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Review article

Hybridoma technology: new developments of practical interest

Sergio R. Samoilovich [1], Charles B. Dugan [1] and Alberto J.L. Macario [1,2]

[1] *Wadsworth Center for Laboratories and Research, New York State Department of Health, and* [2] *School of Public Health Sciences, New York State Department of Health-State University of New York, Albany, NY, U.S.A.*

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Scope

This brief review has a limited scope. It focuses on technical developments of practical interest concerning construction of hybridomas and their expansion for large scale production of monoclonal antibodies.

Some of the procedures surveyed are not yet routinely applied but are promising. Highlighted are tactics to improve fusion efficiency and production of antibodies in vitro, the use of chemically defined culture media, and attempts at generating antibodies other than murine, especially human.

Beginners in hybridoma technology and those planning to use it soon should be aware of current trends beyond the classical technology. These trends should be carefully considered before making basic decisions, for example what cell line to use as a fusion partner.

It should be clear that new avenues are being explored with success. Mouse and rat hybridomas are no longer the only alternatives. Monoclonal antibodies of other species, e.g., human, are within the realm of the possible. The same can be said for other animal species of importance for veterinary sciences.

A deliberate effort was made to point out novelties that will most likely become routine in the immediate future. Relevant key references and sources for reagents and technical information are provided.

Introduction

Since the introduction of hybridoma technology in 1975 (Köhler and Milstein, 1975) considerable effort has been invested in refining production of monoclonal antibodies for basic research and clinical applications. Much has also been learned about the structure and genetics of antibodies by studies with homogeneous populations of these molecules (Teillaud et al., 1983; DePinho et al., 1986). In more recent years the use of monoclonal antibodies in clinical procedures has increased.

In the area of diagnostic testing, monoclonals can be selected to produce reagents with the desired specificities, thus yielding more reliable results. Therapeutic applications, while still in trial stages for the most part, are encouraging and offer the hope of new, more effective treatments for many disorders.

Until recently, antibody-secreting B lymphocytes were prepared almost exclusively by im-

Correspondence to: A.J.L. Macario, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201, U.S.A.

munizing a host with the antigen of interest. The desired cells were recovered from the host by dissociating the spleen or other lymphatic tissues. This procedure has obvious limitations for studies with substances which are not immunogenic in an animal host (e.g., macromolecules normally found in the host) or which cannot, for ethical reasons, be used to immunize humans.

As the study of interactions which occur when a resting B lymphocyte is transformed into a secreting cell have advanced, progress has been made toward developing in vitro immunization procedures. Applications of these procedures have been somewhat limited, but are becoming more common as the need for alternatives to in vivo immunization becomes clearer.

In the early years of hybridoma technology much emphasis was placed upon understanding the structures of antibodies and the mechanisms controlling their production. Animal models and cells were used extensively. The introduction of murine myeloma fusion partners such as Sp2/0-Ag14 (Shulman et al., 1978), which does not produce any heavy or light chains of its own, allowed investigators to produce hybridomas which only secreted antibodies encoded by genes received from the B lymphocyte partner during fusion. For the most part, B lymphocytes were derived from immunized mice (very often BALB/c) to produce mouse × mouse hybrids. Similar investigations with human monoclonal antibodies have been hindered by the lack of a suitable human myeloma fusion partner. Efforts to overcome this limitation are now a significant focus of research in monoclonal antibody technology (Teng et al., 1983; Casali et al., 1986; Wasserman et al., 1986).

Production of monoclonal antibodies in large quantities has traditionally been done in ascitic fluid of mice (usually BALB/c). Although these monoclonals are contaminated by a variety of mouse proteins, they are sufficiently concentrated to be used for some purposes without further purification. However, the ascitic-fluid method is plagued by limitations. In the search for an alternative, some of the expertise gained during the development of bacterial cultivation systems is beginning to be applied to the cultivation of mammalian cells. Closed batch systems and open, continuous-flow systems are being tested. Several new strategies are available to immobilize the hybrid cells while the supernatant, which contains monoclonal antibodies, is collected. Volumes up to several liters can be readily obtained, but special expertise is required to operate these systems. To date the primary limitation of these systems has been the relatively low concentration of antibodies in the exhausted medium and consequently the frequent need for further steps to concentrate them.

Potential contamination of cell lines, particularly with mycoplasma, is of serious concern when cultivating hybridomas in vitro. Mycoplasmas have been shown to alter immunoglobulin synthesis (Hendershot and Levitt, 1985). Continuous monitoring of cultures to detect mycoplasma is imperative and nucleic acid probes have recently been developed for this purpose (Göbel and Stanbridge, 1984; Razin et al., 1984). Diagnostic dyes and molecular probes are becoming readily available from commercial suppliers (for example: Bionique Laboratories, Saranac Lake, NY; Boehringer Mannheim Biochemical, Indianapolis, IN; Gen-Probe distributed by Fisher Scientific, Rochester, NY; Hana Media, Hana Biologicals, Berkeley, CA).

In this brief review we shall attempt to highlight some of the most recent developments in hybridoma technology which seem to offer practical advantages – or which at least show a potential for practical use in the near future. Readers who seek more comprehensive information on current hybridoma technology and monoclonal antibody applications are encouraged to examine any of a number of excellent reviews (Milstein, 1982; Reading, 1982; Denis et al., 1983; Schonherr and Houwink, 1984; Sikora, 1984; Köhler, 1985; Westerwoudt, 1985; DePinho et al., 1986). Those who seek more information on how to generate hybridomas should consult articles detailing that technology (Oi and Herzenberg, 1980; Galfrè and Milstein, 1981; Köhler, 1981; Bastin et al., 1982; Lane et al., 1982; Nakamura et al., 1982; Campbell, 1984; Goding, 1986).

