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STUDIES TOWARDS GENERATION OF CELL LINES AS FUSION PARTNERS
FOR BOVINE MONOCLONAL ANTIBODY PRODUCTION

JOSEPH OLOBO-OKAO

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A THESIS SUBMITTED IN FULFILLMENT FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)
IN THE DEPARTMENT OF ZOOLOGY,
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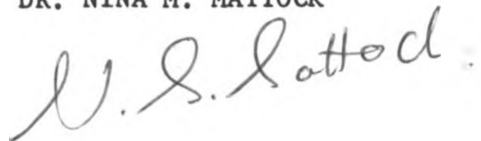
JOSEPH OLOBO-OKAO



This Thesis has been submitted for examination with our
approval as Supervisors.

University Supervisor:

DR. NINA M. MATTOCK



ILRAD Supervisor:

DR. SAMUEL J. BLACK

International Laboratory
for Research on Animal
Diseases)



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ABSTRACT

For the long term production of mouse monoclonal antibodies, normal activated mouse B cells en route to immunoglobulin (Ig) synthesis and secretion are fused to transformed mouse B-cell lines which do not make the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT), and hence do not have a nucleotide salvage pathway for DNA biosynthesis. Fused cells are selected in medium containing hypoxanthine, aminopterin and thymidine (HAT). Unfused normal B-cells die after a few days in culture. Transformed cells lacking the enzyme HGPRT die in the presence of aminopterin, a drug which blocks the de novo biosynthesis of purines and pyrimidines. Fused cells multiply because they possess the enzyme HGPRT re-introduced to the transformed cells with genes from normal cells, and hence can utilise the hypoxanthine and thymidine. Some of the fused cells also continue to produce the specific immunoglobulin to which the normal B-cells were committed.

In this study, the development of a fusion partner that could support bovine monoclonal antibody production was approached in two ways: The first approach involved mutation and selection of bovine leukaemia cell lines BL3 or BL20 to directly obtain aminopterin sensitive cell lines. The second

approach involved fusing the existing HAT-sensitive mouse myeloma cell lines; NS1, x63 or Sp2/0 to Pokeweed mitogen (PWM) activated bovine peripheral blood mononuclear cells (PBM). The resultant rare mouse x bovine hybrid cells were selected for resistance to 8-azaguanine (8AG), HAT sensitivity and expression of many bovine markers. It was hoped that mouse x bovine heterohybridoma fusion partners would have an enhanced ability to retain bovine chromosomes, particularly those with Ig genes, after fusion to activated bovine B-cells.

Studies designed to generate appropriately activated bovine B cells for fusion, showed that culture of 2.5×10^6 bovine PBM/ml of medium containing 2.5 ug/ml of PWM resulted in maximum proliferation and maximum generation of antibody producing cells. The level of response was different in different bovines but the time of peak proliferation was consistent (day four). The same culture conditions permitted B cells in PBM to synthesize IgM and to a lesser extent IgG. Cells with cytoplasmic Ig (cIg) molecules started to appear on day three of culture until day eight (end of culture). Antigen (SRBC) activated bovine lymphocytes producing direct plaques to SRBC could also be obtained by in vitro culture of 2.5×10^6 PBM/ml with 2.5×10^6 SRBC/ml and 0.156-0.625 ug/ml of PWM, or co-culture of 2.5×10^6 SRBC/ml with 2.5×10^6 PBM/ml or spleen cells from animals previously primed with the same antigen (SRBC).

PWM stimulated bovine PBM, biosynthetically labelled in vitro with L(³⁵S)-methionine, showed a linear incorporation of the radioisotope over a four and half hour period. Examination of the culture supernatants by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and autoradiography, revealed bands of radiolabelled IgM and IgG heavy chains (MW 75K and 55K, respectively), bands of light chains (MW 25K), and suspected J-chain (MW 14K). Other secretory molecules (not identified) were also noticed starting on day two or three until day eight of culture.

Bovine leukaemia cell lines BL3 or BL20 were examined for expression of characteristics of potential fusion partners. BL3 cells had aneuploid and an unstable number of chromosomes and stained with few bovine cell markers so it was not studied further. BL20 cells expressed surface bovine IgM and were selected for resistance to 40 ug/ml of 8AG. After this selective procedure, some cells were not killed by HAT treatment. Further selection of the same cells in 6TG containing medium (20 ug/ml) produced a line of cells, designated ATS/BL20, which were completely killed by culture in HAT containing medium in about six days.

ATS/BL20 cells could only clone at 100% efficiency on bovine thymus fibroblast-like feeder cells. Bovine PBM, mouse thymocytes, Con A derived T-cell growth factor, or PWM derived

culture supernatant, were unable to promote the cloning efficiency. With continued selection, a line of ATS/BL20 cells was generated which cloned at a frequency of about 20% without the need of feeder cells or growth factor containing supernatants.

Fusion of ATS/BL20 cells to PWM or antigen activated bovine PBM or in vivo derived spleen cells produced hybrid cells which showed no noticeable proliferation until after about four to eight weeks in culture and which were initially dependent on bovine thymus fibroblast-like feeder cells. The frequency of generation of hybrids was low in each case. On examination, some hybrid cells were positive for cytoplasmic IgM. After cloning on bovine thymus fibroblast-like cells, only those wells with 5, 10 and 20 hybrid cells/well exhibited growth at 1%, 47% and 100% respectively. Further staining revealed no cIgM positive cells indicating that the gene(s) coding for the Ig molecules had been lost or inactivated during the prolonged culture. Analysis of the hybrid cells revealed some which expressed a histocompatibility antigen detected by MAb IL-A7, normally present on donor animal lymphocytes, but absent on ATS/BL20 cells. The modal chromosome number of the hybrid cells was between 56 to 60, with few cases where some cells had about 80 chromosomes. The latter observations were taken as evidence that the growing cells were hybrids.

The initial inhibition of ATS/BL20 x bovine hybrid cell growth, and the eventual growth of cells of probable hybrid origin, but with a normal diploid or near diploid number of chromosomes, was thought to possibly relate to suppression and escape from suppression of tumorigenicity.

PWM activated bovine PBM (day 6 of culture), were fused with mouse myeloma cells X63, NS1 or Sp2/0, using polyethylene glycol (PEG). A few growing X63 x bovine and NS1 x bovine hybrid cells appeared approximately two weeks after fusion. The putative hybrids had a slow initial multiplication time until the seventh week, when NS1 x bovine hybrids started to multiply vigorously. But X63 x bovine hybrid cells were all dead by nine weeks after fusion. Fusion of PWM activated cells to Sp2/0 produced visible hybrids within two weeks, which grew to confluency without any apparent crisis. The efficiency of generating hybrids in these systems was very low.

About 10% of the hybrid cells (NS1 x bovine), expressed bovine cIgM in an indirect immunofluorescence assay. Fluorescence Activated Cell Separator analysis (FACS) revealed that about 10% and 19% of NS1 x bovine and Sp2/0 x bovine hybrid cells respectively expressed surface bovine IgM, as detected by monoclonal antibody (MAb) B5/4, and about eight percent of NS1 x bovine cells expressed a bovine T cell marker

identified by MAb P5. About 20% and 80% of NS1 x bovine hybrid cells stained with the bovine histocompatibility markers IL-A3 and IL-A7 respectively. Karyotype analysis revealed a modal chromosome number of between 62 to 68 and 72 to 110 in hybrids between NS1 x bovine and Sp2/0 x bovine respectively. These latter findings were taken as evidence of hybrid formation between the respective fusion systems.

A proportion of the NS1 x bovine and Sp2/0 x bovine heterohybridoma cells had spontaneous resistance to 8AG. The same cells were killed in HAT medium treatment in about six days, making them potential fusion partners. Surface and cytoplasmic bovine Ig negative and surface bovine Ig (IgM) positive and cytoplasmic bovine Ig negative NS1 x bovine heterohybrid clones were isolated, and fused back to PBM. Putative growing NS1 x bovine x bovine hybridomas appeared on about day nine after fusion, but all the cells were dead by day 15 in three separate fusion experiments where PBM were obtained from PWM culture, implying that the heterohybridomas were unstable.

Comparison of cloning, viability and fusion frequency of parental NS1 or Sp2/0 (primary fusion) versus NS1 x bovine, or Sp2/0 x bovine (secondary fusions) fusion partners, using in vitro antigen activated PBM as donor lymphocytes, gave inconsistent results. The number of hybrid colonies obtained

after each fusion experiment was highly variable, although no growth crisis was experienced, indicating that the in vitro antigen activation system was also unreliable as a source of donor lymphocytes for fusion. This discrepancy is discussed.

The application of mouse NS1, X63 or Sp2/0 or mouse x bovine heterohybridoma cell lines or ATS/BL20 cells as fusion partners for production of bovine monoclonal antibody is discussed in the light of these findings.

GENERAL INTRODUCTION AND LITERATURE REVIEW

1. B Lymphocytes and Antibodies

This thesis concerns studies towards generating cell lines which could be used as fusion partners to produce bovine monoclonal antibodies. To understand the unique properties of monoclonal antibodies and the processes by which they can be generated it is useful to know how antibodies are made by B lymphocytes, and in particular, how the genetic information encoding antibody molecules is stored within the cells.

Antibodies are produced by mature B cells in response to an appropriate stimulus (Section 1.7). Each mature B-cell has on its surface an immunoglobulin (Ig) molecule (an antibody; Fig. 1) which can react with a small component of a single or a limited range of antigenic molecules (Sections 1.2 and 1.3). A schematic diagram of an antibody molecule is presented in Fig. 1, and is discussed in detail in Section 1.2. Cell bound or secreted antibody reacts with an antigenic determinant using a binding site which is created by association of the N terminal amino acids of heavy (H) and light (L) chains (Section 1.2). Secreted antibody can also react with cells or plasma molecules by using components of the constant regions (C) formed by the combined H chains (Section 1.2).

The progeny of an activated B-cell produces antibody with the same antigenic specificity as the original stimulated cell, but other characteristics of the Ig molecule produced by the clonal progeny may change (Section 1.6). The change, "class switch", is detected in parts of the molecules which are not responsible for antigen recognition (constant regions) and which confer other useful biological properties to the molecule. The changes result in the production of antibodies which fall into definable classes. The nomenclature and characteristics of different classes of Ig molecules in bovine are given in Table 1. The mechanisms by which B-cell populations arise, with the capacity to recognize large numbers of different antigenic determinants and to produce different classes of Igs, are introduced below (Sections 1-6), as are methods to isolate and culture B-cells producing Igs of predefined specificity (Section 1.10).

TABLE 1: Nomenclature and Characteristics of Different Immunoglobulin
(Ig) Classes in the Bovine

Ig Class	IgM	IgG1	IgG2	IgE	IgA
Symbol for H-Chain	μ	$\gamma 1$	$\gamma 2$	ϵ	α
Molecular Weight of H-Chain	75-78,000	55-60,000	55-60,000		60,000
of L-chain	22,500	22,500	22,500		22,500
Whole Molecule	900,000	146-163,000	146-150,000		385-430,000
<u>Biological Activities</u>					
Complement activation					
- bovine complement	+	+	+		
- guinea pig complement	+	+	-		
Homologous passive cutaneous anaphylaxis (bovine skin)		+	+		
Heterologous passive cutaneous anaphylaxis (rat skin)		+	-		
Macrophage binding		+	+		

Symbols used: (-) negative reaction; (+) positive reaction;
blank space, not tested.

1.1 Differentiation of B Lymphocytes

Genes encoding antibody molecules exist in a complete form only in mature B-lymphocytes. In the germ cells and in somatic cells other than mature B-lymphocytes the genes are not organised in a functional manner. In these cells the genetic information encoding the variable region (V) components of the H and L chains of antibody molecules is not associated with the genetic information encoding the constant components (C) of the H and L chains. Organisation of the functional genes encoding antibody molecules occurs during maturation of B-lymphocytes and results from rearrangements of the V and C region genes both for H-chains and L-chains of Ig molecules.

During ontogeny of the B-cell system, the first recognisable cells of the B-cell lineage contain only cytoplasmic μ heavy chains (H) with an associated variable (V) region gene product. These pre-B cells are found in the fetal liver (Raff et al., 1976; Burrows et al., 1979; Maki et al., 1981; Landreth et al., 1981) and are presumed eventually to rearrange and then to express light chain (L) genes together with the heavy-chain (H) genes which they are already producing (Burrows et al., 1979; Riley et al., 1981). Pre-B-cells are derived from proliferating, self-renewing progenitor cells (cytoplasmic μ^- , surface μ^-), and there are only one or two further divisions, before the small pre-B-cells undergo non-mitotic maturation to small B-cells (cytoplasmic μ^+ ,

5

surface IgM+) (Vitetta and Uhr, 1975). Although these B-cell maturational events are first detected in fetal liver, the same process of B-cell differentiation later becomes associated with the progeny of pluripotential stem cells, which are located in the bone marrow of the adult (Niewenhuis, 1981).

The immature B cells mature via a stage during which IgM only is expressed, and during which the cells are subject to elimination by clonal abortion (Black et al., 1978; Jemmerson et al., 1982; Nossal and Pike, 1975; Nossal, 1983), to a stage that co-expresses IgM and IgD on the surface (Goding, 1978). Mature B lymphocytes are exported from the bone marrow into the circulation, and ultimately find their way to precise locations within lymphoid organs. B-cells in the follicular areas of lymph nodes and white pulp of the spleen reside in close micro-anatomical proximity to antigen-presenting cells of macrophage lineage and various regulatory T cells (Poppema et al., 1981). These processes of B-cell development are thought to be genetically programmed and not antigen driven. The soluble mediators and surface receptors which direct the homing of B lymphocytes to various areas of lymphoid tissues are not well understood at the present time (Filipovich and Kersey, 1983).

1.2 Immunoglobulin Structure and Antibody Diversity

Antibodies are synthesized and secreted by plasma cells which are derived from activated mature B-cells. An

antibody or immunoglobulin molecule is a glycoprotein which recognises and binds an antigen. The basic unit of an antibody molecule is composed of two identical light (L) chains and two identical heavy (H) chains, held together by non-covalent forces and disulphide bonds (Wu and Kabat, 1970; Fig. 1). Studies involved in the comparison of a number of H-chains of the same class from one or more individuals indicated that they were essentially identical in some amino acid sequences in the constant or C_H region, but that they differed extensively in amino acid sequences in the variable or V_H region (Kabat et al., 1979; Fig. 1). Similar results were obtained when isolated light chains were examined i.e., C_L and V_L regions were recognised.

The V_H and V_L regions of an Ig molecule associate in pairs to form the antigen-binding sites (Wu and Kabat, 1970; Davies et al., 1975; Tonegawa, 1983; Gottlieb, 1980). The C_L and C_H domains join by disulphide bridges to associate heavy and light chains (Koshland, 1975). The C_H regions of the H-chains associate to render the Ig molecule bivalent with respect to antigen-binding sites and to form structures which mediate effector functions such as complement fixation and histamine release from mast cells (Fig. 1). An immunoglobulin molecule has therefore a dual function of antigen recognition and secondary direction of molecules.

