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Determination Of The Time Of Maximum Hybridoma Formation During An Immune Response.

Jefferson Wardlaw Paslay
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DETERMINATION OF THE TIME OF MAXIMUM HYBRIDOMA FORMATION DURING AN IMMUNE RESPONSE

The University of Alabama in Birmingham PH.D. 1980

DETERMINATION OF THE TIME OF MAXIMUM HYBRIDOMA FORMATION DURING AN IMMUNE RESPONSE

by

JEFFERSON WARDLAW PASLAY

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the Department of Microbiology
in The Graduate School, University of Alabama in Birmingham

BIRMINGHAM, ALABAMA

1979

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Title of Dissertation Determination of the Time of Maximum Hybridoma Formation During an Immune Response

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA IN BIRMINGHAM

Degree Doctor of Philosophy Major Subject Microbiology

Name of Candidate Jefferson Wardlaw Paslay

Title Determination of the Time of Maximum Hybridoma Formation During an Immune Response

Conditions influencing the formation of antibody-secreting cell hybrids and how these affect what type of cell hybrids are isolated during an immune response were examined. This investigation included two separate systems to produce parental spleen cells for hybrid formation. One used *in vitro* lipopolysaccharide (LPS)-stimulated spleen cells and the second used spleen cells from Balb/c mice during an immune response to sheep red blood cells (SRBC).

Spleen cells exposed to LPS in culture for up to 120 hours formed cell hybrids with increased frequency at two different times. The major peak was after 72 hours of culture and was coincident with maximum mitotic activity as determined by ^{3}H-thymidine uptake. A second, smaller peak of hybrid production was present after 12 to 24 hours of exposure to LPS. This second peak was not related to mitotic activity. A comparison of cell hybrid formation with the presence of either B cells not producing antibody or plasma cells indicated no dependence of fusion frequency on the relative number of either of these cell populations. This suggested that spleen cells included in

cell hybrid formation might possess a certain membrane configuration which is associated with mitosis or induced by the interaction of LPS with membrane components.

A study of cell hybrids formed with spleen cells from Balb/c mice responding to SRBC indicated that the maximum number were produced prior to maximum PFC development. The immunoglobulin class of the SRBC-specific antibody secreted by isolated hybrids reflected the developing antibody response as measured by serum activity and PFC formation.

Together these studies suggest that the spleen cell which is preferentially included in a polyethylene glycol-induced fusion event is one undergoing proliferation. This may be due to a certain membrane state present on cells after antigenic stimulation but before differentiation into a plasma cell. Therefore, cell hybrids isolated during an immune response are indicative of cells proliferating in response to antigen at the time of fusion.

Abstract Approved by: Committee Chairman J. Claude Bennett

Program Director

Date _____ Dean of Graduate School Emmett J. Rogers

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I. INTRODUCTION

Comprehension of any biological process requires complete understanding of each cell type, the molecules they produce and all cellular interactions which may occur within a given system. The immune response is a collection of complex but distinct systems, each of which is composed of cellular subpopulations with their own array of cell surface markers and secreted products.

The immune system can be separated into two major components: the cellular and humoral responses. Cellular responses are dependent on the activities of thymus-derived lymphocytes, or T cells, while the humoral responses are predominantly dependent on antibody molecules produced by bone marrow-derived lymphocytes, or B cells. T cells have been divided into helper, suppressor or cytotoxic subpopulations based on their stage of differentiation (pre-B, early B, mature B, or plasma cell) and the immunoglobulin class they produce (IgG, IgA, IgM, IgD, or IgE).

Each subpopulation has a definite set of surface markers or secreted products which can be identified serologically or functionally in appropriate assays. There also exists an extensive network of regulatory interactions which function through either cell-cell contact or cell-product-cell interaction. Studies of the immune response have included attempts to isolate various subpopulations, to identify them

by surface markers and to determine their function. Complete understanding of a cell's role or of a secreted product's function will require biochemical characterization of the molecules involved. Such studies necessitate that these molecules be isolated in sufficient quantity. Except in the case of certain tumor cell lines expressing an appropriate molecule or secreting a desired product, this has been impossible.

Studies utilizing somatic cell hybridization between different types of lymphoid cells indicate that any cell of the immune system can be established as a continuous cell line with maintenance of surface markers or secretion of a desired cell product. This technology is based on the work of Kohler and Milstein (1975) who reported the isolation of a B cell hybrid, or hybridoma, producing monoclonal antibody. An understanding of the parameters which enhance the production of hybrid cell lines expressing differentiated functions such as immunoglobulin secretion will allow application of this technique to other biological systems.

Factors which increase the frequency of cell hybrid formation or the recovery of viable hybrids remain undetermined. Lymphocyte hybridomas provide a system where the variables can be studied and maximized for somatic cell hybrid production. Information gained in these studies may be useful in attempts to establish cell lines secreting antibodies, hormones, enzymes and other desirable cell products.

II. BACKGROUND

Somatic Cell Genetics: Its Role in Immunology

Fusion of two parental cell types to produce a hybrid cell offers at least two possibilities for research. First, it allows the investigation of regulatory phenomena as various biochemical pathways of the two parental partners are either extinguished or maintained in the hybrid. Second, a continuous culture line hybrid cell may possess characteristics of one of the original parents which was not a continuous culture line.

Use of Hybrid Cell Lines in Genetic Studies

Somatic cell hybridization procedures have been extensively used to map human genes to their specific chromosome (Ephrussi and Weiss, 1965; Weiss and Green, 1967). The Sendai virus-mediated fusion between somatic cells of human and rodent origin usually yields highly unstable interspecific cell lines which undergo the progressive loss of the human genome. With proper selection techniques, these hybrids evolve rapidly toward a reduced karyotype where only one human chromosome is retained. Hybrid cell lines can be assayed for the presence of a particular function encoded by the human genome; and when this function continually co-segregates with a particular human chromosome, it can be assigned to that chromosome. This offers a unique opportunity for

basic experimentation of molecular biology at the level of single chromosomes.

The emergence of this technique has depended on the development of a selective system for the isolation of hybrids between biochemically marked cells (Littlefield, 1964) and on the increase in the frequency of cell fusion through the use of Sendai virus and chemicals (Okada, 1961; Harris and Watkins, 1965). Recent studies using cell hybridization for analysis include the areas of gene mapping, gene regulation, chromosome replication, and mutation rates.

Hybrid Cell Lines as Used in Immunology

Immunologists interested in the regulation of expression of certain molecules have turned to somatic cell hybridization as a technique for analysis of both cell surface molecules and secreted immunoglobulin.

Expression of Thy-1 and T1a

During the past few years Hyman and co-workers at the Salk Institute have undertaken studies of the steps in the biosynthesis and integration of selected molecules into the plasma membrane and how these steps are regulated. They have chosen to study some of the differentiation antigens of various lymphoid cells. Since all cells of the lymphoid system arise from a common stem cell, there must be a network of structural and regulatory genes that interact during lymphocyte differentiation to produce the characteristic surface phenotypes of certain lymphocyte subpopulations.

A number of Thy-1 loss variants were isolated from three different murine lymphomas by immunoselection. The variants were used to form hybrids with parental lines and other variants. Analysis of Thy-1 expression by these hybrids and parental partners allowed three complementation groups to be identified. Biochemical studies of the Thy-1 proteins expressed on the cell surface of parents and hybrids provided an understanding of the cellular processing of the Thy-1 molecule in terms of the glycosylation requirements for the appearance of an antigenically determined structure (Hyman, 1973; Hyman and Trowbridge, 1976; Trowbridge, Hyman and Mazauskas, 1978).

Similar immunoselection methods allowed the isolation of thymus leukemia antigen (Tla) loss variants, one of which also demonstrated a loss of expression of the mouse major histocompatibility product, H-2. Genetic analysis using somatic cell hybridization has shown the re-appearance of Tla and H-2 of both parental types. This model system suggests the possibility of a common regulatory protein necessary for the expression of both Tla and H-2 which is not present in the variant but can be complemented in hybrids (Hyman and Stallings, 1976; Hyman and Trowbridge, 1976).

Modulation of Tla

Liang and Cohen (1975) investigated Tla expression in a somatic cell hybrid formed between the murine leukemia cell line, ASL-1, (Thy-1^+, Tla^+) and the murine fibroblast cell line, LM(TK^-), (Thy-1^-, Tla^-). H-2 antigens of both parental types and Tla, but not Thy-1, were expressed in the hybrid. Modulation of Tla did not occur in the

hybrid, suggesting that the genetic mechanisms controlling Tla expression are separable from those regulating modulation capacity.

HLA and B_2-microglobulin association

Human-mouse somatic cell hybrids have been used to establish the linkage relationship between genes controlling the expression of HLA and Ia molecules. A hybrid between the human cell line Daudi (HLA^-, Ia^+) and the mouse fibroblast cell line A9 ($H\text{-}2^+$, Ia^-) expressed Ia and H-2 and contained human chromosome 6. Subcloning and karyotyping showed segregation of Ia with chromosome 6. Because the large Ia chain carries serotypically determined specificities, it cannot be determined if both Ia gene loci are on chromosome 6 or if the locus for the larger chain is there while the locus for the smaller chain is located on another chromosome. If the latter is true, then even if the chromosome bearing the small chain locus is lost in the hybrid, the mouse small Ia chain may be induced and complement the human heavy chain for correct surface expression (Barnstable et al., 1976).

Chain complementation in the expression of multichain molecules and possible regulatory mechanisms were examined in intraspecies and interspecies hybrids using the Daudi cell line which does not express HLA or B_2m. Intraspecies hybrids between Daudi and three other human cell lines all expressed new HLA types in addition to those expressed by the normal parental partner. These same new HLA types were expressed on interspecies hybrids between Daudi and mouse fibroblasts which also expressed H-2 and mouse B_2m. Serological studies indicated that this expression of new HLA was in association with mouse B_2m while human B_2m remained undetectable. The results suggest that HLA and

B_2m expression are independent, but that B_2m may be required for correct conformation and glycosylation and that the defect in Daudi may be at the B_2m locus and not at HLA (Barnstable et al., 1976; Jones et al., 1976). The use of cell hybrids provides a method to determine the nature of the relationship between the HLA and B_2m molecules.

Immunoglobulin biosynthesis

Many laboratories have been engaged in studying the regulation of immunoglobulin biosynthesis. The availability of numberous mouse myelomas has provided the necessary model system to allow isolation of sufficient quantities of material to define biochemically certain stages during the synthetic process. Schubert, Munro, and Ohno (1968) used four myelomas of different subclasses which were carried in vivo to isolate secretion variants. Studies using these molecules and existing data using molecules from parental lines provided essential information about the assembly pathway of immunoglobulins and demonstrated the value of variants in these studies.

A major advance in the use of myelomas was their adaptation to cell culture as described by Laskov and Scharff (1970) and Horibata and Harris (1970). In cell culture myelomas are more accessible to manipulation and biochemical assay. Scharff and co-workers and Milstein and co-workers have used cultured myelomas to isolate variants in antibody secretion. These cell lines have been used in somatic cell hybridizations in order to examine the regulation of immunoglobulin expression (reviewed in Margulies, Kuehl and Scharff, 1976; Adetugbo, Milstein and Secher, 1977).

Table I lists a number of somatic cell hybridization studies involving immunoglobulin-producing cells. These studies were performed to determine the genetic control of immunoglobulin production and have been either human-human, human-mouse, or mouse-mouse cell fusions. Extinction of immunoglobulin production occurred only when one parental cell was a fibroblast. In those hybrids where both parents were of lymphoid origin, the immunoglobulin chains of the immunoglobulin producer were usually expressed. Some loss of immunoglobulin synthesis due to chromosome segregation did occur in interspecies hybrids.

The importance of these studies between immunoglobulin-producing cells was that they demonstrated continued production of both parental cell products by the hybrids. This documented the usefulness of this technique in complementation studies of isolated secretion variant cell lines with parental lines and other variants, expression of two different immunoglobulin types within the same cell, and genetic regulation of immunoglobulin production. Most importantly, these studies provided the basis for a method to produce cell lines able to secrete monoclonal, monospecific antibody with specificity for a prechosen antigen.

Production of Specific Antibody by Somatic Cell Hybridization

Investigation in Milstein's laboratory probed the nature and frequency of spontaneous somatic mutations in immunoglobulin structural genes in order to examine the nature and origin of antibody diversity. To accomplish this they have studied secretion variants in the mouse myeloma MOPC 21 using protein and nucleic acid chemistry.

TABLE I

IMMUNOGLOBULIN SYNTHESIS BY
SOMATIC CELL HYBRIDS

Parental Cell Types of Hybrids	Immunoglobulin Expression	Reference
mouse myeloma (IgA, anti-DNP) X mouse fibroblast LMTK$^-$ (Ig^-)	very low levels of IgA and anti-DNP	Periman, 1970
mouse myeloma (IgG2a,k) X mouse lymphoma (Ig^-)	mouse k chain	Mohit and Fan, 1971
mouse myeloma (IgG2a,k) X mouse lymphoma (Ig^-)	mouse k chain and IgG2a	Mohit, 1971
mouse myeloma (IgG2b,k) X mouse fibroblast 3T3Tk$^-$ (Ig^-)	no Ig expression	Coffino et al., 1971
human lymphoblast (light chain) X mouse fibroblast 3T3Tk$^-$ (Ig^-)	human light chain	Orkin et al., 1973
human PBL X mouse myeloma (IgA,k)	mouse IgA, k and human Ig	Schwaber and Cohen, 1973
mouse myeloma (IgG2a,k) X rat myeloma (Ig,k)	mouse IgG2a, rat k, and hybrid molecules	Cotton and Milstein, 1973
human lymphocyte cell line (Ig^+) X human lymphocyte cell line (Ig^+)	human IgM, IgG, k	Bloom and Nakamura, 1974
human PBL X mouse myeloma (IgA,k)	mouse IgA, k human IgG, IgM IgA and k	Schwaber and Cohen, 1974 Schwaber, 1975
mouse myeloma (IgG2a,k) X mouse myeloma (IgG1,k)	mouse IgG1, IgG2a, k, and hybrid molecules	Kohler and Milstein, 1975
mouse myeloma (IgG1,k) X mouse spleen cell	mouse IgG1,k and IgG and IgM specific for antigen	Kohler and Milstein, 1975
myeloma secretion variant X mouse myeloma (IgG2a,k)	mouse IgG2a,k	Kohler and Milstein, 1976

Preliminary Experiments

The full amino acid sequence and location of disulfide bridges of MOPC 21 protein was determined (Svasti and Milstein, 1972). Light and heavy chain mRNA were isolated, analyzed structurally and translated in heterologous cell-free systems (Brownlee et al., 1973; Milstein et al., 1974; Cowan et al., 1976). Using two unique screening techniques described by Cotton and Milstein (1973) and Kohler, Howe and Milstein (1976), nonsecreting, nonproducing, and structural variants were isolated. Four spontaneous variant cell lines produce proteins which are the result of mutations in the constant region of the IgG_1 heavy chain. The mutation events they represent are a "nonsense," a "missense," a "frameshift" and a "deletion." These determinations were made by comparing amino acid sequences and mRNA structural data from the isolated variants and the parent line (Adetugbo, Milstein, and Secher, 1977).

Their data helped clarify many aspects of antibody expression and the reasons why variations might occur, but left open the question of mRNA "scrambling" in antibody producing cells. Immunoglobulins contain variable (V) regions and constant (C) regions, each coded by genes from separate V and C gene pools. The mechanisms of V and C joining remained unknown, and whether the V and C are transcribed as one or separate mRNAs which will later be spliced together was not clear. If the latter situation exists, it would not be seen in normal antibody-producing cells since they express only one V-C pair. To study this question, Cotton and Milstein (1973) produced a cell hybrid between two secreting myelomas, one of rat origin and the other from mouse.

Results obtained after analysis of the secreted products indicated that all parental chains were produced, that new components which appeared were hybrid molecules due to chain mixing, and that no new V-C pairs were detectable. Thus, V and C genes appear to be combined at the DNA level during differentiation from stem cells to plasma cells.

This experiment presented two exciting possibilities. First, by fusing an antigen-specific normal lymphocyte with a myeloma cell, a hybrid cell line could be produced from which variants in the V region could be selected. The frequency and types of mutations in these variants could then be compared to the data from the C region variants. Second, and probably of more importance, this technique could be used to produce a hybrid with predetermined antigen specificity.

Production of Hybrids with Predefined Specificity

Kohler and Milstein (1975) fused spleen cells from a BALB/c mouse, previously immunized with sheep red blood cells (SRBC), with an azaguanine resistant derivative of the MOPC 21 myeloma. After growth in selective media for several weeks, the presence of anti-SRBC antibody-producing cell hybrids was determined by a modified Jerne plaque assay. Some cell hybrids were selected for growth. Isolation and characterization of their secreted immunoglobulin has shown anti-SRBC activity of IgM, IgG_{2b} and IgG_1 classes.

Surviving cell hybrids expressed MOPC 21 myeloma protein and new immunoglobulin derived from the spleen cell parental partner. About 60% of the cell hybrids secreted immunoglobulin chains different

from MOPC 21 and 10% of these were specific for SRBC (Kohler and Milstein, 1976). This suggested selective survival of hybrids resulting from fusions between only a small number of the total B cell population and the myeloma. Hybrid specificity has been shown to be correlated with immunization of the mouse which donates the spleen cells. For example, no anti-SRBC hybrids were isolated from mice immunized with trinitrophenyl-lipopolysaccharide (TNP-LPS) while anti-TNP hybrids were present. It may be that a plasmacytoma preferentially fuses with a plasma cell or that plasmacytoma-plasma cell hybrids are more viable.

An important extension of this particular work with anti-SRBC hybrids has been reported. It is possible to analyze by electrophoretic techniques the secreted products of both the parent myeloma and the hybrid. Such analyses indicated hybrid cell production of mixed molecules comprised of combinations of both parental type chains. Continual screening of the original hybrid allowed selection of subclones producing antibody with greater anti-SRBC activity. Further examination of these subclones often revealed the loss of one or both of the myeloma chains which resulted in the isolation of a cell line with antigen specificity without the presence of any myeloma protein (Kohler and Milstein, 1976).