Modifications of media to support growth of fusion partners and hybridomas

Since the early days of cell culture the necessity for serum supplements has been acknowledged.

TABLE I

LOW-SERUM AND SERUM-FREE MEDIA FORMULAS SUPPORTING GROWTH OF FUSION PARTNERS AND HYBRIDOMA CELL LINES

Name	Supplements added to basal medium	Cell lines supported	Supplier [a]	Reference [b]
None given	Albumin Transferrin	Human × human hybridomas	BoM, CR and Sigma	1
CITTL [c]	Casein Insulin Transferrin Testosterone Linoleic acid	Several	BoM, CR and Sigma	2
SFH	Transferrin Insulin Hydrocortisone Linoleic acid Albumin Trace elements Ethanolamine Ascorbic acid	P3/X63-Ag8, Sp2/0-Ag14 and derived hybrids	BoM, CR and Sigma	3
BM	IMDM Alpha-thioglycerol Progesterone Trace elements	P3/X63-Ag8.653 and derived mouse × mouse and rat × mouse hybrids	ABO, Gibco and as listed in the text	4
KSLM	Insulin Sodium selenite Transferrin Oleic acid Human LDL Diaminoethanol Albumin	P3/NS1/1-Ag4-1 and derived hybrids	BoM, CR and Sigma	5
Nutridoma-SP	Albumin Transferrin Insulin Se	Sp2/0-Ag14 and derived hybrids	BoM	NF
Nutridoma-HU	Ibid.	Human fusion partners and derived hybrids	BoM	NF
Nutridoma-NS	Ibid. with slightly higher protein concentration	P3/NS1/1-Ag4-1 and derived hybrids	BoM	NF
HB-101 [PP]	Insulin Transferrin Albumin	Sp2/0-Ag14 and derived hybrids	Hana	6,7
HB-102	Modified KSLM (see above)	P3/NS1/1-Ag4-1, P3/X63-Ag8.653 and derived hybrids	Hana	NF

(*continued on next page*)

(Table I continued)

Name	Supplements added to basal medium	Cell lines supported	Supplier [a]	Reference [b]
HB-104	Human albumin Human transferrin Human insulin	Human × human hybridomas	Hana	NF
HL-1	Insulin Transferrin Testosterone Sodium selenite Fatty acids Ethanolamine	P3/NS1/1-Ag4-1, Sp2/0-Ag14, P3/X63-Ag8 and derived hybrids	Ventrex	NF

[a] Suppliers: **ABO**, American BioOrganics, N. Tonawanda, NY; **BoM**, Boehringer Mannheim Biochemicals, Indianapolis, IN; **CR**, Collaborative Research, Lexington, MA; **Gibco**, Grand Island Biologicals, Grand Island, Long Island, NY; **Hana**, Hana Biologicals, Berkeley, CA; **Sigma**, Sigma Chemical Co., St. Louis, MO; **Ventrex**, Ventrex BioVentures Group, Portland, ME. These are suppliers with whom the authors are familiar; others may be available. Nutridoma, HB-101, 102 and 104, and HL-1 are only available from the suppliers listed or their authorized distributors.

[b] References are: **1**, Cole et al., 1985; **2**, Darfler and Insel, 1982; **3**, Kovar and Franek, 1984; **4**, Cleveland et al., 1983; **5**, Kawamoto et al., 1983; **6**, McHugh et al., 1983; **7**, Steimer, 1984.

[c] Abbreviations are: **CITTL**, casein, insulin, transferrin, testosterone, linoleic acid; **NF**, no references could be found; **IMDM**, Iscoves's modified Dulbecco Medium; **KSLM**, Kawamoto, Sato, Le, and McClure; **LDL**, low density lipoproteins; [PP], patent pending; **SFH**, serum-free hybridoma.

Efforts to reduce the dependence upon such supplements have had various degrees of success. Work in our laboratory has shown that hybridomas can frequently be grown with less than the 10% serum supplementation often specified in medium formulas. After an appropriate period of acclimation, final serum concentrations of 2.5–5.0% have been successfully utilized for 4 months or more with rare deterioration of culture viability or monoclonal antibody production (Macario and Dugan, unpublished data).

With the advent of hybridoma technology has come a renewed interest in chemically defined media. Many of the reasons are the same as those which have motivated cell culturists, especially the need to eliminate the variations in serum composition between vendors and between lots from the same vendor, to supplement the culture medium with only those components which enhance cell growth, to decrease the probability of introducing contaminants such as viruses and mycoplasma, and to reduce the costs of large-scale operations.

Other concerns specific to the production of monoclonal antibodies from hybridomas include the time and effort required to purify the antibodies from the medium, the risk of introducing components which may inhibit B lymphocyte function before and after hybridization, and the possibility of cross-reactions between the monoclonal antibodies being produced and components in the medium. This last problem is of particular concern in the production of antibodies against compounds which naturally occur in animals and humans such as hormones and enzymes.

The medium formulas presented in Table I favor three compounds: transferrin, albumin and insulin (in decreasing order of frequency cited). To these compounds some formulas add hormones (such as testosterone or progesterone) and sodium selenite. Boehringer Mannheim and Hana Biologicals market media formulated to support two murine myelomas, Sp2/0-Ag14 and P3/NS1/1-Ag4-1, and their derivatives, as well as human × human hybrids. No details are available regarding the composition of these media, and only HB101 has been used in studies we reviewed (McHugh et al., 1983; Steimer, 1984). HL-1 from Ventrex was originally formulated to support the company's murine HL-1 Friendly Myeloma-653 and its derivatives, but broader applications are claimed by

the manufacturer. No references could be located which cite its use.