One antibody can generally bind only one antigenic determinant. Most antigens are complex and express a number of

Figure 1. A Schematic Diagram of an Antibody Molecule at the B-cell Surface

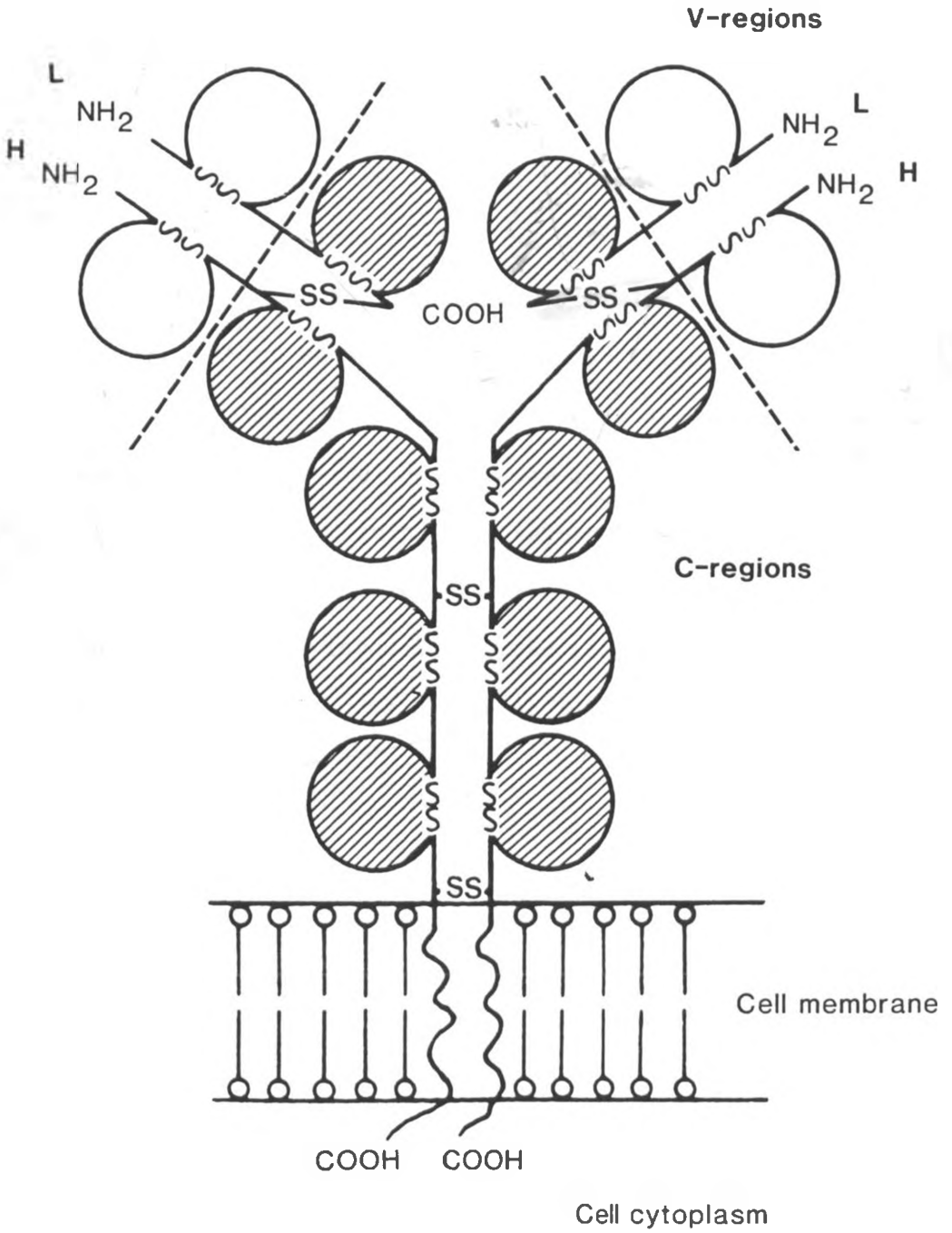


FIGURE 1. A Schematic Diagram of an Antibody Molecule at the B-cell Surface

The diagram consists of two identical heavy (H) and two identical light (L) chains held together by disulphide bonds (-SS- symbols between the chains). There are two identical antigen combining sites, each formed from the N-terminal parts of one H and one L chain. The H and L chains can be divided into domains which show sequence homology (illustrated by circles) including a conserved intra-chain disulphide bond ($\frac{S}{S}$ symbols). The N-terminal domains are called V domains (or regions) because their amino acid sequence varies to give different antibody combining specificities. The other domains are called C domains (shaded) because their sequence is constant for any one L-chain type or H-chain class.

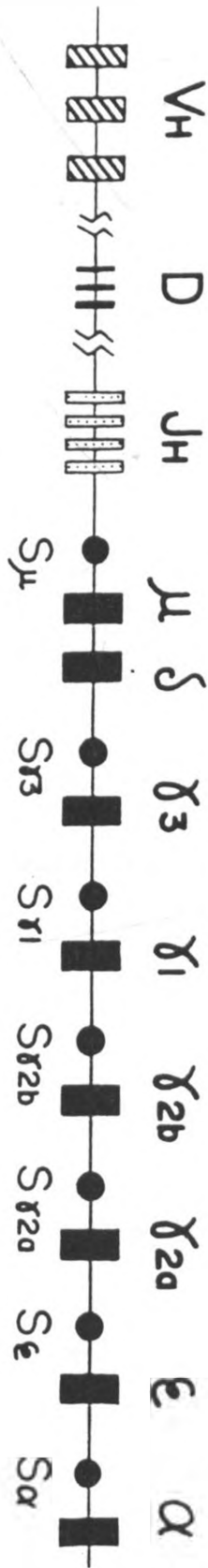
antigenic determinants. B-cell responses stimulated by such antigens are also complex and usually result in the production of a number of antibodies of different classes and specificities (Makela and Cross, 1970). The development of antibodies with different antigenic specificities results from the expression of different V_H and V_L genes in combination with C_H and C_L genes in the population of B cells which secretes the antibodies.

1.3 Immunoglobulin Variable (V) Region Diversity

As discussed above, the immunoglobulin variable region is generated from a combination of Ig V_H and Ig V_L gene products. There are a large number of V region segments in the germ line. Any of these gene segments present on the same chromosome as the C_H region genes can join to a C_H and any of those present on the same chromosome as the C_L region genes can join to a C_L . V_H segments do not associate with C_L , and V_L segments do not associate with C_H , because the gene segments are arranged in three families on three different chromosomes.

A functional Ig L chain V region gene is composed of the V_L joined to J_L (joining segment), which is located close to the C_L gene. Flexibility at the recombination site of V_L and J_L augments the diversity of V_L gene products (Max et al., 1979; Sakano et al., 1979).

Figure 2. Immunoglobulin H-Chain Gene Locus in the Mouse.



In contrast a functional Ig V_H region gene is created by fusion of the V_H to J_H via the D(diversity) segment. The recombinational joining of V_H , (D), and J_H segments has three important characteristics (Fig. 2). First, the combination between different DNA segments seems to be random because the same V_H segments are able to associate with all the J_H segments, as for example in the anti-phosphorylcholine antibody H chain of mouse (Gearhart *et al.*, 1981). Secondly, the recombination reaction has flexibility, i.e. alternative sites for the in-frame recombination at the joining nucleotide. Thirdly, the recombination is accompanied by deletion (Honjo and Kataoka, 1978). Flexibility of the recombination site of V_H and D as well as D and J_H augments the diversity of products which can be generated using a single V_H gene (Early *et al.*, 1980; Kurosawa and Tonegawa, 1982).

1.4 Immunoglobulin Constant (C) Region

In mice and humans there are two separate classes of C_L region genes i.e., C_k and $C\lambda$. These genes are present on different chromosomes and have different J_L segments. Only a single copy of the C_k gene is represented in the mouse and human genomes (Hieter *et al.*, 1982). The distance between the V_k segments and C_k gene is unknown. The $C\lambda$ locus is much more complex than the C_k locus because there are four

C λ genes in the mouse genome. C λ 3 and C λ 1 genes are linked, as are also the C λ 2 and C λ 4 genes (Blomberg and Tonegawa, 1982; Miller et al., 1982). Multiple C λ genes are also found in the human genome (Hieter et al., 1982). Six human C λ genes were physically limited within a 30-kb region. Each human C λ gene is also supposed to have its own J λ segment as in the mouse.

The C_H genes in the mouse are present on a different chromosome to C λ or C_k genes. The C_H gene locus consists of eight genes (μ , δ , γ 3, γ 1, γ 2b, γ 2 α , ϵ and α) and these cluster in a 200-kb region (Shimizu et al., 1981; Honjo and Kataoka, 1978, Fig. 2). Unlike the C_L gene, the C_H genes share one set of J_H segments (Shimizu et al., 1981). The human C_H gene family consists of at least nine genes (μ , δ , γ 1, γ 2, γ 3, γ 4, ϵ , α 1 and α 2). The general organisation of the human C_H gene cluster is similar to that in the mouse, in that the C δ gene is located several kilobases 3' to the C μ gene, and that at least some C γ genes are linked (Ellison and Hood, 1982; Krawinkel and Rabbits, 1982; Rabbits et al., 1981; Takahashi et al., 1982).

1.5 Allelic Exclusion

Limited polymorphism of the Ig constant region genes has been identified and antibodies have been produced which detected polymorphic epitopes on other antibody molecules.

These are referred to as anti-allotype antibodies and have proven very useful in examining the regulation of Ig gene expression. Progeny of parents of different Ig allotypes express both allotypes but never on the same Ig molecule. Furthermore, each mature B-cell present in the progeny expresses either one or other allotype but not both. This is proof that B-cell maturation is associated with the selective expression of Ig genes present on only one of the chromosome pair. The exclusive expression of genes (heavy or light chain) from only one of each chromosome pair is known as "allelic exclusion" (Early and Hood, 1981; Perry et al., 1980). This phenomenon ensures that a single B cell expresses an Ig molecule of only one specificity and not two. In addition to allelic exclusion, functional light (L) chain expression in normal B cells, B-cell tumours or B-cell lines is subject to "isotypic exclusion", so that a single B cell generally expresses either κ or λ L-chains but not both. Rare cloned murine B-cell lines simultaneously expressed κ and λ chains, but it remains to be determined if both kinds of L chains are functional (Alt et al., 1982). A large number of studies from several laboratories have provided molecular descriptions of allelic exclusion for both H- and L-chains as well as L-chain isotypic exclusion (Alt et al., 1980; Korsmeyer et al., 1981; Nottenburg and Weissman, 1981). Analysis of L-chain genes in B cells representing different stages of development have shown that: (1) In about two-thirds of normal or transformed

B-cells producing κ L-chains, one κ allele is functionally rearranged i.e., has a V_L gene in association with a C_L gene, and one allele remains in a germ line state i.e., with V_L and C_L genes separated. (2) In approximately one-third of normal or transformed B cells, one κ allele is functionally rearranged and one allele aberrantly rearranged or deleted. (3) With only a single exception so far, all κ alleles are deleted or aberrantly rearranged in normal or transformed B cells that express functional λ L-chains but not functional κ L-chains. (4) In all B cells that express a functional κ L-chain, λ alleles remain in a germ line configuration. (5) The unexpressed λ alleles are generally aberrantly rearranged in B cells that express a functional λ L-chain (Coleclough *et al.*, 1981; Alt *et al.*, 1980).

Although little is as yet known about allelic exclusion of H-chain expression, some differences and similarities to L-chains are apparent: (1) In over 90% of normal or transformed B cells representing a range of B-cell developmental stages expressing H-chains, both H-chain alleles are rearranged (presumably one functionally and the other aberrantly). (2) In over 90% of virus transformed (Abelson virus) null lymphoid cell lines, all detectable H-chain alleles are rearranged aberrantly or deleted, even though no functional H-chain is expressed. (3) L-chain genes are never present in a rearranged form unless at least one H-chain gene is rearranged.

The following proposals have therefore been made for the regulation of functional Ig gene expression. First, H-chain genes are rearranged at an earlier stage of B-cell development than are L-chain genes (Brown et al., 1981). Second, formation of functional H-chain genes is much less efficient than is formation of functional L-chain genes (Alt et al., 1982). Third, functional H-chain gene formation seems to be necessary before further B-cell development and L-chain rearrangement can occur (Ponte et al., 1981). Fourth, allelic exclusion of H-chain may be an active process in which expression of a functional H-chain prevents additional H-chain V gene-rearrangements (Wallach et al., 1982).

1.6 Immunoglobulin "Class Switching"

After an appropriate stimulation, a B cell produces antibody of defined specificity. The antigen binding properties of the antibody does not change, but the class of the Ig molecule may change. All experimental data are consistent with the concept that development of Ig class diversity is an intraclonal-process that begins with expression of IgM by an immature B lymphocyte and leads to the generation of a family of plasma cells and memory B cells expressing the same V_L and V_H regions in conjunction with each of the different heavy chain classes. This phenomenon is known as "H-chain class switch" (Kataoka et al., 1980; Honjo et al.,

1981), and is of considerable significance due to differences in the biological activities associated with different Ig classes e.g., complement fixation (McNaught et al., 1977), Fc receptor binding on mast cells (Liew, 1982), macrophages (Koski and Kiesel, 1979) and cytotoxic cells (Rouse, 1981), and transport to mucosal surfaces or milk (Butler, 1983).

During differentiation of a single B cell, a given V_H gene is first expressed in combination with the C_μ gene of the same allelic chromosome, and later in the lineage of the B lymphocytes, the same V_H gene is expressed in combination with a different C_H gene. The H-chain class switch results from an event called the S-S recombination. The S region was originally defined as being functionally responsible for the class switch (Kataoka et al., 1980). Nucleotide sequence determination of the region surrounding the S-S recombination site showed that the structural basis of the S region is the tandem repetition of related nucleotide sequences and these S regions are known to be generally conserved in both humans and mice (Nishida et al., 1982; Ravetch et al., 1980). The S-S recombination takes place between two S regions located at the 5' side of each C_H gene. The initial S-S recombination always involves the S_μ region as one of the pair, and the S region of another C_H gene class is involved as the other partner, resulting in the switch of the expressed C_H gene from C_μ to another C_H gene, without alteration of the V region sequence (Fig. 2). The subsequent switch, such as one

from C δ to C ϵ or C α , requires another S-S recombination.

Both types of rearrangements are accompanied by deletion of the intervening DNA segment from the chromosome (Honjo, 1983).

1.7 Immune Response to Antigen

When an animal is infected either naturally or by experimental injection, with a bacterium, virus, parasite, or any other foreign material (i.e., an antigen), the animal's immune system recognises this as an invader, and acts in such a way as to remove the antigen. The host's specific immunological resistance to such an invading body can be divided into; cell mediated immunity (CMI), and humoral responses. Cell mediated responses do not involve secretion of specific antibody, and the effector cells are T lymphocytes. Humoral responses on the other hand involve B lymphocytes with functionally rearranged H and L chain genes as the effector cells and antibody synthesis and secretion occurs. Mature B lymphocytes generally require interaction with T lymphocytes and macrophages in order to proliferate following antigen stimulation, and to generate antibodies (Mosier, 1967).

Antigens that trigger humoral responses have been divided into two groups, on the basis of the additional help they need to drive B cells into optimal antibody production. The "thymus-dependent" (TD) antigens require specific T lymphocytes, as well as non-antigen specific non-lymphocytic

accessory cells, and soluble factors, to help them induce specific antibody production in B cells (Gearhart et al., 1981; Bothwell et al., 1981). In contrast, "thymus-independent" (TI) antigens do not require help from T cells to trigger B cells. On entering the body, the TD antigens are taken up chiefly by mononuclear phagocytes and rapidly catabolised into fragments to be presented in an immunogenic form (Unanue, 1981), and retained either in the form of a component on the macrophage surface, or as an internal pool. Some antigenic components can be released from macrophages as soluble products (Ellner and Rosenthal, 1975). In contrast, the TI antigens have been shown to be high molecular weight polymers and are poorly metabolised (Mosier and Subbarao, 1982).

Only a small population of macrophages can present antigen to T cells (Unanue, 1981). These cells possess easily demonstrable Ia antigens (Todd et al., 1980). Part of the antigen processing step therefore, appears to be the association of antigenic moieties with Ia surface molecules. In order to be stimulated, T-helper cells generally need to see antigen and self Ia determinants simultaneously on antigen presenting cells (APC; Kappler and Marrack, 1978; Todd et al., 1980). However, Ishii et al. (1981), have shown that, some T-helper cell subpopulations, lacking alloreactive cells, were able to interact with MHC non-identical macrophages, suggesting that populations of T cells do exist, that can respond to antigen presented along with foreign Ia. These

studies tended to suggest that the requirement for identity between T-helper cells and APC is apparently more stringent in secondary (memory) responses than in primary responses (Singer, et al., 1981).

