocytes.

dentally' while fusions with EBV^+ partner cells were being attempted (Boyd et al., 1984a; Stricker et al., 1985). These 'cell-driven' transformations appear to lead to more stable cell lines but the mechanisms underlying this phenomenon have never been investigated.

Lymphocyte transformation followed by back fusion

Kozbor et al. (1982) successfully combined the techniques of transformation and cell fusion to produce hybridomas secreting antibodies directed against tetanus toxoid. By combining the two approaches they gained the advantages of both methods and avoided some of the disadvantages. Most of the published work with this technique has used peripheral blood as the source of lymphocytes. The major advantage of transforming the cells before fusing 2–8 weeks later is that the fusion rate rises from 20×10^{-7} to over 100×10^{-7} (Kozbor and Roder, 1984). It has been suggested that the reason peripheral blood fails to fuse well is that the cells are not in an actively dividing state and therefore their nuclei do not fuse with that of the myeloma partner during mitosis (Burnett et al., 1985). Since EBV causes the B lymphocytes to become activated even when transformation does not occur, it allows the lymphocytes to fuse more efficiently resulting in higher yields of hybrids.

The hybrids produced by this approach have the same instability problems as any other hybrids but these can be overcome by early cloning (since such hybrids undoubtedly clone more readily and produce higher levels of specific antibody than transformants), and have been reported as stable from 2.5 to greater than 14 months. The antibodies secreted are representative of the antibodies being produced by the transformed lines at the time of fusion. As a result many of the lines produce IgM although IgGs (especially IgG1) are not uncommon. Using this technique antibodies have been produced against a wide variety of antigens (Table IX).

Feeder layers

A crucial stage in all of the methods described above is during the initial culturing when selection procedure are carried out at reasonably low cell densities. To try to improve the growth characteristics of newly transformed or hybridised cells a wide variety of feeder cells and conditioned media have been used. Feeder layers fall into two distinct groups, cells of the lymphocyte and mononuclear phagocyte lineage of human or mouse origin and human cells predominantly either of embryonic origin or fibroblasts (Table X). Some groups believe they greatly improve the chance of isolating specific antibody-secreting lines (Crawford et al., 1983, 1985; Rosen et al., 1983; Tiebout et al., 1984, Teng et al., 1985a) while others feel they are of limited value (Cote ct al., 1983, 1985; Strike et al., 1984; Schmidt-Ullrich et al., 1986). Unfortunately there have been few well designed experiments to establish the true position. One role which phagocytic cells may play is to clean up cell debris arising from the selection process and to minimise any growth of infectious microorganisms. For example mouse PEC are believed to be very effective at minimising mycoplasma contamination (Doyle et al., 1985).

The expansion of human monoclonal cell lines

The approaches used to grow up human monoclonal cell lines of interest have generally followed those used for mouse monoclonals. They include a variety of in vitro culture procedures together with growth in immunodeficient mice or rats (Table XI). Where relatively small amounts are required stationary culture in flasks is perfectly adequate and has been widely used. Larger amounts have been successfully produced in suspension culture using spinner flasks (Hunter et al., 1982; Thompson et al., 1986) and roller bottles (Wunderlich et al., 1981; Sikora et al., 1983; Thompson et al., 1986). Others have grown human cell lines in immobilised form (Haspel et al., 1985a,b). It is generally accepted that, where extremely large amounts of antibody are required (as for example in therapy), airlift reactors and immobilised cell technologies are the methods of choice. Further details on a number of these procedures can be found in recent reviews (Birch et al., 1985; Duff, 1985; Altschuler et al., 1986).

Scale up in production inevitably introduces problems not least of which is the cost, availability and variability of serum supplements used in medium formulation and their subsequent removal from the final product. These problems

TABLE IX

HUMAN MONOCLONAL ANTIBODIES PRODUCED BY EPSTEIN-BARR VIRUS TRANSFORMATION OF HUMAN LYMPHOCYTES FOLLOWED BY FUSION WITH IMMORTAL PARTNER LINES

Antigen	Lymphoid tissue	Fusion partner		Immunoglobulin isotype secreted	Stability in months	Reference
		Species	Line			
Tetanus toxoid	PBL [a]	Hu [e]	KR4	M(κ)	> 7	Kozbor et al., 1982
Tetanus toxoid	PBL	Hu	KR-12	M	> 10	Kozbor et al., 1984
Pseudomonas aeruginosa	SP [b]	Hu	LTR228	M	–	Siadek and Lostrom, 1985
Pseudomonas aeruginosa	SP	Hu/Mo [f]	F3B6	M	–	Siadek and Lostrom, 1985
Exotoxin	PBL	Mo	SP2/0	M	–	Larrick et al., 1985
Mycobacterium leprae	PBL	Hu	KR4	M	> 12	Atlaw et al., 1985
Gram negative bacteria	SP	Hu/Mo	SHM-D33	M		Teng et al., 1985a
Cytomegalovirus	PBL	Hu	W1-L2	G(λ)	–	Emanuel et al., 1984
Rubella, Rabies, Hepatitis B	PBL (human + chimp)	Hu	Org MHHI	M + G	2.5–6	Van Meel et al., 1985
Rhesus D	PBL	Hu/Mo	SHM-D33	G3(λ)	> 8	Bron et al., 1984
Rhesus D	PBL	Mo	X63-Ag-8.653	M + G3	> 14	Thompson et al., 1986
Rhesus G	PBL	Hu/Mo	SBC-H20	G1	–	Foung et al., 1985
Blood group A	PBL	Hu/Mo	SBC-H20		–	Foung et al., 1985a
Blood group A	PBL	Mo	SP2/0	M	–	Larrick et al., 1985
HLA	PBL	Hu	GM 1500-6TG-OB	M	–	Hulette et al., 1985
Lung carcinoma	PBL, LN, [c] ITL [d]	Hu	KR4	M	> 5	Cole et al., 1984
Breast carcinoma	PBL	Hu	KR4		> 4	Campbell et al., 1986
Prostatic acid phosphatase	SP	Hu/Mo Mo	SHM-D33 SP2/0	M + G	> 6	Yamaura et al., 1985
Rheumatoid factor	PBL	Hu	KR4	M, G + A	–	Haskard et al., 1984

