

From *in vivo* Murine to *in vitro* Human Lymphocyte Immunizations - The changing face of Hybridoma Technology

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1. Introduction

1.1. The immune system and adaptive responses

The immune system is comprised of many different organs and tissues that are found through out the body. The lymphocytes are the central cells of the immune system, constituting 20 - 40 % of the body's white blood cells and are responsible for adaptive immunity and immunological attributes of diversity, specificity, memory, and self/ non self recognition. These lymphocytes circulate continuously in the blood and lymph, and are capable of migrating into tissue spaces and lymphoid organs, thereby providing a high degree of cellular integrity to the immune system. Adaptive immunity comprises of humoral and Cell-mediated responses. Humoral response involves the interaction of the B cells with antigen, resulting in their proliferation and differentiation into antibody secreting plasma cells.

These antibodies serve as the effectors of humoral response facilitating neutralization and/or elimination of the antigens. Cell mediated immunity or cellular immunity is mediated by the effector T cells generated in response to an antigen and their products (such as cytokines). (1, 2, 3). A polyclonal humoral response is comprised of antibodies, derived from various clonal populations having varying specificities (for different

epitopes, on the same immunogen), affinities and classes, and hence provides an effective defense against pathogens. On the other hand, monoclonal humoral response is comprised of antibodies (MAbs) that are derived from a single clone and are hence specific for a single epitope on a complex antigen and have a defined affinity for that epitope. (1,2)

1.2. Antibodies

Antibodies are substances that are produced in response to a particular antigen and are the effectors of humoral immunity. They were identified as serum α globulins by A. Tiselius and E. A. Kabat in 1939. Antibodies are produced in a membrane bound form by B lymphocytes and these membrane bound molecules function as B cell receptors for antigen. Upon interaction of antigen with membrane bound antibodies on naïve B cells, B cell responses are initiated (which constitute the recognition phase of humoral immune response). Antibodies are also secreted by antigen stimulated B cells and in the effector phase of humoral immunity these secreted antibodies bind to antigens and trigger several effector mechanisms that eliminate antigens successfully.

- Polyclonal antibodies - heterogeneous collection of binding sites.
- Monoclonal antibodies - homogenous collection of binding sites

2. Polyclonal Antibodies (PABs) & Monoclonal Antibodies (MAbs)

A polyclonal humoral response is comprised of antibodies with varying specificities for different epitopes, (even on the same antigen), affinities and classes, and hence provides an effective defense against pathogens. Polyclonal antisera are difficult to reproduce because of the variety of antibodies made in the polyclonal response. The level and quality of the antibodies produced however, varies from animal to animal and from a single animal over time. Pabs have a finite availability and can be obtained in a relatively short time (1-2 months). Since they possess different affinities for different epitopes, an overall excellent binding is achieved by adherence to a number of different sites on a complex immunogen or antigen. (1,4)

The development of hybridoma technology by Georges Kohler and Cesar Milstein in 1975, endowed with means for obtaining large quantities of highly specific monoclonal antibodies. Monoclonal antibodies are produced from a single B cell clone and are specific for a single epitope on a complex antigen. All the molecules of a MAb are identical in amino acid sequence and consequently in binding properties, and are secreted continuously by "immortalized" hybridoma cells. Owing to their high degree

of specificity, since they target only one epitope on the complex antigen they minimize cross reactivity.

allow their survival, whereas the non-fused parent cell populations do not survive. The non-myeloma parent cells that do not fuse die

clones are expanded *in vivo* or *in vitro* (5,6,7)

Year	Event
1900	Paul Ehrlich theorizes the possibility of monoclonal antibodies For targeted therapy
1975	Georges Kohler and Cesar milstein invent monoclonal antibodies
1980	First human - mouse IgG produced
1986	First FDA approved mouse mAb produced (Muromonab (OKT-3)
1990	First humanised IgG produced
1997	Rituximab (mouse-human chimeric anti-CD20) becomes the first chimeric mAb approved by the FDA
1997	FDA approves humanized mAb, Adalimumab
2001	First fully human IgG produced
2002	FDA approves first human MAb, Adalimumab

3. An outline of steps involved in the production of MAbs

The critical steps involved in the production of MAbs include

- Immunization
- Choice of fusion partners - splenocytes; myeloma cells
- Fusion/ Somatic cell hybridization
- Cloning of hybrids
- Screening
- Expansion of hybridomas
- Harvest of monoclonal antibodies

Kohler and Milstein immortalized spleen cells from an immunized mouse by fusing them with mouse myeloma cells and the resulting cell line proliferated indefinitely with the continuous production of antibody of single specificity.