Tharakan et al. (1986) compared the growth of murine hybridomas in hormonally defined and serum supplemented media. The hybrids tested grew more quickly in serum supplemented media but tended to produce less antibody (an observation also made by us). Glucose uptake could not be correlated with antibody production rates.This lack of correlation emphasizes the importance of monitoring both, glucose depletion and antibody secretion rates during the optimization of a new medium formula.

Fusion partners

Mouse and rat fusion partners

The mouse and rat cell lines currently being used as partners in mouse × mouse, mouse × rat, rat × mouse, and human × mouse fusions are listed in Table II. Sp2/0-Ag14 produces antibodies encoded by genes received from the B cell partner during fusion. P3/NS1/1-Ag4-1 synthesizes kappa chains but does not secrete them. However, hybrids obtained with P3/NS1/1-Ag4-1 can express different combinations of chains including those of the myeloma and the B lymphocyte.

Sp-2/0-Ag14 and P3/NS1/1-Ag4-1 lines are frequently used. Another line, P3/X63-Ag8, is also commonly used but has the disadvantage of secreting kappa and gamma chains. This line is the origin of P3/NS1/1-Ag4-1 and other clones which have been less widely utilized (ATCC, 1985).

FOX-NY (Taggart and Samloff, 1982) takes advantage of a double deficiency that makes the line sensitive to both hypoxanthine-aminopterin-thymidine (HAT) and adenine-aminopterin-

TABLE II

MOUSE AND RAT CELL LINES USED TO CONSTRUCT HYBRIDOMAS

Species	Designation (short name)	Ig secreted	Hybrid selection medium	Supplier or expert	Reference [a]
Mouse	Sp2/0-Ag14 (Sp2/0)	None	HAT [b]	ATCC [c]	1,2
	P3/X63-Ag8	IgG1 (kappa, gamma)	HAT	ATCC	1,3
	P3/NS1/1-Ag4-1 (NS-1)	None (kappa produced)	HAT	ATCC	1,4
	FOX-NY	None	AAT [d]	Dr. T. Taggart Dept. Molecular Biology and Genetics, Wayne State School of Medicine, Detroit, MI	5
	HL-1 Friendly Myeloma-653	None	AAT	Ventrex Bioventures Portland, ME	NF [e]
Rat	Y3-Ag 1.2.3	Kappa secretor	HAT	ATCC	1,6,7
	YB2/3HL.P2.G11.16Ag.20 (YB2/0)	None	HAT	ATCC	1,8

[a] References are: **1**, ATCC, 1985; **2**, Shulman et al., 1978; **3**, Köhler and Milstein, 1975; **4**, Köhler and Milstein, 1976; **5**, Taggart and Samloff, 1982; **6**, Galfrè et al., 1979; **7**, Lane, 1985; **8**, Kilmartin et al., 1982.

[b] Hypoxanthine-aminopterin-thymidine.

[c] American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD.

[d] Adenine-aminopterin-thymidine.

[e] No references could be found.

TABLE III

HUMAN-CELL FUSION PARTNERS

Type [a]	Designation (short name)	Ig secreted	Hybrid selection medium	Supplier or expert	Reference [b]
L	GM 4672	IgG	HAT [c]	Cell Repository Institute of Medical Research, Camden, NJ	1,2
M	GM 1500 6TG2	IgG	HAT	Ibid.	3,4
L	KR-4	IgG	HAT-O [d]	Consult references	5,6,7
L	UC729-6	IgM	HAT	Dr. Ivan Roisten Univ. of California at San Diego, Cancer Institute, San Diego, CA	8,9
L	UC729-HF_2	None	HAT	Techniclone, Santa Ana, CA	8
L	WIL2/729HF	IgG	HAT	Ibid.	10,11
L	LICR-LON-HMy_2	IgG	HAT, HAzT [e]	Ludwig Inst. Cancer Research, London Branch, Sutton, UK	8,12
M	U-266	IgE	HAT	Consult references	8,13
M	RPMI 8226	Lambda	HAT	ATCC [f]	8,14
L	LTR228	IgM Kappa	HAzT	Cetus Immune Research Labs, Palo Alto, CA	15
M	U266-AR_1 (SKO-007)	IgE	HAT	Dr. Marsha Beaver Stanford Medical Ctr., Stanford, CA	6,7,16
H	HM2.0	None	HAT	Dr. M.R. Posner Rodger Williams Cancer Ctr., Rodger Williams General Hospital, Providence, RI	17
L	HO-323	None	HAT	Consult references	18,19
L	TAW-925	Not reported	HAT	Consult reference	20

[a] **L**, lymphoblastoid, **H**, hybrid, **M**, myeloid

[b] References are: **1**, Croce et al., 1980; **2**, Massicotte et al., 1984; **3**, Eisenbarth et al., 1982; **4**, Reading, 1982; **5**, Kozbor et al., 1982; **6**, Kozbor and Roder, 1983; **7**, Kozbor et al., 1983; **8**, Abrams et al., 1983; **9**, Glassy et al., 1983; **10**, Denis et al., 1983; **11**, Heitzmann and Cohn, 1983; **12**, Edwards et al., 1982; **13**, Nilsson et al., 1970; **14**, Matsuoka et al., 1967; **15**, Buck et al., 1984; **16**, Olsson and Kaplan, 1980; **17**, Posner et al., 1983; **18**, Hashizume et al., 1987; **19**, Ohashi et al., 1986; **20**, Ichimori et al., 1987.

[c] HAT, hypoxanthine-aminopterin-thymidine.

[d] HAT-O, hypoxanthine-aminopterin-thymidine-ouabain.