[a] Peripheral blood lymphocytes.
[b] Splenic lymphocytes.
[c] Lymph node lymphocytes.
[d] Intra-tumoral lymphocytes.
[e] Human cell lines.
[f] Mouse cell lines.

have led to the development of defined 'serum-free' media for the large scale culture of human and mouse monoclonal cell lines. While experience with serum-free media and human cell lines is as yet limited, it is interesting to note that there are reports suggesting that when grown in serum free-media they will produce at least as much, if not more antibody as cells grown in high concentrations of FCS (Cole et al., 1985b; Glassy et al., 1985; Teng et al., 1985a). This has led to suggestions that FCS may contain factors which impair immunoglobulin production (Glassy et al., 1985). Nevertheless, it must be emphasised that the culture of monoclonal antibody-producing cell lines in serum-free, or media containing low concentrations of serum might render secreted im-

TABLE X

FEEDER LAYERS USED IN HUMAN MONOCLONAL ANTIBODY PRODUCTION

Feeder used	References
Cord blood lymphocytes	Sasaki et al., 1984; Melamed et al., 1985
Peripheral blood T cells	Winger et al., 1983; Garzelli et al., 1984
Peripheral blood monocytes	Bischoff et al., 1982; Olsson et al., 1983; Melamed et al., 1985, Andreasen and Olsson, 1986
Allogeneic peripheral blood leukocytes	Kozbor and Roder, 1981; Astaldi et al., 1982; Irie et al., 1982; Crawford et al., 1983; Paulie et al., 1984; Tiebout et al., 1984; Garzelli et al., 1986; Ho et al., 1986 Schmidt-Ullrich et al., 1986
Lymphoblastoid cell lines	Irie et al., 1982; Doyle et al., 1985
Foreskin fibroblasts	Zurawski et al., 1978; Emanuel, 1984; Siadak and Lostrom, 1985
Thymus fibroblasts	Strike et al., 1984
Fibroblasts not specified	Raubitschek et al., 1985; Van Meel et al., 1985
Embryonic lung fibroblasts	Steinitz et al., 1979
Embryonic fibroblasts not specified	Kozbor and Roder, 1981; Satoh et al., 1983; Watson et al., 1983; Rosen et al., 1983
Embryonic kidney	Zurawski et al., 1978
Embryonic lung	Evans et al., 1984
Embryonic amnion	Zurawski et al., 1978
Endothelial cells	Astaldi et al., 1982
Melanoma cells	Watson et al., 1983
Mouse thymocytes	Nowinski, 1980; Olsson et al., 1983, Teng et al., 1985a
Mouse spleen	Kozbor et al., 1981, 1982, 1984; Cole et al., 1984; Atlaw et al., 1985
Mouse peritoneal exudate	Gigliotti et al., 1982, 1984; Kozbor et al., 1982; Cote et al., 1983, 1985; Satoh et al., 1983; Cole et al., 1984; Haskard et al., 1984; Doyle et al., 1985; Melamed et al., 1985; Borup-Christensen, 1986; Carroll et al., 1986; Matsuho et al., 1986; Thompson et al., 1986; Brodeur et al., 1987

TABLE XI

GROWTH OF HUMAN ANTIBODY SECRETING CELL LINES

Procedure	References
Stationary culture	
Wells or flasks	Routinely used
Homogenous suspension culture	
Continuous stirred reactor	Hunter et al., 1982; Thompson et al., 1986
Airlift reactor	
Roller bottles	Wunderlich et al., 1981; Sikora et al., 1983; Thompson et al., 1986
Immobilised cells	
Hollow fibre	Haspel et al., 1985
Microcapsules	
Agarose microbeads	
Ceramic cartridge	
Other	
Ascites	Abrams et al., 1984; Bron et al., 1984; Insel, 1984; Bogard et al., 1985; Burnett et al., 1985; Kozbor et al., 1985b; Olsson et al., 1985; Tiebout et al., 1985; Yamaura et al., 1985; Ware et al., 1985
Lymph perfused bioreactors	

munoglobulin more susceptible to the degradation by endogenous proteases secreted by the hybridoma cell line themselves.

There are a number of reports of attempts to grow human hybrids in nude mice. From these it is apparent that human/human hybridomas do not grow readily in nude mice in spite of their T cell deficiency. This is perhaps not surprising in view of the fact that such mice are capable of rejecting xenogeneic cells by NK and macrophage-mediated mechanisms. Improved take can, however, be achieved by irradiating (350–400 rad) the nude mice prior to inocculation (Bogard et al., 1985; Kozbor et al., 1985), by adopting the cells to in vivo growth (Kozbor et al., 1985) or by mixing the cells with human skin fibroblasts prior to inoculation (Olsson and Brams, 1985). But even with these procedures the take may be as low as 50% (Bogard et al., 1985; Kozbor et al., 1985) or depends on the route of injection (Olsson and Brams, 1985).

Fortunately the position with respect to heterohybrids appears to be better several groups reporting that such cells will grow in non-irradiated mice without adaptation (Bron et al., 1984; Insel et al., 1984; Burnett et al., 1985; Tiebout et al., 1985; Yamoura et al., 1985). This observation is perhaps not surprising in light of previous observations that human/mouse B cell hybrids express the H-2 haplotype of the murine parent but fail to express the human HLA antigens which would evoke immune rejection (Raison et al., 1982). It is, perhaps, also worth stressing that in our hand such hybrids normally grow better in vitro.

A number of other observations on the growth of human hybridomas in mice are worthy of comment. Recent reports suggest that human/human hybridomas grow much better in SCID mice (i.e., mice with severe combined immunodeficiency syndrome) than in nude mice (Ware et al., 1985; Effros et al., 1986). Unfortunately such mice have to be housed in a special hood and fed sterile diet.