Lines of myeloma cells, some secreting immunoglobulin and others not, have been established in long-term culture for several species. By fusing such cells, using inactivated Sendai virus or polyethylene glycol, with another population (traditionally spleen cells from mouse immunized *in vivo* or *in vitro*) it is feasible to produce immunoglobulin secreting hybrids of which a wide number secrete a specific antibody. Hybridomas formed by fusion of two different cells inherit characteristics that

naturally in culture, but the non-fused parent myeloma cells, are eliminated by growth of the mixed culture in a selective medium. The most critical step is the screening of hybridoma culture supernatants for production of the specific monoclonal antibody desired. The wells containing such hybridomas are identified, sub cloned and the

3.1. Advantages of MAbs

- Indefinite supply of antibodies with constant characteristics.
- Mono-specific antibodies can be obtained from impure immunogen
- Affinity and fine specificity are defined and can be selected to suit application
- Availability of antibodies for multiple different and discrete epitopes on a given antigen.
- Easy purification in large quantities by methods that do not damage immunoreactivity
- Clean reagents in immunoassays, giving low non-specific binding and background.
- Often do not inhibit biological activity of the antigen (e.g. enzymes)

A comparison between monoclonal and polyclonal antibodies is given in Table 1.2.

Monoclonal Antibodies	Polyclonal Antibodies
<ul style="list-style-type: none"> • Derived from a single clone 	<ul style="list-style-type: none"> • Derived from multiple clones
<ul style="list-style-type: none"> • Specificity high; Homogeneous: one antibody type, identical antibodies specific to only single epitope. 	<ul style="list-style-type: none"> • Heterogeneous; serum contains a complex mixture of antibodies of different affinity
<ul style="list-style-type: none"> • Effect of change on a single epitope alters specificity significantly 	<ul style="list-style-type: none"> • Effect of change on a single or small number of epitopes is less significant in altering the specificity
<ul style="list-style-type: none"> • Unstable over a wide pH 	<ul style="list-style-type: none"> • Stable over a wide pH and salt conc.
<ul style="list-style-type: none"> • Production- expensive and time consuming 	<ul style="list-style-type: none"> • Production-Cheaper and large quantities of antibodies can be produced rapidly.
<ul style="list-style-type: none"> • Do not show cross reactivity 	<ul style="list-style-type: none"> • Show cross reactivity
<ul style="list-style-type: none"> • Mabs is useful in evaluating changes in molecular conformation, protein-protein interactions, and phosphorylation states, and in identifying single members of protein families. 	<ul style="list-style-type: none"> • Owing to its cross reactivity they are best suited for agglutination and precipitation reactions.
<ul style="list-style-type: none"> • Poor precipitins and agglutinins. 	

Source: Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications and information resources by Lipman NS, Jackson LR, Trudel LJ, Weis- Garcia F.

3.2. Applications of Monoclonal Antibodies

MABs prove useful in three major aspects: the isolation and purification of rare antibodies and antigens, diagnosis, and treatment.

- Monoclonal antibodies to the blood group and human leukocyte antigens improve Cross-matching and tissue-typing facilities.
- Monoclonal antibodies to specific cell surface antigens of both normal and abnormal (carcinomatous) cells provide improved identification, separation, and classification of cells and provide means for diagnosis in parasitic, autoimmune and malignant disease states.
- They prove useful in treatment regimens requiring passive immunization.
- Wide spread application in Cancer immunotherapy where they are aimed at targeting cancer cells.
- Fluorescent or enzyme conjugated monoclonal antibodies are used in immunotechniques like ELISA or RIA.
- Used for measuring and monitoring protein and drug levels in serum
- In identification and quantification of hormones
- Identification of auto antibodies thus helping in the diagnosis of autoimmune disorders.
- In the identification of infectious agents.
- Used in adjunct with epitope matching and molecular modeling, these MABs enable antigenic profiling and visualization of macromolecular surfaces.
- MABs raised against Organ specific antigens, aid in diagnosis of nature of primary tumor Eg- CA-125. (8,9)

4. A transition in the hybridoma technology - *in-vitro* immunizations:

It is not in all diseases that lymphocytes secreting antibodies against a particular antigen are found continuously in circulation.