The interaction of T-helper cells with antigen presented on the surface of APCs presumably initiates T-cell differentiation and proliferation. However, maintenance of clonal expansion also appears to require non-antigen specific regulatory molecules such as interleukin 1 (IL 1), and interleukin 2 (IL 2), which are produced by stimulated macrophages and lymphocytes respectively. Interleukin 1 or IL1-like molecules induce a subpopulation of T cells to produce IL2, which aids the continued proliferation of antigen/mitogen activated T-cell subsets. IL1 and IL2 have no effect on unprimed cells (Smith and Ruscetti, 1981; Unanue, 1981). Once T-cell help has been generated, direct contact between effector T helper cells and B cells, brought about by antigen bridging (Mitchison, 1971), is the likely initiation signal for B-cell activation. However, helper T cells also release antigen-specific soluble factors which can take the place of T-helper cells in generating antibody responses in vitro (Howie et al., 1979), and these may be important mechanisms of delivery of T-cell help. Although they are antigen-specific, these soluble factors do not possess Ig constant region epitopes, but they share idiotypic determinants with antibodies (Mozes and Haimovich, 1979), and have Ia determinants, which

could be responsible for the MHC restricted activity found with some of these factors (Shiozawa et al., 1977). How these factors interact with B cells is unknown. An antigen bridge could focus a factor on to the B-cell surface. Alternatively, macrophages or other accessory cells may be involved, since accessory cells appear to be at least desirable if not obligatory for collaboration between antigen-specific T cells and B cells (Howie and Feldmann, 1978).

Non-antigen specific soluble factors are thought to finally drive clonally expanded B cells to terminal differentiation of Ig synthesis and secretion (Pure et al., 1981; Martinez and Coutinho, 1981). The role of non-antigen specific lympho-stimulatory mediators on proliferation and differentiation of B cells is less well studied than for T cells. However, a great deal of experimental data has recently been accumulating, assessing the role of antigen non-specific factors in B-cell triggering (Moller, 1984; Hoffman, 1980; Huber et al., 1977; Schimpl and Wecker, 1975; Singer et al., 1981). These studies have attempted to identify the pertinent lymphokines, elaborated as a result of T-helper:accessory cell and T-helper:B-cell interactions, involved in activating responding B cells. A variety of such factors include IL1, IL2, B-cell growth factor (BCGF) and T-cell replacing factor (TRF) (Moller, 1984). It still remains unclear at this time whether some, or all of these factors, are involved in B-cell triggering, and it still remains controversial in what sequence

these factors act on responding B cells (Singer and Hodes, 1983). Active suppression of B-cell activation by T-suppressor cells is also mediated by a subset of T cells. In the human system, it was shown that T lymphocytes expressing surface antigens reactive with monoclonal antibody OKT3 (anti-pan mature T-cells), and OKT5/8, were responsible for suppressive activity (Reinherz and Schlossman, 1980).

1.8 Polyvalent Antisera: Uses and Limitations

Polyvalent antisera generated by mammals in response to complex antigens, have been widely used to classify organisms, to study their evolutionary relationships, and to detect the microheterogeneity of proteins (Pollock et al., 1984), etc. The usefulness of conventional antisera however, has been complicated by the difficulty of repeatedly generating large amounts of antibodies with restricted specificity. An additional problem is presented by the fact that the spectrum of antibodies changes during the course of an immune response (Pollock et al., 1984). Thus, antisera taken at different times, could have differences in the titre as well as in the ability to perform certain functions, such as complement fixation, or precipitation and agglutination of the antigens. In addition, if the antibodies to a particular antigenic determinant are lost, the loss may go undetected, because the vast majority of antibodies may still bind the complex

antigen. This situation may complicate assays where related but not identical antigens are being studied. To a large extent the use of polyvalent antibody preparations has now been replaced by using monoclonal antibodies which are products of an expanded clone of B-cells.

1.9 Long Term B-Cell Lines Producing Homogeneous Antibodies

Most of the insights into Ig gene arrangements and rearrangements reported above (Sections 1.1-1.6) came from studies which employed cloned cells of B-cell origin which produced monoclonal antibody. The exploitation of monoclonal antibody technology started with the realisation that naturally occurring myeloma tumours were antibody forming cells, and that each myeloma tumour represented the proliferation of a single cell of plasma cell origin, and secreted a unique Ig (Potter, 1972). This soon led to the widespread use of monoclonal myeloma proteins for studying antibody structure (Edelman *et al.*, 1973), and for identifying isotypic, allotypic and idiotypic markers (Natvig and Kunkel, 1973) i.e., serologic markers of C regions and V regions respectively. The development of serologic tools to identify pieces of Ig molecules together with the development of recombinant DNA technologies led naturally to investigations into regulation of Ig gene expression. In addition, the findings that antibodies secreted by some spontaneous human and chemically induced

murine myelomas were specific for defined, often simple antigenic determinants, such as haptens and polysaccharides (Natvig and Kunkel, 1973, Potter, 1977), provided systems for analysing antigen/antibody interactions and the structure of the antigen combining sites of Ig molecules.

Many approaches have been used in attempts to obtain cells producing homogeneous antibodies of defined specificity. Attempts have been made to transform B cells with viruses. SV 40 virus has been successfully used to obtain B-cell lines from a rabbit secreting antibody to pneumococcal type III polysaccharide (Strosberg et al., 1974). Abelson virus which transforms mouse lymphocytes, has also been used in attempts to obtain long term B cell lines, but with little success (Premkumar et al., 1975). Epstein-Barr virus transformation was also used to generate lymphoblastoid human cell lines producing Igs specific for tetanus toxoids (Zuranski et al., 1978), the nitrophenyl-hapten (Steinitz et al., 1977), and human RBC antigens (Koskimies, 1980). But most of these EBV lines were known to secrete relatively very low amounts of antibodies. The disadvantage with these techniques was the limited range of antigens to which they could be applied, and the viruses showed species restriction.

Polyclonal activation of B cells, eg., with Pokeweed mitogen, has also been used in attempts to obtain long term B-cell lines; but polyclonal activators only permit analysis of the early events in Ig synthesis (Melchers et al., 1975).

Recent studies have however shown that long term culture of immunocompetent B cells is feasible using supernatants containing T-cell replacing factor (TRF; Howard et al., 1981). Although investigators continue to study new methods for the generation of monoclonal antibody producing B-cell lines, the system of hybridoma production provides the most straight forward method by which this can be achieved (Kohler and Milstein, 1975).

1.10 Monoclonal Antibodies Produced by Cell Fusion:
Generation, Uses, and Applications

The development of the hybridoma technology (Kohler and Milstein, 1975) marked the beginning of a new era in immunological research, and showed that somatic cell hybridization could be used to generate a continuous hybrid cell line producing a monoclonal antibody. The fusion experiments involved mouse myeloma tumour cells fused to spleen cells derived from a mouse that had previously been immunised with an antigen.

The mouse myeloma cell line used as fusion partner was deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), an enzyme that is coded by a gene on the X-chromosome. HGPRT negative cells have therefore no salvage pathway for DNA biosynthesis, and hence die when cultured in

aminopterin containing medium, a folic acid antagonist which blocks de novo DNA biosynthesis.

The cell (HGPRT-ve) can however be rescued by fusion with another normal cell which supplies the missing enzyme that allows only the hybrid cells to grow in aminopterin containing medium. Normal lymphocytes for fusion are obtained from animals, usually mice, that have been immunized with a specific antigen. The lymphocytes contribute specific antibody-producing genes in addition to the HGPRT gene to the hybridomas; the myelomas provide genes that allow the hybridoma to divide indefinitely and continue to express the immunoglobulin genes.

To obtain a continuously growing B-cell hybrid with functional antibodies, the fused cells have to retain the gene(s) for the salvage pathway for DNA synthesis, a functionally rearranged L-chain gene, a functionally rearranged H-chain gene and a gene that allows the hybridoma to divide continuously in addition to house keeping genes i.e., genes encoding structural proteins, receptors for nutrients and hormones, enzymes etc. Once generated, the B cell hybridomas provide a long lasting source of a unique antibody. With repeated cloning of the hybridomas, a stable cell clone which secretes antibody of desired specificity can be obtained (refer results, Section 3).

Monoclonal antibodies have been useful as diagnostic (Mitchell 1979; Pratt and David, 1981), therapeutic (Miller

and Levy, 1981; Ritz et al., 1981), and cell typing reagents (Reinhertz et al., 1980; Lalor, 1983; Reviewed by Kohler, 1986). These reagents have been used in studies of parasitic diseases, to map antigenic determinants, to isolate and study proteins which are useful in the diagnosis and epidemiology of parasite diseases, and in attempts to develop parasite vaccines (Pollock et al., 1984; Mitchell and Anders, 1982; McBride, 1983; Cain, 1984; Gamble, 1984). In addition, monoclonal antibodies provide convenient tools to study public and private markers (idiotypes) on the variable or antigen combining region of Ig molecules (Capra, 1977). Anti-idiotopic monoclonal antibodies have been used to selectively modulate immune responses (Rajewsky and Takemori, 1983) and in attempts to examine idiotope based (Jerne 1974, 1976) models of immune response regulation (Herzenberg et al., 1980; Benacerraf, 1980).

Although a very powerful tool in serology, monoclonal antibodies have also some inherent disadvantages. Precipitation studies with these antibodies are often difficult, since precipitation depends on extensive antigen cross-linking to form insoluble lattices. A monoclonal antibody binds to only one determinant on a monomeric antigen, such as a protein, and so this determinant must be re-iterated on the target protein to permit extensive cross-linking (Yelton and Scharff, 1981). It usually takes about six months to generate a stable hybridoma, whereas production of conventional

antiserum requires less time, energy and expense. Studies with monoclonal reagents must also be interpreted with caution, since the restricted specificities of each antibody might result in cross-reactivity with antigens, that are largely dissimilar, but share minor structural domains that are serologically indistinguishable (Milstein and Lennox, 1980). However, the main advantage of using monoclonal antibodies as opposed to polyvalent anti-sera, is the isolation of a unique B-cell product in very large quantities, which clearly offsets the difficulties in production and use of the monoclonal antibodies.

The use of cell fusion to generate monoclonal antibody secreting cell lines was initially developed in mice (Kohler and Milstein, 1975). The extension of monoclonal antibody technology to species (eg., human and bovine) other than the murine would be desirable, and have advantages over the murine system, e.g., for: (1) immunotherapy including anti-idiotypic modulation of the immune response; (2) for studying the spectrum of B-cell responses directed against antigens in the particular species at the cellular level.

In the human system, many attempts have been made to generate monoclonal antibodies. Many strategies have been employed, but none has yet gained widespread application. Epstein-Barr virus (EBV) transformation offers an alternative to somatic cell hybridization for the immortalization of human antibody-secreting cells. EBV transformation has been used successfully in many laboratories to produce permanent cell

lines secreting human monoclonal antibodies against viruses, bacteria and autoantigens (Boylston et al., 1980; Steinitz et al., 1984; Crawford et al., 1983). Unfortunately, EBV-transformed normal B lymphocytes often grow inefficiently at low cell density and hence are difficult to clone. Furthermore, EBV infection of unfractionated peripheral blood mononuclear cells (PBM) triggers vigorous antiviral responses consisting of α and γ interferon release and the generation of cytotoxic T cells (Tsoukas et al., 1982).

Attempts to produce Ig secreting human cells have also been made by fusing mouse myeloma cells to human B cells. Using this approach antibody-secreting mouse x human heterohybridomas to several antigens have been produced. These studies have utilised diverse donor lymphocytes (eg., from peripheral blood, spleens, tonsils), and several different murine myeloma cell lines (NS1; X63 or Sp2/0; Nowinski et al., 1980; Gigliotti and Insel, 1982; Kozbor et al., 1982a; Butler et al., 1984; Sikora et al., 1982). In most of these studies, the heterohybridomas ceased to secrete specific antibodies after only one to two months in culture. Some of the factors contributing to the decline in immunoglobulin production were the preferential loss of human chromosomes, and a poorly understood specific suppression of human immunoglobulin secretion (Raison et al., 1982).

Because of the instability of mouse x human heterohybridomas, investigators tried to isolate human lymphoblastoid and myeloma cell lines as alternatives for cell

fusion (Edwards et al., 1982; Togawa et al., 1982; Abrahams et al., 1983; Ritts et al., 1983). Some of these cell lines have been fused to human B cells to produce specific antibodies to bacterial and viral antigens (Chiorazzi et al., 1982), tumour antigens (Sikora et al., 1982; Glassy et al., 1983a; Houghton et al., 1983), and autoantibodies (Shoenfeld et al., 1982; Isenberg et al., 1984). The major advantage of human x human hybridomas is their relative stability. However, the human parental cell lines isolated so far are still not ideal. Problems with human cell lines have included; (1) poor hybridization and cloning efficiency, (2) production of only very small amounts of monoclonal antibody, (3) presence of EBV genes capable of transforming unfused B-cells, (4) slow growth of the human x human hybridomas in nude mice.

In an attempt to combine the favourable characteristics of both human and murine parental cell types, mouse x human heterohybridoma cell lines have been rendered aminopterin sensitive and used for further fusion with activated human B-cells (Teng et al., 1983; Fong et al., 1984). Such mouse x human fusion partners were found to be superior to the parental mouse myeloma cell lines X63 or Sp2/0 in test fusions with human B-cells, presumably as a result of enhanced retention of human chromosomes.

1.11 Attempts to Derive Bovine Monoclonal Antibodies

The requirements for production of hybrid cell lines

secreting bovine monoclonal antibodies are similar to those of any other species i.e. a permissive, selectable, myeloma or lymphoblastoid cell line, for fusion to antigen or mitogen activated B cells.

Except for an isolated case of the appearance of Bence-jones proteins in the bovine, (Rodkey and Kimmel, 1972), there have been no other reports to date of bovine cells producing myeloma proteins. Neither has a bovine fusion partner for production of bovine monoclonal antibodies been generated.

In view of this barrier, few workers have attempted to generate bovine monoclonal antibodies by fusing mouse myeloma cells to bovine B cells. Srikumaran et al. (1983/1984), have recently reported obtaining hybrid cell lines secreting bovine monoclonal antibodies to DNP by fusion of the mouse myeloma cell line Sp2/0 to bovine spleen cells. Earlier on, Davidson et al. (1982), fused bovine leukaemia virus (BLV) infected leucocytes with mouse myeloma cell line NS1 or x63 and generated hybrid cells secreting bovine immunoglobulins. The drawbacks encountered in the above two fusion attempts included; a low amount of antibody production by the hybrid cells, lack of specificity of secreted antibodies in the case where BLV infected lymphocytes were used as donor cells, and lack of stability of the hybrid cells due presumably to chromosome loss.

There is therefore a continued need for the development of stable hybrid cell lines capable of generating

high yields of viable bovine hybridomas, and high levels of bovine monoclonal antibodies. Theilen et al. (1968), and Morzaria et al. (1982) have isolated bovine lymphoblastoid cell lines designated BL3 and BL20 respectively, from cases of bovine leukosis. These cell lines have not been shown to produce any myeloma proteins, but perhaps could be rendered suitable for use as fusion partners by selection for aminopterin sensitivity.

An approach which has been developed to produce a fusion partner for human monoclonal antibody production and which could also be tried for the bovine system would be to generate a mouse x bovine heterohybridoma fusion partner. Mouse x human fusion partners were shown by Teng et al. (1983), Fong et al. (1984) in back fusion experiments with human lymphocytes, to form more stable hybrids and also to give higher levels of human monoclonal antibodies as compared to primary fusions. The approach combined the characteristics of human B lymphocytes and the commonly used mouse myeloma cell lines NS1, x63 or Sp2/0.