There have also been conflicting reports on the amounts of immunoglobulin produced in ascites. According to Kozbor et al. (1985), cells adapted for growth in nude mice by in vivo passage exhibited an increase in both tumorigenicity and immunoglobulin production, properties which were associated with a change in morphology. This change was transient as the cells reverted to their original phenotype on return to in vitro culture. The differentiation observed in vitro was attributed to growth factors released during the inflammatory process induced by pristane treatment. In contrast, others have observed that hybridomas adapted for growth in ascites may not secrete large amounts of immunoglobulin (Olsson and Brams, 1985).

Finally the problems of growing human cell lines in nude mice are further compounded by those of viral contamination, a situation aggravated by the extreme susceptibility of such mice to infection. In view of the aforementioned difficulties it is inevitable that the large scale production of human monoclonal antibodies for prophylactic, therapeutic and diagnostic use (such as imaging) will be dependent upon bulk culture either in airlift fermenters or immobilised cell systems.

A survey of human monoclonal antibodies

In spite of the many difficulties encountered in human monoclonal antibody production the success achieved to date has served to reinforce the potential of this technology. This success is illustrated in Table XII which lists many of the human monoclonals described so far. As is to be expected the majority of these monoclonals have been developed with prophylaxis and therapy in mind and are therefore directed against a wide range of bacterial, viral, erythrocyte and tumour antigens. Of particular interest are those derived from normal individuals or individuals with autoimmune diseases which react with autoantigens, both internal and cell surface. The basic strategies employed in the production of many of these antibodies is summarised in Tables VII–IX and elsewhere in the text.

While the range of human monoclonals described to date is large, it should be recognised that many of the antibody-secreting cell lines described have proved to be unstable. Furthermore many of the antibodies have not been adequately characterised and their specificity is open to question. This is especially true of the monoclonals reputed to act with tumour and autoantigens.

TABLE XII

A SURVEY OF HUMAN MONOCLONAL ANTIBODIES

Target	References
Bacterial antigens	
Diphtheria toxin	Gigliotti et al., 1984
Gram-negative endotoxin	Teng et al., 1983, 1985a
Exotoxin A	Larrick et al., 1985
Haemophilus influenzae	Hunter et al., 1982; Gigliotti et al., 1984; Brodeur et al., 1987
Mycobacterium leprae	Atlaw et al., 1984; Foung et al., 1985a
Neisseria meningitides	Brodeur et al., 1987
Pneunomoccus polysaccharide	Schwaber et al., 1984
Pseudomonas aeruginosa	Sawada et al., 1985; Siadak and Lostrom, 1985
Tetanus toxoid	Zurawski et al., 1978; Kozbor and Roder, 1981, 1982; Chiorazzi et al., 1982; Larrick et al., 1983; Teng et al., 1983; Boyd et al., 1984b; Tiebout et al., 1984, 1985; Ho et al., 1985; Ichimori et al., 1985
Tetanus toxin	Gigliotti and Insel, 1982
Chlamydia	Rosen et al., 1983
Viral antigens	
Cytomegalovirus	Emanuel et al., 1984; Matsumoto et al., 1986; Redmund et al., 1986
Epstein-Barr	Koizumi et al., 1986
Hepatitis A	Van Meel et al., 1985
Hepatitis Bs	Burnett et al., 1985; Ichimori et al., 1985; Stricker et al., 1985; Van Meel et al., 1985
Hepatitis Bc	Furuya et al., 1982
Herpes simplex	Seigneurin et al., 1983; Evans et al., 1984; Masuho et al., 1986
HTLV-1	Matsushita et al., 1986
Influenza virus nucleoprotein	Crawford and Callard, 1983b; Ostberg and Pursch, 1983
Rubella	Croce et al., 1980; Ritts et al., 1983; Van Meel et al., 1985; Hilfenhaus et al., 1986
Rabies	Van Meel et al., 1985
T cell leukemia	Matsushita et al., 1986
Varicella zoster	Foung et al., 1985a,b
Other infectious agents	
Plasmodium falciparum	Schmidt-Ullrich, 1986; Udomsangpetch et al., 1986
Red cell and white cell antigens	
Blood group A	Foung et al., 1985a; Larrick et al., 1985; Raubitschek et al., 1985
Blood group i	Hirohashi et al., 1986
Rhesus D	Boylston et al., 1980; Astaldi et al., 1982; Crawford et al., 1983; Bron et al., 1984; Doyle et al., 1985; Melamed et al., 1985; Paire et al., 1986; Thompson et al., 1986.
Rhesus G	Foung et al., 1986
Forssman	Nowinski et al., 1980
Sheep red blood cells	Strike et al., 1984
Chicken red blood cells	Winger et al., 1983
HLA	Hulette et al., 1985; Effross et al., 1986
Tumour antigens	
Bladder	Paulie et al., 1984
Brain glioma	Sikora et al., 1982, 1983
Breast	Wunderlich et al., 1981; Cote et al., 1983, 1984, 1985, 1986; Campbell et al., 1986; Strelkauskes et al., 1987a
Chronic lymphocytic leukaemia	Abrams et al., 1984
Colorectal carcinoma	Hirohashi et al., 1982b; Haspel et al., 1985a,b; Borup-Christensen et al., 1986

(continued on next page)

(Table XII continued)