Materials and Methods Which Made the Production of Monoclonal Cell Lines Possible

Kohler and Milstein's method for deriving monoclonal, monospecific antibody from cell cultures may be used to establish permanent cell lines, producing reagents which are valuable for diagnosis and treatment as well as the analysis of complex antigenic structures such

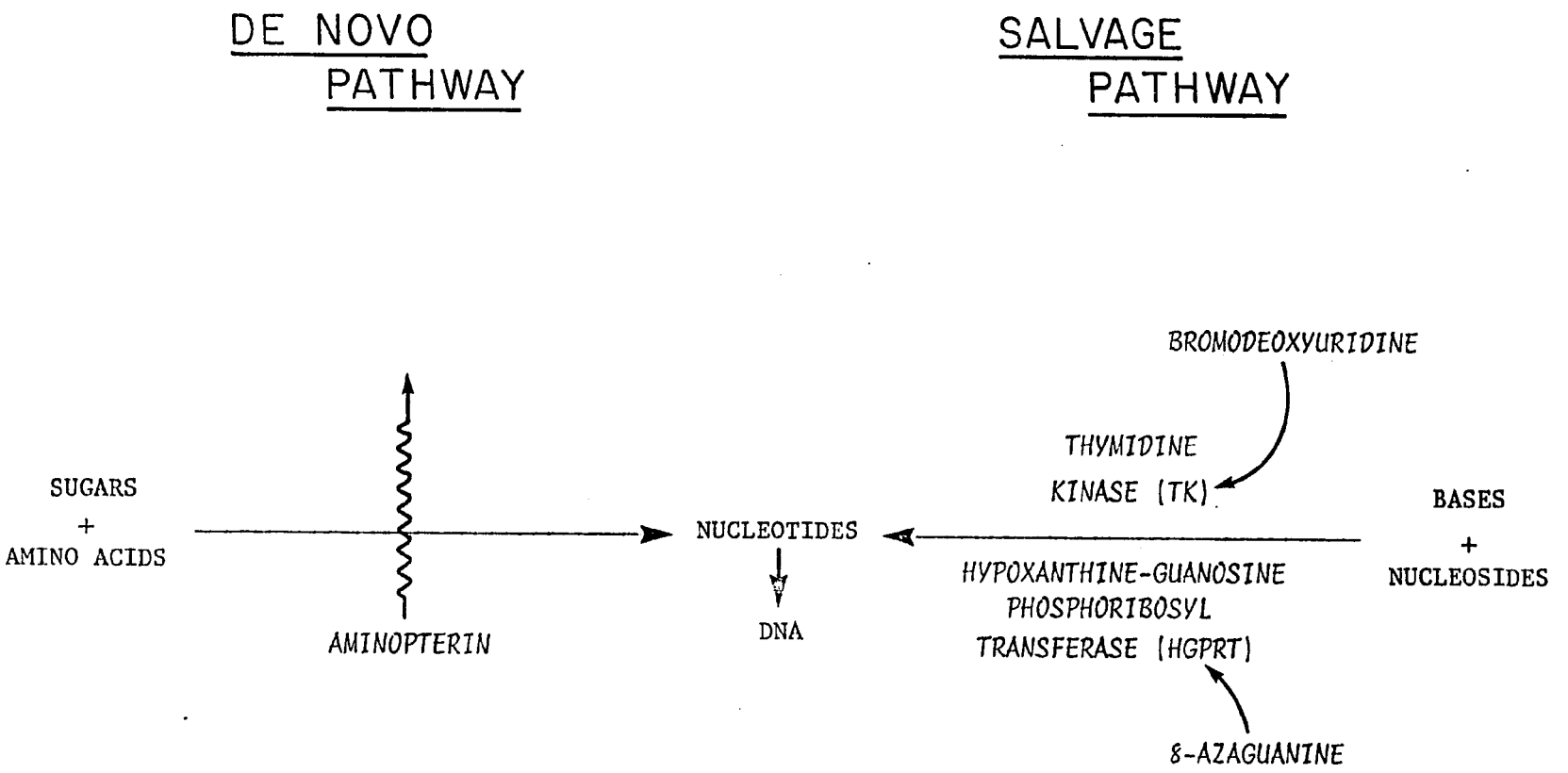
as cell surface molecules. The development of this technique was facilitated by a number of other advances.

Information provided in Table I makes it obvious that the recovery of immunoglobulin production depends on the use of an immunoglobulin-producing cell. The availability of such a line was due to the isolation of induced or spontaneous tumors in mice (Potter, 1977) and the adaptation of these tumors to growth in cell culture (Horibata and Harris, 1970).

The fusion of mouse spleen cells with a mouse myeloma results in three hybrid products--spleen cell-spleen cell, myeloma-myeloma, and spleen cell-myeloma--plus unfused spleen cells and myeloma cells. Spleen cell-spleen cell hybrids and unfused spleen cells will die within a few days in culture, but myeloma-myeloma hybrids, myeloma-spleen cell hybrids and unfused myeloma cells continue to grow. Without a proper selective technique the recovery of desired spleen cell-myeloma hybrids would be difficult. Littlefield (1964) introduced a selection method based upon a biochemical defect in parental cells which requires complementation in the hybrid to allow survival. Figure 1 illustrates the biochemical pathways involved in the genetic selection and the selection process.

Deriving an enzyme-deficient cell line is accomplished by growing it in the presence of cytotoxic analogs of thymidine or guanine. Uptake of the analog by a specific salvage pathway enzyme eventually leads to cell death; mutant cells deficient in that enzyme survive and can be isolated and used as parents in hybridization experiments. Selection requires the addition of hypoxanthine (H), aminopterin (A), and thymidine (T) to the culture medium (HAT medium).

Figure 1. HAT Selection of Hybrids. Biochemical pathways and inhibitors involved in the production of enzyme deficient cell lines and the selection of somatic cell hybrids.


DE NOVO PATHWAY
SALVAGE PATHWAY
BROMODEOXYURIDINE
THYMIDINE KINASE (TK)
SUGARS + AMINO ACIDS
NUCLEOTIDES
BASES + NUCLEOSIDES
AMINOPTERIN
DNA
HYPOXANTHINE-GUANOSINE PHOSPHORIBOSYL TRANSFERASE (HGPRT)
8-AZAGUANINE

Aminopterin inhibits the major pathway by blocking the activity of dihydrofolate reductase which is required to convert dihydrofolate to tetrahydrofolate.

Normal cells survive this inhibition by shifting DNA synthesis to the salvage pathway and utilizing the excess hypoxanthine and thymidine in the HAT media while genetically deficient cells perish. Therefore, in spleen cell-myeloma hybridizations, the myeloma is thymidine kinase negative, TK^-, or hypoxanthine-guanosine phosphoribosyl transferase negative, $HGPRT^-$, and after fusion the cultures are exposed to HAT selection. Only myeloma cells complemented by TK^+ or $HGPRT^+$ spleen cells following fusion will survive selection. The myeloma cell provides the ability to survive in culture indefinitely plus the cellular machinery for massive antibody production while the spleen cell parent complements the genetic deficiency and donates the new antibody specificity.

Subsequent Hybrids Produced by Milstein and Co-Workers

Following the initial success of Kohler and Milstein in 1975, many laboratories have established antibody-producing cell hybrids against numerous antigens.

Hybrid cell lines secreting antibody specific for the major histocompatibility antigens of the rat were isolated by Galfre et al. (1977). Two important new aspects of cell hybridization were introduced in that report. First, polyethylene glycol was used as a fusing agent in obtaining spleen cell-myeloma hybrids. Its use appeared to increase greatly the frequency of hybrid recovery. Second, an

interspecies hybrid, rat-mouse, was derived which produced antibody and was stable with respect to that property.

Using xenoimmunization, hybrids have been isolated which are specific for rat thymocyte differentiation antigens (Williams, Galfre, and Milstein, 1977), human thymocyte antigen (McMichael et al., 1979), human erythrocyte antigens, and one specific for all human HLA-A, B, and C chains (Barnstable et al., 1978). Taking advantage of the ability to produce rat-mouse hybrids, Springer and co-workers (1979) isolated a battery of rat antibodies specific for various surface molecules of mouse lymphocytes. Stern et al. (1978) screened a number of these antibodies and isolated one specific for embryonic carcinoma which reacts with preimplantation embryos. In an attempt to produce a hybrid specific for mouse H-2, Pearson et al. (1977) isolated a hybrid producing antibody specific for mouse IgD.

Hybrid Production by Other Laboratories and Their Uses

A number of facts indicate the interest in this technique and the desire for antibodies produced from hybrid cell lines. In 1978, a workshop on lymphocyte hybridomas was held at the National Institutes of Health to discuss progress up to that time in hybrid production (Melchers, Potter, and Warner, 1978). It was obvious from that meeting that the use of this technology was expanding and was rapidly becoming a general laboratory tool. This was also implied by the inclusion of a chapter on cell hybrids (Herzenberg, Herzenberg and Milstein, 1978) in the most recent edition of a respected immunological methods book. Finally, a number of commercial firms now sell monoclonal antibodies,

and one company has recently been formed specifically for the commercial production of hybrids (Dickson, 1979). Table II gives a partial list of interesting antibodies produced by cell hybrids in a number of different laboratories. Until now the major use of monoclonal antibodies has been in characterization of cells and antigenic molecules. Some recent reports indicate the future usefulness of these products.

Willison and Stern (1978) used a monoclonal antibody specific for preimplantation embryos (Stern et al., 1978) to study the distribution of a single antigen during various stages of mouse embryogenesis. Monoclonal antibodies specific for SRBC but differing in antibody class have been used in functional assays to determine the presence of various surface receptors for the Fc portion of the antibody molecule on macrophages (Diamond, Bloom and Scharff, 1978). Stocker and co-workers (1979) have described a method for cell separation using a xenogeneic monoclonal antibody in conjunction with a rosetting technique.

Sunderland, McMaster and Williams (1979) reported the use of a mouse anti-rat thymocyte monoclonal antibody on an affinity column for the purification of a leukocyte common antigen from rat thymocyte membranes. Large scale culture of a murine thymoma expressing H-$2K^k$ coupled with purification of their solubilized membrane components on an affinity column constructed from monoclonal antibody specific for H-$2K^k$ (Oi et al., 1978) has produced milligram quantities of H-$2K^k$ for sequence analysis (Mole et al., 1979). Hunter et al. (1979), using the same system, are investigating a high molecular weight protein which is recognized by both conventional and monoclonal H-$2K^k$ specific antisera.

TABLE II

HYBRIDS PRODUCED BY VARIOUS LABORATORIES

Laboratory Designation	Specificity	Reference
PA 2.1 PA 2.2	human HLA-A2 most human HLA-A,B,C	Parham & Bodmer, 1978
N 24	human B_2m	Trucco, Stocker & Ceppellini, 1978
3.1, 3.2, 3.3	human melanoma antigens	Yeh & co-workers, 1979
PI 125, PI 153	human neuroblastoma antigens	Kennett & Gilbert, 1979
AB 14, AC 15	human lymphokine osteoclast factor	Luken, Mohler & Nedwin, 1979
HK-PEG-1	influenza virus	Koprowski, Gerhard and Croce, 1977
101, 102, 103	rabies virus	Wiktor & Koprowski, 1978
A25.1#1B3	SV 40 virus T antigen	Martinis & Croce, 1978
PO 772 C2	peroxidase	Zagury & co-workers, 1979
11-4.1 11-2.12,11-5.2,11-1.23 10-2.16,10-3.6,11-3.5	murine $H\text{-}2k^k$ murine I-A(2) murine I-A (17)	Oi & co-workers, 1978
HO-22-1 HO-13-4	murine Thy-1.1 murine Thy-1.2	Marshak-Rothstein & co-workers, 1979
5R4, 27R9, 30R3	mouse $H\text{-}2^k$	Lemke & co-workers, 1978
B16-146 B16-167	murine Qat-4 murine Qat-5	Hammerling, Hammerling & Flaherty, 1979
I3/2	mouse lymphocyte	Trowbridge, 1978
M1/70	glycoprotein, T200 murine macrophage antigen	Springer & co-workers, 1979

Parham, Barnstable and Bodmer (1979) have used a monclonal antibody which recognizes a non-polymorphic determinant on most HLA-A,B,C molecules (Barnstable et al., 1978) to purify these surface proteins. This same antibody has been used in conjunction with another monoclonal antibody specific for human thymus antigen (HTA) (McMichael et al., 1979) by Zeigler and Milstein (1979) to demonstrate that the small molecular weight molecule associated with HTA may not be B_2m. Parham, Sehgal and Brodsby (1979) used a panel of monoclonal antibodies specific for HLA to study evolutionary similarity between the species as related to products of their major histocompatibility complexes.

The Fusion Technique, Modifications and Recent Advances in Its Use

The general technique as originally applied to produce cell hybrids between mouse spleen cells and myeloma cells is illustrated in Figure 2. In simple terms, it consists of mixing the two parental lines together, exposing them to a fusing agent, and resuspending the cells in growth medium followed by distribution into growth vessels. Selection of hybrids by including HAT in the growth medium is performed for 10 to 14 days and then the supernatants of surviving hybrids are assayed for antibody and/or specificity. Chosen hybrids are cloned to verify their monoclonal origin and then grown for antibody production as tumors in mice or in cell culture. Most laboratories producing monoclonal antibodies by hybridization use slight variations of this technique as introduced by either Galfre et al. (1977) or Gefter, Margulies and Scharff (1977). These two methods differ only in minor manipulations, but both introduce PEG as a fusing agent.

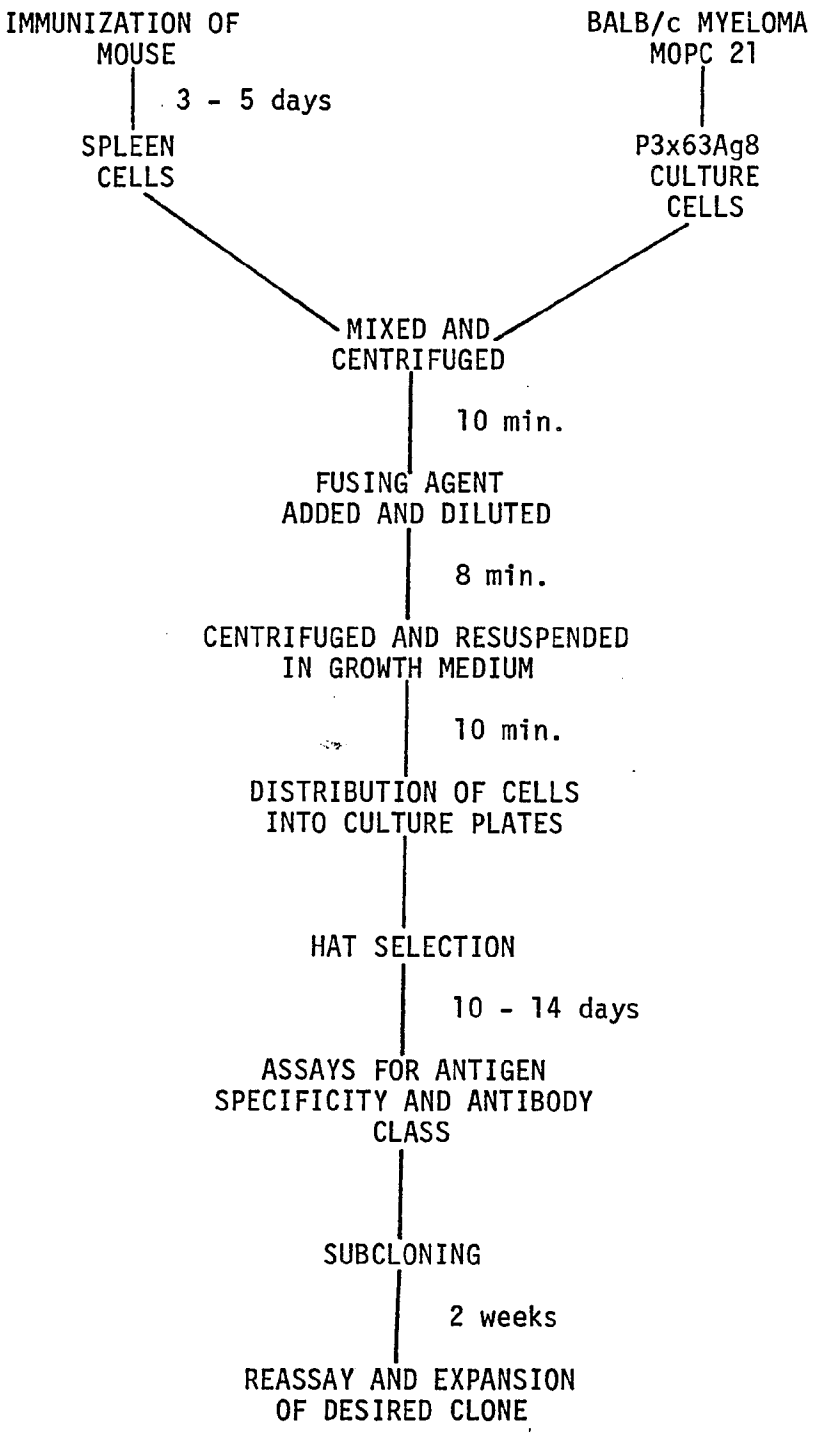


Figure 2. General Fusion Technique.

PEG As A Fusing Agent

Sendai virus has been the major agent utilized to increase the frequency of cell fusion since its introduction by Okada (1961) and Harris and Watkins (1965). This agent, although useful, has the disadvantages of being laborious to produce (Klebe, Chen, and Ruddle, 1970), having variable activity from batch to batch, possessing different activity with different cell lines being fused, and contaminating cells with viral products. PEG appears to be a more advantageous agent because it is consistent from lot to lot, easier to prepare and use and less expensive.

PEG was first introduced as a fusing agent for plants (Kao, 1974) but has been used subsequently for intraspecies, interspecies and even interkingdom hybridization. The nature of its fusing ability is unknown, but the ease of fusion may be due to similar activities with all biological membranes and a lack of dependence on a specific cell surface receptor.

Pontecorvo (1975) introduced PEG as a means of producing hybrids between mammalian cells. A number of parameters affecting PEG-induced fusion have been investigated by Davidson and co-workers (Davidson and Gerald, 1976; Davidson, O'Malley and Wheeler, 1976) who found that molecular weight, concentration of PEG, length of exposure, pH, temperature and cell density all affected cell hybridization. Maximum fusion frequency requires optimizing these variables which either control the interaction of PEG with the cell membrane or reduce the naturally toxic effects of PEG. Norwood, Zeigler and Martin (1976) reported that the use of dimethyl sulfoxide (DMSO) enhanced PEG-induced fusions.

A major factor involved in PEG-induced fusion is the length of time required for fusion to occur. Steplewski, Koprowski and Leibovitz (1976) showed that fuions at 37°C induced cellular bridges within five to seven minutes while none appeared at room temperature. Pontecorvo, Riddle and Hales (1977) studied the time and mode of fusion in human fibroblasts and found that fusion was usually complete within ten to twenty minutes, that fusion occurs at the time of addition of PEG or is initiated then, and that in subconfluent monolayers fusion commences at ends of pseudopodia.

Techniques for the use of PEG to fuse cells in suspension have been reported by Hales (1977), O'Malley and Davidson (1977), and Gefter, Margulies and Scharff (1977). The latter technique is simple, short and applicable to fusions of myeloma cells; therefore, it has been used by many investigators to produce mouse spleen cell-myeloma hybrids. The use of PEG in those experiments increased the frequency 100- to 300-fold over Sendai virus fusions.

New Cell Lines

In producing hybridomas to secrete monoclonal antibody, one problem is the inclusion of parental myeloma immunoglobulin chains in the secreted product. This problem was recognized by Kohler and Milstein (1975) and a nonsecreting variant, NSI, of their original line, P3x63xAg8, was isolated and shown to fuse with other myelomas (Kohler, Howe and Milstein, 1976) and with spleen cells (Kohler and Milstein, 1976). It synthesizes light chain but only secretes it after incorporation into hybrid molecules after fusion. Presently, a number

of different myeloma lines are available for fusion partners (Table III). No study has been reported which compares fusion frequency, relative stability of hybrids, or degree of chain mixing.

Derivation of T Cell Hybridomas

Once it was obvious that normal spleen B cells could be "immortalized" by somatic cell hybridization with myeloma cells, a number of laboratories turned to the question of whether T cell hybrids could be established. Previous experiments by R. Hyman at the Salk Institute (Hyman, 1973; Hyman and Stallings, 1976; Hyman and Kelleher, 1975) demonstrated the loss of the T cell marker, Thy-1, in fusions between a thymoma and a fibroblast. They had been able to demonstrate Thy-1 expression in a hybrid between an immunoselected Thy-1 negative variant and a lymphoma, and the expression of both parental Thy-1 types when two different thymomas were fused. These results suggested using a T cell line in attempting to rescue a T cell function.