This restricts the antigens against which antibodies can be raised. The technique of *in vivo* immunization refers to the administration of the specific antigen into an organism. Mice and rats are commonly used for this purpose. A number of animals need to be sacrificed in order to obtain organs such as the spleen, lymph nodes etc. which are the source of antibody producing lymphocytes.

Also, *in vivo* stimulations of lymphocytes are limited by the failure to stimulate adequate numbers of antigen specific B lymphocytes. This may be due to tolerance (antigen specific non-responsiveness) or to an antigen hierarchy response (selective responsiveness to one or a few components of the immunogen preparation) rather than to a lack of antigen-reactive precursor cells. (10) An enhanced ability to overcome suppression and break tolerance is seen in *in vitro* systems. Breaking tolerance *in vivo* may lead to an impaired viability in immunized organisms. (11) Regulation of the cellular immune responses to self antigens and conserved structures that are characteristic of an *in vivo* response does not occur *in vitro*, resulting in stronger responses to such antigens. Another major advantage is that *in vitro* immunizations require lesser amounts of antigen and are less time consuming. The time required for the immunization of lymphocytes in culture is usually around 4-5 days. It is also difficult to raise antibodies against self antigens or toxic antigens *in vivo*. Weakly immunogenic antigens also fail to result in efficient amounts of antibodies. The size and nature of antigen also limits *in vivo* stimulations. Antigens of molecular weight above 10,000 daltons, usually globular proteins elicit optimum responses. With respect to immunizations of humans, ethical considerations severely restrict the antigens that can be deliberately introduced *in vivo*. (11) The use of *in vitro* immunizations would help overcome the problems associated with *in vivo* techniques and help produce therapeutically valuable antibodies.

Monoclonal antibodies have potential applications across the fields of diagnostics, therapeutics and research. Optimization of methods to produce large quantities of specific monoclonal antibodies has been the goal of numerous researchers. The production

of murine antibodies by both *in vitro* and *in vivo* immunizations has been achieved successfully over the last two decades. Human MAB production however has been limited by the lack of a reproducible technique. *In vivo* immunization with specific antigen or the use of lymphocytes from patients suffering from certain diseases has been used for immortalization procedures *in vitro*. Although these methods have met with a fair amount of success in producing MAB, they have several limitations. Recently, a lot of the monoclonal antibody production methods have involved antibody engineering. These methods seem promising and because of the superior technology, are expected to produce quality antibodies. However, chimeric human antibodies that contain mouse variable regions and human constant regions still have 25% non-human proteins that may elicit a human anti-antibody response. Grafting only the CDRs onto human framework regions results in antibodies of lowered potential and still contains 6-10% mouse proteins. Antibodies from transgenic mice containing human immunoglobulin genes can be considered completely human with respect to the protein components. But, since they are produced through immunizations in a highly dissimilar species, their epitope specificity and glycosylation patterns are a cause for concern when considering their effectiveness and immunogenicity, respectively *in vivo*. (12)

Considering these drawbacks, the process of *in vitro* immunizations of human lymphocytes seems to be the only fool proof method of generating antibodies that can be used for varied applications. *In vitro* immunization refers to the induction of an antigen specific primary response of B lymphocytes in culture. The development of monoclonal antibodies by *in vitro* immunization is dependant on several aspects sources of the B lymphocytes, conditions that are required for antigenic stimulation of the cells, immortalization of the antigen primed cells, stability of the hybrids and the production of sufficient quantities of the purified antibodies. It holds great promise as a technique because of the several advantages it offers.

5. Advantages of *in vitro* immunizations over *in vivo* immunizations

To develop therapeutically valuable human monoclonal antibodies, lymphocytes from individuals suffering from an infectious disease are obtained and immortalized *in vitro*. For example, it is known that B cells producing antibodies circulate in the peripheral blood of HIV patients. The lymphocytes obtained from such individuals can be transformed *in vitro* using EBV to produce monoclonal antibodies against gp41 and p24 HIV proteins. (13) *in vivo* immunizations have yielded several important antibodies. The most commonly used antigen for injection *in vivo* is the

keyhole limpet haemocyanin (KLH), because it is a potent immunogen and elicits large amounts of antibody production. Bacterial components such as capsular polysaccharides (14) and attenuated bacterial toxins such as tetanus toxoid have also been used.

6. Murine *in vitro* immunizations

The possibility that murine spleenocytes could be activated *in vitro* came from the work of Anderson *et al.* They conducted experiments to increase the stimulation of murine spleenocytes by culturing them with normal thymus cells along with a B cell mitogen. (15). The addition of T dependent antigens to unprimed dissociated mouse

spleen cells does not activate the B lymphocytes and no significant production of antibody is seen.