[e] HAzT, hypoxanthine-azaserine-thymidine.

[f] ATCC, American Type Culture Collection.

thymidine (AAT) selection. The RBF/Dn mice required to obtain optimum results with this line are more expensive than the BALB/c mice commonly used and are not available in large quantities without a significant delay. Only one study was found that had used FOX-NY since its introduction in 1983, and in that work BALB/c mice were used with the FOX-NY cells (Lane, 1985).

The HL-1 Friendly Myeloma-653 is relatively new and is quite similar to FOX-NY. According to the supplier, this line can be used either with BALB/c mice in a traditional HAT selection system or with RBF/Dn mice in an AAT selection system. We found no studies utilizing these cells, but the supplier claims that they are used by several large companies as well as by groups at NIH. These cells have been available for a short time and may not yet be widely known.

The YB2/0 rat myeloma is a nonsecreting derivative of a fusion between the Y3-Ag 1.2.3 line and AO spleen cells. The cells are somewhat larger and appear to grow slightly better than the original Y3-Ag 1.2.3. line (ATCC, 1985). Proponents of rat myelomas for rat × rat and rat × human hybridizations claim greater stability and antibody yield (Clark et al., 1983). In spite of these claims hybridomas made with rat myeloma cells are not being widely utilized. This may be due in part to difficulties encountered when trying to grow rat hybridomas (Galfrè and Milstein, 1981; Goding, 1981). Van Snick et al. (1986) recently isolated a T cell-derived lymphokine which was found to be necessary for in vitro cultivation of most of their rat × mouse hybrids. A similar requirement for a macrophage-derived factor to support the in vitro growth of plasmacytoma cells was also reported by Nordan and Potter (1986). Understanding the factors which regulate in vitro growth of rat hybridomas may contribute to making this system more widely utilized.

Human-cell fusion partners

Development of cell lines for use in preparing human × human hybridomas has been an area of great interest, not only because of the potential for clinically significant applications, but also because interesting genetic problems can be studied with the human × human system (Hunter, 1985).

The lines which have become available thus far are primarily lymphoblastoid in origin, rather than myelomas, or are hybrids with more characteristics of lymphoblasts than of mature malignant cells (Table III). Notable exceptions are GM 1500 6TG2, U-266 and its derivatives, and RPMI 8226. These lines have been available for years. Lines GM 1500 6TG2 and U-266 continue to be useful in generating human × human hybrids up to this day, however, the ability of RPMI 8226 to form stable hybrids has been questioned (Abrams et al., 1983; Kozbor et al., 1983). Each of the remaining lymphoblastoid lines has its supporters, but none are widely utilized.

Except for UC729-H_2 all of the human-cell fusion partners suffer the disadvantage of secreting parental heavy or light chains or antibodies. UC729-H_2 is a nonsecretor and produces stable hybrids which grew well for months (Abrams et al., 1983).

The HM2.0 line represents a break from the more traditional approach, in which new lines of fusion partners are developed from clones of established lines. HM2.0 is a hybrid between a human myeloma which did not secrete antibodies when fused to lymphocytes, and a plasma cell leukemia. This fusion produced a line which does not secrete heavy or light chains or antibodies of its own (Posner, 1983). Additional cell lines of this type are being developed which have high fusion efficiencies and maintain antibody production for more than 1 year (Posner, personal communications). Similar efforts have been made to produce nonsecreting hybrid fusion partners from murine myelomas and human cells (Ostberg and Pursh, 1983; Teng et al., 1983).

All cell lines now being used rely on a defect in the hypoxanthine phosphoribosyl transferase salvage pathway. Some increase in the number of antibody-secreting hybrids has been obtained by substituting azaserine for aminopterin (Edwards et al., 1982; Buck et al., 1984) or by adding ouabain (Kozbor et al., 1982; Kozbor and Roder, 1983).

Manipulations to improve fusion efficiency

In rodent systems immune lymphocytes can usually be generated by injecting a host with the desired antigens. After sufficient time and proper

boosting to enhance the response, the spleens of these animals will contain antibody-secreting B lymphocytes. The cell suspensions thus obtained usually do not need to be enriched before being fused to a myeloma line.

In some cases however, the immunogenicity of a particular determinant is low. Essentially three strategies have been developed to improve the response to weak antigens or to a minor determinant of a complex immunogen and thus increase the number of relevant B lymphocytes. The immunogen can be adsorbed onto a matrix such as nitrocellulose which is then introduced into the host (Sternick and Sturmer, 1984; Knudsen, 1985).

Splenocytes harvested from an immunized donor can be reinjected, along with antigen, into a syngeneic, irradiated recipient. The spleen cells of the second host are harvested for fusion with the myeloma partner. This procedure yields more antibody-secreting hybrids than can be obtained with the usual technique (Siraganian et al., 1983).

An enhanced response against a particular determinant in a mixture can also be developed in animals by depressing the response toward determinants which are not of interest. This selective reduction of response can be achieved either by injecting a cytotoxic agent, such as cyclophosphamide, with the immunogen (Matthew and Patterson, 1983) or by passively immunizing the recipient with antibodies directed toward the undesired determinants (Thalhamer and Freund, 1985).

Due to ethical considerations, immune human B lymphocytes cannot be prepared as in animal systems. Many antigens of interest cannot be used to immunize patients; and even in those cases where immunization is acceptable, the primary source of antibody-secreting B lymphocytes is most frequently peripheral blood.