Methods for the primary in vitro immunisation of bovine lymphocytes with mitogens and/or antigens to obtain terminally differentiated B cells for monoclonal antibody production are not well defined, although bovine PBM have been shown to proliferate in the presence of various mitogens (Pearson et al., 1979; Lazary et al., 1974b). The development of an in vitro immunisation protocol of bovine lymphocytes with antigen would therefore provide a reliable system for

generating bovine B cells for fusion which could be applied to several antigens.

1.12 Objectives of This Study

In view of the foregoing introduction and literature review, the objectives of this study were:

1. To establish reproducible conditions for in vitro activation of bovine peripheral blood mononuclear cells (PBM) with Pokeweed mitogen (PWM) and/or antigen i.e. sheep red blood cells (SRBC) to be used as a source of B lymphocytes for fusion (refer results, Section 1).
2. To examine and select for an aminopterin sensitive bovine lymphoblastoid cell line e.g., BL3 or BL20 as a fusion partner for intraspecies specific bovine monoclonal antibody production (refer results, Section 2).
3. To use the knowledge gained from attempts to generate mouse x human fusion partners, to generate mouse x bovine heterohybrid cell lines/clones and select for aminopterin sensitive mouse x bovine cell mutants to be used as fusion partners for bovine B lymphocytes, (refer results, Section 3).

MATERIALS AND METHODS

2.1 Animals

2.1.1 Bovines

Bovines employed in the experiments were of Boran breeds (Bos indicus) aged 4 months to 3 years and were reared and maintained at ILRAD.

2.1.2 Mice

BALB/c mice were originally purchased from the Laboratory Animal Centre (Carshalton, England), expanded as an inbred colony at ILRAD, and used at 2 weeks to 6 months of age.

2.2 Cell Preparation

2.2.1 Isolation of Bovine Peripheral Blood Mononuclear Cells (PBM)

Peripheral blood was obtained from the jugular vein and diluted in an equal volume of Alsever's solution (Appendix 1). The peripheral blood mononuclear cells (PBM)

were isolated over Ficoll-Paque (Pharmacia; Pharmacia Fine Chemicals Uppsala, Sweden) according to the method of Boyum (1968). A ratio of seven mls of blood to three mls of Ficoll-Paque was used and the Ficoll-Paque was introduced beneath the blood using a pasteur pipette. The tubes containing the blood and Ficoll-Paque were centrifuged at 700-750g for 30 minutes at 4°C. Cells at the interface were carefully removed and washed once by centrifugation at 200-250g for 10 min in Alsever's solution and twice in RPMI-1640 medium (Gibco, Ltd, Paisley, Scotland) containing 10% heat inactivated foetal bovine serum (FBS; 56°C for 30 min), 100 ug/ml streptomycin, 100 units/ml penicillin, 5×10^{-5} M 2-mercaptoethanol (2-ME) and 2 mM/ml L-glutamine (complete RPMI-1640 medium). Viability was determined by staining the PBM with 0.4% trypan blue dye and counting unstained (living) and blue stained (dead) cells within three minutes in a Neubauer improved haemocytometer.

2.2.2 Exteriorisation of Bovine Spleen

The surgical operation was performed by Dr. T. Jordt of ILRAD. The spleens of weaner calves were exteriorised to ease isolation of the cells. The bovine was starved for 24 hours before surgery to reduce the size of the rumen, ruminal tympany and the risk of regurgitation of rumen contents during anaesthesia. An intramuscular (I.M) injection of 0.07 mg/kg

body weight of xylazin (Rompum, Bayer Germany) was given prior to anaesthesia. Anaesthetic induction was done by an intravenous (I.V.) injection of 6mg/kg body weight thiopento-sodium (Intraval, May and Baker, U.K.) and the bovine placed on the right side before inserting the endotracheal tube. The anaesthetic maintenance during surgery was on a closed circuit anaesthetic machine (Komesaroff, Australia) working on a mixture of halothane (Hoechst, Germany) and methoxyfluorane (Metofane, Jaussen, Le Brun, France). The area over the 12th rib on the left side of the bovine was prepared for aseptic surgery by clipping off the hair and disinfecting with Savlon (Macclesfield, Cherise, UK). An incision of about 15-20 cm. long was then made over the 12th rib extending from the costo-chondrial junction and dorsally. The tissues over the rib were incised and dissected free, without freeing the periosteum. The rib was then transected at the dorsal limit (5-10 cm from the head of the rib) using an embryology wire. The distal end was dislodged at the costo-chondrial junction and the rib removed. The peritoneum was incised at the area of the resected rib and the caudo-ventral extremity of the spleen was located and freed by careful blunt dissection and pulled out through the abdominal incision. The skin on the caudo-ventral aspect of the abdominal incision was dissected free from the underlying tissue in order to accommodate the freed end of the spleen (7-12 cm). The lateral aspect of the splenic capsule was sutured to the cranial border of the

peritoneum with uninterrupted Chrom-catgut (USP 2/0, Braum Germany) suture material, and the medial aspect of the splenic capsule was sutured to the caudal border of the peritoneal incision in a similar manner, i.e. closing the peritoneal incision around the spleen. The skin was closed over the exposed spleen with interrupted Supramid (USP 3, Braum, West Germany), with the mattress suture pattern. The wound was sprayed with embacycline aerosol (May and Baker UK), followed with an intramuscular injection of penicillin and streptomycin (1 ml/15 kg, Maxbrook, Ireland). Suture material on the skin was removed 10 days post surgery.

2.2.3 Isolation of Bovine Spleen Cells

Calves with exteriorised spleens (Section 2.2.2) were used to supply spleen cells. The area above the spleen was disinfected with Savlon (Macclesfield, Cheshire, UK) and the cells were obtained aseptically using a sterile needle (18g) fitted to a disposable syringe containing 10-15 mls of Alsever's solution. The needle was pushed along the width and/or length of the spleen and an equal volume of splenic aspirate was mixed with Alsever's solution. Spleen tissues were at times obtained from animals slaughtered at LLRAD and placed in petri dishes containing Alsever's solution. The cells were released from the tissue material into Alsever's solution by mincing with a pair of scissors and pressing gently

with the blunt end of a syringe plunger. The suspension was transferred to a sterile centrifuge tube and clumps were allowed to settle at the bottom of the tubes for 5-6 minutes. The suspension containing the spleen cells was centrifuged once in Alsever's solution to isolate the cells, at 200 g for 10 minutes. The cell pellets were washed two times in complete RPMI-1640 (Section 2.2.1) by centrifugation as above, before determining the cell number and viability as described (Section 2.2.1).

2.2.4 Bovine Thymus Biopsy

Thymus tissue material, for isolation of bovine thymocytes was obtained from 4-6 month old weaner calves. Calves were prepared for aseptic surgery as described for bovine spleen exteriorisation (Section 2.2.2). A 10 cm long (skin and muscle) incision was made starting mid head-shoulder and extending caudally parallel with and along the ventral aspect of the left jugular vein. A careful blunt dissection towards the trachea, ventral of the jugular vein, aorta carotis communis, ventral recurrens and dorsal of sterno-mandibularis muscle was performed. After exposing part of the thymus, a small piece (1 x 1 x 2cm) was cut and placed in a sterile container with Alsever's solution. Thymus cells were isolated as described for spleen cells (Section 2.2.3). The blunt dissected connective tissue was sutured with non-interrupted

Chrom-catgut (USP 2/o Braum West Germany) suture material. The skin was closed with Supramid (USP3, Braum, West Germany) using an interrupted mattress pattern and the bovine was treated as described earlier (Section 2.2.2).

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2.2.5. Isolation of Bovine Thymus Fibroblast-Like Cell
Monolayers

Freshly isolated bovine thymus cells (Section 2.2.4) were seeded at 10^6 /ml in complete RPMI-1640 medium containing 10% FBS, and dispensed in 20-30 ml volumes in 150 cm^2 Costar tissue culture flasks (Costar, Broadway, Cambridge Massachussets, USA). Cultures were incubated at 37°C in an atmosphere of 7-10% CO_2 in air. Adherent cells were confluent after 10-14 days. Passages were done weekly using trypsinisation medium (Appendix 1). Briefly, the confluent cells were washed twice in calcium and magnesium free Hank's balanced salt solution (HBSS, Washing Medium; Gibco Europe Ltd.; Appendix 1). After removing the washing medium, trypsinisation medium which was just enough to cover the cell surface, was introduced into the flasks. The cells were incubated at 37°C until they were completely detached from the bottom of the flasks. The cells were then washed once in complete RPMI-1640 medium by centrifugation (200 g for 10 min), dispensed at 10^5 /ml in the same medium before being distributed in tissue culture flasks/wells. The adherent cells

were used as feeder cells within 4-48 hrs of plating. Excess cells were resuspended in a mixture of 10% dimethyl sulfoxide (DMSO, Merck) in foetal bovine serum (FBS, v/v) and stored in liquid nitrogen (N_2). Cells in freezing vials were left in the vapour phase of liquid N_2 for about 24 hrs before sinking the vials into liquid nitrogen.

2.2.6 Isolation of Mouse Spleen Cells

Mice were killed by anaesthetising with diethyl ether solution vapour and disinfected by immersing them in a container with 70% alcohol (v/v). Each mouse was fixed by its legs to a soft board with the ventral aspects facing upwards using 25 g needles. The spleen was removed aseptically by slitting open the abdominal cavity using sterile scissors and forceps, and placed into a Petri dish containing RPMI-1640 medium without serum. Splenocytes were isolated as described for bovine spleen cells (Section 2.2.3).

2.2.7 Isolation of Mouse Thymus Cells

Mice (2-3 weeks old) were prepared as described (Section 2.2.6). The thoracic cavity was opened with a pair of scissors upto the anterior end, to expose the thymus, and the lobes were excised and placed in a container with RPMI-1640 medium without serum. Single thymus cells were isolated as in Section 2.2.3.

2.2.8 Isolation of Sheep Red Blood Cells (SRBC)

Sheep red blood cells were isolated from the venous blood of adult sheep which were reared on ILRAD farms. The blood was mixed at a 1:1 ratio with Alsever's solution and the red cells were obtained by centrifugation at 300 g for 15 minutes and stored in Alsever's solution at 4°C for a maximum of two weeks. Prior to each experiment, the cells were washed three times in RPMI-1640 medium by centrifugation as above.

2.3 Irradiation of Cells

Peripheral blood mononuclear cells (PBM) derived as in Section 2.2.1 were depleted of plastic adherent cells before irradiation. The cell density was adjusted to 5×10^6 /ml in RPMI-1640 medium containing 10% FBS and cells were dispensed into Costar flasks at volumes of 5-8 mls/flask (25 cm^2) or 20-30 mls/flask (75 cm^2). The flasks were placed in an incubator at 37°C in an atmosphere of 7 - 10% CO_2 in air for two hours. Non-adherent cells were recovered by aspirating the supernatant and centrifuging at 200g for 10 minutes. Thymus cells (mouse), prepared as described (Section 2.2.7), continuously growing cell lines obtained at logarithmic phase of growth and non-adherent PBM were diluted to 5×10^6 /ml in RPMI-1640 medium and dispensed into Costar flasks as above. The flasks containing the cells were placed on ice and

irradiated with gamma (γ) rays from a ^{137}Cs source at a total dose of 600-5000 rads (R) depending on the cell type. After irradiation, the cell suspension was centrifuged (200 g for 10 min) and the medium was drawn off before use.

2.4 In vitro or In vivo Stimulation of Lymphocytes with Mitogen/Antigen

2.4.1 Stimulation of Bovine PBM With Pokeweed Mitogen (PWM).

Ficoll-Paque purified bovine PBM (Section 2.2.1) were resuspended in complete RPMI-1640 medium at concentrations ranging from $5 \times 10^5/\text{ml}$ to $8 \times 10^6/\text{ml}$. 100 μl aliquots of the cell suspension were dispensed into duplicate wells of a 96-well, flat bottomed microtitre plate (Costar Broadway, Massachusetts, USA). A stock solution of Pokeweed mitogen (PWM, Phytolacca americana, Sigma Chemical Co., 1mg/ml in sterile PBS) was further diluted in complete RPMI-1640 and dispensed in 50 μl volumes to the different cell concentrations to make final dilutions of .625 $\mu\text{g}/\text{ml}$ to 10 $\mu\text{g}/\text{ml}$ of PWM and 3×10^5 cells/ml to 5×10^6 cells/ml respectively. Control cultures received the medium alone. The cells were incubated at 37°C in an atmosphere of 7 - 10% CO_2 in air. The incorporation of [^{125}I] iodo 2-deoxyuridine ([^{125}I]UDR) into deoxyribonucleic acid (DNA) was examined on different days of culture (1-6 days).

2.4.2 Preparation of PWM-Induced Growth Factor Containing Culture Supernatants.

The production of PWM-induced growth factors was performed according to the method of Sredni et al. (1981), but with slight modifications. Isolated bovine PBM (Section 2.2.1) were seeded at 2.5×10^6 cells/ml in RPMI-1640 medium containing 10% FBS and 2.5 ug/ml of PWM. The cell suspensions were dispensed in 20-30 ml volumes in 75 cm² Costar tissue culture flasks. The cells were placed at 37°C in a CO₂ incubator. After three days of incubation the culture was harvested and the cells removed by centrifugation (200-250g for 15 min. at 4°C). This supernatant preparation, which was designated growth factor containing culture supernate, was sterilised by filtration through 0.22-um filters (Nalge, Sybron, Rochester, New York) and stored at -80°C until needed.

2.4.3 Immunisation of Calves Against Sheep Red Blood Cells (SRBC) (conducted with Ms. V. Lutje)

Calves with exteriorised spleens (Section 2.2.2) were primed with 10^{11} sheep red blood cells (SRBC) in saline, by subcutaneous (SC) injections at several sites along the back. The calves were boosted with the same antigen (10^{11} SRBC) by injection at similar sites 3-4 weeks later. Another schedule of immunisation consisted of a booster injection of 10^{11} SRBC

in saline into the exteriorised spleen. The spleen cells were isolated from the immunised calves as previously described (Section 2.2.3).

2.4.4 Immunisation of Mice Against SRBC

Female BALB/c mice of 6-8 weeks old (Section 2.1.2) were injected intraperitoneally (I.P.) with 10^7 SRBC diluted in one ml of saline. Three days post immunisation the mice were killed by exposure to diethyl ether vapour and the spleen cells were isolated as before (Section 2.2.6).

2.4.5 In Vitro Immunisation of Bovine PBM with SRBC.

Ficoll-Paque isolated bovine PBM (Section 2.2.1) were resuspended in RPMI-1640 medium supplemented with 10% FBS which had been adsorbed (x3 at 10% v/v) with SRBC. The cells were distributed at 2.5×10^6 /well in duplicate in 24 well Costar plates. To each well containing the cells was added different concentrations of PWM and SRBC, diluted in RPMI-1640 medium, to make final dilutions ranging from 0.040 ug/ml to 2.5 ug/ml and 2×10^5 cells/ml to 2×10^8 cells/ml respectively. The total volume of mixture per well was one ml. Control cultures consisted of PBM alone, PBM plus SRBC or PBM plus PWM. In other experiments, PBM from bovines previously immunised against SRBC (Section 2.4.3) were cultured at 2.5×10^6 PBM/ml of medium in the presence of the same antigen

(2.5×10^6 SRBC/ml). The cultures were incubated at 37°C in an atmosphere of 7-10% CO_2 in air for 1-8 days. Cells were harvested from duplicate wells at different times of culture, washed two times in RPMI-1640 medium by centrifugation (200 g for 10 min), and brought to the appropriate concentration for plaque assays.