Target	References
Tumour antigens	
Gastric carcinoma	Hirohashi et al., 1982b; Sugiyama et al., 1986
Lung carcinoma	Hirohashi et al., 1982a; Cote et al., 1983, 1984, 1985, 1986; Cole et al., 1984, 1985a; Murakami et al., 1985
Lymphoma	Carroll et al., 1986
Melanoma	Irie et al., 1982; Wallenius et al., 1983; Watson et al., 1983; Kan-Mitchell et al., 1986
Myloid leukaemia	Andreasen and Olsson, 1986
Renal carcinoma	Cote et al., 1983, 1984, 1985, 1986
Vulva	Glassy et al., 1985
Autoantigens	
Astrocytes	Simpson et al., 1986
Cytoskeletal proteins (actin, myosin, tubulin)	Cote et al., 1983, 1986; Cote and Houghton, 1985
DNA (ss and ds)	Schonfield et al., 1982; Teng et al., 1983; Massicote et al., 1984; Sasaki et al., 1984, 1985
Endothelial cells	Hamburger et al., 1985
Glial fibrilary acid protein	Cote and Houghton, 1985
Golgi	Cote et al., 1986
Myelin-associated glycoprotein	Murray et al., 1984
Nuclear antigens	Satoh et al., 1983; Cote et al., 1986; Simpson et al., 1986; Someya and Kondo, 1986
Nucleolar antigens	Cote et al., 1986; Simpson et al., 1986
Nerve axons	Garzelli et al., 1984
Neurones	Simpson et al., 1986
Neurofibrillary plaques	Simpson et al., 1986
Neurofibrillary tangles	Simpson et al., 1986
Pancreatic islet cells	Eisenbarth et al., 1982; Satoh et al., 1983; Garzelli et al., 1984
Pancreatic duct cells	Satoh et al., 1983; Someya and Kondo, 1986
Parathyroid gland	Garzelli et al., 1984
Pituitary	Satoh et al., 1983
Platelets	Schonfield et al., 1982
Prostatic acid phosphatase	Yamoura et al., 1985
Ribosomal RNA	Teng et al., 1983
Sperm	Winger et al., 1983
Sperm coating protein	Kyurchiev et al., 1986
Stratified squamous epithelium	Garzelli et al., 1984
Thyroglobulin	Dighiero et al., 1983
Thyroid follicle	Satoh et al., 1983
Vimentin	Cote and Houghton, 1985
Rheumatoid factor	Haskard and Archer, 1984
Others	
Bombesin	Ho et al., 1985
2,4-dinitrochlorobenzene	Olsson and Kaplan, 1980
2,4-dinitrophenol	Teng et al., 1983; Bieber et al., 1987
4-Hydroxy-3,5-dinitrophenacetic acid	Steinitz et al., 1977, 1979
Keyhole limpet haemocyanin	Lane et al., 1982; Bieber and Teng, 1987
Phosphorylcholine	Yoshie and Ono, 1980
Sperm whale myoglobin	Bieber and Teng, 1987

Note. For precise details of the specificity of these antibodies the readers should consult the original references. It must be stressed however that in many instances the antibodies have not been adequately characterised.

The application of human monoclonal antibody technology

It is the widespread recognition of the marked potential of human monoclonal antibodies that has sustained work in this area despite major technical difficulties. The advantages of the technology have been emphasised on numerous occasions and in certain instances realised. In brief they include the possibility of new or improved diagnostics, prophylactics and therapeutics and novel ways of probing the B cell repertoire in health and disease. These various uses are summarised in Table XIII and some are commented on in further detail below. For a detailed survey of the potential therapeutic application of human monoclonals, the readers should consult a recent review by Larrick and Bourla (1986).

Attempts to develop human monoclonals of diagnostic importance have usually been prompted by difficulties experienced in producing mouse monoclonals of the desired specificity. This has been attributed to the failure of the mouse to recognise the relevant antigens against a background of relatively immunodominant epitopes. Obvious examples in this area are monoclonals to the Rhesus D antigen and HLA determinants. The human approach has, of course, been given further impetus because of the possible therapeutic value of monoclonal antibodies to the antigens in question.

Understandably it is the prophylactic and therapeutic potential of monoclonal antibodies which has attracted most attention to date with the main emphasis being in the infectious diseases and cancer fields. The possibilities in these areas are quite varied and can readily be illustrated with reference to infectious diseases. The most obvious use is in the passive therapy of infections or in the passive enhancement of the response to vaccines. As stressed by others this form of therapy is of considerable interest in those cases where the infectious agent in question is either resistant or proving increasingly difficult to manage by conventional forms of therapy, e.g., infection with Gram-negative microorganisms (Sasawada et al., 1985; Teng et al., 1985a) and *Malaria falciparum* (Schmidt-Ullrich et al., 1986).

Immortalising B cells from either actively immunised individuals or individuals suffering or recovering from infection also provides valuable information on the ontogeny of the immune response and on the antigens which can elicit protective antibody. This knowledge can be of paramount importance in developing vaccines from either recombinant or natural sources and cannot be readily obtained using murine-derived monoclonals. These human monoclonal antibodies can also be used to purify immunogenic peptides for use in vaccines and as anti-idiotype vaccines themselves. More recently there has been interest in the possibility of using monoclonals of this kind to target drugs to microorganisms.

Many of the aforementioned applications have also stimulated the intensive programme of work on human anti-tumour monoclonals. Of particular interest in this area has been their use in identifying putative tumour-specific antigens, as anti-idiotype vaccines and as reagents for imaging and targeting tumours.

There are of course other important therapeutic applications some of which have been prompted by the success already achieved with monoclonals of murine origin. Of especial importance in this context are monoclonals which can be used to modulate the immune response. This includes antibodies against human lymphocyte subsets, antigens of the major histocompatability complex and soluble mediators of immunity including interleukins which might be used to reverse graft rejection and treat autoimmune diseases.

More recently the potential of human monoclonal antibodies as probes for identifying candidate antigens for contraceptive vaccines has also been emphasised (Kyurchiev et al., 1986). To date most work in this area has been performed with mouse monoclonals and as with other vaccines it remains to be established that the mouse recognises the relevant epitopes.