Techniques have been developed for handling and preparing peripheral blood lymphocytes (PBL) for fusion. Some of these techniques are summarized in Table IV. Most PBL are isolated from whole blood of donors by centrifugation in a lymphocyte separation medium such as Ficoll-Hypaque (Pharmacia, Piscataway, NJ). Each subsequent step increases the risk of culture contamination, but the increased production of hybrids and the greater stability of the antibody secretion may outweigh the risk. Further enrichment of the B

TABLE IV

MANIPULATIONS OF HUMAN LYMPHOCYTES FOR IMPROVING FUSION EFFICIENCY AND VIABILITY OF MONOCLONAL ANTIBODY-PRODUCING CELLS

Method	Advantage	Disadvantage	Reference [a]
Enrichment for B cells	Lessens T cell inhibition of B cell growth	Requires manipulations of the cells which may increase the probability of bacterial contamination	1,2,3
In vitro immunization or boosting	Homologous immune lymphocytes can be selectively stimulated; especially useful when antigen is scarce or when initial response is poor	Increases possibility of bacterial contamination	3,4,5,6,7,8
Pokeweed mitogen or lipopolysaccharide	Selective stimulation of certain B lymphocyte subpopulations	IgM-producing clones predominate	9,10,11,12
Epstein-Barr virus infection	Long term stability of hybridomas	Risk of cell-transforming DNA contamination of the antibody product; dominance of IgM-producing clones	1,10,13,14,15,16,17

[a] References are: **1**, Casali et al., 1986; **2**, Gigliotti et al., 1984; **3**, Maeda et al., 1986; **4**, Borrebaeck, 1986; **5**, Ho et al., 1985; **6**, Reading, 1982; **7**, Wasserman et al., 1986, **8**, Schelling, 1986; **9**, Denis et al., 1983; **10**, Heitzmann and Cohn, 1983; **11**, Kozbor and Roder, 1983; **12**, Westerwoudt, 1985; **13**, Effros et al., 1986; **14**, Kozbor et al., 1982; **15**, Kozbor and Roder, 1984; **16**, Roome and Reading, 1984; **17**, Thompson et al., 1986.

lymphocyte population, although not always essential, can be achieved by using the adherent (Maeda et al., 1986) or rosetting (Gigliotti et al., 1984) properties of the various subgroups of lymphocytes to increase the number of antibody-secreting hybrids produced by fusion. Recent work with mouse cells by Hadas and Theilen (1987) suggests that the use of low hybrid-cell concentrations in the primary culture after fusion tends to favor the development of antibody-secreting clones. In some instances this approach may be helpful in reducing the number of purification steps required prior to fusion.

When host immunization is impossible or the response to the immunogen must be heightened, PBL can be stimulated in vitro by exposing them to antigen for various lengths of time. This procedure often requires complex media and additives, such as dextran sulfate, to enhance the response of the PBLs (Schelling, 1986), or thymocyte-conditioned medium to improve the response of murine spleen cells (Takahashi et al., 1987). Extensive reviews of in vitro immunization are available (Reading, 1982; Borrebaeck, 1986). Early work has shown that primary and secondary long-term responses can be elicited in vitro in animal models (Macario et al., 1973; Conway de Macario and Macario, 1978). More recently in vitro methods have been applied to nonhuman systems where the solubility of the immunogen was low (Van Ness et al., 1984) or the immunogenicity was weak (Rathjen and Underwood, 1985).

Mitogen-activated B lymphocytes have been used for hybridization in animal model systems. Incubation of PBL with activators such as pokeweed mitogen (Denis et al., 1983) has been helpful in some instances to stimulate quiescent B cells prior to fusion. B lymphocytes can also be activated by incubation with Epstein-Barr virus, a commonly used technique for preparing immortal B lymphocytes which is discussed at greater length in other works (Kozbor et al., 1982; Kozbor and Roder, 1983b, Roome and Reading, 1984; Casali et al., 1986; Velez et al., 1986).

Fusogens

In their original work Köhler and Milstein used Sendai virus to fuse antibody-secreting B lymphocytes to myeloma partners (Köhler et al., 1975), but in later years polyethylene glycol (PEG) has been used instead of the virus. Objections have been raised to the use of PEG because of its toxicity to the cells, and alternatives have been suggested. Nonetheless, a review of recent literature shows widespread use of some form of PEG for generation of hybridomas, and major reports which detail procedures specify fusing with PEG (Oi and Herzenberg, 1980; Galfrè and Milstein, 1981; Köhler, 1981; Goding, 1986).

The mechanisms involved in PEG-mediated cell fusion continue to be studied. The necessity for close contact between the cells being fused has been documented (Knutton and Pasternak, 1979; Wojcieszyn et al., 1983). PEG appears to promote the close apposition of cell membranes while additives contained in the commercial preparations actually stimulate the fusion process (Wojcieszyn et al., 1983). This may help to explain why fusing efficiencies differ so much when various brands, molecular weights and lots of PEG are used (Fazekas de St.Groth and Scheidegger, 1980; Lane et al., 1984). Compounds such as dimethyl sulfoxide have been used to modify the cell membranes and enhance fusion (Fazekas de St.Groth and Scheidegger, 1980). Synchronization of the cell cycle with colcemid to produce myeloma cells in the M stage have been reported (Miyahara et al., 1984). None of these techniques has been widely adopted.

Immunochemical methods have been applied to fusion mixtures in an effort to guide the immune lymphocytes to the myeloma cells. The latter cells are coated with antigen (Kranz et al., 1980) or haptens (Lo et al., 1984) and then reacted with immune lymphocytes with or without PEG (Bankert et al., 1980). While apparently elegant, these techniques have not found frequent use.