2.5 Measurement of Proliferation or Antibody Synthesis and Secretion by Mitogen/Antigen Stimulated Bovine Lymphocytes

2.5.1 $[^{125}\text{I}]$ UDR Incorporation into Deoxyribonucleic Acid (DNA) of Bovine PBM Activated with PWM

To each culture well to be examined (Section 2.4.1), was added 0.5 μCi ($5\text{-}^{125}\text{I}$) iodo 2-deoxyuridine [(^{125}I) UDR; 5 Ci/mg; 0.5- $\mu\text{Ci}/\text{ml}$; Amersham, I.M. 355] in 25 μl of RPMI-1640 medium and a further incubation was carried out at 37°C in an atmosphere of 7-10% CO_2 in air for 4-6 hours. The cells were harvested onto glass fibre filters using a Titertek Cell Harvester (Flow Laboratories). The filters were air dried and the radiolabel associated with the filters was counted in a Packard autogamma spectrometer. Results were expressed as counts per minute (cpm) versus duration of cells in culture.

2.5.2 Analysis of Anti-SRBC Specific Antibodies by the
Direct Plaque Forming Single Cell Assay (PFA)

Spleen cells from bovines previously immunised against SRBC (Section 2.4.3), or cell suspensions from cultures stimulated with PWM or PWM plus SRBC as in Sections 2.4.1 and 2.4.5 respectively, were assayed for direct plaque formation against SRBC by a modification of the Cunningham et al. (1966) method, employing chambers prepared from glass microscope slides (76 x 25 mm). A row of clean grease-free slides were placed side by side on a flat surface and lined against a straight edge. Three strips of six mm wide, double sided self-adhesive tapes (Scotch, Tape No. 410-Minnesota Mining and Manufacturing Co.), were applied in parallel strips across the ends and middle of individual slides. After peeling off the tape backing, another layer of slides was placed on top of the first ones and glued firmly together. The double slides formed were separated by breaking the tapes. Each double chamber in the slides had a capacity of about 100 ul. The density of viable cells to be tested for plaque formation was adjusted to 10^7 /ml in RPMI-1640 containing 10% SRBC adsorbed FBS (Section 2.4.5). To each tube was added 500 ul of RPMI-1640 medium, 125 ul of 15% SRBC in RPMI-1640 medium, and 75 ul of guinea pig serum, which had previously been absorbed thrice with 10% (v/v) SRBC and bovine PBM by incubation at 4°C for 30 min. The contents of the tubes were mixed and 100 ul samples were removed, and added to equal volumes of the cells (10^6) to be

tested. After mixing again, 100 ul of the suspension was removed (5×10^5 cells) and introduced into the double chambers in the slides. The chambers were sealed immediately, with paraffin wax-petroleum jelly at 150°C. The slides were incubated at 37°C for 1 1/2-2 hours and the number of single cell plaques were counted by the naked eye and on an inverted microscope.

2.6 Cell Lines and Culture Conditions

2.6.1 Bovine Leukaemia Cell Lines

Two long term cultured bovine leukaemia cell lines designated BL3 (Theilen et al., 1968) and BL20 (Morzaria et al., 1982) were used in these studies. Bovine leukaemia cell line BL3 was from an adult steer with lymphosarcoma (not known whether sporadic or enzootic bovine leukosis). The line was adapted to culture conditions by Theilen et al. (1968), and had aneuploid chromosome constitution in number and kind. The cell line was passed to me by Dr. Jan Naessens of ILRAD. He had obtained the line from Dr. Santos Karr of ILRAD who in turn had obtained it from Dr. C.G.D. Brown of Edinburgh. BL3 cells were grown in Leibovitz's L15 medium containing 10% FBS, 10% tryptose phosphate broth, 100 units/ml penicillin, 100 ug/ml streptomycin, 2mM/ml L-glutamine and $5 \times 10^{-5}M$ 2-mercaptoethanol and incubated at 37°C without CO₂ gas. They were passaged twice a week and harvested at logarithmic

phase of growth for all experiments. Bovine leukaemia cell line BL20 was a gift from Dr. Morzaria from ILRAD. The cell line was isolated from the bronchial lymph node of a heifer calf with sporadic bovine leukosis in its multicentric form and established in culture by Morzaria et al. (1982). No viral particles were detected in the cell line nor did the line have receptors for peanut agglutinin (PNA, Arachis hypogaea (Morzaria et al., 1982). The cells were maintained in continuous culture in complete RPMI-1640 medium supplemented with 10% FBS (Section 2.2.1) and incubated at 37°C in an atmosphere of 7-10% CO₂ in air. Passages were done twice a week and the cells were used at logarithmic phase of growth.

2.6.2 Mouse Myeloma Cell Lines P3/NS1/1-Ag-4-1(NS1), X63-Ag-8.653 (X63) and Sp2/0-Ag14 (Sp2/0)

Mouse myeloma cell lines P3/NS1/1-Ag-4-1 (NS1; Kohler et al., 1976) and X63-Ag-8.653 (X63; Kearney et al., 1979) are descendants of MOPC 21, a myeloma cell line which secretes IgG1 molecules and k light chains. The MOPC 21 line was adapted to culture conditions by Horibata and harris (1970) and renamed P3K. An 8-azaguanine resistant and aminopterin sensitive subline of P3K was established by Kohler and Milstein (1975) and named P3 x 63-Ag-8. A variant of P3 was subsequently produced by Kohler et al., (1976) and designated P3-NS1-Ag-4-1. This line lacks the IgG1 heavy chain. When cultured alone, it synthesises k light chains, which are not

secreted but are internally degraded. A subclone of P3-X63-Ag8 was produced by Kearney et al., (1979) and designated X63-Ag-8.653. It is a clone which does not express immunoglobulin heavy or light chains. Sp 2/0-Ag 14 was isolated as a re-clone of Sp 2/HL-Ag, itself derived in several steps from Sp 2/HLGK, a hybrid between a BA1B/c spleen cell contributing a γ 2b(H) and k(L) chain with anti-sheep red blood cell activity and the mouse myeloma cell line X63-Ag8 (γ (G) and k(K))². Sp 2/0-Ag14 is resistant to 20 ug/ml of 8-azaguanine, dies in HAT supplemented medium and synthesizes no Ig molecules. It has about 73 chromosomes (Shulman et al., 1978). The mouse myeloma cell lines were maintained in the laboratory in complete RPMI-1640 medium supplemented with 10% FBS at 37°C in an atmosphere of 7-10% CO₂ in air. The cells were passaged twice a week and used at logarithmic phase of growth for all experiments unless indicated otherwise.

2.6.3 Limiting Dilution Cloning of Cells

Cells to be cloned were collected at the logarithmic phase of growth and resuspended at the desired density in (RPMI-1640 or L15) medium supplemented with 15% FBS. Most cloning experiments utilised cell concentrations ranging from 1 cell/100 ul to 10,000 cells/100 ul of medium delivered to individual wells of a round or flat-bottom 96-well microtitre plate. Feeder cells were in some instances included in cloning

experiments to test their ability to improve the cloning efficiency. Bovine thymus fibroblast-like feeder cells were seeded 4-48 hours prior to cloning, in 96 well plates, as described in Section 2.2.5. Other cell types tested for their ability to improve the cloning efficiency of some cell lines included mouse thymocytes (Section 2.2.7) and irradiated bovine PBM (Section 2.3.1). These latter cells types were seeded (10^6 /well) together with the cells to be cloned. Two batches of concanavalin A (Con A; Canavalia ensiformis) induced T-cell growth factor (TCGF) kindly provided by Kathleen Logan and Bruno Goddeeris of ILRAD were also tested in separate assays at 10, 20 and 50% dilution, for the ability to enhance cloning of some cell lines. In some assays, PWM-induced growth factors (Section 2.4.2) were tested for the ability to promote growth of cells. Cells resuspended in RPMI-1640 medium were incubated at 37°C in the presence of an atmosphere of 7-10% CO₂ in air while the cells in L15 medium were incubated at 37°C without CO₂ gas. The cloned cells were fed once a week by removing about half of the spent medium and replacing with an equal volume of fresh medium. The wells were inspected for cell growth twice a week by light microscope examination. Cells from selected wells with significant growth were transferred to larger wells for continued expansion.

2.7 Chromosome Analysis

Metaphase chromosome spreads were prepared according

to the standard techniques (Moorhead, et al., 1960; Worton and Duff, 1979). Continuously growing cell lines/clones derived at logarithmic phase of growth, or PBM which had previously been stimulated with PWM for three days (Section 2.4.1), were cultured in the appropriate growth medium containing 10% FBS and N-Desacetyl-N-methyl colchicine (Colcemid; 0.3-0.5 ug/ml; Grand Island N.Y., U.S.A.), at 37°C in an atmosphere of 7-10% CO₂ in air for 4-24 hours, depending on the length of the cycle of the cell line/clone. After harvesting and pelleting the cells by centrifugation at 200 g for 10 minutes, the supernatant was decanted and the pellet was resuspended in two mls of 0.5-1% tri-sodium citrate (w/v; BDH; Analar) and incubated at RT for 10-30 minutes. The hypotonic sodium citrate solution was decanted after centrifugation as before, and the cells were fixed by addition of 15-20 mls of freshly prepared cold methanol/acetic acid (3:1; v/v) and incubated on ice for 15 minutes. The fixation procedure was repeated twice before chromosome spreads were prepared on chilled microscope slides (76 x 25 mm), dried at RT, and stained with 4% Giemsa for 10 min. Individual chromosome spreads were examined using a high power objective (X100; oil immersion) on a Leitz microscope (Leitz, Wetzlar, Germany) equipped with a 35-mm Leitz camera. A green filter was used to photograph chromosome spreads on black and white films (Kodak; Panatomic-X, Eastman, N.Y.). Enlarged prints were made on 12.5 x 17.5 cm Kodak photographic papers (Eastman Kodak Company, N.Y.), and the

modal chromosome numbers for each cell line/clone was obtained from a mean of 50-100 different cell spreads.

2.8 Antibodies and Staining Procedures

2.8.1 A list of Antibodies Used in This Study

TABLE 2

Antibody	Specificity	Reference
Monoclonal antibody B5/4 (MAb)	Epitope on bovine IgM	Pinder et al., (1980)
MAb A ₂	Epitope on bovine IgG ₁ and IgG ₂	Wells and Karr (unpublished)
MAb P5	Bovine T cells and a subset of monocytes	Lalor (1983) Lalor <u>et al.</u> , (1986)
MAb IL-A17	Class I restricted T cells	Ellis <u>et al.</u> , (1986)
MAb K6	Polymorphic antigen on bovine T cells	Barry (unpublished)
MAb IL-A12	Class II restricted T cells	Baldwin <u>et al.</u> , (1986)
MAb R1	Monomorphic epitope on bovine leukocyte antigen (BoLA) class II molecule	Lalor <u>et al.</u> , (1986)
MAb P8	Monomorphic epitope on bovine macrophages	Lalor <u>et al.</u> , (1986)
MAb IL-A3	Monomorphic epitope on BoLA class I molecule	Gift from Dr. A. Teale of ILRAD

Antibody	Specificity	Reference
MAb IL-A4	Polymorphic epitope on BoLA class I molecule	Goddeeris <i>et al.</i> , (1986)
MAb IL-A7	Probably polymorphic epitope on BoLA class I molecule	Gift from Dr. A. Teale of ILRAD
Mouse anti H ₂ D ^b	Polymorphic epitope on H ₂ class I	Litton Bionetics Kensington M.D. USA.
FITC rabbit anti-mouse Ig (FITC-RAmIg)	Heavy (H) and Light (L) chain of mouse Ig	Nordic Immunological Laboratories, Tilburg, Netherlands.
FITC sheep anti mouse Ig. (FITC-ShmIg)	Heavy (H) and light (L) chain of mouse Ig	Nordic Immunological Laboratories, Netherlands.
FITC rabbit anti-bovine IgA (FITC-RABIGa)	H and L chain of bovine IgA	Miles Laboratories Naperville, USA.

All antibodies were diluted in phosphate buffered saline (PBS) containing 2.5-5% normal rabbit serum (NRS) with or without 0.1% sodium azide (IFA buffer). The optimal dilution of each antibody for use in direct and indirect immunofluorescence assays was established in a checkerboard titration before each experiment. Diluted antibodies were stored at 0-4°C and used within four weeks.

2.8.2 Direct and Indirect Immunofluorescence Staining of Cells for Fluorescence Activated Cell Separator (FACS) Analysis.

Cells to be stained (PBM, or continuously growing cells etc.) were collected at logarithmic growth phase and washed once by centrifugation (200 g for 10 minutes) in IFA buffer. The cells were resuspended at 10^7 /ml in the same buffer (IFA buffer) and 50 ul aliquots were dispensed in U or V shaped wells of 96-well microtitre plates (Flow laboratories; Hamden Connecticut). The cells were centrifuged (150 g for 5 min.), the supernatant was removed and the pellet was resuspended in residual buffer by gentle tapping. To each well on the plate containing the cells was added 100 ul of RPMI-1640 medium containing 10% normal rabbit serum (NRS; Gibco, Paisley Scotland). The cells were incubated at 37°C in an atmosphere of 7 - 10% CO₂ in air for 60 minutes and centrifuged as above to remove the supernatant. Incubation of cells with 10% NRS was observed in preliminary studies to reduce non-specific or Fc-mediated binding of antibodies.

For direct immunofluorescence, 25 or 50 ul aliquots of an optimal concentration of Fluorescein isothiocyanate (FITC) conjugated antibody, diluted in IFA buffer, was dispensed to each well and mixed. Further incubation of the cells was conducted at 4°C for 30-45 minutes. The cells were then washed twice by centrifugation at 150 g for 5 minutes in IFA buffer prior to analysis. For indirect immunofluorescence, the

staining protocol was similar to that used for direct immunofluorescence, i.e. cells to be stained were first incubated with an optimally diluted antibody in IFA buffer (first step antibody), washed twice and incubated further with a FITC-conjugated second antibody directed against the first step antibody, before performing two more cell washes as before. A list of antibodies used for staining is given in Section 2.8.1 (Table 2).

Prior to analysis, the cells were centrifuged at 150 g for 5 minutes, the supernatant removed and the cell pellets were diluted in one ml of isotonic saline (Appendix 1) and passed through a 0.45 micron nylon screen filter (Nybolt, Swiss Silke Bolting Cloth Manufacturing Co. Zurich Switzerland) to remove clumps and debris. On some occasions stained cells were not examined immediately, but were centrifuged (150 g for 5 min), the supernatant removed, and the pellets in the microtitre plates were resuspended in 2% formalin in phosphate buffered saline (PBS; Appendix 1).

The plates containing the cells were sealed with parafilm and aluminium foil and stored at 4°C in the dark, until analysis (24-48 hrs later). Fixed cells were treated as above before analysis.

2.8.3. Cytoplasmic Immunoglobulin (cIg) Staining of Cells

Pokeweed mitogen (PWM), or antigen activated bovine lymphocytes (Sections 2.4.1, 2.4.5), or continuously growing

cells, were obtained and resuspended in 100% NRS, at 10^8 cells/ml. Five μ l samples were removed and applied onto clean coverslips (22 x 22 mm). Smears were made by placing a second coverslip on top of the first one and sliding the two in opposite directions. The smears were air dried and fixed in ethanol/acetone (1:1; v/v) at -20°C for 10 minutes. The fixative was allowed to evaporate at room temperature (RT) and the coverglasses were mounted with DePex (Serva, Heidelberg, West Germany) on to microscope slides (76 x 26 mm) with the cell smears facing upwards. The mountant was allowed to harden at room temperature (RT) overnight (o/n). Thin cell smears on coverslips mounted on to the slides were rehydrated by immersing in a bath of PBS containing 2.5% NRS and 0.1% sodium azide for 10 min. Excessive moisture on the edges of the cell smears was blotted dry with filter paper. 25 or 50 μ l of mouse monoclonal anti-bovine IgM or IgG, or FITC-rabbit anti-bovine IgA (for direct staining) (Section 2.8.1) diluted in IFA buffer, was applied to the cell smears on the coverslips mounted on the slides, and the slides were incubated at RT in a humid chamber for one hour. The antibodies were washed off by immersing the slides in two changes of the IFA buffer for 10 minutes, and excessive moisture was blotted dry with filter paper taking care not to dry the areas with cell smears. A second step antibody (FITC-conjugated ShMIg; Section 2.8.1) which was appropriately diluted in IFA buffer was applied (25 or 50 μ l volumes) to the cells and incubation, washing and drying of slides repeated as before. Stained cells were

mounted under coverslips in a mixture of 90% glycerol in PBS (v/v; Mounting buffer; Appendix 1.). For microscopic examination a Leitz Orthoplan fluorescence microscope equipped with a ploemopak 2 vertical illuminator, a HBO 100 mercury lamp and a 35 mm camera was used (Leitz, Wetzlar, Germany).