While the aforementioned uses have been largely responsible for sustained interest in human monoclonal antibody technology, what is perhaps less well appreciated is the contribution it has made, and will continue to make, to our understanding of the B cell repertoire in health and disease (Glassy et al., 1983, 1986; Winger et al., 1983; Cote and Houghton, 1985; Cote et al., 1986). The ability to clone, expand and hopefully im-

TABLE XIII

PROPOSED USES OF HUMAN MONOCLONAL ANTIBODIES

Example	Reference
(A) Diagnostic and monitoring	
(1) Viral infections, e.g., cytomegalovirus	Emanuel et al., 1986
HTLV-1	Matsushita et al., 1986
(2) Other infections, e.g., chlamydia	Rosen et al., 1983
(3) Malignancies, e.g., leukaemia, colorectal carcinoma, melanoma, etc.	Glassy et al., 1983; Cole et al., 1984;Olsson et al., 1984; Haspel et al., 1985a,b; Matsushita et al., 1985; Andreasen et al., 1986; Kan-Mitchell et al., 1986; Smith et al., 1987; Strelkauskas, 1987a
(4) Red cell typing, e.g., Rhesus incompatibility	Crawford et al., 1983a; Bron et al., 1984
(5) Tissue typing, e.g., HLA antigens	Steinitz et al., 1977; Hulette et al., 1985; Effros et al., 1986
(6) Immunopathology, e.g., antibodies to pathological and normal cell components	Haspel et al., 1985a,b; Imam et al., 1986; Simpson et al., 1986; Kan-Mitchell et al., 1986
(B) Prophylactic and therapeutic	
(1) General comment on value as prophylactic and therapeutic	Gigliotti and Insel, 1982; Glassy et al., 1983 Ichimori et al., 1985; Koizumi et al., 1986; Larrick and Bourla, 1986
(2) Treatment of infectious diseases	
(a) identifying immunogenic epitopes for use in vaccines	Atlaw et al., 1985; Matsushita et al., 1986; Schmidt-Ullrich et al., 1986
(b) purifying immunogenic peptides for use in vaccines	Schmidt-Ullrich et al., 1986
(c) investigating regions of genome coding immunogenic peptides	Schmidt-Ullrich et al., 1986
(d) as anti-idiotype vaccines	Atlaw et al., 1985; Matsushita et al. 1986; Schmidt-Ullrich et al., 1986
(e) passive therapy of infections, e.g., Gram-negative, CMV, HTLV-1, *haemophilus influenzae*	Hunter et al., 1982; Emanuel et al., 1984
(f) passive enhancement of response to vaccines	Atlaw et al., 1985
(3) Treatment of malignancies	
(a) identifying immunogenic peptides for vaccines	Kan-Mitchell et al., 1986
(b) as anti-idiotype vaccines	Gaffar et al., 1986a
(c) as therapeutic agents (either alone or drug conjugates)	Glassy et al., 1983; Sikora et al., 1983; Cole et al., 1984; Haspel et al., 1985a,b; Matsushita et al., 1985; Borup-Christensen et al., 1986; Andreasen et al., 1987; Sikora et al., 1987; Smith and Teng, 1987; Strelkauskas, 1987a
(d) imaging or localisation of tumours	Sikora, 1983; Glassy et al., 1985; Borup-Christensen et al., 1986; Burnett et al., 1987; Sikora et al., 1987; Strelkauskas et al., 1987a
(4) General application as immunomodulators e.g. antibodies to suppressor cells, interleukins, anti idiotypes	Dorfman et al., 1985 Sasaki et al., 1984
(5) Therapy of autoimmune diseases	Croce et al., 1980; Sasaki et al., 1984; Chiorazzi, 1986
(6) Management of transplant patients e.g., antibodies to histocompatibility antigens	Hulette et al., 1985; Effros et al., 1986
(7) Prevention of Rhesus incompatibility	Crawford et al., 1983; Bron et al., 1984
(8) In contraception	
(a) as contraceptive per se	Kyurchiev et al., 1986
(b) in identifying immunogenic peptides for use in contraceptive vaccines	

(continued on next page)

(Table XIII continued)

Example	Reference
(C) Investigating the immune system in health and disease	
(1) Studying the normal B cell repertoire in health and disease	Glassy et al., 1983; Winger et al., 1983; Cote et al., 1985, 1986
(2) Examining the specificity/activity of B cells in micro environments e.g. tumour, synovial cavity	Glassy et al., 1983; Watson et al., 1983; Winger et al., 1983
(3) Analysing the relevance of humoral immunity in situations of clinical importance	
(a) infection	Matsushita et al., 1986; Redmond et al., 1986.
(b) malignancy	Sikora et al., 1982; Watson et al., 1983; Paulie et al., 1984; Glassy et al., 1985, 1987; Andreasen et al., 1986; Cote et al., 1986; Kan-Mitchell et al., 1986; Smith and Teng, 1987
(c) autoimmunity and tolerance	Eisenbarth et al., 1982; Shoenfield et al., 1982; Glassy et al., 1983; Satoh et al., 1983; Sasaki et al., 1984; Chiorazzi, 1986
(d) allergic disorders	Chiorazzi, 1986
(4) Characterising and isolating antigens of clinical relevance	
(a) antigens for use in vaccines – see above	
(b) tumour antigens	Smith and Teng, 1987
(c) autoantigens	Satoh et al., 1983
(d) red cell antigens	Bron et al., 1984
(5) Studying the molecular biology of the immunoglobulin locus	Carrol et al., 1986

mortalise specific antibody-secreting cells not only facilitates the analysis of the humoral immune response to microorganisms and tumours (referred to above) but also permits the more detailed investigation of such basic immune phenomena as autoimmunity and tolerance. This approach is especially advantageous when one wishes to investigate the properties of B cells in distinct microenvironments such as tumours, synovial cavities, etc.

Studies to date have shown that within the human B cell repertoire there are precursors capable of recognising and responding to a wide variety of normal and abnormal cell components (Table XII). Furthermore it would seem that the majority of these B cell precursors are programmed against intracellular components rather than cell surface components (see Cote et al., 1984, 1985, 1986). As suggested by Cote et al. (1985) this could reflect differences in susceptibility to tolerance induction of cell surface and intracellular antigens. It is readily apparent that further studies in this area will both increase our understanding of basic immune phenomena and provide a wide range of valuable reagents for use in other studies.

Limitations of current technology

Throughout this review we have emphasised the problems in relation to the supply of suitable immune lymphocytes for fusion and transformation together with the low efficiency of the immortalisation processes themselves. The other major limitation of human antibody-secreting cell lines is the low levels of immunoglobulin synthesised together with the all too frequent loss of this function.