The growth supporting conditions can be provided by the culture of young thymocytes or by mixed lymphocyte cultures which provide B cell growth factor (BCGF), B cell differentiation factor (BCDF), IFN- γ , IL-1 and IL-2. Later, improved techniques were developed to provide optimum conditions for *in vitro* activation such as the use of phorbol myristate acetate ester stimulated EL-4 thymoma cells. EL-4 derived lymphokines alone had the ability to support an *in vitro* immunization 2-3 times better than mixed lymphocyte culture derived lymphokines. (16) Adjuvant peptide N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) and dextran sulphate have also been reported to have been used to improve the yield of specific hybridomas. (17) Over 50 monoclonal antibodies of murine origin have been developed from *in vitro* immunized spleenocytes.

It is even possible to raise antibodies by *in vitro* immunizations of mouse spleenocytes against whole complete cells such as collecting duct principle epithelium. Such procedures avoid solubilization and extraction of surface proteins of the cells, yet antibodies against the plasma membrane antigens can be effectively produced. (18)

7. From Murine to Human MAbs

7.1. Limitations of murine MAbs

One of the main applications of monoclonal antibodies is its use in the treatment of various human disorders. When antibodies produced in murine systems are used for this purpose, the main disadvantages are that there is a fast elimination of the antibodies out of the bloodstream, and a triggering of an immunological response to the mouse protein to neutralize it, thus rendering it ineffective. There is also a possibility of a hypersensitivity response with severe symptoms. Side effects of using mouse monoclonal antibodies in humans include fever, rashes, vomiting, urticaria, bronchospasm, tachycardia, dyspnea etc. Such responses can be greatly reduced by using syngenic antibodies. (19)

Table. 3 Comparison between *in vivo* and *in vitro* immunizations used in producing MAbs

Characteristics	<i>in vitro</i> immunization	<i>in vivo</i> immunization
Antigen		
Molecular weight	Low	High
Chemical nature	Proteins, peptides, nucleic acid, lipids etc	Preferably proteins
Toxicity	Even highly toxic substances can be used	Non toxic substances alone can be used
Self antigens	Mab can be raised to autologous antigens	Cannot be raised
Weak immunogens	Strong immune response is evoked	Impossible to evoke an immune response
Concentrations	< 1 μ g(usually in ng)	In mg
Quantity administered(usually)	in μ l	in ml
Immunization		
Done	<i>in vitro</i> in culture plates	<i>in vivo</i> in animal
Booster doses	Not required	Must
Adjuvant	Not required	Must
Time taken	One week	Several weeks
Antibodies Produced		
Isotype produced	Predominantly IgM	Predominantly IgG
Antibody specificity	Broader repertoire of specificities	Comparatively narrow specificities
Affinity maturation	Contributed only by antigenic selection (as 4-5 days in culture is too short for somatic mutation to contribute significantly)	Contributed by antigenic selection and somatic mutation
Time taken for MAb production	1.5-2 months	4-6 months

Table 4 Characteristics of a model <i>in vitro</i> immunization schedule	
Characteristics	<i>in vitro</i> immunization schedule
Antigen	Low molecular weight substances Autologous antigens Weak immunogens
Toxicity	For even highly toxic antigens
Choice of animal	Highly immunogenic strain- Balb/c
Immunization schedule	
Antigen Dose	Very little amount of antigen is used-in μ l
Antigen Concentration	in μ g (or even nanogram)
Immunization	Splenocytes immunized <i>in vitro</i>
Fusion	Done within a week of immunization
Fusion Partners	
Lymphocytes	Mouse lymphocytes stimulated <i>in vitro</i>
Myeloma cells	Syngenic fusion partner- Sp2/0 cells
Type of response	Mimics Primary response
Isotype of Ab that predominates	IgM
Specificity	Broader repertoire of specificities
Total time of production	1.5- 2 months

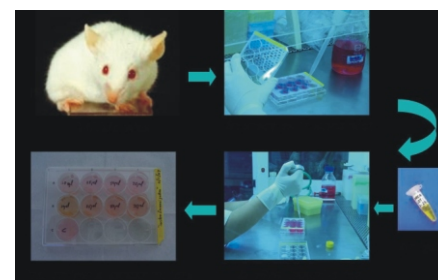


Fig. 1 Overall schematic design for *in vitro* immunizations

7.2. Human Monoclonal Antibodies (hMAbs)

Human monoclonal antibodies recognize epitopes that antibodies produced in other organisms may not be able to. Xenogenic immunizations predominantly produce antibodies that react mostly with immunodominant structures, such as blood group antigens, monomorphic framework epitopes on the MHC etc. hMAbs on the other hand, tend to recognize polymorphic MHC epitopes, tumor-associated antigens- 'altered self' antigens - and other determinants