Photographic records were made on Kodak photographic papers (Kodak Tri-x-pan, Eastman, N.Y.). A total of 50-100 cells were counted per sample before determining the percentage of positive cells.

2.8.4 Indirect Immunofluorescence Staining of Bovine Thymus Fibroblast-Like Cells

Bovine thymus fibroblast-like cells were obtained from cultures by trypsinisation (Section 2.2.5) and washed two times in RPMI-1640 medium containing 10% FBS by centrifugation (200 g for 10 min). The cells were resuspended in RPMI-1640 medium at 10^5 /ml and 200 ul aliquots (2×10^4 cells) were dispensed into each chamber of a Lab-Tek tissue culture chamber slide (Miles Scientific, Mile Laboratories, U.S.A.). The cells were allowed to adhere and form a monolayer by incubating at 37°C in an atmosphere of 7-10% CO₂ in air for 4-48 hours. Before staining, the medium above the monolayers and also the plastic chambers from the slides were removed. The cells were allowed to dry at room temperature (RT) for 10-15 minutes before they were fixed in methanol for five minutes. The fixative was allowed to evaporate and the cells were rehydrated by immersing

the slides in a bath of PBS containing 2.5% NRS and 0.1% sodium azide for 10 min. After removing excess moisture by blotting with filter paper, the procedure followed for indirect staining was the same as that described for cytoplasmic Ig staining of cells (Section 2.8.3). A panel of antibodies employed for staining the cells is described in Section 2.8.1. All analyses were conducted using a Leitz fluorescence microscope (Section 2.8.3).

2.8.5. Analysis of Stained Cells by Fluorescence Activated Cell Separator (FACS II)

Stained cells in suspension (Section 2.8.2) were analysed on the FACS II cell separator (Becton Dickinson FACS Division, Mountainview, USA) as described by Bonner et al. (1972) and Loken et al. (1979) but with a few modifications. Cells were processed on the FACS II at a rate of 1000 - 1500 per second for analysis. All experiments were performed with the argon ion laser set at 300 mW, 488 nm (for excitation of FITC), the photomultiplier tube (Model QL-30, EMI-Genco Inc., Plainview, New York, USA) at 450v, the light scatter gain at two and the fluorescence gain at two. The FACS II was interfaced with a X-Y recorder (Model 7040A/7041A, Hewlett Packard, San Diego California, USA), and a microprocessor (Dr. Olaf Ahrens, Hamburg W. Germany), for print out and processing of all fluorescence profiles. To record the cell fluorescence versus scatter profile dot plots, polaroid photographs were

taken using a C-5A Oscilloscope Camera (Tektronix, Beaverton Oregon, USA). At the commencement of each experiment conducted on different days, the FACS II was programmed to analyse the fluorescence signals from viable cells only, as defined by light scattering properties (Herzenberg and Herzenberg, 1978).

In brief, non fixed cell preparations were incubated at 5×10^5 cells/ml of IFA medium, at room temperature (RT) in the presence of 0.2 ng/ml of fluorescein diacetate (FDA, Sigma Chemical Co.) for 1-2 minutes . The cells were processed by the FACS II at a rate of 1000 cells/second and lower scatter windows were selected to exclude channels in which non-fluorescent (dead) cells resided. All subsequent analyses were done using the selected scatter window setting. Two types of cell parameter profiles generated by FACS II analysis were recorded in the results as:

- a) histograms relating the fluorescence intensity of the antibody-stained cells to the relative number of cells;
- b) histograms of relative cell size versus the fluorescence intensity on the cells.

2.9. Biosynthetic Labelling of Cells and Analysis of Cell Associated and Secreted Proteins

2.9.1. Biosynthetic Labelling of Cells

Amino acid depleted (methionine), bicarbonate buffered, RPMI-1640 medium was prepared from a Selectamine Kit

(Gibco, Grand island, New York, USA). The medium was supplemented with 2.5% FBS and warmed to 37°C prior to use (labelling medium). The cells to be labelled were resuspended at 10^6 /ml in the labelling medium. 100 uCi of L(35 S)-methionine (21.83 mCi/ml; 1335 Ci/mmol; Radiochemical Centre Amersham; S.J. 235) was added per ml of the cell suspension and cultures were incubated at 37°C in an atmosphere of 7-10% CO₂ in air for up to four and half hours.

2.9.2. Analysis of Cell Associated and Secreted Proteins

In Vitro

Duplicate samples (25 ul each) containing putative L(35 S)-methionine labelled cells (2.5×10^4 ; Section 2.9.1) were resuspended gently and harvested at 0, 1 1/2, 3 and 4 1/2 hours of incubation and dispensed in one ml of cold PBS in 10 ml sterilin centrifuge tubes (Feltham, Middlesex, U.K.). The cells and corresponding supernatants were separated by centrifugation at 200 g for 10 minutes. The supernatants were transferred to other tubes containing bovine serum albumin (BSA, Fraction V; BDH Chemicals Ltd, Poole, England) as carrier protein, to a final concentration of 0.25 mg/ml. Acid insoluble proteins in the cell pellets and supernatants were precipitated with 10% trichloroacetic acid (TCA, Merck Darmstadt Germany). A stock solution of 20% TCA was added slowly while vortexing to an equal volume of the supernatant or, a 10% solution of TCA was added to the cell pellets. The

precipitates were collected on 0.45 um millipore filters (Millipore, S.A. Molsheim, France) and washed two times with a total of 20 mls of 10% TCA using a millipore manifold (Millipore Corporation, Bedford, Massachussets, USA). The filters were dried and immersed in four ml of Aquasol (New England Nuclear, Boston, Massachussets) and the radiolabelled proteins collected on the filters were counted in a Packard Tri-Carb liquid scintillation spectrometer. Results were expressed as mean counts per minute versus duration of labelling.

2.9.3. Preparation of Secreted Proteins for Analysis by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Aliquots of culture medium (500 ul) containing 5×10^5 biosynthetically labelled (L (^{35}S) methionine) cells (Section 2.9.1) were removed after gentle resuspension at 0 and three hours of labelling. The cell suspension was placed in 10 ml sterilin tubes and centrifuged at 200 g for three minutes. The supernatants were transferred to eppendorf tubes and proteins were precipitated by adding 500 ul of 20% TCA while vortexing to make a final concentration of 10% TCA. Precipitates were then pelleted by centrifugation in an eppendorf centrifuge (Eppendorf 5412; 10,000 g) for 10 minutes, and the supernatant was discarded in a radioactive container. A second wash of the precipitates was done in one

ml of cold acetone, by centrifugation in the eppendorf for two minutes. The samples were dried by a water pump or in an oven at 37°C. The TCA precipitated, acetone washed, secreted products were solubilised in equal volumes (50-100 ul) of reducing or non-reducing sample buffer (Appendix 1) by boiling at 100°C on a multi-blok heater (Lab-Line Instruments Inc. USA) until completely dissolved. Solubilised samples were analysed immediately or stored at -80°C until electrophoresis.

2.9.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Autoradiography of Cell Secreted Proteins.

Electrophoresis of L(³⁵S)-methionine labelled secreted proteins (Section 2.9.3) was performed on flat vertical slab gels, moulded on an apparatus which was commercially obtained from Albert Einstein College of Medicine, (Bronx, N.Y., U.S.A.). Details of the apparatus have previously been described by Studier (1973), O, Farrell (1975) and Jones (1980). The gel consisted of a lower gradient resolving and an upper stacking gel. The resolving gel was prepared using a gradient gel mixer (LKB 11300, Ultrograd). The concentration ranged from 7.5-17.5% acrylamide while the stacking gel was three percent acrylamide (Maizel, 1971; Appendix 1) unless stated otherwise. The ratio of acrylamide to N, N'-methylenebisacrylamide (Serva, Heidelberg, Germany) was

30/0.8 (Appendix 1). The polymerised gel had approximate measurements of 140mm x 125mm x 0.75 mm.

The upper and lower reservoirs of the gel apparatus were filled with sodium dodecyl sulphate (SDS) running buffer (Appendix 1; Laemmli, 1970) and samples (5-75 μ l) were carefully loaded using Hamilton pipettes into slots formed in the stacking gel by teflon combs. Electrophoresis calibration kit for low and high molecular weight proteins, purchased from Pharmacia (Pharmacia Uppsala, Sweden), and purified bovine IgM and IgG from Miles Laboratories (Miles, Naperville, USA), were included in every run.

The standard molecular weight proteins were sometimes labelled with ^{14}C -formaldehyde (4.6 mCi/ml; 14 mCi/mmol.; Radiochemical Centre Amersham; CFA 343; Rice and Means 1971). Samples were electrophoresed at 15 mA per gel until the bromophenol blue line reached the interface between the stacking and resolving gel. The current was then increased to 25 mA during separation in the resolving gel until the end of the run. After electrophoretic separation, the proteins were visualised by staining with 0.25% Coomassie Brilliant Blue R250 (Merck, Darmstadt, Germany, Appendix 1) at RT (Fairbanks et al., 1971) overnight (o/n). The gels were destained in 7.5% acetic acid (Appendix 1), dried on a slab gel dryer (Bio-rad Labs.) for 90 minutes, and autoradiographed using Kodak X-Omat AR films (Eastman Kodak Company) for varying amounts of time, depending on the amount of radioactivity in the gel.

2.10 Generation of Hypoxanthine, Aminopterin and Thymidine (HAT) Sensitive Cell Lines and Subsequent Fusion to Bovine B Cells

2.10.1. Generation of 8-Azaguanine (8AG) or 6-Thioguanine (6TG) Resistant Cell Lines

The method of Irigoyen et al. (1981) was used with some minor modifications for generation of 8-azaguanine (8AG) and/or 6-Thioguanine (6TG) resistant cell lines (Fig. 3). The cell lines to be selected were obtained at logarithmic phase of growth and seeded at 2×10^5 cells/ml in RPMI-1640 medium containing 10% dialysed FBS, and 2-Amino-6-hydroxypurine (8-Azaguanine; Sigma Chemical Co.), or 2-Amino-6-mercaptapurine (6-Thioguanine; Sigma Chemical Co.) ranging from 2.5 ug/ml to 40 ug/ml. The cells were incubated at 37°C in an atmosphere of 7-10% CO₂ in air for five days. Viable cells were determined daily by trypan blue dye test and examined within three minutes of staining. These preliminary studies were designed to determine if any cells were spontaneously resistant to either of the two purine analogues. Cell line(s) found to have some cells spontaneously resistant to either 8AG or 6TG, were expanded in medium containing either of the purine analogues while those cell lines not showing spontaneously resistant cells were subjected to irradiation treatment, in order to increase the frequency of mutation (Section 2.3). After irradiation, the cell suspension was

FIGURE 3 GENERATION OF 8-AZAGUANINE OR 6-THIOGUANINE
RESISTANT CELL LINES

- 1 LOG PHASE CELLS

- 2 MUTAGEN (CHEMICAL OR PHYSICAL)

- 3 EXPAND (2-3 POPULATION DOUBLINGS)

- 4 SEED IN INITIAL CONCENTRATION OF 8 AG OR TG AND
CULTURE FOR 3 DAYS

- 5 WASH IN MEDIUM WITHOUT DRUG

- 6 EXPAND IN MEDIUM WITHOUT DRUG (2-3 POP. DOUBLINGS)

- 7 NEXT LOG PHASE

- 8 SEED IN NEXT CONC. OF 8AG OR 6TG

- 9 REPEAT 1-8 UNTIL CELLS GROW IN 20-40 ug/ml OF 8AG
OR 6TG.

washed by centrifugation (200g for 10 min) and the supernatant discarded. The cells were then allowed to multiply in RPMI-1640 medium containing 15% non-dialysed FBS at 37°C in a 7-10% CO₂ incubator. At the next logarithmic phase of growth, the cells were seeded at 2×10^5 /ml of RPMI-1640 medium supplemented with 10% dialysed FBS at an initial concentration of 2.5 ug/ml of either 8AG or 6TG (Fig. 3). After incubation for three days as above, viable cells were recovered and washed once in complete growth medium and the population was expanded as before in RPMI-1640 medium containing 15% non-dialysed FBS without 8AG or 6TG (Fig. 3). Further selection and expansion of the cells in gradually increasing concentrations of the purine analogues (5, 10, 20 and 40 ug/ml) was continued until a concentration of 40 ug/ml of 8AG, or 20 ug/ml of 6TG was achieved (Fig. 3). Resultant cells were routinely maintained in medium containing 8AG (40 ug/ml) or 6TG (20 ug/ml) depending on the drug they were selected in.

2.10.2. Determination of Sensitivity of 8AG and/or 6TG

Resistant Lymphoblastoid Potential Fusion Partners to HAT Containing Medium

8-azaguanine and/or 6-Thioguanine resistant potential lymphoblastoid fusion partners, or mouse myeloma cell lines NS1 X63 or Sp2/0, were resuspended at $2-5 \times 10^5$ /ml in medium containing 15% FBS and hypoxanthine, aminopterin and thymidine

(HAT medium, Sigma Chemical Co., Appendix 1; Szybalska and Szybalski, 1962; Littlefield, 1964; Pearson et al., 1980). The concentration of aminopterin was sometimes varied from 4×10^{-7} M to 1×10^{-6} M while the concentrations of hypoxanthine and thymidine were always left constant at 10^{-4} M and 3×10^{-5} M respectively. The cells were maintained at 37°C in a 7-10% CO₂ incubator. Cell viability was assessed every two days for the next 14 days by staining the cells with 0.4% trypan blue dye and counting in an haemocytometer within three minutes.