An entirely different approach to fusing cells, electrofusion, was proposed in the late 1970s (Zimmerman and Scheurich, 1981; Bischoff et al., 1982). Cells were oriented by a nonuniform electric field and then fused by applying a pulse of high-frequency energy. Like immunochemical methods, electrofusion has not become popular. We found only one paper that reported successful construction of antibody-secreting hybridomas by electrofusion alone (Bischoff et al., 1982). How-

ever, electrofusion has been successfully combined with immunochemical methods (Lo et al., 1984) and a biotin/avidin technique to direct B lymphocyte-myeloma cell aggregation to increase hybridoma yield (Wojchowski and Sytkowski, 1986). The high cost of equipment necessary for this technique may partly explain its rather limited utilization.

Feeder cells

Feeder cell layers are routinely used in hybridoma technology to support the growth of newly fused hybrids and single cells during cloning. Peritoneal macrophages (Fazekas de St.Groth and Scheidegger, 1980), splenocytes (Goding, 1986), or thymocytes (Oi and Herzenberg, 1980) are employed to provide unknown factors which enhance the growth of the hybrid cells. Although the enhancing effect of auxiliary feeder cells on long-term antibody synthesis in vitro has been reported for various systems including the rabbit lymph node model (Conway de Macario and Macario, 1976), the introduction of these cells in hybridoma technology raises several concerns. Since most feeder cells are prepared from tissues removed from animal donors, the possibility that the hybridomas will become contaminated by bacteria, fungi or mycoplasma introduced with the feeder cells increases. Feeder cells will compete with hybrid cells for metabolites and increase the depletion rate of the medium. The additional cell number will increase the rate of metabolic waste accumulation which may prove to be inhibitory to the hybrids. Feeder cells are usually normal somatic cells and do not divide or survive for long periods of time in culture. Slow-growing hybridoma cells will require the feeder layer to be replenished with new cells. This will increase the probability of contamination. It will also increase the probability that dead cells left in the cultures will release autolytic enzymes which may damage either the hybridoma cells or the antibodies.

The development of alternatives to feeder cells has been slow in coming. Recent studies have shown that conditioned medium from established macrophage cell lines effectively supported the in vitro growth of plasmacytoma cells (Nordan and Potter, 1986). The same conditioned medium enhanced the establishment of hybridomas after fusion and also the cloning efficiency of hybrids cloned by limiting dilution (Rathjen and Greczy, 1986). Furthermore, Van Snick et al. (1986) have reported the isolation of a T-cell derived lymphokine which supports the in vitro growth of rat × mouse hybrids which otherwise failed to survive. Continued investigations along these lines should lead to the development of well defined additives. The latter could be included in serum-free media formulas to improve performance at all stages of hybridoma cultivation.

Production of monoclonal antibodies in vivo

Production of high concentrations of monoclonal antibodies in ascitic fluid is a well-established technique (Oi and Herzenberg, 1980; Galfrè and Milstein, 1981; Bastin et al., 1982). In our laboratory this system yields antibodies at concentrations averaging 10–50 mg/ml in 3–5 ml of ascitic fluid per mouse. Ascites usually develops within 2 weeks.

Murine hosts for the hybridomas are most often BALB/c and are prepared by a single intraperitoneal injection of Pristane (2,6,10,14-tetramethylpentadecane). For some mouse × mouse hybridomas the use of Pristane can be eliminated without deminishing the concentrations of antibodies recovered (Macario and Dugan, unpublished data).

With the increasing need for large quantities of nonmurine antibodies, elaborate methods have been developed to prepare the host and the hybridomas (Table V). One problem with in vivo generation is host rejection of the hybridoma. To minimize rejection mice have been irradiated. Although the optimal radiation dose varies with the system used, suppression of the host immune system in this manner appears to have alleviated both host rejection of the hybridoma and immune response to the antibodies (Truitt et al., 1984; Kozbor et al., 1985; Weissman et al., 1985). Another problem encountered with in vivo generation is the need for hybridomas to adapt to the complex environment of the host peritoneal cavity. Passage as a subcutaneous tumor (Truitt et al.,

TABLE V

PRODUCTION OF MONOCLONAL ANTIBODIES IN VIVO

Host	Host pre-treatment	Hybrids grown	Reference [a]
Mouse	Pristane Hydrocortisone Gamma radiation (600 rad)	Mouse × mouse Human × mouse Rat × mouse	1
	Pristane Cyclophosphamide	Calf × mouse Porcine × mouse	2 3
	Pristane Gamma radiation (600 rad)	Human × Human [b]	4,5
Nude mouse	Pristane	Rabbit × mouse Rat × mouse	6 7
Nude rat	None Pristane	Rat × rat [c] Rat × mouse	8 7

[a] References are: **1**, Weissman et al., 1985; **2**, Raybould et al., 1985a; **3**, Raybould et al., 1985b; **4**, Kozbor et al., 1985; **5**, Truitt et al., 1984; **6**, Yarmush et al., 1980; **7**, Noeman et al., 1982; **8**, Hirsch et al., 1985.

[b] Cells adapted by subcutaneous implantation in irradiated mice prior to intraperitoneal injection for ascites production.

[c] Cells adapted by intravenous injection in rats prior to intraperitoneal injection for ascites production.

1984; Kozbor et al., 1985) or by growth in liver tissue (Hirsch et al., 1985) has proved useful for establishing hybridomas in vivo and for stabilizing the secretion of antibodies in ascitic tumors.

Even when the difficulties of producing monoclonal antibodies in ascitic fluid can be resolved, purity may remain a problem. Subsequent purification techniques must be applied whenever the ascitic-fluid impurities are an inconvenience (e.g., in therapeutic trials). These techniques vary in complexity, but in general the more steps incorporated to attain the desired level of purity, the lower the final yield (Dalchau and Fabre, 1982; Bazin et al., 1984; Macario and Dugan, unpublished results). Use of large numbers of animals to produce substantial volumes of ascitic fluid requires appropriate facilities and specialized staff.