Table 5. Comparison of <i>in vitro</i> Immunization protocol adopted by a few researchers				
Characteristic	Takahashi <i>et al</i> (27)	Boer <i>et al</i> (32)	Bonwick <i>et al</i> (33)	McMahon <i>et al</i> (34)
Immunogen	Purified LH, TSH, Jack bean urease	Human TG Phe Ox (haptens)	Sulcofuran Flucofuron	G IgG
Adjuvant	no	no	no	no
Immunogen concentrations per ml	μ g	μ g	Ng	-
Host animal	Balb/c	Balb/c	Balb/c	Balb/c
Age, gender	Female, 6-8 weeks	?, 2-4 weeks	?, 6 weeks	?, 6-8 weeks
Medium during immunization	Supplemented DMEM	Serum free DMEM	DMEM with serum	DMEM
Immunization culture capacity	6 well plate	6 well plate	T-75 flask	T-25 flask
Supplements 1	Mixed TCM	TCM	TCM	EL-4 CM
Supplements 2	PMA- TCM	EL-4 CM	-	-
Supplements 3	PMA/Con A- TCM	T-24 CM	-	-
Days of fusion	5th -8th day	3rd-7th day	4th day	5th day
Medium for fusion	DMEM	DMEM	DMEM	DMEM
Ratio of fusion partners (splenocytes: myeloma)	1:1	5:1	2:1	10:1
Fusogen employed	50% PEG	38% PEG	-	50% PEG
Limiting dilution medium	sDME-HAT	HAT	HAT	HAT
Days post fusion	9-14 days	10-14 days	-	8 days
Screening technique	ELISA	ELISA	ELISA	Eli spot
Antibody isotypes obtained	IgM (IgG)	IgM	IgM	IgM

LH -luteinizing hormone; TG- thyroglobulin; TSH- Thyroid stimulating hormone; Phe- Ox 2-Phenyl 5-oxazolone; G IgG-Goat IgG

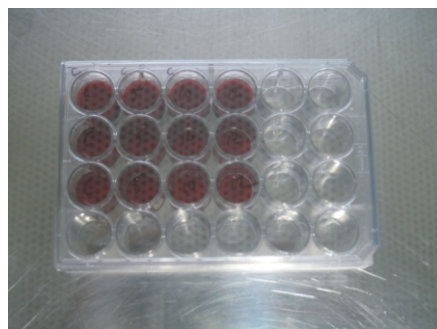


Fig. 2 *in vitro* immunizations can be conveniently performed in culture plates with minimal animal and antigenic usage.

neglected by the xenogenic immune systems. (19) The carbohydrate sequences of the murine antibodies are less compatible to the Fc receptor on human effector cells compared to the asparagine-linked human sequences. This prevents the opsonization of antigens and hence phagocytosis. (16)

The antibodies generated by *in vitro* immunizations are generally of the IgM type and only few of the IgG isotype. It was also seen that when murine monoclonal antibodies were used in cancer therapy, there was limited penetration into tumor sites, short half life of the antibodies within the human body and a failure to recruit host effector functions. (20) hMAbs would serve best to diagnose and treat human diseases. For example, human monoclonal antibodies to HIV could provide standards for diagnostic tests to measure absolute and not relative levels of anti-HIV antibodies in body fluids. They may also serve as the basis for development of anti-idiotype vaccines and also will help define immunodominant regions of HIV that humans recognize regions that may differ from those recognized by other species. (13)

7.3. *in vitro* immunizations of Human Lymphocytes

The use of *in vitro* immunizations for the development of hMAbs has met with moderate success despite the huge efforts by several laboratories. (21) The first *in vitro* immunization resulting in the production of human monoclonal antibodies was by Strike *et al* in 1984. Sheep RBCs were used to stimulate tonsillar lymphocytes. Although the ability of sheep RBC to function as a true antigen has been questioned, the method that was established has been followed and applied to the production of antibodies against other antigens.