Kohler et al. (1977) produced hybrids between a myeloma and a thymoma which secreted the myeloma protein, expressed H-2 products of both parents, but did not express the Thy-1 surface marker of the thymoma. Similar restriction was found by Schwaber (1977) in attempting to produce hybrids between a mouse myeloma and human B cells from normal patients, patients with X-linked agammaglobulinemia without B cells, and patients with common variable agammaglobulinemia with B cells which did not synthesize or secrete antibodies. Hybrids were isolated only when B cells were present in the population of human cells. These two studies suggest that the expression of specific

TABLE III

CELL LINES AVAILABLE FOR HYBRID PRODUCTION

Designation	Immunoglobulin Production	Biochemical Marker	Reference
P3x63Ag8[a]	IgG_1, k	8-Azaguanine^r	Kohler and Milstein (1975)
P3-NS1/1-Ag4-1[a]	Intracellular k	8-Azaguanine^r	Kohler, Howe and Milstein (1976)
MPC11-x45-6TG[b]	IgG_{2b}, k	6-Thioguanine^r	Yelton et al. (1978)
P3x63Ag8.653[a]	None	8-Azaguanine^r	Kearney et al. (1979)
Sp2/0-Ag14[c]	None	8-Azaguanine^r	Shulman and Kohler (1978)

[a]Derived from BALB/c MOPC 21 myeloma.
[b]Derived from BALB/c MPC 11 myeloma.
[c]Isolated subclone of hybridoma from fusion of BALB/c spleen cells and P3x63Ag8, selected for loss of antibody production.

properties of normal cells in hybrid lines may depend on the use of a tumor line closely differentiated along a pathway similar to the cell type to be immortalized.

Using the AKR mouse thymoma BW5147, a number of laboratories have produced T cell hybridomas. Assay systems for successful fusions require determination of cell surface markers of the spleen T cell parent (H-2, Thy-1 and others) or recognition of some functional property assigned to T cells. Goldsby et al. (1977) and Kohler et al. (1977) used mixed lymphocyte cultures as a source of spleen T cell parents while Hammerling (1977) used nylon wood-purified, mitogenically activated and antigen-stimulated spleen T cells as parental cells. All of these experiments produced T cell hybrids but none exhibited functional properties. This problem was addressed by Melchers (1978) when he suggested several possible causes: (1) the BW5147 cell line may be differentiated in that even with successful T cell hybrids it suppresses some functions, (2) cytotoxic lines may be difficult to obtain because during the fusion process the cytotoxic cell parent could kill the tumor cell parent, and (3) assay systems may not be sensitive enough to ascertain certain functions present in low levels.

One T cell function which has recently been maintained in T cell hybrids is suppressive activity. Ruddle (1978), Simpson and co-workers (1978), Taniguchi and Miller (1978), and Newport-Sautes and co-workers (1979) have reported T cell hybridomas which mediate antigen-specific suppression assayed in vivo or in vitro, or with cell surface characteristics which can be used for structural studies. But more importantly, it has provided some insight into the mechanisms restricting the successful rescue of highly differentiated functions. This vital

information may provide the key to obtaining hybrids in other biological systems which also express highly differentiated functions, such as hormone or enzyme secretion.

Objective

The preceding sections review evidence that monoclonal, monospecific antibody produced by hybridomas is an extremely powerful tool. Literature reports demonstrate the varied systems in which this technique is the method of choice and it is probable that this explosion in its use has just begun.

Numerous fusions and mass screenings have been so successful that extensive investigation into details of the fusion process have not been demanded. Since first described, the technique has undergone few major changes. Investigators usually immunize several times and after the animal is responding, it is given a final injection and used for hybridization within three to five days. Most laboratories have thus produced the specific hybridoma desired, or one with another specificity worthy of continued study.

Even with its widespread success, a few major questions still remain unanswered. Is there a particular time after immunization which is optimal for fusion? Do certain spleen cell populations preferentially enter into the fusion event? Can the fusion frequency be increased? These questions all concern a central factor, the exact state of differentiation of the antigen-specific normal spleen cells which are chosen as fusion partners in preference to others.

The lack of answers to these questions has not restrained the use of the technique, but without them it may not reach its full

potential. If the fusion process were better understood, it might be possible to isolate antigen-specific hybrids of a desired immunoglobulin class and with a desired binding affinity.

The objective of this work has been to answer the questions presented above. Two model systems have been used in this research. The lipopolysaccharide-induced polyclonal activation of murine spleen cells in vitro has provided a method to study cell type and differentiation states of responding B cells in relation to hybrid formation. Investigation of hybrid formation during an in vivo immune response used the well characterized response of mice to sheep erythrocytes. This system provides a number of excellent assays and sufficient background data to allow correlation of hybrid formation with both serum activity and responding cell populations.

III. PROCEDURES

General

Materials

Animals

Female mice of the inbred strains Balb/cCum and AKR/Cum were purchased from Cumberland View Farms, Cumberland, Tennessee. Mice were maintained in the animal facilities of this institution during all experiments with continual access to food and water.

Myeloma cell line

The myeloma cell line P3x63Ag8 (P3) was obtained from the Salk Institute through the courtesy of Dr. Milstein. This line secretes IgG_1, k immunoglobulin.

Media

Myeloma cell lines and the products of all fusion experiments were maintained in Dulbecco's Modified Eagle Medium, DME, with 4500 mg of glucose per liter. Complete DME (DME-FCS) was prepared from DME by supplementing it with 15% FCS, 2mM glutamine, non-essential amino acids, and Gentamycin (50ug/ml). DME-HAT was prepared from DME-FCS by adding hypoxanthine, aminopterin, and thymidine as described by Littlefield (1964).

Culture conditions

All cell lines, fusion experiments and LPS cultures were maintained at 37°C in 8% CO_2 in a humid incubator.

Cell counting

Determination of both cell number and viability was performed by counting cells on a hemocytometer in the presence of the vital dye, trypan blue.

PEG

Polyethylene glycol (PEG), molecular weight 1000 (Sigma, St. Louis, Missouri) was prepared as described by Pontecorvo (1975). Prior to use the pH was adjusted to 7.6-7.8 with 0.1N NaOH.

Methods

Spleen cell preparation

Mice were sacrificed by cervical dislocation and the spleens removed under sterile conditions. Spleen cell suspensions were prepared by perfusing the excised spleen with 4 ml of phosphate-buffered saline containing 5% FCS using a syringe with a 22 gauge needle. The cells were gently expressed from the capsule by using the edge of the needle. The suspension was allowed to settle for five minutes in an ice bath and the supernatant transferred to a second tube. For plaque-forming assays, nucleated cells were counted on a hemocytometer in the presence of 2% acetic acid, diluted to the desired concentration and kept in an ice bath. Cells used in fusions were washed twice in DME by centrifugation.

Cell fusion

Spleen cell-myeloma hybrids were formed by the fusion techniques described by Galfre et al. (1977), with the following modifications. A 40% PEG concentration was employed instead of 50%. After PEG addition, the cells were diluted to only 10 ml versus 50 ml. The ratio of spleen cells to myeloma cells was 2:1. Following fusion, the cells were re-suspended in DME-FCS at a density of 10^5 spleen cells per drop (0.075 ml), and were plated by adding one drop to each well of a 96-well microtiter tissue culture plate. Twenty-four hours later, one drop of 2X DME-HAT was added to each well. The medium was then removed and replaced by fresh DME-HAT every 3 to 5 days.

This plating technique and use of these cell densities have several advantages. Delaying addition of HAT for 24 hours allows any possible hybrid to double prior to selection. This allows establishment of the salvage pathway enzymes and provides two cell progeny from each fusion event. This may increase the chances of survival during the strenuous culture conditions imposed during HAT selection. Growth by P3 cells during the first 48 hours conditions the medium and serves the purpose of a feeder layer. At the cell density used, there is usually not more than one hybrid per well which facilitates the assay of individual hybrid products without the need for sub-cloning. Finally, the chances of any type of clonal competition or suppression are reduced at these cell densities.

Scoring, sampling and storage of hybrid supernatants

After 10-15 days of HAT selection, visually detectable colonies were present. These were marked and the wells filled with medium. Two days later 0.2 ml of culture supernatant was removed aseptically and stored at -20°C in a rigid V-bottom microtiter plate sealed with an adhesive plastic cover to prevent evaporation.

Enzyme-linked immunosorbent assay (ELISA)

Hybrid supernatants were screened for the class of antibody they produced using an ELISA as described by Kearney et al. (1979). Class-specific antisera were the generous gift of Dr. Kearney.

Lipopolysaccharide Studies

Materials

Lipopolysaccharide (LPS)

Bacterial lipopolysaccharide from E. coli 0111:B4 was obtained from Difco Laboratories, Detroit, Michigan. It was dissolved in PBS and stored frozen as a 50X stock solution (2500 mcg/ml). All experiments were performed with the same lot of LPS which had been freshly thawed.

Medium

LPS cultures were normally established in RPMI 1640 with 20% FCS. Substitution with DME did not seem to alter the pattern of culture development.

Methods

LPS cultures

All cultures were established according to Kearney and Lawton (1975) using the recommended culture materials and cell densities.

Radiolabel uptake

Concentrations of $5x10^5$ cells from fresh spleen cell preparations or from LPS cultures were incubated with tritiated-thymidine or tritiated-leucine (New England Nuclear, Boston, Massachusetts) in microtiter plates with one uCi of the desired label per well for six to eight hours at 37^oC. Cultures were harvested using an automated sample harvester and the samples counted on a liquid scintillation counter.

Fluorescent staining

Cultured and uncultured spleen cells were stained for cytoplasmic or surface immunoglobulin of the various classes by methods described by Kearney and Lawton (1975). Class-specific antisera and technical advice and assistance were kindly provided by Drs. Kearney and Burrows.

Sheep Red Blood Cell Studies

Materials

Sheep red blood cells (SRBC)

Blood from the same animal (Microbiological Associates, Bethesda, Maryland) was used for all experiments. SRBC were stored in Alsever's Solution at 4^oC for at least two weeks prior to use.

Cunningham slide chambers

Slide chambers for the plaque-forming assay were prepared as described by Cunningham and Szenberg (1968) except that only two chambers were formed per slide.

Guinea pig complement

Lyopholyzed guinea pig complement was purchased from GIBCO, Grand Island, New York. It was reconstituted at full strength and stored in aliquots until needed at -70°C.

Developing sera

Goat anti-mouse 7S Ig antibody was purchased from Litton Bionetics, Kennsington, Maryland. It was titered and found to produce maximum indirect plaques at a 1:300 final dilution. A correction to the number of indirect plaques was performed to compensate for suppression of direct plaques by the developing sera at this dilution.

Methods

Immunization

Mice were injected intraperitoneally with 0.1 ml of a 2% suspension of SRBC in 0.9% saline. The cells were washed three times with saline before resuspension to 2% ($5x10^8$/ml).

Plaque-forming assays (PFC)

Direct and indirect assays were performed using the method of Cunningham and Szenberg (1968) as modified by Silver and Winn (1973).

Serology

Hemagglutination and cytotoxicity assays were performed according to Silver, McKenzie and Winn (1972).

Antigen specificity

Hybrid supernatants were screened for anti-SRBC activity by using cells bound to plastic plates as described by Stocker and Heusser (1979). The method of detection was by an ELISA.

IV. RESULTS

Preliminary Experiments

Conditions Which Minimize Variability in the Fusion Technique

Cell line

The parental myeloma cell line used in this work was P3x63Ag8 (P3), an IgG_1, k producer. Attempts to use P3-NS1/Ag4 (NSI), a non-secreting myeloma line, usually produced fewer hybrids and exhibited more variability from experiment to experiment when compared with P3. This precluded its use in the following studies, even though its non-secreting characteristic made it more desirable.

Growth curve analysis of P3 indicated that cells could be maintained in log phase growth when they were cultured at a density of $4x10^5$ to $1x10^6$ cells/ml (Figure 3). Cells used in fusion experiments were harvested at a density between these two points. Better growth and easier harvesting were possible if P3 was maintained in regular petri plates (bacteriological) instead of those plates treated for tissue culture.

PEG concentration

Figure 4 illustrates the toxicity of PEG on normal mouse spleen cells. Cells were exposed to the various PEG concentrations for eight minutes, diluted with medium and their viability determined. A 40% concentration was chosen for use in subsequent studies because even with

Figure 3. P3x63Ag8 Growth Curve. Cell cultures were initiated at $2x10^5$ cell/ml. Samples were taken from triplicate cultures at various times after initiation. Cell number and viability were determined by counting on a hemacytometer in the presence of trypan blue dye.

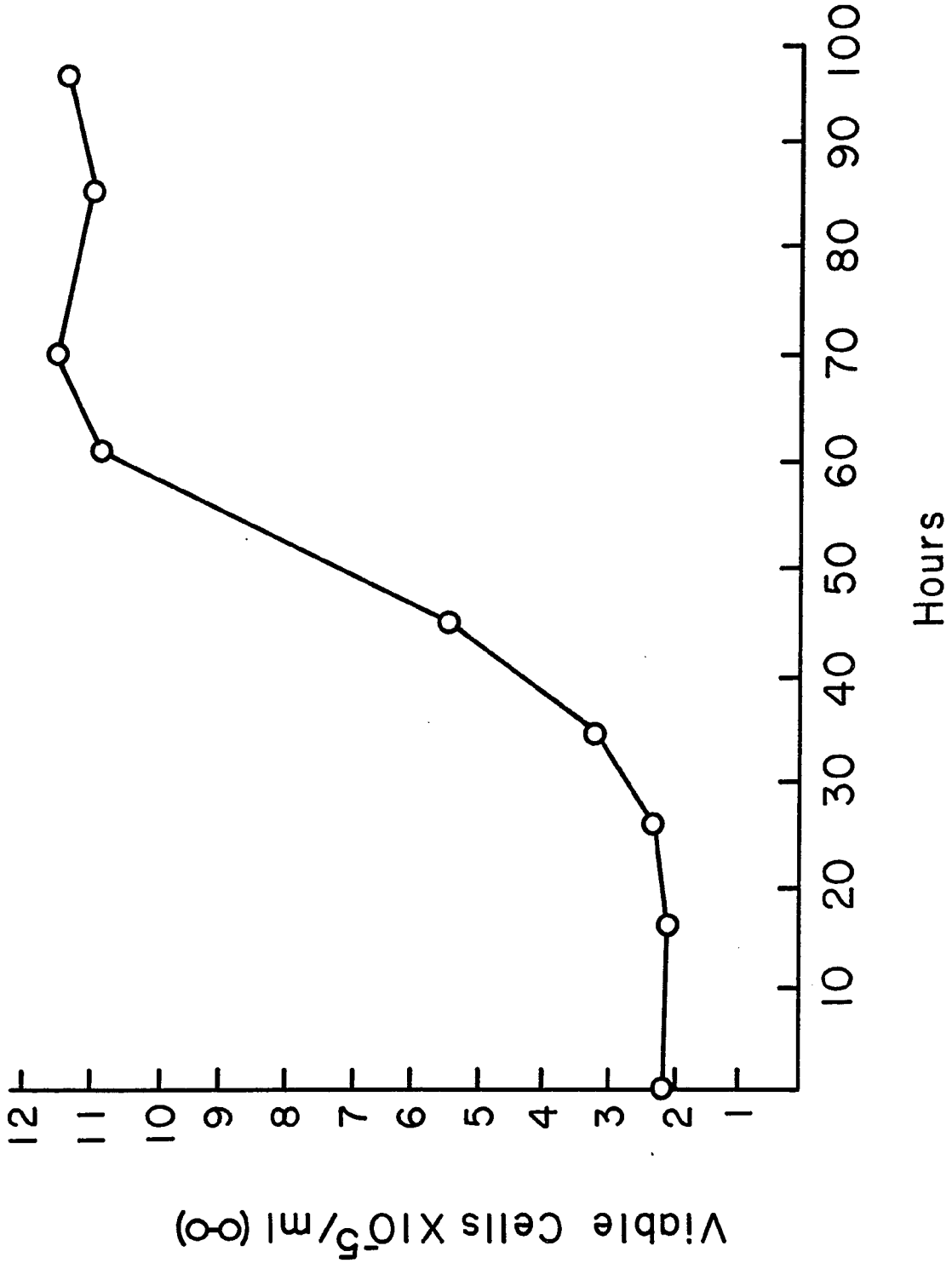
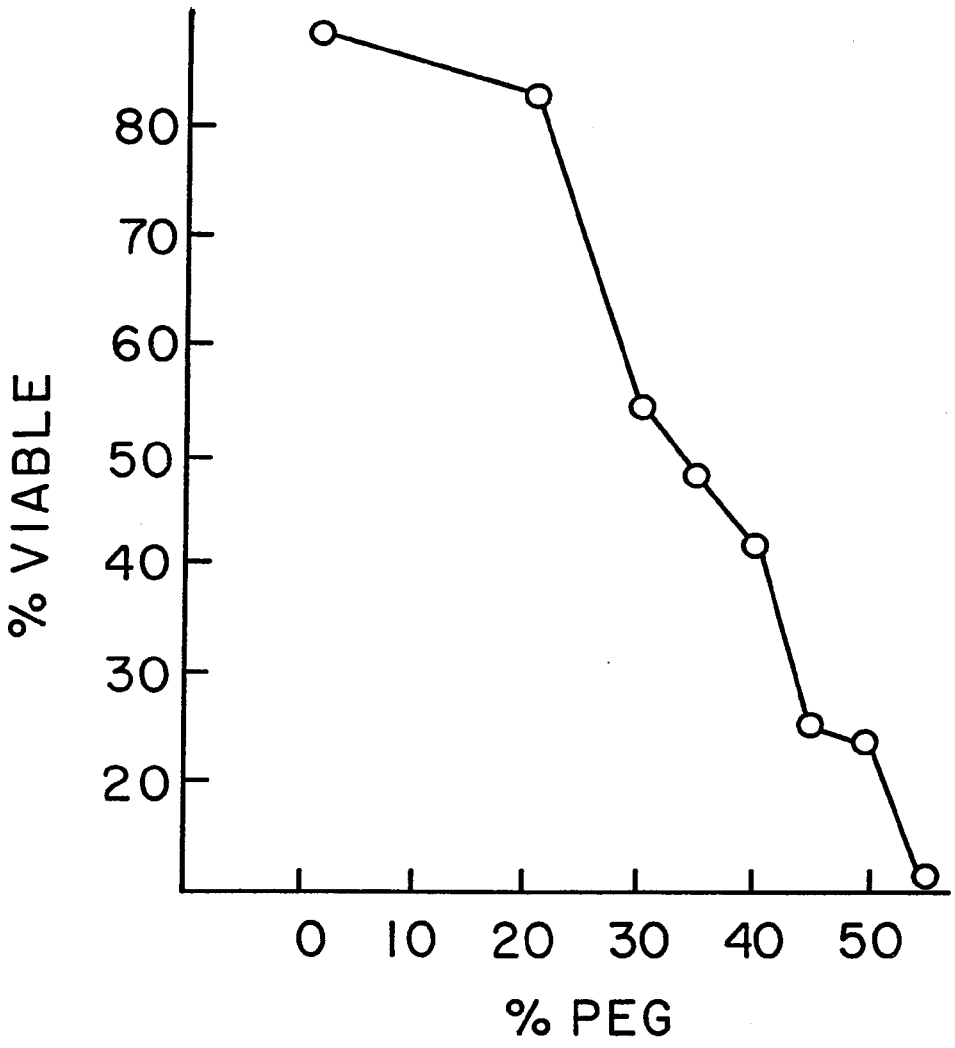

Viable Cells X10⁻⁵/ml (oo)
12
11
10
9
8
7
6
5
4
3
2
1
10
20
30
40
50
60
70
80
90
100
Hours

Figure 4. Toxicity of Various PEG Concentrations. 10^8 spleen cells were exposed to various concentrations of PEG for eight minutes. The cell suspension was then diluted and a sample taken to determine viability by trypan blue dye exclusion.