These problems are common to transformed cell lines, hybrids and heterohybrids and are generally attributed to either overgrowth by non-secreting lymphocyte 'contaminants' or to the loss of structural genes coding for immunoglobulin. The former explanation is perhaps most appropriate following EBV transformation where cells are polyclonally activated and cloning in most hands is difficult. On the other hand the loss of regulatory genes is believed to be particularly relevant to heterohybrids which are regarded as being chromosomally unstable, preferentially segregating human chromosomes (Kozbor and Croce, 1985; Teng et al., 1985). Indeed as previ-

ously remarked the instability of human lymphocyte heterohybrids has been used by Croce and others to map genes to chromosomes (see, for example, Croce et al. 1980b; Erikson et al., 1981). In this context it is interesting to note that karyotype analysis reveals that heterohybrids preferentially retain chromosome 14 coding for the heavy chain and lose chromosome 2 which codes for the κ light chain (Croce, 1980b). Furthermore they usually have five times as many mouse as human chromosomes (Raison et al., 1982; Teng et al., 1985b). It has also been suggested that stable translocations of human immunoglobulin genes onto mouse chromosomes may occur following fusion (Cote et al., 1984, 1985). While this is an interesting possibility, to the best of our knowledge it still remains to be demonstrated.

Unfortunately in most instances the reasons for the decline in antibody secretion by human monoclonal cell lines has not been adequately followed up. In almost every case synthesis has been assessed by measuring either total immunoglobulin or specific antibody in culture supernatants, the possible presence of immunoglobulin in the cytoplasm being ignored. As a result it is difficult to distinguish between defects in synthesis and secretion. From a consideration of the literature it is now apparent that the loss of immunoglobulin secretion observed with most cell lines, together with the low levels they secrete initially, can be attributed to a variety of factors (Table XIV). These include deficits in growth and differentiation factors and their receptors, structural and regulatory gene defects and imperfections in the synthetic and secretory machinery of the cells. The latter are believed to be largely due to quantitative and functional deficiencies in rough endoplasmic reticulum, polyribosomes and the Golgi apparatus (Kozbor et al., 1983; Sikora et al., 1983; Kozbor and Croce, 1985). A number of these suggested defects are worthy of further comment.

An area which has been overlooked is that of defects unrelated to the expression of immunoglobulin genes. These include shortages in endogenous or exogenous growth and differentiation factors (Winger et al., 1983) or their receptors (Melamed et al., 1985). In this context it should be noted that the problem with many human antibody-secreting cell lines is not one of proliferation but inadequate immunoglobulin secretion. Furthermore attempts to improve antibody secretion by co-cultivation with HLA-Dr-matched specific helper T cells or antibody to HLA-Dr, which is believed to substitute for T cell help, have not been successful (Crawford, 1985).

Of especial interest are proposed mechanisms relating to the expression of structural genes for immunoglobulin. The theories that have advanced

TABLE XIV

POSSIBLE REASONS FOR LOW OR ABSENT IMMUNOGLOBULIN SYNTHESIS BY HUMAN MONOCLONAL CELL LINES

Suggested defect	References
Inhibitory effect of mycoplasma or other microorganisms	Van Meel et al., 1985
Overgrowth of secreting cells by non-secretors	Zurawski et al., 1978
Shortage of relevant growth and differentiation factors	Winger et al., 1983
Absence of appropriate receptors for growth and differentiation factors	Melamed et al., 1985
Loss or inappropriate incorporation of structural genes for immunoglobulin H and L chain	Schwaber et al., 1984; Teng et al., 1985b
Insufficient copies of structural (and regulatory genes)	Schwaber et al., 1984; Sahagan et al., 1986
Inappropriate H and L chain combination resulting in hybrid molecules	
Failure/loss of relevant regulatory genes or other defects in regulation	Raison et al., 1982; Schwaber et al., 1984; Kozbor and Croce, 1985
Defects in the synthetic machinery resulting in impaired transcription, translation or assembly	Sikora et al., 1983; Kozbor and Croce, 1985; Teng et al., 1985
Defects in the secretory machinery of the cell	Kozbor and Croce, 1985; Teng et al., 1985; Gaffar et al., 1986b

include the loss of such genes, their inappropriate integration or inadequate gene dosage (Table XIV). As the secretion of heavy chain is dependent upon the presence of functional light chains, immunoglobulin secretion might fail if the relevant light chain genes are preferentially lost or fail to function (Köhler et al., 1976; Pepe et al., 1986). If such a mechanism is involved one would expect heterohybridomas to produce a preponderance of human monoclonals with λ light chains in view of reports that the chromosome 2 carrying the κ chain gene is preferentially lost (Croce et al., 1980b).

Although the selective loss of structural genes is a simple and hence attractive proposition, there is evidence to indicate that it is not without its limitations. For example isoenzyme-mapping studies on non-secreting human/mouse heterohybrids indicate that they still carried the heavy chain gene chromosome (nos. 14) and could be induced to re-express immunoglobulin by stimulation with LPS (Raison et al., 1982). Furthermore Schwaber and his colleagues (1984) have shown that back fusion with normal peripheral blood lymphocytes may also re-activate secretion, an observation we have confirmed in our own laboratory. These observations are highly suggestive that the defect in many human monoclonal cell lines is due either to a failure of regulatory genes or some other aspect of regulation (Raison et al., 1982; Schwaber et al., 1984).

Finally the morphology of the human cell lines generated to data should not be overlooked. Generally speaking they have been found to have a less well developed rough endoplasmic reticulum than mature plasma cells and have numerous free polyribosomes (Kozbor et al., 1983; Larrick et al., 1983). These structural abnormalities could lead to impaired translation, assembly and secretion of immunoglobulin.

Strategies for the future

From our previous comments it is obvious that the production of human monoclonals is still a chance affair involving considerable effort and dedication. It is also equally apparent that at the present time there is no simple answer to the many problems which beset this work. Nevertheless there are areas which are worthy of more detailed consideration and these are listed in Table XV; some are also discussed in further detail below.