The Current Challenge

Optimizations of monoclonal antibody production by *in vitro* immunizations have and continue to be a challenge. First, there is no consensus on the optimum source of lymphocytes for immunization. Tonsillar lymphocytes, spleen and peripheral blood are the most commonly used sources, each having advantages and disadvantages. Second, the immortalization procedures vary greatly. Different methods of immortalization have met with varying degrees of success. The stability of hybridomas formed out of the immortalization procedures also varies greatly. There is also a poor understanding of the kinetics of *in vitro* antibody production mechanisms leading to class switch and

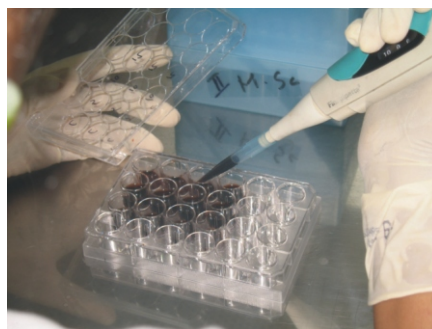


Fig.3 Antigenic withdrawal being performed by washing the entire cell mixture in plain medium and re-plating to increase the efficiency of *in vitro* immunizations

affinity maturation have not been delineated so far. Finally, the optimization of the purification of produced antibodies has not been achieved. (19) All of these problems have to be tackled individually in order to establish favourable and reproducible techniques for generating human monoclonal antibodies from lymphocytes immunized *in vitro*.

7.4. Source of lymphocytes for *in vitro* immunizations

The spleen is the most popular organ for *in vitro* immunizations (refer table). It has been shown that the spleen is a more satisfactory source of antigen specific B cells rather than routine tonsillectomy specimens. (22) The spleenocytes are fractionated into T and B cell populations and then used for immunization cultures. The methods of separation vary from resetting to separation using specific antibodies or FACS methods. A combination

of T helper cells and B cells in the ratio of at least 0.4 is then cultured in medium containing serum along with the required lymphokines and the antigen. (23). Methods have also been described where the cells are not separated into fractions and allogeneic, endogenous stimulation is used in the place of supplementation with purified lymphokines. Immunizations of spleenocytes usually results in the production of IgM. However, if memory cells in the spleen get stimulated, then IgG may be produced. The memory cells may have been developed due to the exposure to an antigen similar to that used for the *in vitro* immunization. This cross reactive stimulation is known as 'original antigenic sin' which occurs *in vitro* due to the loss of suppressive and regulatory influences. (24)

7.5. Strategies for the *in vitro* immunizations of human peripheral blood lymphocytes

The spleen or tonsil removal after immunizations is necessary after immunizations, which are less accessible organs. It is known that the peripheral blood is a source of circulating B lymphocytes secreting antibody which is the most readily available. It permits easy and repeated sampling. Density gradient centrifugation using Ficoll, Percoll and lymphoflot is the method commonly employed to isolate lymphocytes from peripheral blood. From 100 ml of blood it is possible to isolate about 10⁸ lymphocytes which is the optimum amount of cells for fusion. (21) Theoretically, from 10⁸ lymphocytes, 10³ should be clonotypic for any given antigen. Selection and clonal proliferation of the activated B cells over a period of 6-7 days and a doubling time of 20 hours would lead to approximately 1-3X10⁵ lymphocytes secreting antibodies

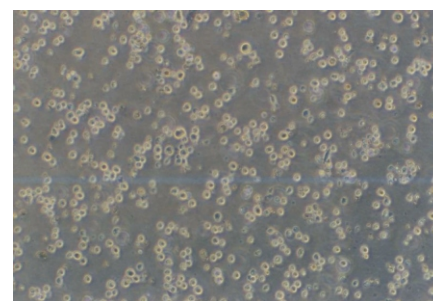


Fig. 4 U266, a human myeloma cell line secretory for IL2, that can be used for conditioning medium and also as fusion partners for developing human hybridomas.

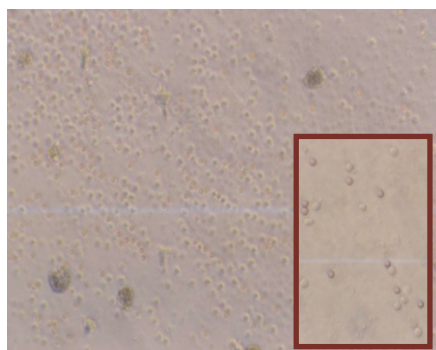


Fig. 5 A mixed human lymphocyte culture that can be used for *in vitro* immunizations to develop human hybridomas.

against the antigen. (19) Attempts to standardize a protocol for peripheral blood lymphocyte immunizations *in vitro* have met with moderate success. It is known that the culture conditions under which immunizations are to be performed is extremely important. But, the experiments on understanding the optimal conditions have met with contradictory technical findings.