% VIABLE
80
70
60
50
40
30
20
0
10
20
30
40
50
% PEG

prolonged exposure, as compared to one minute in normal fusion techniques, 40% spleen cell viability was maintained. When used in hybrid production, this concentration routinely gave 60 to 75% spleen cell viability and had little effect on the viability of the myeloma cells.

Attempts to Produce Monoclonal Antibodies Specific for a Murine MHC Product

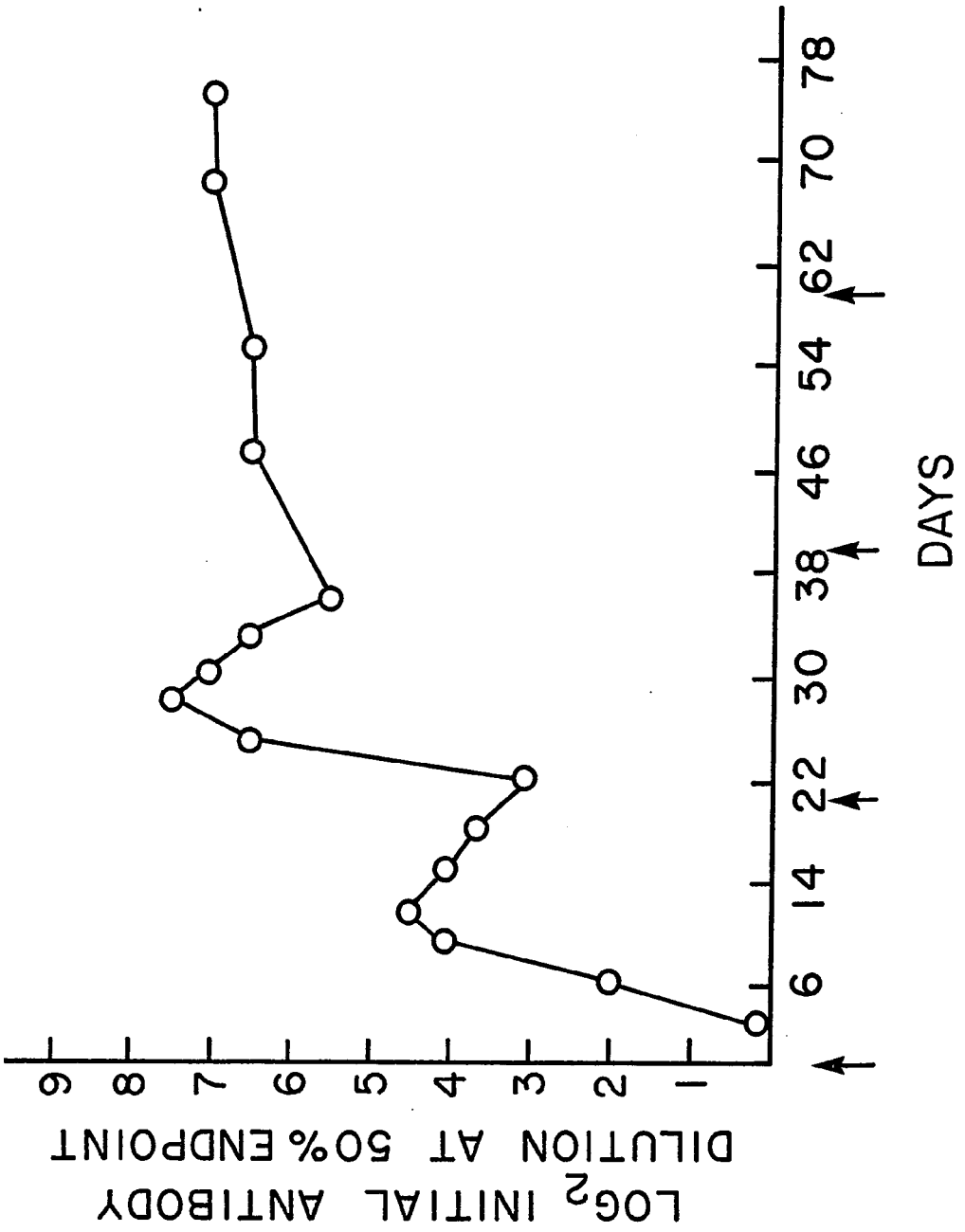
Spleen cells from hyperimmunized mice (five to six injections) were used for fusion with P3 in an attempt to produce hybrids secreting antibody specific for murine H-$2K^k$. In four different fusions, over 400 independent hybrids were produced and analyzed for anti-H-$2K^k$ specificity. All clones were negative for anti-H-$2K^k$ activity.

Examination of the serum antibody response to this antigen (Figure 5) indicated that at the time of fusion a high serum titer specific for H-$2K^k$ existed. Other experiments using SRBC as the antigen routinely produced hybrids with as many as 23% being antigen-specific when fusions were performed two to five days after a secondary immunization. Fusions performed eight to ten days after the secondary injection produced few hybrids with none secreting antigen-specific antibody. This raised the questions of whether hyperimmunization was the optimal method for preparing spleen cells to be used in hybrid production and whether plasma cells were the predominant cell type included in hybrid formation to yield antibody-producing cell lines.

Hybrid Production During Primary Responses

The experiments with SRBC indicated that specific hybrids could be isolated from fusions performed two to five days following a secondary

Figure 5. Titer of Anti-H-2K^k Antisera (D3b/UAB II). (C3H.OH x 129)F1 female mice were injected intraperitoneally with $4x10^5$ lymphocytes from C3H female mice. Arrows indicate time of injection. Antibody titer was determined by a radiobinding assay using C3H red cells and ^{125}I-protein A.


LOG2 INITIAL ANTIBODY
DILUTION AT 50% ENDPOINT
1
2
3
4
5
6
7
8
9
6
14
22
30
38
46
54
62
70
78
DAYS

immunization. To determine if specific hybrids could be produced during a primary response, three different antigen systems were used. These included trinitrophenol (TNP), a hapten; purified Mason-Pfizer virus, a particulate antigen, and SRBC, a complex array of cellular antigens. The results (Table IV) demonstrate that hybrids could be produced during a primary response to these antigens when the generation of specific plasma cells probably would not have reached its maximum value. These results suggest that the plasma cell was not the only, and might not be the predominant, partner in PEG-induced fusion events.

Hybrid Formation with In Vitro Lipopolysaccharide-stimulated Spleen Cells

LPS is a polyclonal activator which induces the growth and differentiation of resting small B lymphocytes into antibody-secreting plasma cells of the major immunoglobulin classes. By harvesting spleen cells cultured in the presence of LPS for various periods of time, it is possible to obtain B cell populations enriched for different developmental types. Hybrids were produced from fusions between P3 and LPS-stimulated spleen cells to investigate the possible relationship between cell fusion and the various stages of lymphocyte differentiation.

Experimental Protocol

AKR or CBA mouse spleen cells were established in LPS cultures at 24-hour intervals, harvested simultaneously and used as fusion partners with P3. Small aliquots of each culture were used for radiolabelled precursor incorporation and for fluorescent staining to determine surface and cytoplasmic immunoglobulin. After fusion cultures

TABLE IV

HYBRID PRODUCTION TO VARIOUS ANTIGENS
AFTER A PRIMARY INJECTION

Antigen	Days Post 1° Injection	Growth Positive Wells (%)	Antigen-Specific Wells
TNP-HRBC[a]	2	55/272 (20)	2/55 anti-TNP 35/55 anti-HRBC
	4	93/272 (34)	7/93 anti-TNP 40/93 anti-HRBC
Mason-Pfizer[b] Virus	3	7/272 (3)	0/7
	5	254/272 (90)	12/54
	7	272/272 (100)	8/272
SRBC	3	58/268 (22)	N.D.[c]
	4	42/44 (95)	N.D.

[a]Performed in cooperation with S. Jackson. Mice were immunized with TNP-HRBC and hybrid supernatants were assayed on TNP-SRBC, HRBC and SRBC for hemagglutination.

[b]Performed in cooperation with E. Hunter. Hybrid supernatants were assayed by both a radioimmune assay and an ELISA on infected target cells fixed to microtiter plates.

[c]N.D.= Not determined.

were exposed to HAT selection for ten to fourteen days, wells were scored for growth and the supernatants from positive wells assayed for antibody production using an ELISA.

Characteristics of LPS Cultures

Cell types

A composite of several experiments which demonstrate the kinetics of LPS-induced polyclonal activation is presented in Figure 6. Surface IgM^+ cells compose about 40 to 50% of the initial culture and progress to about 85% after 96 hours. Cytoplasmic IgM^+ cells progress from an initial 1 or 2% to 30 or 40% after 120 hours. IgG^+ cells, both surface and cytoplasmic, comprise 1 to 2% of the initial culture, begin proliferation at 48 hours and constitute about 20% of the culture at 120 hours.

Metabolic activity

Synthesis of protein and DNA was determined by the uptake of leucine and thymidine, respectively (Figure 7). Protein synthesis in the experimental cultures becomes greater than the control cultures (0 hour) as early as 24 hours after initiation and increases steadily to 120 hours. This increase is coincident with the differentiation of small B cells to plasma cells (Figure 6) and probably indicates the rise in antibody production. Decline of protein synthesis after 120 hours of culture suggests depletion of culture medium with resulting loss of antibody secretion.

Maximum thymidine uptake, an index of mitotic activity, occurs at or just prior to 72 hours. An increase in cell number also commences

Figure 6. Kinetics of LPS Cultures. CBA or AKR spleen cells were cultured in the presence of LPS for various periods of time. Cultures were harvested simultaneously and samples used for thymidine incorporation or for fluorescent staining. Relative thymidine incorporation, 0---0; surface IgM, □---□; cytoplasmic IgM, Δ---Δ; surface IgG, ■---■; cytoplasmic IgG ▲---▲. Fluorescence data are expressed as percent of total cell population.

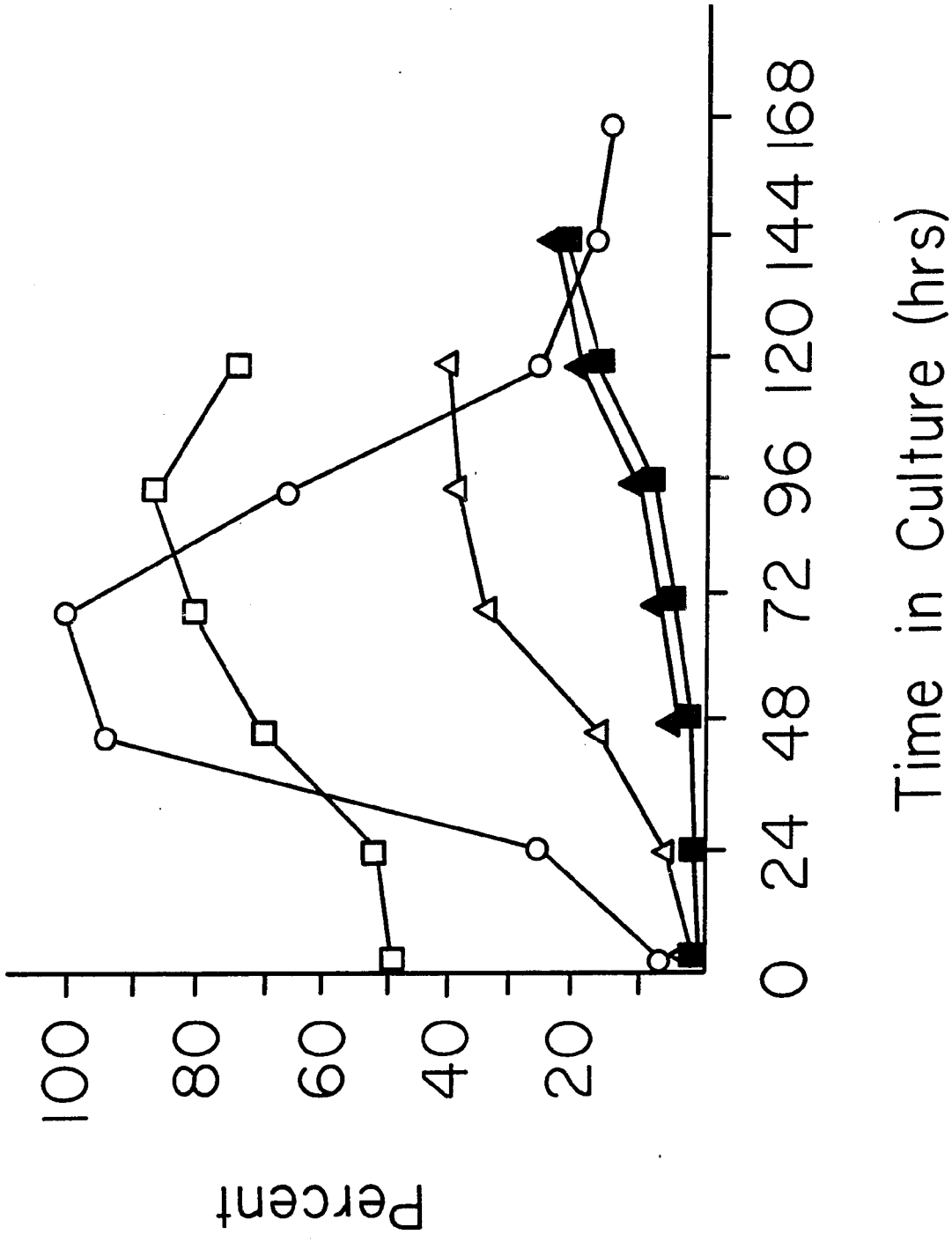
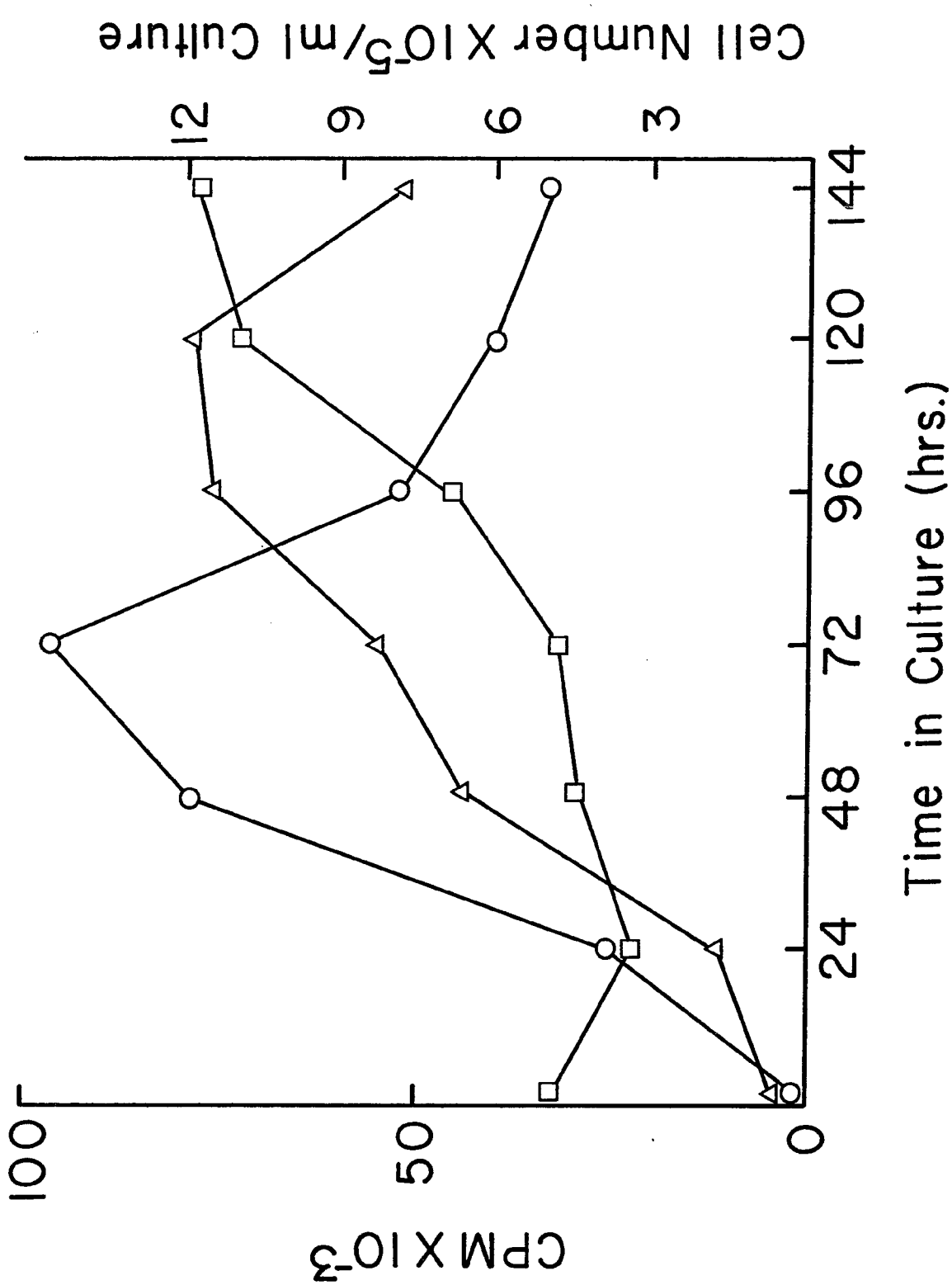

Percent
100
80
60
40
20
0
24
48
72
96
120
144
168
Time in Culture (hrs)

Figure 7. Metabolic Activity of LPS Cultures. CBA or AKR spleen cells were cultured in the presence of LPS for various periods of time. Cultures were harvested simultaneously and samples used for thymidine or leucine incorporation and to determine viability. Thymidine incorporation, 0---0; leucine incorporation, Δ---Δ ; cells/ml of culture fluid, □---□ .


CPM X 10⁻³
100
50
0
24
48
72
96
120
144
Time in Culture (hrs.)
12
9
6
3
Cell Number X 10⁻⁵/ml Culture

at this time and continues to about 144 hours of culture. Alterations in culture conditions can shift the mitotic activity and the resulting increase in cell number to a slightly earlier or later time depending on whether the conditions enhance or inhibit mitogenesis (Table VI, Exp. C).

Studies on Five Day LPS Cultures

Hybrid frequency

Table V presents data on hybrid production with spleen cells cultured for 0, 24, 48, 72, 96, or 120 hours with LPS. Positive wells were determined by visual inspection using a microtiter reader on days 10 and 15. This method allows detection of clones of about 50 to 75 cells. The percent of positive wells is the number of wells with cell growth divided by the number of wells seeded with 10^5 spleen cells. The mean number of hybrids per well was calculated using the Poisson relation. The results indicate maximum hybrid production at 72 hours of culture with a frequency of 1.3 hybrids formed per 10^5 cultured spleen cells.