Supply of immune lymphocytes

As emphasised elsewhere in this review this still represents a major obstacle with respect to human monoclonal development. However, we believe that improvements in this area are possible both with regards to immunisation protocols and lymphocyte selection procedures. In situations where active immunisation is feasible we need to optimise injection and bleeding protocols. This will undoubtedly require a better understanding of the factors influencing the activation, proliferation, differentiation and circulation of committed B cell precursors. Furthermore, where feasible, it will be interesting to establish whether adjuvants such as BCG can be used to increase the circulating pool of committed B cell precursors or their activated progeny.

It is obvious that vital information in this and other areas (see below) will be obtained by detailed cell surface phenotype analysis using specific monoclonal antibodies in conjunction with flow cytometry or immunocytochemical techniques. Of particular value in this respect will be monoclonals against B cell differentiation antigens, growth factor receptors and specific markers of activated B cells (Susuki et al., 1986). Whatever advances are achieved in in vivo immunisation we firmly believe like others that the future of human monoclonal antibody technology will be largely dependent upon advances in in vitro immunisation procedures (Dorfman, 1985; Borrebaeck, 1986). This approach permits both the specific expansion of B cell precursors and the development of antibodies against immunogens which cannot be safely administered in vivo. The value of this approach, like its in vivo counterpart, will be significantly enhanced as our knowledge of the events associated with lymphocyte triggering, proliferation and differentiation, increases and as techniques for affecting class switching are evolved. However, before this approach can be effectively exploited a number of factors will require extensive investigation. These include the

TABLE XV

KEY AREAS FOR FUTURE RESEARCH IN HUMAN MONOCLONAL ANTIBODY PRODUCTION

Improved immunisation strategies	In vivo In vitro
Selection of immune B cells	Tissue source Specificity Differentiation status Proliferative capacity
Immortalisation procedures	Optimisation of existing technologies Improved B cell lines More efficient fusion procedures, e.g., electrofusion Alternative immortalisation strategies, e.g., transfection
Lymphocyte cloning techniques	Alternative cloning techniques, e.g., micromanipulation, fluorescence activated cell sorter Use of recombinant growth and differentiation factors
Regulation of B cell growth	Growth factor effects Growth factor receptor expression
Regulation of immunoglobulin synthesis and secretion	Expression of immunoglobulin structural genes Role of regulatory genes
Production by recombinant techniques	Human antibody molecules Hybrid antibody molecules Mixed antibody protein molecules Site-directed antibody mutants

source of lymphoid cells, the dose and form of immunogen, the influence of mitogens and adjuvants, the value of growth and differentiation factors and the optimum culture time.

Of general importance with respect to the outcome of both in vitro immunisation and immortalisation steps is the cellular composition of the lymphocyte suspensions used. It is generally accepted that the chance of obtaining specific antibody-secreting hybrids is improved by enriching the relevant antibody-secreting precursors and such procedures should be followed where feasible. Of additional importance is the non-B cell content of the reaction mixture. Thus the outcome of in vitro immunisation will depend on the close interaction of B cells, antigen-presenting cells and helper and suppressor T cells and as stressed earlier the outgrowth of transformed B cells is impaired by cytotoxic T cells. However, what is perhaps less well appreciated is that NK cells can inhibit the proliferation and immunoglobulin-secreting capacity of B cells including those transformed by EBV (James and Ritchie, 1984; Kuwano et al., 1986). Further studies on the effects of specific enrichment of plasma cell precursors and the removal of inhibitory cells (such as Tc and NK) should lead to improvements in both in vitro immunisation techniques and immortalisation procedures.

Immortalisation procedures

The most widely used immortalisation procedures in the immediate future will still continue to be EBV transformation and cell fusion or a combination of these procedures. Like others we strongly believe that these procedures are far from optimised and require further basic investigation, especially of the cell populations most susceptible to transformation or fusion.

As stressed earlier there appears to be some confusion over the subset of B lymphocytes which is immortalised by EBV and activated to secrete immunoglobulin. Thus according to Aman et al. (1984) it is a small high density resting population while Chan et al. (1986) claim that it is a large cycling population. It is also worth noting that

twice as many B cells are induced to secrete immunoglobulin as are immortalised (Tosato et al., 1985), an observation of importance when screening for stable antibody-secreting hybrids. In spite of the above the enrichment of the 'relevant' B cell population (along with the removal of Tc and NK cells) would obviously facilitate the production of monoclonals by this procedure.

The alternative cell-driven EBV transformation procedure also requires further evaluation (Siadak and Lostrom, 1985). This technique which involves cocultivation of normal B lymphocytes with a HAT-sensitive EBV-transformed B cell line is reputed to be simple, reproducible and more efficient than conventional transformation and fusion procedures.

The most obvious limitation as far as the fusion process itself is concerned still remains the lack of suitable non-secreting fusion partners capable of producing stable hybrids secreting high levels of antibody. While much effort has been directed towards the development of suitable fusion partners, few, if any, perform like their murine counterparts. Nevertheless, what is encouraging from the authors' viewpoint is the success achieved to date with mouse and mouse/human fusion partners. As stressed earlier a number of investigators have found them to fuse better, and the resultant hybrids clone easier and secrete more antibody, than human hybrids and contrary to general opinion, they have proved to be relatively stable (Tables VI and VII). Furthermore, as previously indicated, they grow better in nude mice and we have also found them easier to grow in tissue culture (personal observations). Thus until better human cell lines are developed, we would stress that mouse and mouse/human fusion partners should not be ignored.

Almost without exception the fusion process itself has been performed with PEG. In view of the low efficiency of this process, the wide variation in fusion efficiency noted between PEG from different manufacturers and from batch to batch, and its relative toxicity, alternative methods of fusion should also be considered. As stressed by Dorfmann (1985) more efficient procedures would be a great advantage, especially where the numbers of lymphocytes available is limited, as for example may be the case following in vitro immunisation or when the lymphocytes have been isolated from, say, a tumour. One process which might be suitable for fusing small numbers of cells is the electrofusion technique developed by Zimmerman and co-workers (Bischoff et al., 1982; Zimmerman and Vienken, 1982). Although this technique has not been widely used to date it is reputed to be 100 000 times more effective than the PEG method (Dorfmann, 1985) and has also been applied to the production of T cell hybridomas (Gravekamp et al., 1985). Furthermore its potential has been enhanced by the recent development of avidin-biotin techniques for 'linking' specific antibody-forming B cells and myeloma cells, prior to electrofusion (Lo et al., 1984; Wojchowski and Sytkowski, 1986). It is also conceivable that some time in the near future procedures will be developed involving the fusion of single cells, followed by the expansion of the hybrid in carefully formulated material containi. g growth and differentiation factors produced by recombinant techniques. This procedure could take advantage of the sophisticated single cell procedures developed by Nossal and his colleagues (see for example Nossal and Pike, 1983; Pike et al., 1984).