2.10.3. Cell Fusion

Cells were fused in the following combinations using essentially the method of Kohler and Milstein (1975) as modified by Pearson et al. (1980). Mouse myeloma cells NS1, X63 or Sp2/0 with bovine PBM; 8 azaguanine resistant and HAT sensitive mouse x bovine hybrids with bovine PBM; 8-azaguanine and 6-Thioguanine resistant and HAT sensitive BL20 cells (ATS/BL20) with either bovine PBM or spleen cells. Bovine peripheral blood mononuclear cells (PBM) were isolated as in Section 2.2.1 and cultured in the presence of PWM (Section 2.4.1) for six days, before fusion. Dead cells and debris were removed over Ficoll-paque (7 mls of cell suspension: 3 mls of Ficoll-paque) by centrifugation at 250 g for 20 minutes. Viable PBM were recovered from the interface using a sterile pipette. Spleen cells or PBM from calves previously immunised

against SRBC (Section 2.4.3) were obtained for fusion on day three after the in vivo or in vitro booster respectively with the antigen. Continuously growing lymphoblastoid/ myeloma cell lines/clones fusion partners were obtained at logarithmic phase of growth. The cells to be fused were washed twice by centrifugation (200g for 10 min) in serum and hydroxyethyl piperazine ethane sulphonic acid (HEPES) free RPMI-1640 medium or PBS before fusion. In the final wash (3rd) the fusion partners were mixed at 1:1 or 1:2 ratio in 50 ml Falcon centrifuge tubes before pelleting as above. The medium was removed completely and the pellet was eased by gentle tapping of the tubes. Fusion was performed for one minute each using one ml each of polyethylene glycol one and two (PEG, 1550, Serva Feinbiochemica, Heidelberg, Germany or PEG 4000, Merck, Darmstadt, Germany and DMSO; Pearson et al., 1980; Appendix 1) prewarmed to 37°C. After fusion, the cell mixture was diluted slowly, to 50 mls with serum and HEPES free RPMI-1640 over a period of 10 minutes; and pelleted by centrifugation at 150g for 15 minutes. The fusion products were gently resuspended at the desired density in RPMI-1640 medium supplemented with 15% FBS, 25 mM Hepes, 5×10^{-5} M 2-ME, 100 units/ml penicillin, 100 ug/ml of streptomycin and 2 mM/ml L-glutamine (Fusion medium). The cell suspension was distributed in appropriate aliquots to each of the wells of Costar plates (96 or 24 well plates) in the presence or absence of feeder cells or growth factor containing culture supernatants (Section 2.6.3). After an overnight incubation at

37°C in an atmosphere of 7-10% CO₂ in air, about one-half of the fusion medium was removed and replaced with an equal volume of HAT medium with or without growth factor containing culture supernatants (Sections 2.4.2. and 2.6.3.). Selection of fused cells was continued by replacing the HAT medium every two or three days for the next 7-14 days. Thereafter the cells were expanded in hypoxanthine, thymidine (HT) medium (Appendix 1) for seven days and in fusion medium containing 15% FBS.

RESULTS

SECTION 1

ESTABLISHMENT OF CONDITIONS FOR OBTAINING ACTIVATED BOVINE B
LYMPHOCYTES EN ROUTE TO Ig SYNTHESIS AND SECRETION FOR FUSION.Introduction

A major requirement for monoclonal antibody production in any species is a reliable source of activated B cells en route to immunoglobulin synthesis and secretion for use as donor lymphocytes for fusion (Kohler and Milstein, 1975; Oi et al., 1978; Stevens et al., 1979; Goding, 1980; Volkman et al., 1981). The activated B lymphocytes can be obtained by in vivo or in vitro stimulation of spleen, lymph node, PBM, or tonsillar cells, etc., with antigen or mitogen. The donor cells must be fused to the lymphoblastoid/plasmacytoma cell fusion partner in order to obtain the maximum yield of antibody producing hybrids at a narrowly defined, although not always easily determined, time, following the activation of the cells with the antigen/mitogen (Olsson et al., 1983). The application of this principle allows many variations in the immunisation/activation schedules and the choice of anatomical location of lymphocytes harvested for fusion.

Induction of primary in vitro immune responses following antigenic activation of mouse cells has allowed for

in depth probing of the mechanisms underlying B-cell activation (Mishell and Dutton, 1967). Similar studies using antigen for in vitro activation of lymphocytes have recently been reported in the human system (Fauci and Pratt, 1976; Morimoto et al., 1981; Lane et al., 1982). Such in vitro systems, that are antigen specific and that require no polyclonal activators, yet can be easily reproduced by many laboratories, are only just emerging for domestic animals (Fillion et al., 1984). Efforts at ILRAD to obtain specific responses of bovine cells to in vitro activation with antigen have met with limited success (Lutje, personal communication), and it has generally been opted to induce B-cell responses with mitogens rather than antigens in order to obtain a large number of activated bovine B-cells for fusion for the first part of these studies.

Lymphocytes of several mammalian species have been shown to proliferate, and to be polyclonally activated when stimulated with a variety of agents. These agents include: plant lectins; e.g., concanavalin A (Con A); phytohaemagglutinin M (PHA-M); peanut agglutinin (PNA); Wheatgerm agglutinin (WGA) (Andersson et al., 1972; Pearson et al., 1979; Stadler et al., 1980; Waldmann and Broder, 1982), and constitutive elements of bacterial cell walls of Staphylococcus aureus Cowan 1 and Salmonella typhimurium (Dziarski et al., 1980; Jelinek and Lipsky, 1983).

In contrast to antigens, the above agents evoke lymphocyte proliferation on a non-immune or non-specific basis, and are known collectively as mitogens. In the mouse system,

mitogens such as Con A and PHA-M stimulate DNA synthesis in T-lymphocytes, and other mitogens such as PWM and LPS stimulate B lymphocytes (Andersson et al., 1972; Greaves and Janossy, 1972). Induction of DNA synthetic responses by stimulation of bovine PBM with mitogens has also been reported by Lazary et al. (1974b), Muscoplat et al. (1974a), Pearson et al. (1979). The interaction of lymphocytes in culture, and the kinetics of the reaction of mitogens with lymphocyte membranes, is complex. But generally, plant lectins react with specific carbohydrate moieties (Sharon and Lis, 1972), and presumably bind glycoproteins and glycolipids which constitute the external hydrophilic portion of cell membranes (Sharon and Lis, 1972). The degree of reaction of cultures varies with the area of the culture system (Leventhal and Oppenheim, 1967; Moorhead et al., 1967), the concentration of the lectin, and the ratio of lectin molecules to the lymphocytes. The time of peak response varies not only with the cell types but also with the particular lectin. Thus the establishment of optimal conditions for an assay of lymphocytes with lectins is necessarily empirical, and requires that attention be paid to these variables.

Pokeweed mitogen (isolated from Phytolacca americana, a poisonous plant) has been shown to induce polyclonal activation in human and mouse B-cells (Waldmann and Broder, 1982). Studies with this lectin have provided valuable information on the cellular interactions and immuno-regulatory events which affect humoral responses in the above species. It

has also been reported that PWM activated human B cells gave rise to immunoglobulin secreting hybrids when fused with either GM 4672 or RH-L4 or SKO-007 human lymphoblastoid cell lines (Schoenfeld et al., 1982; Olsson et al., 1983).

Methods which have been used to assay the effect of activators on B cells are: [125 I]UDR incorporation; enumeration of Ig-producing cells in a reverse haemolytic plaque assay (Fauci and Pratt, 1976); detection of cytoplasmic immunoglobulin in plasma cells by immunofluorescence (Gathings et al., 1977); incorporation of radiolabelled amino acid precursors into Ig-synthesizing and secreting cells (Gutman et al., 1978); Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of radiolabelled proteins (Saxon et al., 1977); enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) of secreted proteins (Saxon et al., 1977).

To define the conditions for activation of bovine B lymphocytes en route to Ig synthesis and secretion, bovine lymphocytes were cultured in vitro with PWM and sometimes with the particulate antigen, sheep red blood cells (SRBC) in combination with PWM. In other instances, PBM or spleen cells from animals previously primed with an antigen (SRBC) were further boosted with the same antigen in vitro. In this section, it is shown that, under appropriate culture conditions of cell density and PWM or antigen concentration (SRBC), bovine B lymphocytes can be driven to synthesize and secrete IgM and to a lesser extent IgG.

3.1 Yield of Bovine Peripheral Blood Mononuclear Cells (PBM) on Ficoll-Paque

Bovine peripheral blood obtained from the jugular vein yielded between 1 to 4×10^6 viable cells/ml of blood. The average yield was about 2×10^6 PBM cells/ml and the viability was usually above 95% as determined by trypan blue dye exclusion. The PBM yield per ml of blood varied from bovine to bovine and also from a single bovine on different days of isolation.

3.1.1 Cell Types Present in Bovine PBM

Peripheral blood mononuclear cells from 11 bovines were stained in suspension by the indirect immunofluorescence method using MAb P5 which detects bovine T cells, MAb B5/4 which reacts with an epitope on bovine IgM, MAb A₂ which reacts with an epitope on bovine IgG₁ and IgG₂ and MAb P8 which detects bovine macrophages. Stained cells were analysed on a FACS II.

The results recorded in Table 3, and Fig. 4 (representative histogram) show that cells expressing the T cell marker P5 ranged between 25-56% of total PBM. Monoclonal antibody P5 defined two T-cell subpopulations on the basis of fluorescence intensity; the so called P5^{h1} and P5^{low} subpopulations. The percentage of bovine peripheral blood cells expressing the B-cell marker B5/4 was found to be in the

range of 7-30% (Table 3, representative histogram Fig. 4). The macrophage population as determined by staining with MAb P8 ranged from 1-4% (Table 3, representative histogram Fig. 5). The percentage of PBM which could be stained with MAb A₂ was between 1-4% (Table 3, representative histogram Fig. 5). Between % and 73% of total PBM could be identified by the antibodies. The remainder may belong to the null cell population. The method of isolation of PBM excluded polymorphonuclear leukocytes which by Giemsa-staining of air-dried methanol-fixed smears of isolated PBM constituted less than one percent of the total population.

3.1.2 Pokeweed Mitogen (PWM) Induced Proliferative Response of Bovine PBM

PBM were cultured in complete RPMI-1640 medium at concentrations ranging from 3×10^5 cells/ml to 5×10^6 cells/ml at 37°C in a 7-10% CO₂ in air atmosphere for 1-6 days. Pokeweed mitogen was added to the different cell densities to make final concentrations of 0.625 ug/ml to 10 ug/ml. At daily intervals, the cells in duplicate wells were pulsed with 0.5 uCi [¹²⁵I]UDR and after incubation for another 4-6 hours, the cells were harvested onto filters using a Titertek Cell Harvester and the radiolabel associated with the filters was counted in a Packard autogamma spectrometer. As shown in Fig. 6; 2.5×10^6 cells/ml in combination with 2.5 ug/ml of PWM gave the maximum incorporation of [¹²⁵I]UDR,

indicative of a proliferative response, on day four of stimulation. All other culture conditions on different days gave comparatively lower responses (Figs. 7 and 8).

TABLE 3. Percent of Bovine Peripheral Blood Mononuclear Cells (PBM) Stained by Different Antibodies Recognising Different Cell Subpopulations.

Animal Number	Antibody Designation				Total % of Cells Identified
	B5/4	A2	P5	P8	
C440	22	4	32	1	59
C279	30	3	25	2	60
C705	12	2	34	4	52
C706	8	2	49	4	63
C711	15	3	29	1	48
C712	23	1	40	1	65
C713	14	1	48	1	64
C714	23	2	28	1	54
C717	7	1	42	1	51
C719	19	1	30	1	51
C720	15	1	56	1	73

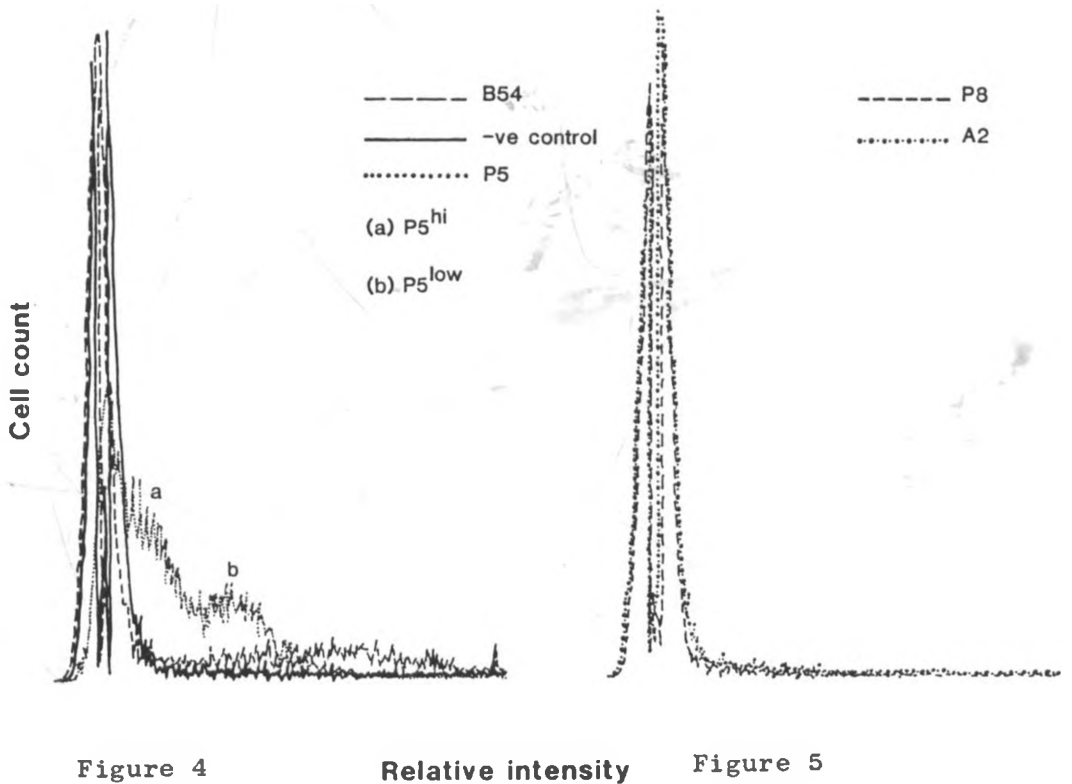


Figure 4 Relative intensity Figure 5

Representative Histograms of Bovine PBM Stained with Antibody B5/4 or P5 or Normal Mouse Serum as Negative Control (Figure 4; a and b Represent the Biphasic Populations of P5^{hi} and P5^{low} Cell Subsets Respectively), and P8 or A₂ (Figure 5).

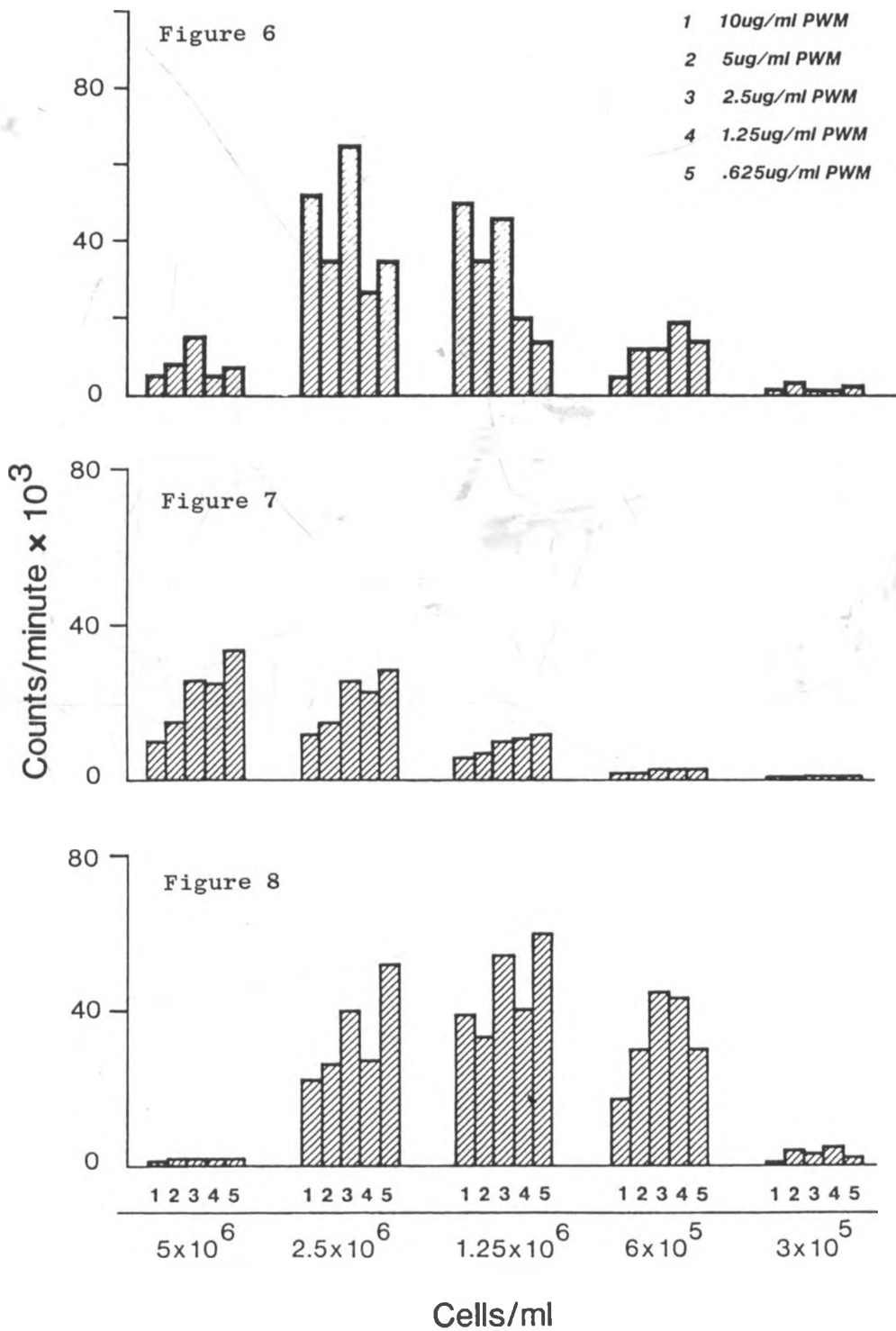
When similar studies were conducted on PBM from 11 bovines and examined on day four of culture, the PBM responses were found to be of variable magnitude (Fig. 9). Control cultures established without PWM registered less than 100 cpm in all experiments (Fig. 9). We concluded from these studies that 2.5×10^6 PBM cells/ml in combination with 2.5 ug/ml of PWM gave the best proliferative response of the cells on day four of culture.