Influence of cell type

To answer the question of whether a particular spleen B cell subpopulation is preferentially included in PEG-induced fusions, hybrid frequency has been compared with two states of differentiation present in LPS-activated cultures. Figure 8 illustrates the relationship of hybrid formation to either surface IgM^+ or cytoplasmic Ig^+ cells. In both of these graphs the percent of each cell type in the culture at various times has been provided as a reference. There is no apparent

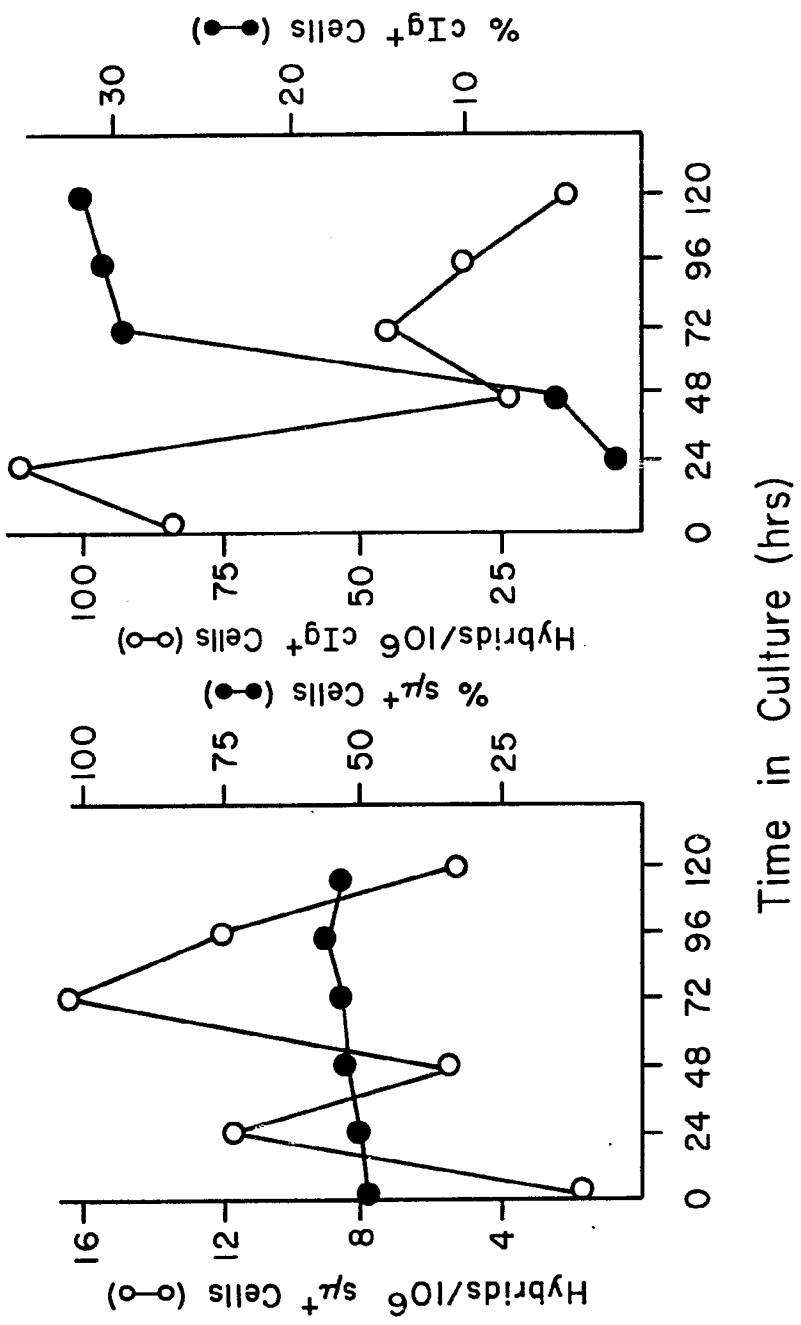
TABLE V

FREQUENCY OF HYBRID PRODUCTION WITH SPLEEN CELLS STIMULATED IN FIVE DAY LPS CULTURES

	Time in Culture (hrs.)					
	0	24	48	72	96	120
Positive wells	32	141	205	528	478	280
Wells seeded with 10^5 cells	384	288	720	720	720	720
% Positive	8	49	29	73	66	39
Mean hybrids per well[a]	0.08	0.67	0.35	1.30	1.08	0.49

[a]Mean calculated by Poisson relation $P(0) = e^{-x}$, where P(0) equals percent of negative wells.

Figure 8. Hybrid Production in Relation to B Cell Differentiation.


Hybrids/10⁶ sμ⁺ Cells (o—o)
16
12
8
4
% sμ⁺ Cells (●—●)
100
75
50
25
Hybrids/10⁶ cIg⁺ Cells (o—o)
100
75
50
25
% cIg⁺ Cells (●—●)
30
20
10
0 24 48 72 96 120
0 24 48 72 96 120
Time in Culture (hrs)

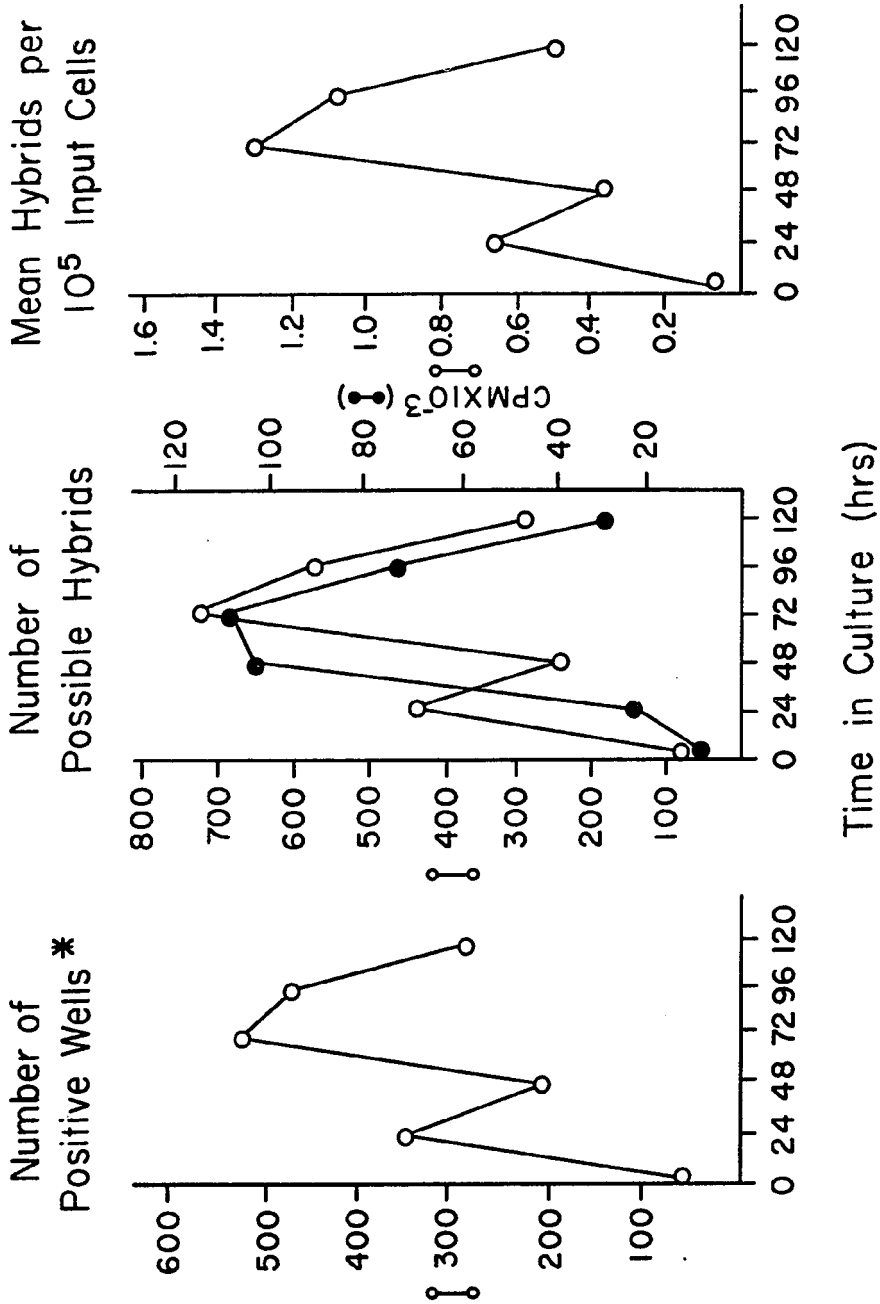
correlation between the lines on either graph, which indicates that hybrid frequency is not related to the relative number of either B cells not producing antibody (surface IgM^+, cytoplasmic Ig^-) or antibody-producing plasma cells (cytoplasmic Ig^+).

Influence of mitotic activity

Figure 9 illustrates the number of positive wells, the number of possible hybrids and the mean number of hybrids per 10^5 spleen cells in relation to time in culture. Positive wells and the mean number of hybrids were determined as already described. The total number of possible hybrids indicates the number of discrete colonies that could be detected visually, which ranged from 0 to 5 per well. The theoretical number of hybrids that was possible at each time was calculated by multiplying the mean number of hybrids per well by the number of wells seeded. A plot of these data closely agrees with the number of hybrids determined visually and indicates that each visually discrete colony probably represented a single hybrid.

The three graphs are very similar, each demonstrating two peaks of hybrid formation, one at 72 hours and the other at 24 hours. A plot of thymidine uptake is superimposed over the graph of the possible number of hybrids and demonstrates that the major peak of hybrid formation coincides with maximum mitotic activity at 72 hours. To confirm this observation, two additional experiments were conducted which used spleen cells stimulated for up to five days with LPS in culture (Table VI, Experiments B and C). A comparison of the hybrid frequencies from these experiments and the experiment presented above (Table V and Table VI, Experiment A) indicates that maximum hybrid

Figure 9. Hybrid Production in Relation to Mitotic Activity.



* Normalized to 720 wells

TABLE VI

HYBRID PRODUCTION BY SPLEEN CELLS STIMULATED IN FIVE DAY LPS CULTURES

Exp.	Time in Culture (hrs.)	Positive Wells	Wells Seeded	% Positive	Mean[a]
A	0	32	384	8	.08
	24	141	288	49	.67
	48	205	720	29	.34
	72	528	720	73	1.31
	96	478	720	66	1.08
	120	280	720	39	.49
B	0	6	480	1	.01
	24	50	480	10	.11
	48	185	480	39	.49
	72	275	480	57	.84
	96	70	480	14	.15
	120	30	480	6	.06
C	0	40	480	8	.08
	24	170	480	35	.43
	48	253	480	53	.76
	72	220	480	46	.62
	96	185	480	39	.49
	120	100	480	21	.24

[a]Mean calculated by Poisson relation $P(0) = e^{-x}$, where P(0) equals the percent of negative wells.

production occurred at 72 hours in two of the three and at 48 hours in the other. In the latter experiment, maximum thymidine uptake had shifted toward 48 hours. No early peak of hybrid production was found at 24 hours. Together these experiments show that maximum hybrid frequency is correlated with the mitotic activity of the spleen cell parent.

Antibody production by hybrids

Supernatants from positive wells were screened for eight IgG or IgM secretion by an ELISA. Determination of IgG secretion was limited to the subclasses IgG_{2a}, IgG_{2b} and IgG_3 because the parent myeloma, P3, produces IgG_1. In the initial experiment only an ELISA for IgM was established, but in a subsequent experiment (Table VI, Experiment B) an ELISA for both IgG and IgM was used (Table VII).

The percent of hybrids which produce IgM increases from about 60% at 24 hours to 70% to 80% at 72 hours and then decreases to about 40% during the following 48 hours. This decrease in IgM-producing hybrids occurs during the period of culture in which cytoplasmic IgG^+ cells are proliferating (Figure 6), and is reflected in the rise in the percentage of hybrids producing IgG during the last 36 hours of culture. The high frequency of IgG-producing hybrids at 48 hours of culture may be related to the number of mitotically active IgG precursors at this point.

Studies on 48-Hour LPS Cultures

The initial 5-day LPS study indicated peaks of hybrid production at 24 and 72 hours. The latter is presumably due to mitotic activity

TABLE VII

ANTIBODY PRODUCTION BY HYBRIDS FROM SPLEEN CELLS STIMULATED IN LPS CULTURES

Exp.	Time in Culture	%IgM[a]	%IgG[b]	Total[c]
A	0	0	N.D.[d]	--
	24	58	N.D.	--
	48	40	N.D.	--
	72	71	N.D.	--
	96	63	N.D.	--
	120	42	N.D.	--
B	24	60	0	60
	48	63	16	79
	72	78	4	82
	96	59	7	66
	120	40	12	52

[a]Percent of growth-positive hybrids. In experiment A 96 hybrids were assayed at each time except 0 hour where only 24 were available. 192 hybrids were checked at each time in experiment B.

[b]Assayed for IgG_{2a}, G_{2b}, and G_3.

[c]Total of IgM plus IgG.

[d]N.D. = Not determined.

as described above. In order to determine if a definite early peak did occur, LPS-induced spleen cell cultures were established at 12-hour intervals during a 48-hour period. Cells were harvested, used as parents for fusion with P3, and subsequently assayed for hybrid frequency and antibody production (Table VIII).

Though not as prominent as the original 24-hour peak, an increased number of hybrids did occur at 12 hours compared to 0-, 24- and 36-hour cultures. The large number of hybrids formed at 48 hours plus data from a thymidine uptake study suggested a shift in mitotic activity toward 48 hours may have moved the 24-hour peak to 12 hours in this experiment and in two other 5-day LPS studies (Table VI, Experiments B and C).

Analysis of the antibody produced by hybrids isolated in this experiment reveals that IgM secretors constituted 60-70% of all hybrids produced during the 24- to 48-hour period. The high frequency of IgG secretors previously observed at 48 hours is again evident. Double antibody producers were detected at both 36 and 48 hours of culture, and calculation of the mean number of hybrids per well at these time points indicated that these wells most likely contained a single hybrid.

Hybrid Production During the Immune Response of Balb/c Mice to Sheep Red Blood Cells

The studies described in the previous section utilizing LPS-induced spleen cells to fuse with P3 indicated that mitotically active B cells were the predominant cell type included in PEG-induced fusions. Therefore, hybrids isolated at any given time during the development

TABLE VIII

CHARACTERISTICS OF HYBRIDS PRODUCED BY SPLEEN CELLS STIMULATED IN 48-HOUR LPS CULTURES

Time in Culture (hrs)	Positive[a] Wells	IgM^+ Wells (%)	IgG^+ Wells (%)	IgM^{+b} IgG^+
0	6	0 (0)	1 (16)	0
12	68	30 (44)	0 (0)	0
24	26	18 (70)	0 (0)	0
36	52	30 (58)	2 (7)	2
48	302	185 (61)	36 (12)	2

[a]480 wells established at each time.

[b]Number of wells which produced both immunoglobulin classes.

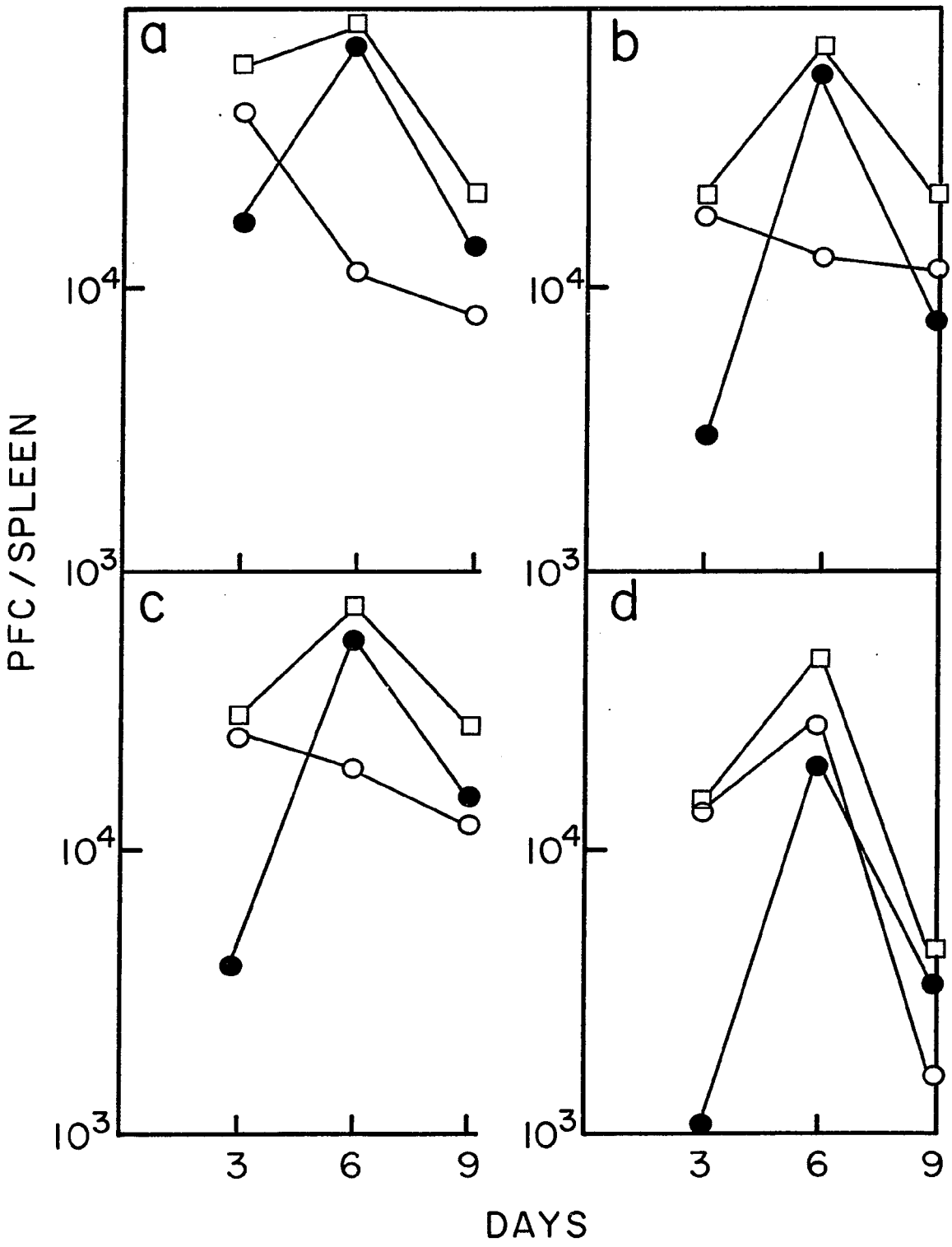
of an immune response might reflect the nature of the cells undergoing clonal proliferation. In this study, spleen cell-myeloma hybrids were formed at various times after immunization of Balb/c mice and later analyzed to determine their relationship to several parameters of the in vivo immune response.

Experimental Design

Balb/c mice were injected intraperitoneally on days 0 and 21 with $5x10^7$ SRBC. Every three days after the primary injection and every day after the secondary injection two mice were bled and spleen cell suspensions prepared. Serum was separated from the whole blood and stored frozen until determination of hemagglutinating and hemolytic antibodies. A small aliquot of spleen cells was assayed for plaque-forming cells (PFC) and the remainder was used in fusions with P3. After 10-14 days of HAT selection, wells were scored for growth and supernatants from hybrids tested for antibody class and specificity for SRBC.

For better comparisons of the antibody class and specificity of hybrids with the serum antibody and PFC data, it was necessary to quantitate the IgM and IgG responses independently. Initial direct and indirect PFC responses of Balb/c mice to various doses of SRBC were determined (Figure 10). The total PFC response of all the doses used was similar in magnitude and development, but each was the summation of different direct and indirect components. A dose of $5x10^7$ SRBC was chosen for use in all subsequent experiments since it produced maximum separation on both days 3 and 6.

Figure 10. PFC Response of Balb/c Mice to Various Doses of SRBC. Balb/c mice were injected intraperitoneally with SRBC. a. 5×10^8, b. 5×10^7, c. 5×10^6, and d. 5×10^5. PFC assays were performed on the spleen cells of two mice from each group on days 3, 6, and 9. Total PFC, □---□ ; direct PFC, 0---0; indirect PFC, ●---●.


a
b
c
d
10^4
10^3
PFC / SPLEEN
3
6
9
DAYS

It was desired that the influence of the P3 parent on the fusion process be constant. Therefore, the P3 cell line was maintained in log growth throughout the entire experiment (Figure 11).