Production of monoclonal antibodies in vitro

Development of in vitro cultivation techniques for production of monoclonal antibodies is becoming a major endeavor in hybridoma technology. The current availability of low-serum and serum-free media (discussed above) makes optimization of the cultivation systems feasible. Potential parameters for modification include gas exchange, mechanical forces acting on the cells (fluid dynamics), rates of nutrient consumption, means for regulating feeding, waste product accumulation and effects on cell growth and secretion, and the effects of temperature and pressure (Feder and Tolbert, 1983; Glacken et al., 1983; Gruenberg and Walker, 1983; Birch et al., 1985; Reuveny et al., 1986a,b). It must be reiterated that the goal of optimization is high product secretion (i.e., monoclonal antibodies) *not* rapid growth of the producing cells.

In general the procedures in use (Table VI) can be divided into small-scale systems, laboratory-scale units, and commercial, mass production. Small-scale production includes systems such as stationary culture flasks and roller bottle techniques. These are readily available in laboratories which do tissue culture and are effective for producing small quantities (< 1 liter) of antibody-containing media from several clones simultaneously (Garberi et al., 1985). They are also frequently used to maintain hybrid lines.

Laboratory-scale units are in a state of rapid development. Units such as the Vitafiber II (Amicon), Dynacell Culture System (Millipore), and ACUSYST-Jr (Endotronics) are intended for production of 1–3 liter batches of antibody-containing medium over a period of 1–4 months, with medium changes weekly or biweekly. In general, commercial literature claims laboratory-scale units produce 5–10-fold higher concentrations of antibody than the stationary flask system. For the Dynacell System we have found this claim to be true (Macario and Dugan, unpublished data). Also included in this category are the smaller airlift fermentors and stirred culture systems, which are being developed for bench-top operation. Control of these units is becoming very sophisticated (Wernerspach, 1986). Laboratory-scale units are particularly suited to expansion of a small number of hybrids of particular interest.

Development of the larger commercial-scale units is based mostly upon experience with cultivation of bacteria. Production runs in excess of 50

TABLE VI

PRODUCTION OF MONOCLONAL ANTIBODIES IN VITRO

Method	Main characteristics	Supplier [a]	Anticipated antibody concentration	Reference [b]
Stationary culture	Sedimentary monolayer in culture flasks	Corning, Costar, Falcon	10–100 μg/ml (usually < 50 μg/ml)	1,2,3
Roller culture	Rotating cylindrical bottles	Corning, Costar, Falcon	50–1 000 μg/ml (usually < 200 μg/ml)	3,4,5
Stirred reactor	Cells grown in suspension or attached to beads or microcarriers. Batch or continuous feed possible.	QS, NBS, LL, CP	> 200 μg/ml (may be higher)	6,7,8
Fluidized cell suspension	Cells kept in suspension by upward flow of air or medium.	FS, LL, NBS, QS, Ventrex	100–500 μg/ml	9
Entrapment of cells	Cells entrapped in agarose or polymer then cultured in roller bottles or fermentors.	FMC, Difco (see above for fermentors and roller bottles)	< 10 μg/ml (probably more in most cases)	10,11
Perfusion reactors	Cells entrapped in hollow fibers, between membranes, or in a ceramic core while medium is pumped through the reactor.	AC, CRBS, EI, MC	500–1 000 μg/ml	12,13,14

[a] Suppliers are: **AC**, Amicon Corp., Danvers, MA; **Corning,** Corning Laboratory Sciences, Corning, NY; **Costar,** Costar, Van Nuys, CA; **CP**, Cole Parmer Instrument Co., Chicago, IL; **CRBS,** Charles Rivers Biotechnical Services, Wilmington, MA; **Difco,** Difco Laboratories, Detroit, MI; **EI**, Endotronics, Coon Rapids, MN; **Falcon,** Falcon Labware, Becton Dickinson, Oxnard, CA; **FS**, Fisher Scientific, Rochester, NY; **FMC**, FMC Corp., Marine Colloids Division, Rockland, ME; **LL**, Lab Line Bioengineering, LTD., Melrose, IL; **MC**, Millipore Corp., Bedford, MA; **NBS**, New Brunswick Scientific, Edison, NJ; **QS**, Queue Systems, Parksburg, WV; **Ventrex,** Ventrex BioVentures Group, Portland, ME. These are suppliers with whom the authors are familiar; others may be available.

[b] References are: **1**, Cleveland et al., 1983; **2**, Galfrè and Milstein, 1981; **3**, Ostberg and Pursch, 1983; **4**, Bodeus et al., 1985; **5**, Sjögren-Jansson and Jeansson, 1985; **6**, Reuveny et al., 1986a; **7**, Reuveny et al., 1986b; **8**, Velez et al., 1986; **9**, Birch et al., 1985; **10**, Nilsson et al., 1983; **11**, Scheirer et al., 1984; **12**, Seaver et al., 1984; **13**, Weimann et al., 1983; **14**, Altshuler et al., 1986.

liters are not uncommon. Airlift or stirred culture systems are usually employed with special equipment and techniques to maintain uniform temperature, pH, and nutrient supply and to remove cell wastes and/or undesirable products (Klausner, 1983; Bloom, 1986). These systems are not intended for the average research laboratory. Production of monoclonal antibodies for diagnostic or therapeutic applications must also comply with government regulations, which may require modifications to equipment and/or procedures (Zimmerman et al., 1985). Hence in choosing an in vitro cultivation system one must consider the volumes of product desired and the intended use of the product, as well as the space and funds available.