7.6. *in vitro* stimulation of *in vivo* primed lymphocytes

Lymphocytes from individuals who have been sensitized to a particular antigen *in vivo* have been stimulated *in vitro*. Stimulation can be achieved using a polyclonal B cell activator such as pokeweed mitogen or the specific antigen itself. The triggering of cells *in vitro* after booster immunization has been found to increase the efficiency of monoclonal antibody production. (25) In such experiments, it was found that the kinetics of the circulating lymphocytes was very important. 6-8 days after booster immunizations, antibody producing cells can be found in peripheral circulation. But, at 14 days, the number of these cells shows a rapid decline to baseline values. It is seen that as long as the antigen specific B cells that are formed due to *in vivo* immunizations are found in culture, they can be triggered *in vitro* to produce antibodies of appreciable titer. Further, it has also been postulated that every antigenic stimulation event is associated with a polyclonal response. (26) The dichotomy between antibodies produced by stimulation with mitogens and with specific antigen is determined by several conditions such as the antigenic concentration, cell density and cell culture surfaces. There is a marked antigen

specific suppression of antibody production by the *in vivo* primed lymphocytes stimulated

- The cell line should preferably not secrete any immunoglobulin

Cell line	Parent Cell line	Type	Ig secretion
U-266-8AZ-r	U-266	Myeloma	IgE()
SK-007	U-266	Myeloma	IgE
FU-266	U-266	Myeloma	
GM15006TG-A12	GM1500	ARH77 Myeloma	IgG2()
KR-4	GM1500		
LICR LON/Hmy2	ARH77	Lymphoblastoid	
WI-L2-729-HFZ	WIL-2	Lymphoblastoid	
LTR 228	WIL-2	Lymphoblastoid	
H35.1.1	WIL-2	Lymphoblastoid	
RPMI-8226-AR	RPMI 8226	Myeloma	
Rh-L4-AR1	RH-L4	Lymphoma	IgG()

in vitro at higher concentrations. (27) These experiments do not however shed light on the mechanisms of activation of the B cells *in vitro*. Also, there has been no speculation regarding whether or not a polyclonal response involves the activation of both naïve and memory B cells.

7.7 A few important study approaches that led to better understanding of *in vitro* stimulation of human lymphocytes

- Stimulation of B cells using a Marbrook chamber importance of cell concentrations
- Separation of lymphocytes into T, B and accessory cells
- Effect of growth factors on *in vitro* immunizations
- *in vitro* cytotoxic lymphocyte suppression of B cells
- Role of CD 40 in the activation of B cells

7.8. Immortalization of peripheral blood lymphocytes

- Transformation with Epstein Barr virus (EBV)
- Electrofusion
- Chemical fusion

7.9. Fusion partners

Good characteristics of a fusion partner include:

- It should be easy to clone and produce stable quantities of antibodies
- It should possess an appropriate selection marker

Human fusion partners are not of myeloma phenotype but are usually lymphoblastoid in nature. Hence they produce lesser quantities of antibodies. The human fusion partner cell lines also usually lack an appropriate selection marker. The specifications under which human lymphocyte-human myeloma fusions are performed vary greatly, contributing to the difficulty in raising antibodies by this method. The table 1.6 shows the available fusion partners for human lymphocytes. Improved cell lines for fusion can be produced by incubating in the presence of 8-azaguanine thus producing a cell line that was 8-azaguanine resistant and HAT medium sensitive. (28)

Fusion with murine myelomas

Interspecies hybrids, such as the fusion of human lymphocytes with mouse myeloma cells have been reported. These hybridomas have certain advantages such as short doubling time and survival at low concentrations. They were however found to be unstable. After a few passages, the human-mouse hybridomas lose immunoglobulin production, due to the segregation of human chromosomes out of the mouse genome. The

Cell line	Species	Immunoglobulin production	
		Heavy	Light
P3-X63-Ag.8	Mouse	+	+
NS1/1-Ag.4.1	Mouse	-	+*
Sp2/0-Ag.14	Mouse	-	-
Y3-Ag.1.2.3	Rat	-	+

+* produced but not secreted

Source: Secher DS. Monoclonal antibodies by cell fusion; Immunology today.

other drawbacks of this method, is that it results in a mixture of proteins yielding heterogeneous immunoglobulin products. Early detection of positive hybrids and cloning repeatedly are necessary to maintain the stability of a human-mouse hybrid. The ratio of lymphocytes to myeloma (preferably 2:1) and optimum initial cell densities also affect the efficiency of fusion. (25) However, these hetrohybridomas can be used as a fusion partner for human lymphocytes. Such triomas were found to be highly stable and the cell lines could produce antibodies for over 22 months.