Hybrid Formation During a Primary and Secondary Immune Response

Antibody-forming cells

Direct and indirect plaques were assayed during the primary and secondary responses (Figure 12). Direct plaques (IgM PFCs) reached a peak four days after the primary injection and declined thereafter. Following the secondary injection, IgM PFCs also reached a peak four days later (day 25) and declined to a low level on day 28. The magnitude of the primary IgM PFC response was twice that of the secondary.

Indirect plaques (IgG PFCs) were at a maximum on day 10 after the primary and on day four (day 25) after the secondary. IgG PFCs quickly declined in the primary response but remained in large numbers for five days during the secondary.

The IgM PFC and IgG PFC peaks were well separated during the primary response but coincided during the secondary. A reversal of the IgM to IgG ratio occurred between the primary and secondary responses.

Serum activity

Sera prepared from bleedings during both primary and secondary responses were assayed for hemolysins and hemagglutinins before and after treatment with 2-mercaptoethanol (2-ME) (Figure 13). In these assays IgM is 2-ME sensitive and IgG is 2-ME resistant. Both the cytotoxic and hemagglutination titers were largely due to IgM in the

Figure 11. P3X63Ag8 Growth Pattern During a 33-Day Study. Samples of the P3X63Ag8 myeloma cell cultures used during the entire 33 day study were counted daily. Viability was determined using trypan blue dye. Growth during the 24-hour period prior to a fusion experiment, ●---●.

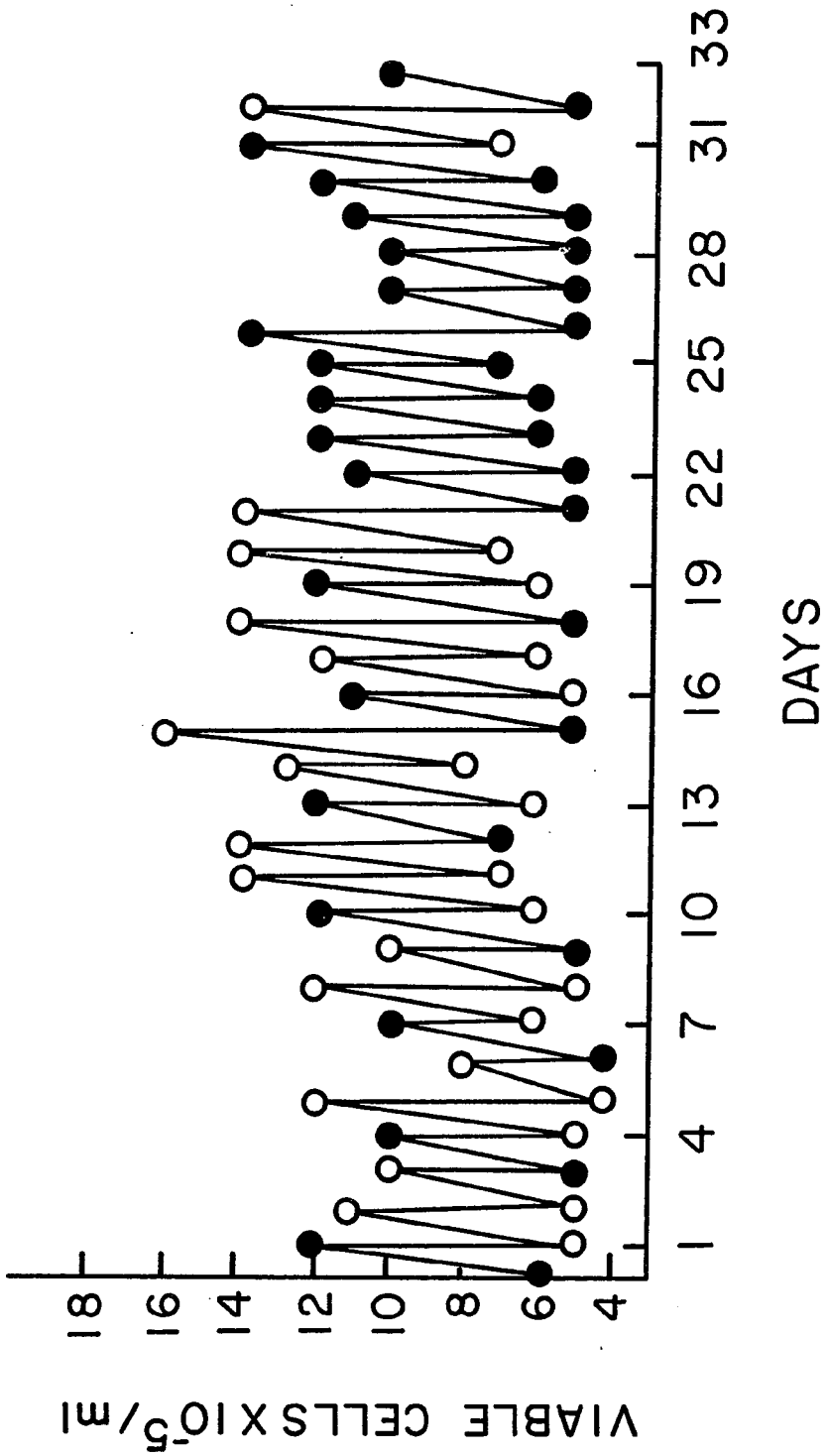

VIABLE CELLS X 10^{-5}/ml
18
16
14
12
10
8
6
4
1
4
7
10
13
16
19
22
25
28
31
33
DAYS

Figure 12. PFC Response of Balb/c Mice to SRBC During a 33-Day Study. PFC assays were performed on samples of spleen cells used for fusion. Direct (IgM) PFC, 0---0; indirect (IgG) PFC, ●---●. Arrows indicate the time of injection of $5X10^7$ SRBC.

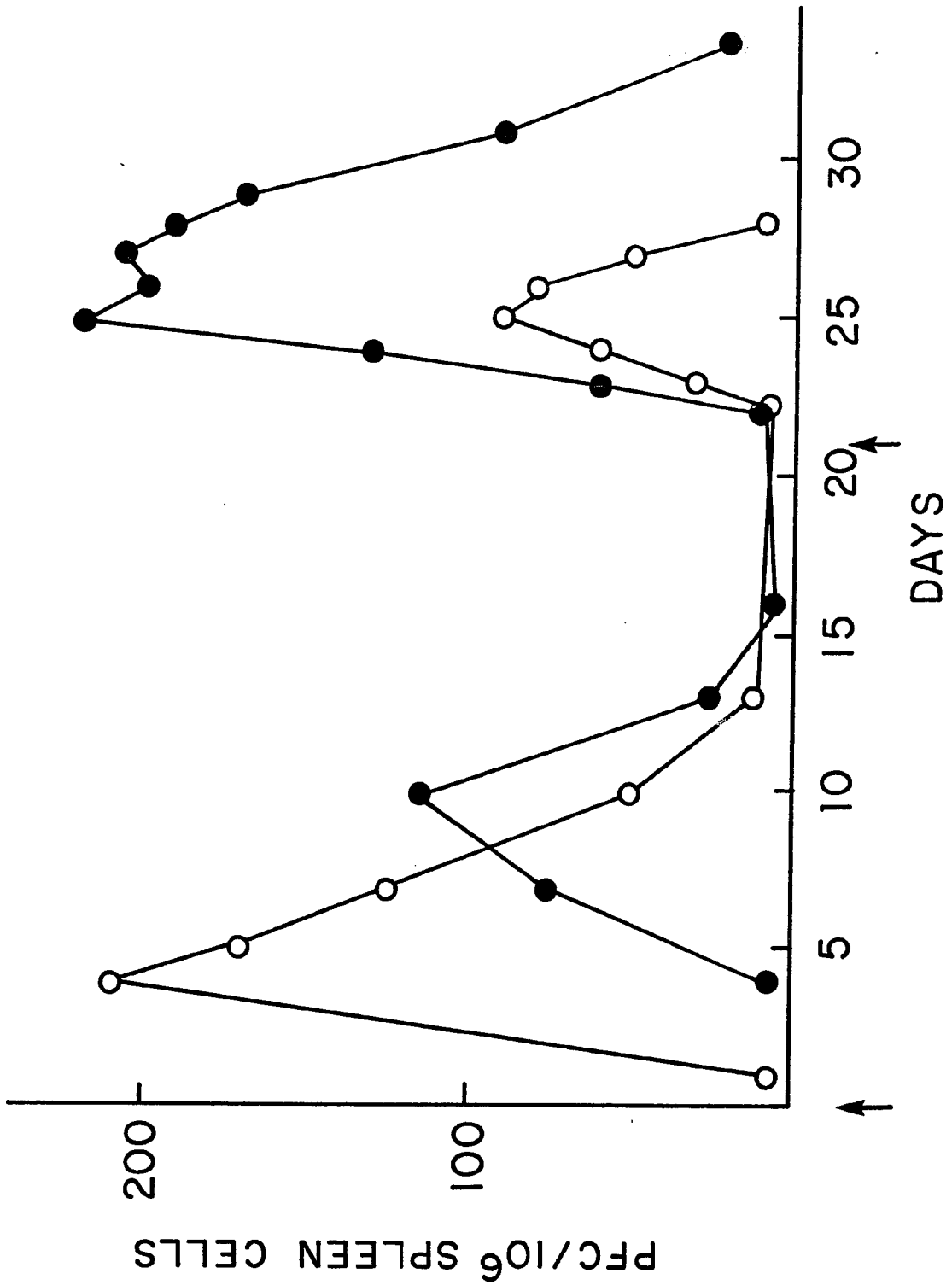
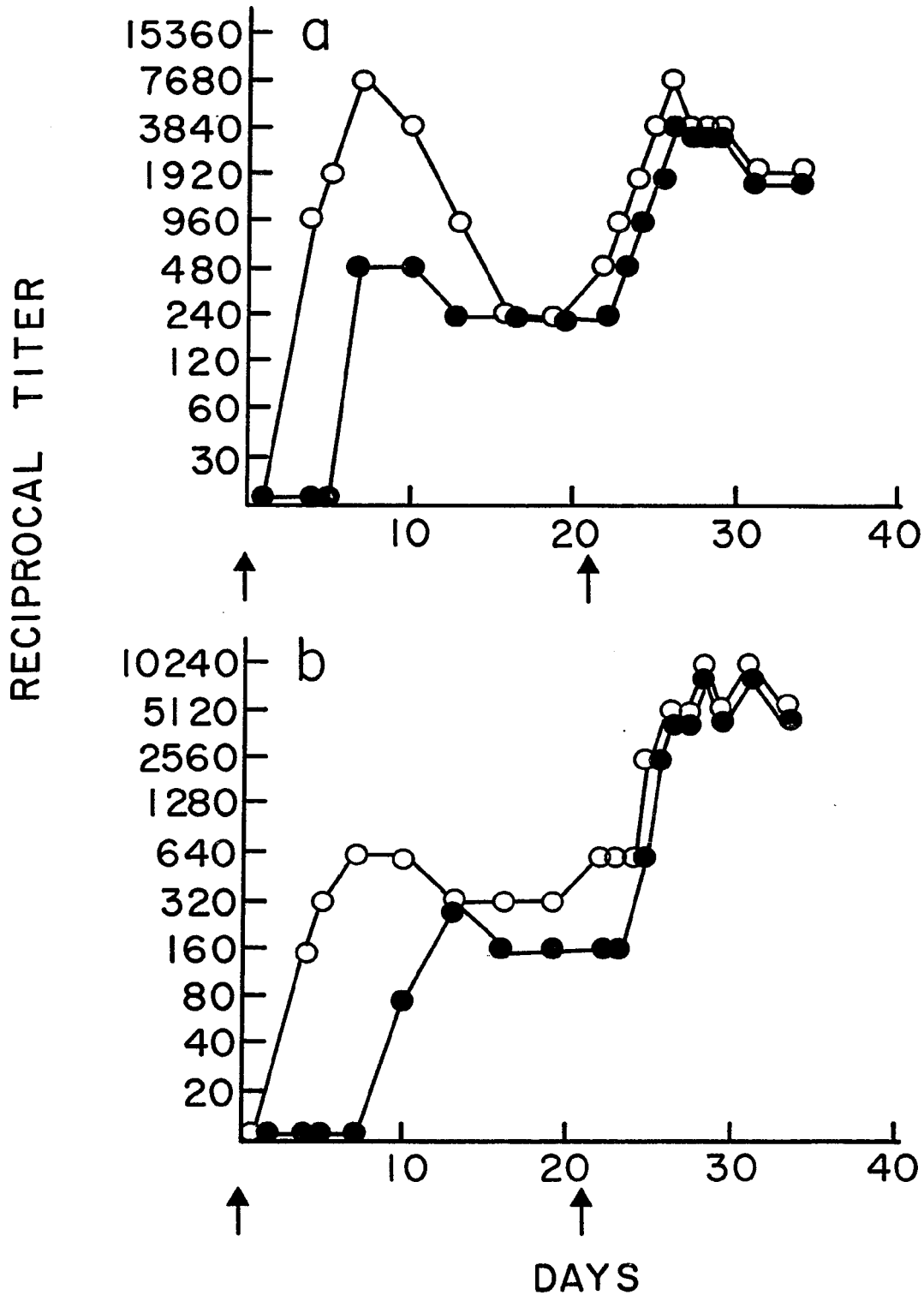

PFC/10⁶ SPLEEN CELLS
200
100
5
10
15
20
25
30
DAYS

Figure 13. Hemagglutination and Cytotoxic Serum Activity During a 33-Day Response of Balb/c Mice to SRBC. Hemagglutination (a) and cytotoxic (b) assays were performed on serum samples from mice whose spleen cells were used for fusion. Assays were conducted with, 0---0, or without, ●---●, 2-ME present. Arrows indicate the time of injection of $5X10^7$ SRBC.


RECIPROCAL TITER
15360
7680
3840
1920
960
480
240
120
60
30
a
10
20
30
40
10240
5120
2560
1280
640
320
160
80
40
20
b
10
20
30
40
DAYS

first 10 days of the primary response. This is in agreement with the predominance of IgM PFCs during the first 10 days. From days 10 through 21 the IgG activity becomes a major constituent of both the hemagglutinating and cytotoxic serum activity. Following the secondary injection, IgG becomes the largest part of both the hemagglutinating and cytotoxic response. IgM plays only a small role in the hemagglutinin titer during the first five days of the secondary response, after which IgG constitutes almost 100% of the response. This coincides with the rapid rise in IgG PFCs after the second immunization.

Hybrid production

Spleens from Balb/c mice were used for fusion on days 1, 4, 7, 10, 13, 16 and 19 during the primary response and days 22 through 28, day 31 and day 33 during the secondary response. Two peaks of hybrid production occurred during the primary response, one at day 4 and the other at day 16 (Figure 14). There were also two peaks evident in the secondary response, on days 22 and 28. The magnitude of the peaks using the secondary response was three to four times larger than those of the primary response. Very few PFCs were observed on the day that the second peak of hybrid production occurred during the primary response, and the second peak found during the secondary response occurred after the time of maximum PFC.

Antibody production and hybrid specificity

Supernatants from positive wells from each day during the primary and secondary responses were screened by ELISAs to determine the class of antibody secreted (Figure 15) and the number of hybrids specific for SRBC (Figure 16). IgM-secreting hybrids were present

Figure 14. Hybrid Production by Balb/c Mouse Spleen Cells During a 33-Day Response to SRBC. Arrows indicate the time of injection of $5X10^7$ SRBC.

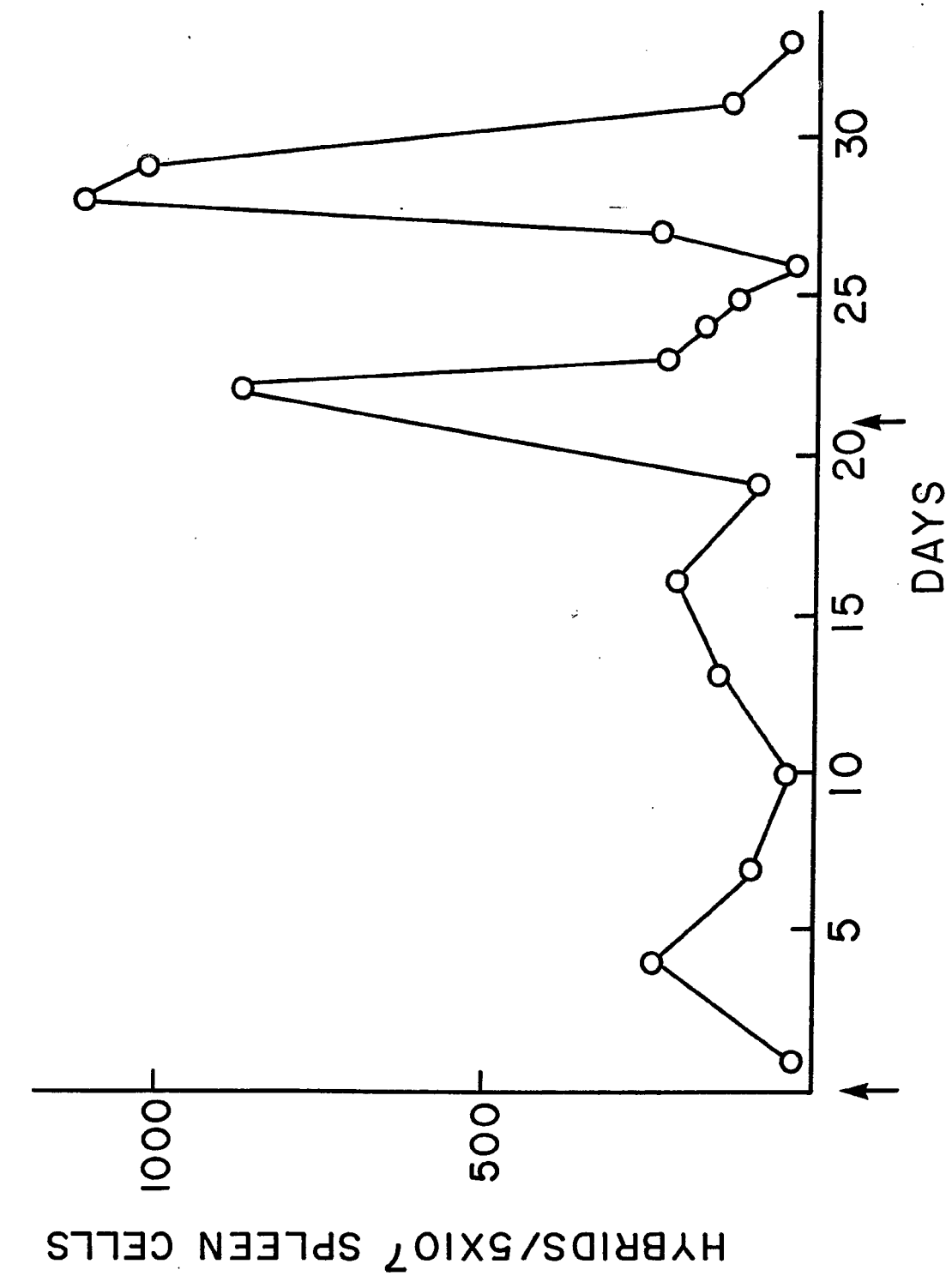

HYBRIDS/5X10⁷ SPLEEN CELLS
1000
500
5
10
15
20
25
30
DAYS

Figure 15. Antibody Production by Hybrids Isolated During a 33-Day Response of Balb/c Mice to SRBC. Supernatants from wells with hybrids present were sampled and assayed by an ELISA to determine the class of antibody being secreted. IgM-secreting hybrids, O---O; IgG-secreting hybrids, ●---●.