With current immortalisation procedures cloning will still remain a major problem. Transformed cells undoubtedly clone less readily than hybrid cells and human hybrids less efficiently than their murine counterparts. Cloning procedures will obviously be facilitated as highly purified growth factors become more readily available and as our knowledge of their mode of action increases. In the meantime there are alternatives to the widely used dilution and soft agar cloning techniques. These include the micromanipulation of single cells (Thompson et al., 1986) and as advocated by Shay (1985) fluorescent-activated cell sorter-based cloning techniques.

Improving stability and immunoglobulin secretion

Considerable effort will have to be directed towards improving the levels of antibody secreted by human cell lines and to ensuring that high levels of secretion are sustained. While this might be partly solved by improvements in immunisation, selection and immortalisation, other strategies will be necessary. The most obvious approach

is to frequently enrich antibody-secreting cell lines and to pursue a rigorous cloning policy. The former procedure has been successfully applied to cell lines secreting anti-red cell antibodies (see, for example, Crawford, 1985; Doyle et al., 1985).

Other approaches would seem appropriate in view of the observations that cell lines which have stopped secreting immunoglobulin can be re-activated either by fusion with normal lymphocytes (Schwaber et al., 1984) or by stimulation with LPS (Raison et al., 1982). As previously stressed these observations, which have been largely overlooked, suggest that the failure of human cell lines to secrete immunoglobulin may be a consequence of defects in the function of regulatory genes or synthetic and secreting mechanisms.

In view of this possibility any techniques for enhancing gene expression would appear worthy of investigation. This might simply involve back fusion, stimulation with LPS or treatment with phorbol esters. The latter approach has been shown to enhance receptor expression and immunoglobulin secretion by B cells (Polke et al., 1986) and is of considerable importance in view of recent suggestions that senescence in human antibody-secreting cell lines might be associated with a decline in growth factor receptor expression (Melamed et al., 1985). More sophisticated approaches would involve transfecting cells with specific enhancer sequences (Teng et al., 1985b) or by gene-silencing techniques involving demethylation of the genome (Olsson and Brams, 1985).

Whichever approach is used to improve specific antibody secretion we believe that when important cell lines cease to secrete immunoglobulin, gene probes and specific antisera should be used to establish if the basic defect is due to the loss of the relevant structural genes, defects in transcription or translation or a failure of assembly and secretion. A better understanding of the nature of the defect could eventually lead to improved corrective measures. It should be stressed, however, that these approaches will only be of value when the fusion partner also lacks the relevant structural genes.

Gene-cloning strategies

In this review we have been concerned with conventional approaches to human monoclonal antibody production. We have also indicated that genetic engineering procedures might be used to improve the secretion of specific antibody by immortalised cell lines. It should be stressed that genetic engineering procedures have already been successfully applied to the cloning and expression of immunoglobulin genes themselves. Although there are still problems to overcome the achievements to date are extremely encouraging and worthy of comment for they open up the way to establishing stable cell lines producing antibodies with unique properties (Aguila et al., 1986). Thus so far complete immunoglobulin molecules have been produced by expressing mouse H and L chain genes in *E. coli* (Boss et al., 1984), yeast (Wood et al., 1985) and myeloma cell lines (Neuberger, 1985), using conventional recombinant procedures employing vectors.

A less sophisticated approach has been to either transfect antibody-secreting cells with calcium-precipitated DNA from permanent cell lines (Jonak et al., 1984) or alternatively, permanent cell lines with similar preparations from antibody-producing cell lines (Strelkauskas et al., 1987b).

There are also reports on the cloning and expression of human heavy and light chain genes using recombinant procedures. However, of particular interest are the reports describing the construction, cloning and expression of genes coding for hybrid molecules containing mouse variable and human constant region domains. This approach, which has recently been reviewed by Oi and Morrison (1986), enables one to produce 'humanised' monoclonal antibodies against immunogens which cannot be injected into humans or which fail to elicit a good immune response in vitro or in vivo. The genes have been introduced into myeloma cells by both spheroplast fusion (Jones et al., 1986; Sharon et al., 1986) and electroporation techniques (Sahagan et al., 1986). The immunoglobulin produced by these recombinants retains the specificity of the original mouse antibody and it will intrinsically be less immunogenic in man than the original mouse antibody. Using this approach chimeric antibodies have been produced against haptens (Boulianne et al., 1986), tumour antigens (Sahagan et al., 1986) and other

antigens (Takeda et al., 1985).

More recently the potential of the technology has been further advanced. Thus Jones et al. (1986) have replaced the genes coding for the hypervariable (complementarity-determining) region of the H chain with genes coding for the hypervariable region of a murine monoclonal heavy chain. Exploitation of this kind of approach should lead to hybrid molecules of even weaker immunogenicity. Sharon et al. (1986) on the other hand have shown how the site-directed mutagenesis approach can be used to alter the specificity and idiotype expression of murine monoclonals. This approach involves the insertion of specific oligonucleotide sequences into heavy chain variable region genes and could enable the development of antibodies with novel specificities.

Finally the potential of this technique for producing antibodies with novel effector functions should not be overlooked. Thus Neuberger and his colleagues (Neuberger et al., 1984; Neuberger, 1985) have expressed in myeloma cells genes coding for the $F(ab')_2$ region of mouse immunoglobulin linked to genes for either enzymes or c-*myc* antigenic determinants. Human constructs of this type could prove extremely useful in diagnosis, targeting and imaging.

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