Heteromyelomas

To overcome the instability of human-mouse hybridomas and the absence of efficient human myelomas, heteromyelomas came into existence. Heteromyelomas are the fusions of a mouse myeloma with a human myeloma. These can then be fused with human lymphocytes to form a human-heteromyeloma trioma - [human × (human × mouse)]. The mechanism underlying the stability of fusion between hetrohybrid cell lines and human lymphocytes is not yet known. It has been suggested that human chromosomes, or their fragments, retained in

the partner line after the first fusion, modify the intracellular environment in such a way that the human lymphocyte chromosomes or fragments following the second fusion are stabilized. Kalantarov *et al.* have described a method to perform serial fusions of a murine myeloma with human lymphoid cells. (12) They proposed that the increased number of human chromosomes can enhance the ability of the fusion partner to fuse with human cells and enable it to yield more stable hybridomas. They fused mouse myeloma cell line X63.Ag8.653 with the human myeloma RPMI8226 which resulted in a clone B6B11 that was efficiently used for fusion with lymphocytes from the lymph node. Then, they fused the resulting B6B11-human lymphocyte (from lymph node) to peripheral blood lymphocytes resulting in high efficiency hybridization and subsequent antibody production.

Conclusion

The revolutionary Hybridoma Technology as described by Kohler & Milstein required their understanding and application of various

Table 9: A chronological listing of USFDA approved Monoclonal Antibodies for human health care.

S.No.	MAB	Year of Approval	Type of MAb	Brand Name	Application
1.	Muromonab-CD3	1986	murine	Orthoclone OKT3	Transplant rejection
2.	Abciximab	1994	chimeric	ReoPro	Cardiovascular disease
3.	Basiliximab	1998	chimeric	Simulect	Transplant rejection
4.	Daclizumab	1997	humanized	Zenapax	Transplant rejection
5.	Rituximab	1997	chimeric	Rituxan, Mabthera	Non-Hodgkin lymphoma
6.	Infliximab	1998	chimeric	Remicade	Inflammatory diseases (mostly auto-immune disorders)
7.	Palivizumab	1998	humanized	Synagis	Viral infection (especially Respiratory Syncytial Virus (RSV))
8.	Trastuzumab	1998	humanized	Herceptin	Breast cancer
9.	Gemtuzumab ozogamicin	2000	humanized	Mylotarg	Acute myelogenous leukemia (with calicheamicin)
10.	Alemtuzumab	2001	humanized	Campath	Chronic lymphocytic leukemia
11.	Adalimumab	2002	human	Humira	Inflammatory diseases (mostly auto-immune disorders)
12.	Efalizumab	2002	humanized	Raptiva	Inflammatory diseases (psoriasis)
13.	Ibritumomab tiuxetan	2002	murine	Zevalin	Non-Hodgkin lymphoma (with yttrium-90 or indium-111)
14.	Tositumomab	2003	murine	Bexxar	Non-Hodgkin lymphoma
15.	Bevacizumab	2004	humanized	Avastin	Colorectal cancer
16.	Cetuximab	2004	chimeric	Erbix	Colorectal cancer
17.	Omalizumab	2004	humanized	Xolair	Inflammatory diseases (mainly allergy-related asthma therapy)
18.	Ranibizumab	2006	humanized	Lucentis	Macular degeneration
19.	Panitumumab	2006	human	Vectibix	Colorectal cancer
20.	Natalizumab	2006	humanized	Tysabri	Inflammatory diseases (mainly autoimmune-related multiple sclerosis therapy)
21.	Eculizumab	2007	humanized	Soliris	Inflammatory diseases including paroxysmal nocturnal hemoglobinuria

biological phenomena and experimental protocols. A few which were documented prior to the Hybridoma technology were selection markers, somatic cell hybridizations, genetic compatibilities, antibody secretory natures of b-lymphocytes. Now, many decades after the discovery, Hybridoma Technology has seen refinements and developments. Infact, this area of experimental biology has been one of the most dynamic technologies as Monoclonal Antibodies are becoming increasingly indispensable for human health care and research.

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