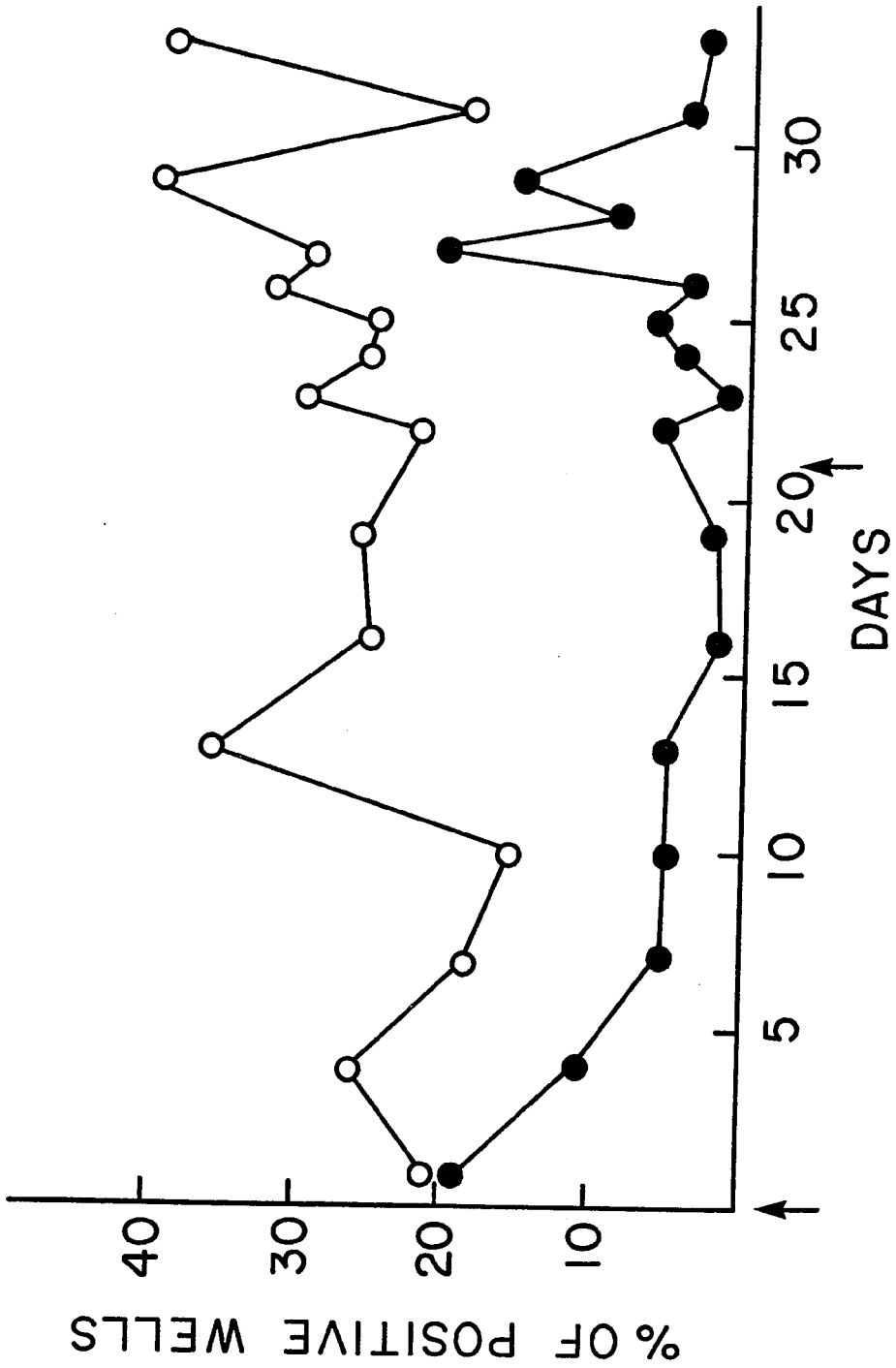
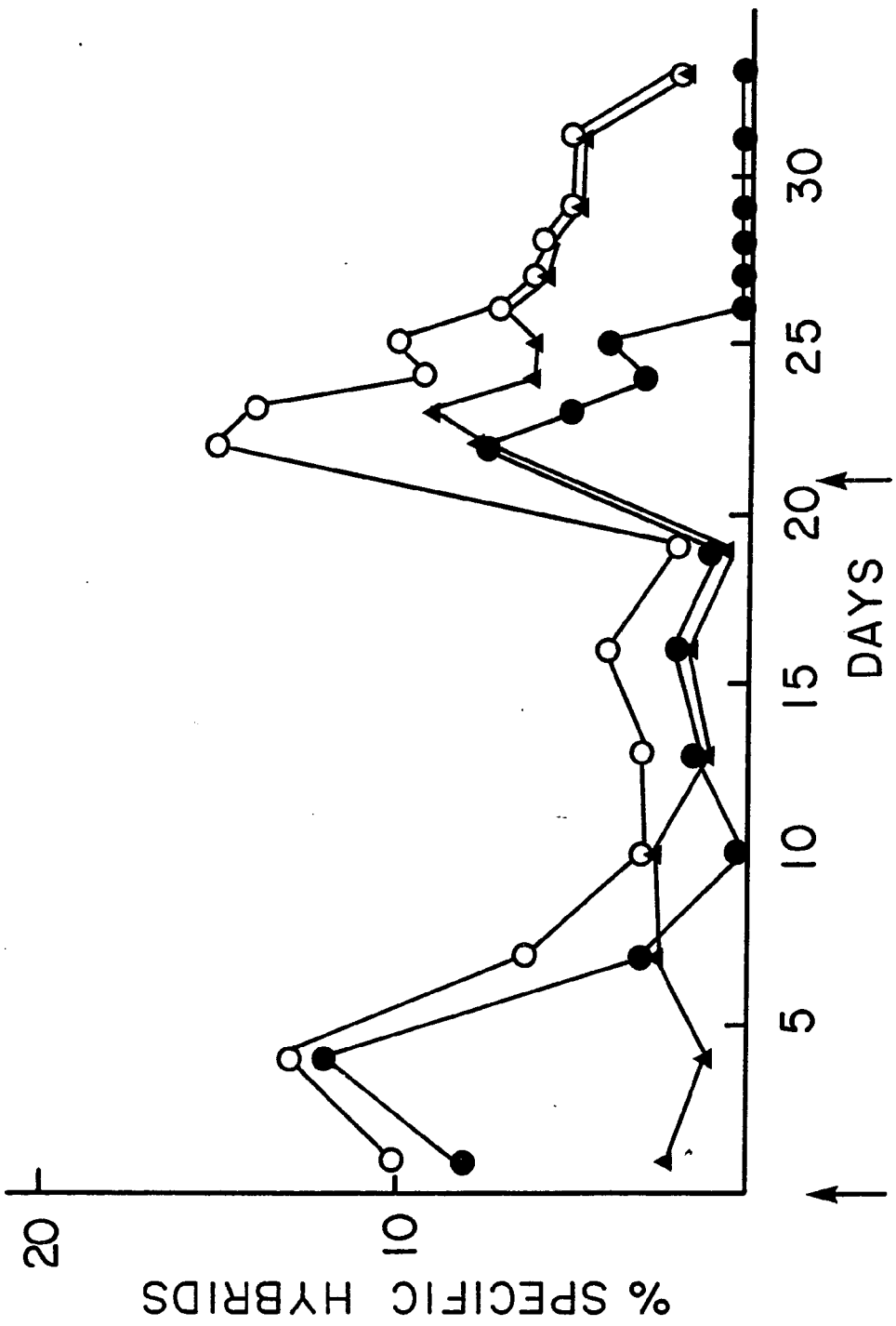

% OF POSITIVE WELLS
40
30
20
10
5
10
15
20
25
30
DAYS

Figure 16. Antigen Specificity of Hybrids Isolated During a 33-Day Response of Balb/c Mice to SRBC. Supernatants from wells with hybrids present were sampled and assayed for anti-SRBC activity by a modified ELISA. Percentage of hybrids specific for SRBC secreting IgM, ●---●; secreting IgG, ▲---▲ ; total, O---O.


% SPECIFIC HYBRIDS
20
10
5
10
15
20
25
30
DAYS

throughout the entire experiment ranging from 16% to 40% of all hybrids with peaks on days 4, 13 and 29. IgG-secreting hybrids were present in the highest percentage on days 1, 4 and 27 through 29.

The largest percentage of hybrids with anti-SRBC specificity was found on days 1 and 4 during the primary response and on days 22 through 25 of the secondary response (Figure 16). Those formed on days 1 and 4 were predominantly IgM secretors while those produced during days 22 to 25 were predominantly IgG secretors.

The results of these experiments indicate that the formation of specific hybrids took place prior to maximum PFC development in both the primary and secondary responses. Hybrids isolated at these times were indicative of the class, IgM or IgG, which was undergoing development. The second peak of hybrid formation occurred during both responses, was unrelated to PFC generation and produced few antigen specific hybrids.

Other experiments suggested that the peak of hybrid formation in the primary response may have occurred prior to day 4. To test this possibility and clarify the relationship between hybrid production and a developing immune response, a second experiment was conducted. The first 13 days of the primary response were repeated, therefore, taking advantage of the IgM and IgG class development and also possibly verifying the existence of the second peak.

Hybrid Formation During the Primary Response of Balb/c Mice to SRBC

Analysis of serum and PFCs

Balb/c mice were injected on day 0 and bled on days 1 through 10. The serum hemagglutination and cytotoxic titers were determined

and found to be very similar to the first 10 days of the previous experiment (Figure 13).

Direct and indirect PFCs were assayed on small aliquots of the spleen cells prepared for use in fusions (Figure 17). The IgM PFC peaked on day 4 and then declined. Its overall magnitude was slightly higher than those values obtained in the earlier experiment. The IgG PFC response peaked on day 7 at 165 PFCs per 10^6 spleen cells and then declined to 41 PFCs per 10^6 spleen cells on day 10. This curve demonstrates a shift of about two to three days when compared to the previous primary response, but the IgM and IgG responses remained separate.

Hybrid formation

Spleen cells from two mice were pooled and used for fusion with P3 on days 1 through 7, 10 and 13 after the primary injection (Figure 18). On day 1, 426 hybrids per $5x10^7$ spleen cells were formed. A slow decrease in hybrid production occurred over the next six days. After day 7 there was a rise in hybrid formation on both days 10 and 13. The large number of hybrids produced on days 1, 2 and 3 in this experiment suggests that peak production was prior to day 4 in the last experiment. The increases on days 10 and 13 may represent the ascending side of the second peak seen in the more extensive previous study (Figure 14).

Antibody production

An ELISA was used to screen for IgM- and IgG-secreting hybrids. The greatest percentage of IgM secretors occurred on day 1 (70%) and thereafter declined to day 10 with a slight increase on day 13.

Figure 17. PFC Response of Balb/c Mice During a 10-Day Study. PFC assays were performed on samples of spleen cells used for fusion. Direct (IgM), 0---0; indirect (IgG), ●---●.

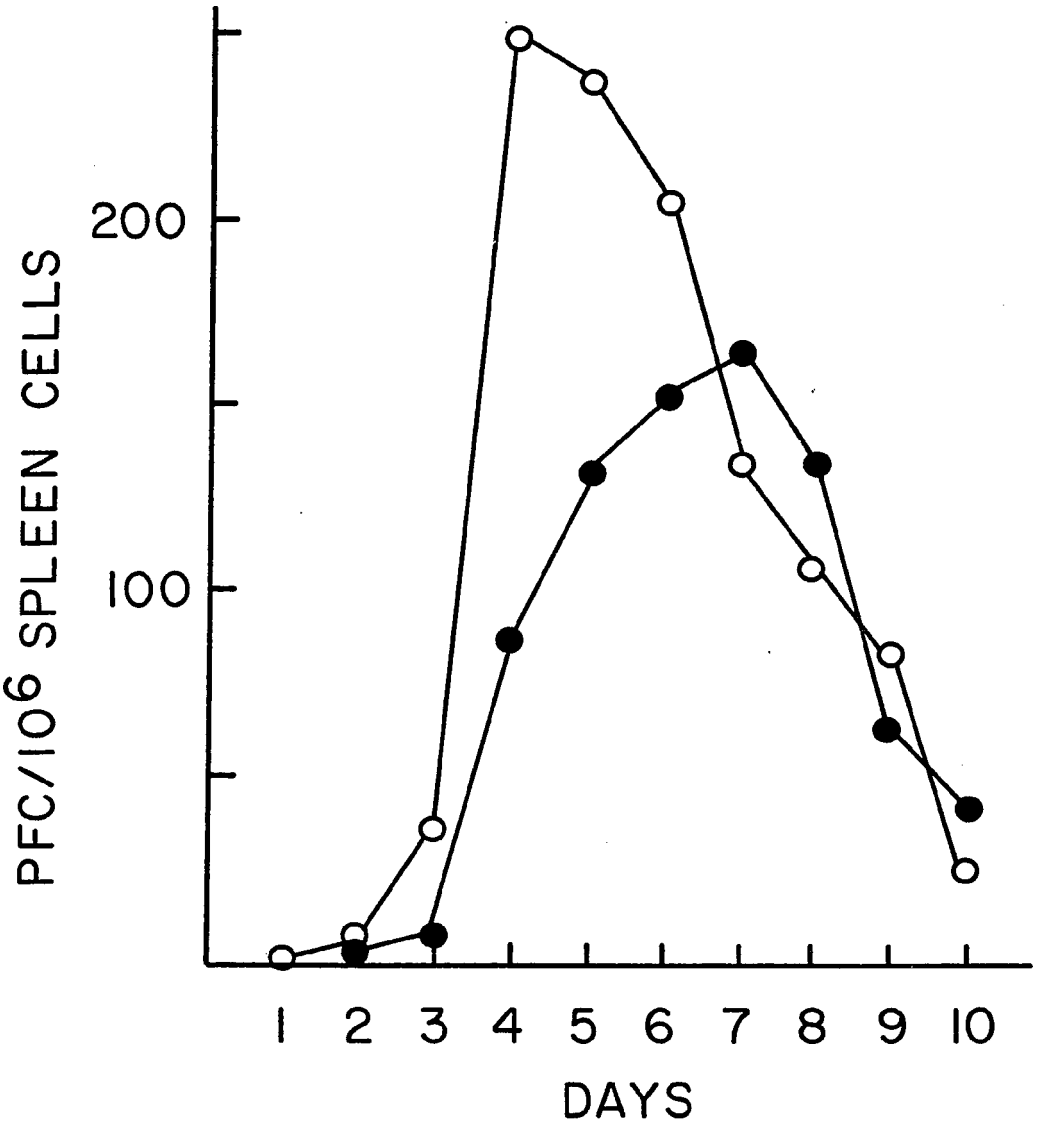
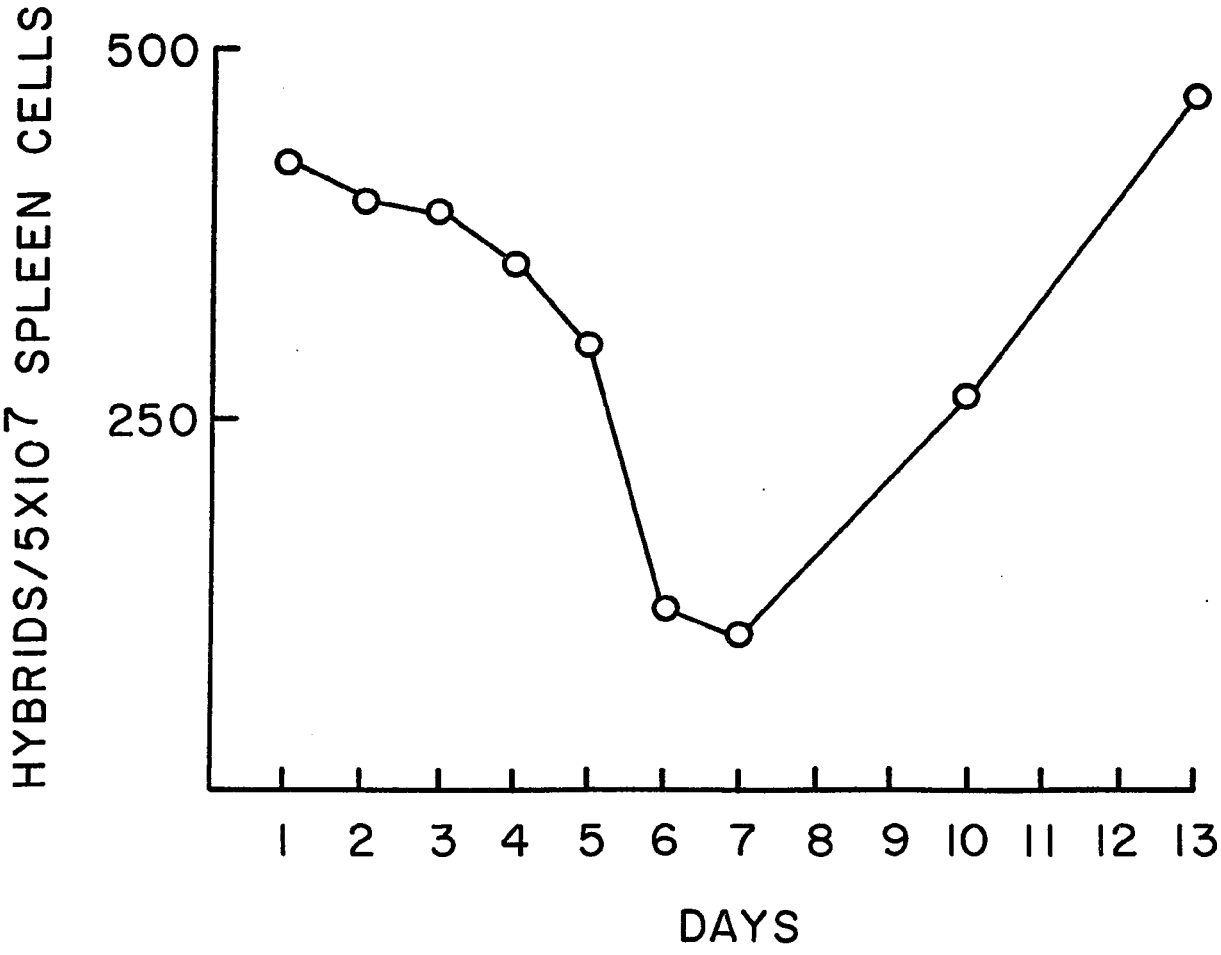

PFC/10⁶ SPLEEN CELLS
200
100
1 2 3 4 5 6 7 8 9 10
DAYS

Figure 18. Hybrid Production by Balb/c Mouse Spleen Cells During a 10-Day Response to SRBC.


HYBRIDS/5X10^7 SPLEEN CELLS
500
250
1 2 3 4 5 6 7 8 9 10 11 12 13
DAYS

IgG-secreting hybrids ranged between 10% and 20% of all hybrids, being most prominent on day 4 (Figure 19).

Antigen-specific hybrids

Figure 20 shows the results of an ELISA assay to determine the number of hybrids secreting antibody specific for SRBC. There is a slow decline in the percentage specific from day 1 to day 4, followed by a more rapid decline through day 7. On days 1 to 3 the majority of SRBC-specific hybrids secrete IgM, while on day 4 and thereafter the majority of specific hybrids are secretors of IgG or some other class. No IgM producers were identified among specific hybrids isolated on days 10 and 13.

Figure 19. Antibody Production by Hybrids Isolated During a 10-Day Response of Balb/c Mice to SRBC. Supernatants from wells with hybrids present were sampled and assayed by an ELISA to determine the class of antibody being secreted. IgM-secreting hybrids, 0---0; IgG-secreting hybrids, ●---●.