Examples of human × mouse hybridomas

Generation of human × human hybridomas has been hindered to some degree by the absence of an appropriate fusion partner which grows well, produces stable hybrids, does not secrete antibodies of its own, but does secrete high levels of antibody when fused to an immune lymphocyte (Hunter, 1985). To avoid some of these problems encountered with the human fusion partners, some investigators have produced heterologous hybrids by using the well established murine fusion partners (Table VII). Such interspecies hybrids are often plagued by instability resulting from chromosomal incompatibility. Human chromosomes 2 and 22, both of which are important to sustained

production of monoclonal antibodies, appear to be particularly susceptible.

To reduce the possibility of such losses some investigators have fused the murine myeloma to a nonimmune human cell prior to using the hybrid as a fusion partner with immune lymphocytes (Ostberg and Pursch, 1983; Teng et al., 1983). Such hybrid fusion partners require considerable time to generate, but they appear to produce stable hybridomas with slightly higher secretion rates than nonhybridized murine myelomas. Whether the use of murine fusion partners to produce human × mouse hybrids favors the development of more IgG-secreting clones cannot be determined, since most publications report the screening of hybridization products for only a single class of antibodies.

Examples of hybridomas producing antibodies other than human, mouse, and rat

Although the murine hybridoma system is the most widely used model for producing and studying hybridomas and their products, other animal systems have also been investigated (Sherman and Markham, 1986). Murine myelomas, most often Sp2/0-Ag14, and a mouse × human heteromyeloma have been used as fusion partners with immune lymphocytes of bovine, porcine, ovine, rabbit, monkey, and chimpanzee origin (Table VIII) all animals of scientific, veterinary, and possibly clinical importance. The animal hosts were immunized in vivo, and no additional stimulation was used prior to fusion. Hybrids with non-primate immune lymphocytes showed stable antibody pro-

TABLE VII

HUMAN × MOUSE HYBRIDS FOR PRODUCTION OF HUMAN MONOCLONAL ANTIBODIES

Fusion partner [a]		Antibody			Reference [b]
Designation	Origin	Specificity	Class	Secretion rate or concentration (duration)	
D-33	Murine P3/X63-Ag8.653 × human FU-266	Tetanus toxoid, 2,4-dinitrophenyl, DNA, others	IgM	2–36 μg/ml/10^6 cells/day (over 7 months)	1
P3/X63-Ag8.653	Murine	Tetanus toxoid, diphtheria toxoid	IgG	1.6–5.9 μg/ml (over 7 months)	2
		Hepatitis B surface antigen	IgG	10 μg/ml/10^5 cells/day (over 9 months)	3
		Rhesus-positive erythrocytes, PBL from patients with autoimmune disorders	IgG, IgM and IgA	Data not given	4
SP-1	Murine	Keyhole limpet hemocyanin	IgM	< 30 μg/ml (over 8 months)	5
SPAZ-4	Murine Sp2/0-Ag14 × human peripheral blood lymphocytes	Human influenza	IgG	25–50 μg/ml (over 22 months)	6
SP2/0-Ag14	Murine	Not given; various B-cell malignancies were fused	IgM	Data not given	7

[a] In all cases the cell lines listed were fused to immune human lymphocytes.

[b] References are: **1**, Teng et al., 1983, **2**, Gigliotti et al., 1984; **3**, Maeda et al., 1986; **4**, Thompson et al., 1986; **5**, Lane et al., 1982; **6**, Ostberg and Pursch, 1983; **7**, Weissman et al., 1985.

TABLE VIII

HYBRIDS PRODUCING MONOCLONAL ANTIBODIES OTHER THAN HUMAN, MOUSE OR RAT

Myeloma	Animal source of immune lymphocytes	Antibody		Reference [a]
		Specificity	Class	
Sp2/0-Ag14	Bovine	Bovine enteric coronavirus	IgG_2	1
	Porcine	*Escherichia coli* strain B41	IgG	2
	Rabbit	Not reported	Ig [b]	3
P3/X63-Ag8	Rabbit	Not reported	Ig	3
P3/X63-Ag8U1	Rabbit	Not reported	Ig	3
45.6TG1.7	Rabbit	Not reported	Ig	3
P3/NS1/1-Ag4-1	Rabbit	Not reported	Ig	3
	Sheep	Testosterone	IgG	4
SBC-H20	Rhesus monkey and chimpanzee	Murine monoclonal antibody OKT4 and allogenic chimp cells	IgG and IgM	5
Org MHH.1	Chimpanzee	Hepatitis A virus	IgG IgM	6

[a] References are: **1**, Raybould et al., 1985a; **2**, Raybould et al., 1985b; **3**, Yarmush et al., 1980; **4**, Groves et al., 1987; **5**, Van Meurs and Jonker, 1986; **6**, Van Meel et al., 1985.
[b] Ig, chains of various allotypes.

duction for several months and could be cultured in BALB/c mice to produce antibodies in ascitic fluid. Murine antibodies against the monoclonal bovine and porcine antibodies in these ascitic fluids proved to be significant contaminants. Hybrids of primate lymphocytes fused to murine myelomas were much less stable than when fused to heteromyelomas (Van Meel et al., 1985; Van Meurs and Jonker, 1986).

Monoclonal antibodies directed toward other species can be easily envisioned to have applications in veterinary medicine similar to the applications of human monoclonal antibodies (Sherman and Markham, 1986). The production of hybridomas secreting primate monoclonal antibodies may also have important clinical applications. Immunotherapy using monoclonal antibodies of murine origin tends to elicite an immune response against the therapeutic agent. Applications such as immunosuppressive therapy often require extended exposure to these antibodies and become less effective as the patient produces antibodies against the murine antibodies. Antibodies of primate origin may prove to be far less immunogenic particularly in those cases where Fab fragments are used.

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