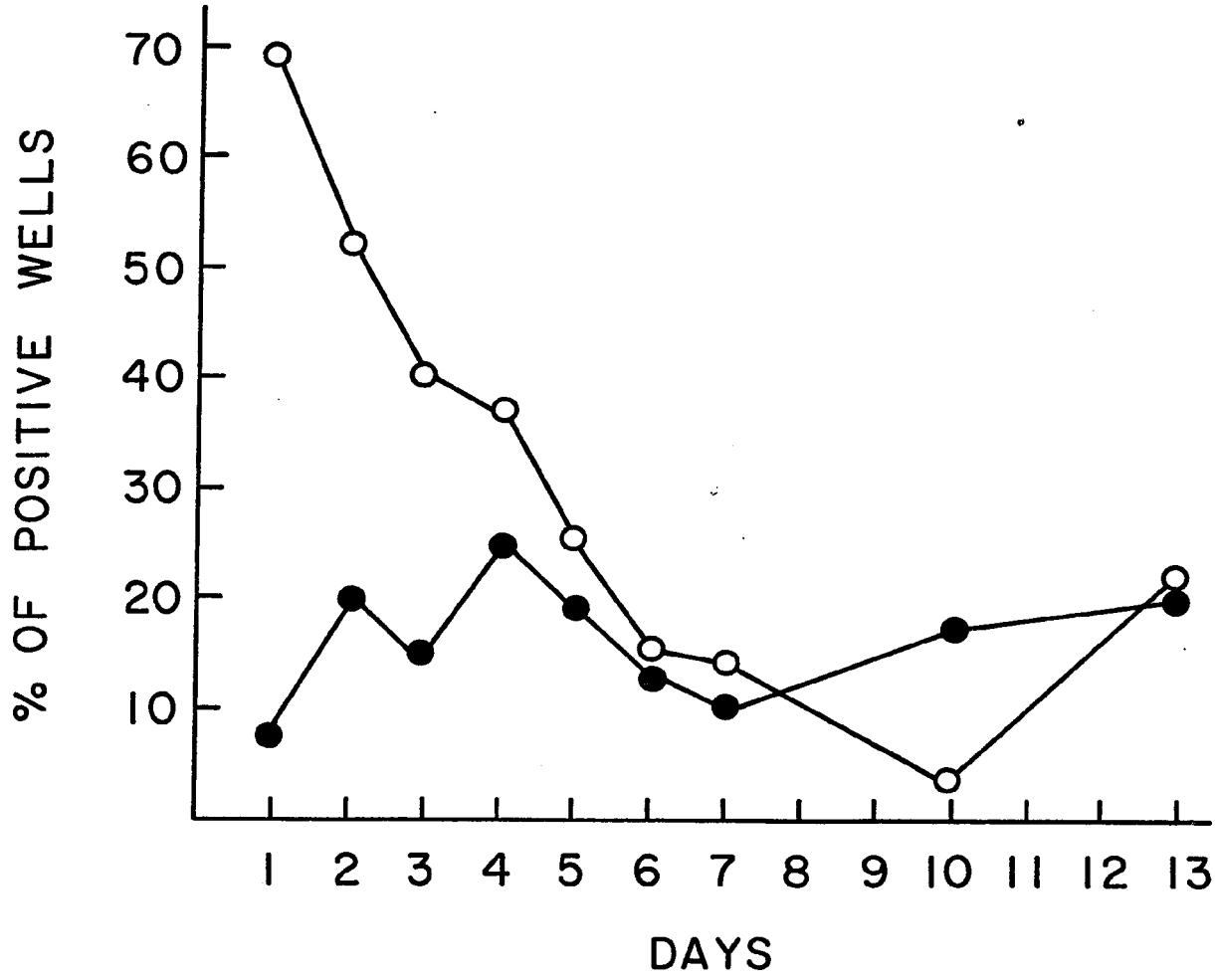
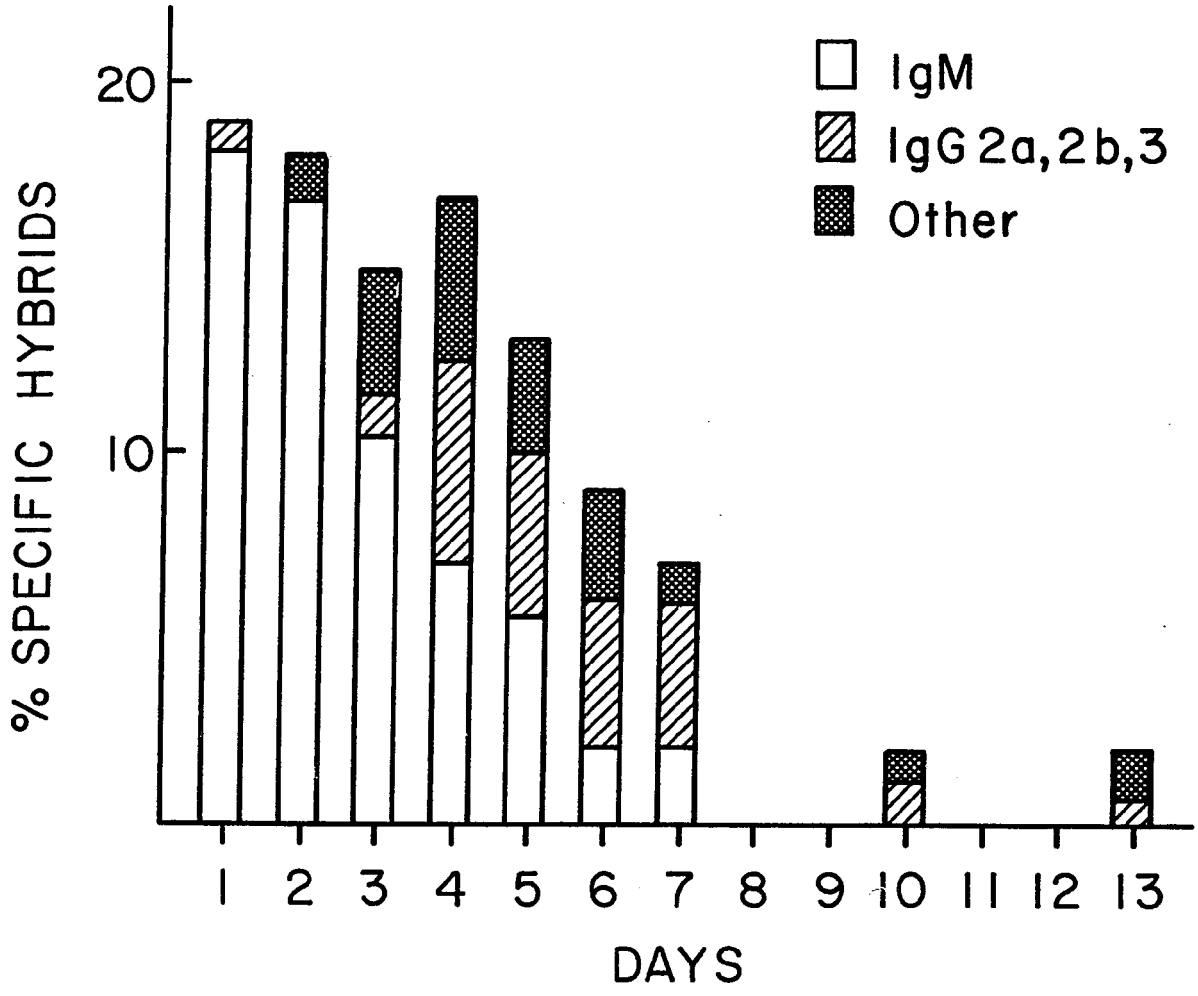

% OF POSITIVE WELLS
70
60
50
40
30
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13
DAYS

Figure 20. Antigen Specificity of Hybrids Isolated During a 10-Day Response of Balb/c Mice to SRBC. Growth positive wells were assayed for anti-SRBC activity by a modified ELISA. Expressed as the percentage of total hybrids.


IgM
IgG 2a, 2b, 3
Other
% SPECIFIC HYBRIDS
20
10
1 2 3 4 5 6 7 8 9 10 11 12 13
DAYS

V. DISCUSSION

The results of the preliminary experiments raised two important points about hybrid production using spleen cells from an immune animal as one parental cell type. First, the presence of a high serum antibody titer specific for the antigen of interest might not be the best indicator of the optimum fusion time. Second, it was demonstrated using various antigens that specific hybrids could be isolated early in a primary response at a time prior to the expected peak PFC expression. These studies suggested that high frequencies of hybrid formation by PEG were not dependent on the presence of antibody-secreting plasma cells. Possible explanations for these observations include the possibilities that serum activity may reflect antibody production (plasma cell activity) in an organ other than the one used for fusion, or that plasma cells were present but were not the cell partner of choice in PEG-induced fusions.

Kohler and Milstein (1975, 1976) noted that some selective mechanism may be operating which preferentially included antigen-specific antibody-forming cells from the total spleen cell population in hybrids. This observation was based on the fact that they isolated more antigen-specific hybrids than the frequency of spleen cell precursors to that antigen would have suggested. With this in mind, a number of investigators used hyperimmune animals and produced hybrids

specific for interesting molecules, IgD (Pearson et al., 1977) and a rat thymocyte differentiation antigen (Williams, Galfre and Milstein, 1977). But it was clear from their immunization schedules and their past research interest that these were not the specific monoclonal hybrids originally desired. Two other laboratories attempting to produce anti-H-2 specific hybrids were successful only after changing their immunization protocol from one of hyperimmunization to one of only two injections followed by fusion 3-5 days later (Lemke et al., 1978; Oi et al., 1978).

Isolation of hybrids producing antigen-specific antibody at a time previous to maximum PFC formation in the primary response has been reported by Claflin and Williams (1978). These investigators suggested that fusion might involve cells at a preplasma cell stage rather than mature plasma cells. Recently, hybrids specific for the Thy-1 molecule have been isolated in fusions after a primary injection (Marshak-Rothstein et al., 1979). These reports support the data on hybrid production during a primary response presented in this work and suggest that a stage of B cell maturation after antigen recognition is particularly susceptible to PEG-induced fusion and that it does not appear to be the plasma cell.

The use of LPS-induced polyclonal activation of cultured spleen cells allowed the investigation of B cell maturation and its role in hybrid formation without some of the difficulties inherent in a heterogeneous in vivo immune response. In studies using LPS-stimulated spleen cells as one of the parental cell types, it was found that the highest frequency of hybrid production coincided with the period of maximum

mitotic activity. In those experiments where peak mitotic activity was at an earlier time than usual, there was also a commensurate shift of maximum hybrid production.

Anderson and Melchers (1978) have also suggested that the blast form of B cells may be the major cell involved in hybrid production. This conclusion was based on comparisons of the fusion efficiency of a small-cell and large-cell pool of normal splenic lymphocytes isolated by gravity sedimentation. The large-cell pool contained cells which synthesized DNA, divided, and secreted IgM, and it was this pool which gave maximum hybrid production. These data are difficult to interpret because in addition to mitotic cells, plasma cells were present.

Two possibilities exist which might explain why mitotic cells are selectively included in PEG-induced fusions. First, it might be that nuclear fusion is the most important event in successful hybridization and mitotic activity increases the chance of nuclear fusions. Second, the interaction at the cell membrane might be the crucial factor and this interaction might be dependent on a certain membrane state more prevalent during mitotic activity.

Some of the highest frequencies of hybrid formation have been reported when two rapidly growing cell culture lines have been used (Gefter, Margulies and Scharff, 1977). Most techniques for fusion with PEG specify that cells should be in log phase growth, indicating the importance of mitotic activity. Hansen and Stadler (1977) reported an increased frequency of hybrid production in PEG-induced fusions of mitotic cells and attributed this to the rearrangement of intrinsic membrane proteins. PEG-induced fusion was decreased in the presence of colcemid, an inhibitor of mitosis and the assembly of microtubules

which may control glycoprotein movement in the lipid bilayer. This suggested a relationship between mitotic membrane configuration and PEG-mediated fusion frequency.

In the LPS studies reported in this work a smaller peak of hybrid formation was present between 12 and 24 hours which was not associated with mitotic activity. A similar situation was reported by Kennett et al. (1978) who used neonatal spleen cells stimulated with LPS to produce hybrids. They reported maximum hybrid production at 6 hours when compared to 1, 24 and 48 hours of stimulation. No 12-hour stimulation data were presented nor was there a comparison of neonatal versus adult spleen cells. Regardless of these differences, there seems to be some stage in early LPS-induced activation which also enhances hybrid formation. It does not appear to be dependent on either mitosis or the presence of plasma cells.

This increase in hybrid production which occurs after a short exposure to LPS might be better understood if we look at another substance which also increases fusion frequency. The chemical DMSO has been reported to enhance the frequency of fusion (Norwood, Zeigler and Martin, 1976). It was suggested that the mode of DMSO action might involve either (1) an alteration of the aqueous environment around the membrane to allow better PEG-cell contact, or (2) an induction of alterations in the cell membrane which not themselves cause fusion but increase fusion by PEG. This implied that DMSO might produce a favorable membrane state for fusion. The interaction of LPS with its putative receptor (Forni and Coutinho, 1978) may also cause similar membrane alterations conducive to fusion. This could account for the early peak

of hybrid formation at 12-24 hours after LPS culture reported in this work and the work of Kennett et al. (1978).

The studies with LPS indicated that the fusion process using PEG may select those cells going through proliferation at a point between antigen recognition and the final plasma cell stage. Therefore, hybrid cell lines established at various times after immunization might reflect the characteristics of the responding cells. In two experiments, one covering both a primary and secondary immunization and the other covering only a primary immunization, the response of Balb/c mice to SRBC was studied with hybrid formation.

In a 33-day study covering a primary and secondary response, two peaks of increased hybrid cell production were present in both the primary and secondary responses. The first peak in both responses appeared prior to maximum anti-SRBC PFC expression, again indicating that it was the proliferative stages of B cell development which were preferentially included in a fusion event. The second peak occurred at a time of few PFCs in the primary response and after maximum PFCs in the secondary response. Antigen-specific hybrids were most prevalent among the hybrids isolated during the first peaks of both responses, but most were IgM producers in the primary while the secondary contained more IgG producers. This was in accordance with the ongoing development of the response in terms of PFCs and serum activity.

The desired analysis of IgM and IgG development was difficult in the first experiment because the activity peaks of these two antibody classes coincided in the secondary response, while the time points were too separated in the primary. In a second experiment, covering 10 days

of a primary response, a good separation of the IgG and IgM response occurred.

The data from the second experiment clearly indicated that maximum hybrid production preceded the maximum PFC response. In fact, minimum hybrid formation occurred at the point of maximum specific PFC expression. The percentage of hybrids specific for SRBC also decreased after the first day. A most revealing fact was seen when comparing the development of IgM PFCs (days 2-4) and IgG PFCs (days 3-7). The ratio of these two classes in SRBC-specific hybrids follows very closely a shift in the number of responding PFCs of each class. Also during days 3 through 6, a large percentage of the specific hybrids were neither IgM, IgG_{2a}, IgG_{2b}, nor IgG_3 and were probably IgG_1 or IgA. Seman, Chevalier, and Stanislawski (1978) reported that the IgG response of Balb/c mice to SRBC was 71% IgG_1 and 30% IgG_2 at day 6 after a primary injection. The data presented in the present investigation indicate that on the day of peak PFC response (day 7) only 20% of the SRBC-specific hybrids were possible IgG_1, but that on days 3 through 6 a number of hybrids were isolated which could have been IgG_1. This should be the case since those cells proliferating on days 3-6 would give rise to the plasma cells present on day 7.

The decrease in the percentage of hybrids identifiable by the ELISA in these studies may be due to the rise in IgG_1- and IgA-producing hybrids. Dresser (1978) has suggested that SRBC acts as a polyclonal stimulator, inducing both a specific response to its own antigens and a subsequent autoantibody response. He found that only about 20% of IgM PFCs produced in the response to SRBC were specific for SRBC and that a

large proportion were anti-immunoglobulin. Although this helps explain the number of non-specific hybrids in the first peaks of the primary and secondary response, it does not explain the second peaks.

These peaks could be due to: (1) a response to new antigens released after damage to SRBC during the first response peak, or (2) an anti-self response associated with idiotypes. If the first possibility is true, no PFC or SRBC-specific hybrid supernatants would be detected in association with the second peak of hybrid formation. This was the case in both experiments presented in this work. The second possibility could also be true based on a report by Cozenza (1976). He described a system in which the antigen-specific response was followed very closely by an anti-idiotypic response which appeared to regulate the specific response, If the same mechanism were operating in this anti-SRBC system, separation of the antigen-specific response peak and the anti-idiotypic response peak might be expected. This could occur because the heterogeneous anti-SRBC response would require a time delay before any one idiotype reached sufficient concentration to elicit an anti-idiotypic response.

To test this possibility, four IgM anti-SRBC positive hybrids were chosen from days 2 and 3 of the primary response. The supernatants of these hybrids were screened against 200 IgG_2 nonspecific hybrids from the second peak in a modified ELISA. None of the 200 hybrids tested were specific for the IgM anti-SRBC antibiodies. Identifying such a relationship, however, might prove difficult for the following reasons. First, as mentioned above, the response to SRBC is polyclonal and very heterogeneous with numerous idiotypes being expressed. Second, the

assay here requires the binding of the IgM to the SRBC, thus blocking some binding sites and restricting any recognition by anti-idiotype to those specific for determinants outside the antigen-combining site.

Claflin and Williams (1978) have also reported that maximum specific hybrid production occurs prior to the peak PFC response. Their observations are consistent with the data presented in this work. These reports differ from Kohler and Milstein's (1976) observation that specific hybrids were isolated on the day of peak PFC expression. This difference could be due to the different agents used to produce hybrids. Kohler and Milstein were using Sendai virus at the time they performed their experiments. Sendai virus might operate by attachment to molecules on the cell surface and holding the cells in close proximity to allow membrane fusion. The maximum number of specific hybrids would be produced when the largest number of antigen-specific cells was available, such as PFC. PEG, as the present studies indicate, is more dependent on mitotic cells and induces higher numbers of hybrids when the spleen cells are in a preplasma stage.

The present report is the first in which an immune response has been studied by forming hybrids at numerous times in both the primary and secondary responses. Two other laboratories (Reth, Hammerling and Rajewsky, 1978; Ju et al., 1979) have used hybrid formation to investigate idiotype expression. They both found disparities between sera and hybridoma idiotypes. A possible explanation is that during these experiments fusions were done at only a few time points during the primary and/or secondary responses. They probably isolated only a small fraction of the total cells responding, which may not be reflective of

the complete response. This fact is well documented in the work presented in this thesis. Another report (Trucco, Stocker and Ceppellini, 1978) stated that hybrids produced four days after a primary injection were all IgG producers. This puzzled these investigators because on that day IgM expression is usually at a maximum. If their antigen is similar to SRBC, then the data in the present work would lead to the prediction that on day 4 the IgG response should be commencing and a number of IgG^+ hybrids should be isolated.

In summary, the results of both the *in vitro* and *in vivo* experiments presented in this paper suggest that cell hybrid formation preferentially involves proliferating cells at some stage of development following antigen stimulation but before the plasma cell stage. Hybrid formation during an immune response indicated that hybrids secreting antibody of a particular immunoglobulin class could be isolated if the time of development of cells producing the various antibody classes in response to an antigen were known. The knowledge gained from these studies not only improves the probability of recovering hybrid cell lines secreting antibody specific for a desired antigen, but also of choosing the class of antibody and its relative affinity. The data presented on factors which increase cell hybrid formation may enhance attempts in other areas of biology to establish continuous cell lines with differentiated functions.

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