The in vitro response of bovine PBM cultures in the presence of 2.5 ug/ml of PWM and 4×10^{-7} M aminopterin was examined for 1-6 days. The results are shown in Fig. 10. The incorporation of [125 I]UDR by the cells was reduced to about 25% in the presence of the drug as compared with cells in control cultures with PWM alone (Fig. 10).

3.1.3 Pokeweed Mitogen Induced Ig Synthesis by Bovine PBM

Peripheral blood cells obtained over Ficoll-Paque were cultured for 1-8 days in complete RPMI-1640 medium at a concentration of 2.5×10^6 cells/ml in combination with 2.5 ug/ml of PWM. At daily intervals, cells were collected from the cultures and examined, after staining of fixed smears, for the presence of cytoplasmic IgM (cIgM) using MAb B5/4, or the presence of cIgG using MAb A₂, or the presence of cIgA using FITC-conjugated rabbit anti-bovine IgA.

Cells with cIgM and to a lesser extent cIgG were observed to arise about day three of culture (Table 4). The



Incorporation of (125 I)UDR by Different Densities of PBM
 During Stimulation with Varying Concentrations of PWM.

Day Four (Figure 6); Day One (Figure 7); Day Six (Figure 8).

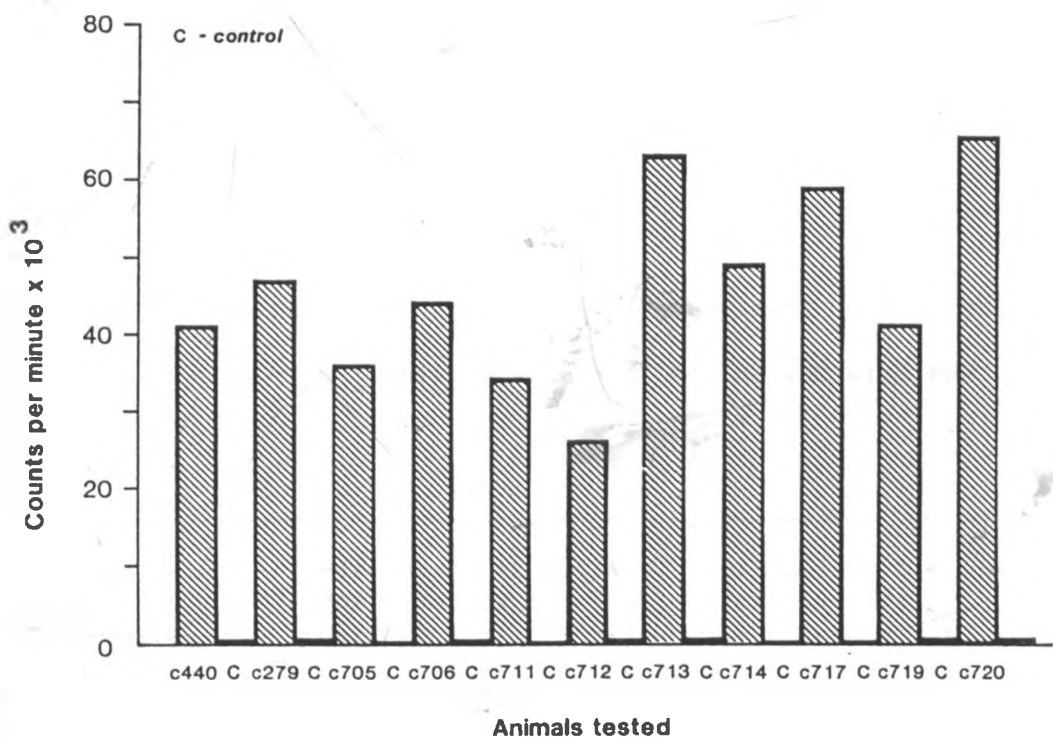


Figure 9. Incorporation of (¹²⁵I)UDR by PBM from 11 Bovines on Day Four of Culture with PWM.

percentage of cIgM positive cells increased from 11% on day three of culture to 20% on day eight (end of culture; Table 4; Fig. 11). Between 2 and 5% of the cells examined

TABLE 4. Kinetics of Appearance of Cytoplasmic IgM (cIgM) During In Vitro Culture of Bovine PBM with PWM.

Day of Culture	Percentage of Cells Positive for Surface IgM	Percentage of Cells Positive for Cytoplasmic IgM
1	20	0 (-)
2	20	0 (-)
3		11 (+)
4		10 (+)
5		15 (++)
6		15 (+++)
7		17 (++++)
8		20 (++++)

A minimum of 50-100 cells were counted per sample.

(-) and (+) denote degrees of fluorescence

(-) Non-fluorescent

(+) Weakly fluorescent

(++) Moderately fluorescent

(+++ Strongly fluorescent

(++++ Very strongly fluorescent

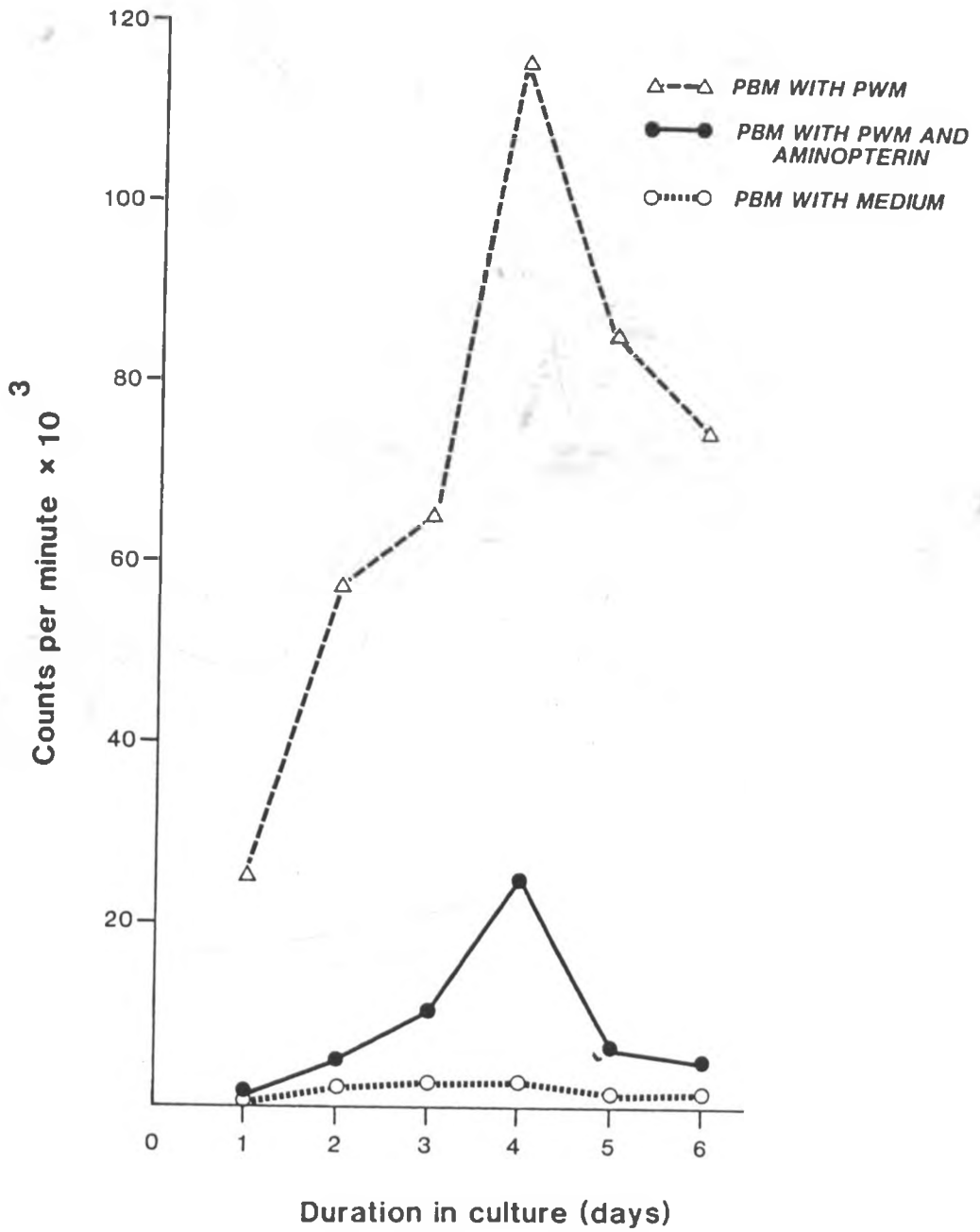


Figure 10. Incorporation of (^{125}I)UDR by Bovine PBM Cultured in the Presence of PWM and Aminopterin.

expressed cIgG starting around day three until the end of culture period (day 8), with no apparent increase in the percentage of positive cells. No cIgA positive cells were observed under this culture condition. The intensity of fluorescence of cIgM and cIgG positive cells also increased with increased time in culture (Table 4). Results from similar studies for expression of cIgM in PBM from 11 bovines after six days of culture with PWM are shown in Table 5. Different bovines responded differently in the expression of cIg with ranges varying from 2.5-20%. When PBM from the same bovines were examined three weeks later, the percentage of cIgM positive cells varied from 1-20% (Table 5). Cytoplasmic IgG positive cells ranged from 0-5% (data not shown).

3.1.4 Pokeweed Mitogen Induced Protein Synthesis and Secretion by Bovine PBM in vitro

Bovine PBM in PWM cultures (1-8 days) were harvested daily and cultured in methionine depleted, bicarbonate buffered RPMI-1640 medium, containing 2.5% FBS and 100 uCi/ml of L-[³⁵S]-methionine. Cultured cells and their corresponding supernatants were harvested at one and half hour intervals for four and half hours, and macromolecules were precipitated with TCA. The precipitates were collected on millipore filters and the filters were dried and counted in a Packard Tri-Carb liquid scintillation spectrometer. Incorporation of L-[³⁵S]-methionine by cells was linear over the four and

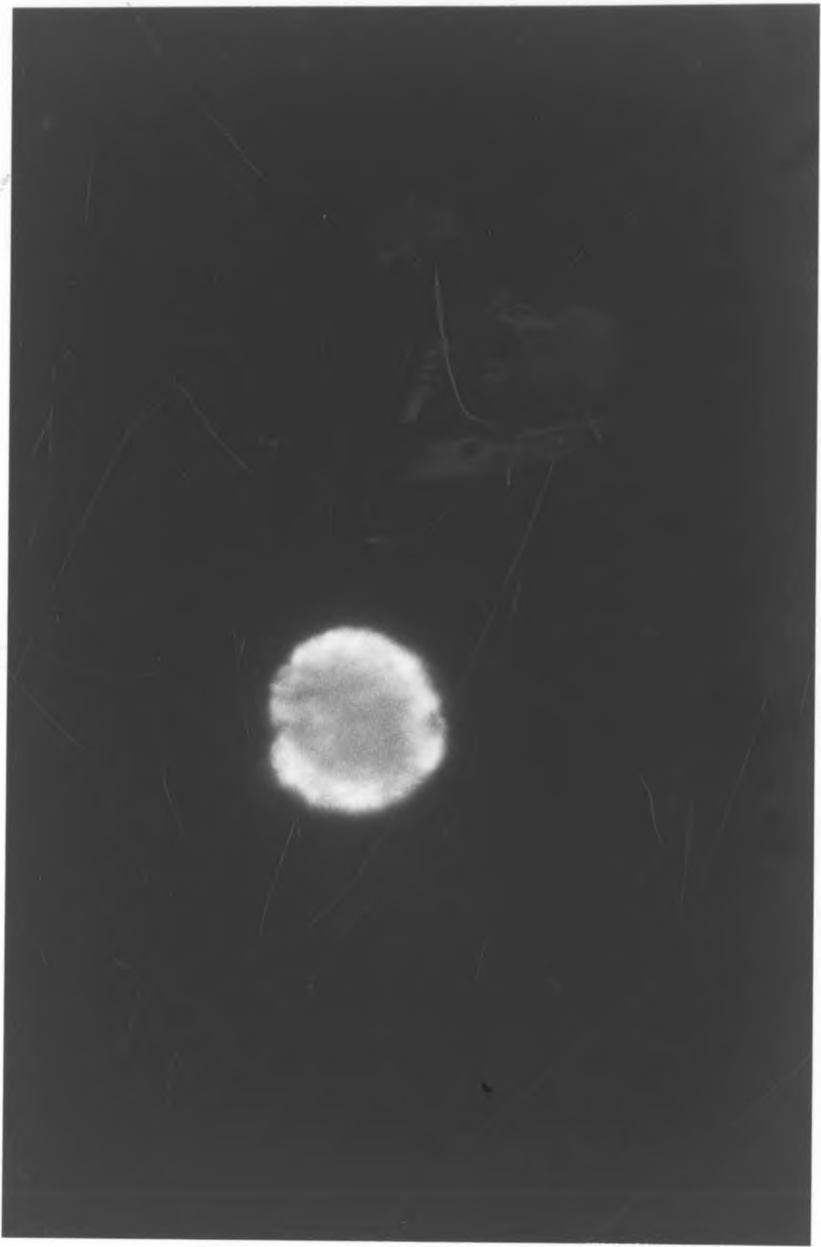


Figure 11. A Plasma Cell in Bovine PBM Cultured in the Presence of PWM and Stained with MAb B5/4 for Intracellular IgM.

half-hour period. Maximum incorporation of the radiolabel by the cells was seen on day four of culture (results for only days 1, 4 and 8 are shown in Fig. 12). L-[³⁵S]-methionine containing protein was released into the culture medium by the cells (only days 1, 4 and 8 shown in Fig. 12) in a linear

TABLE 5. Expression of cIgM by PBM From 11 Bovines During Culture with PWM for Six Days

Animals Tested	1st Test		2nd Test (3 weeks later)	
	Percent resting PBM +ve for sIgM	Percent blasted PBM +ve for cIgM	Percent resting PBM +ve for sIgM	Percent blasted PBM +ve for cIgM
c 440	27	20	29	20
c 279	30	13	36	3
c 705	10	8	14	2.5
c 706	20	7.5	9	9
c 711	26	12	23	6
c 712	18	4	17	15.5
c 713	11	2.5	11	4.5
c 714	18	16	15	2
c 717	8	11	10	2.5
c 719	12	7	18	1
c 720	10	16	17	8.5

manner. Culture medium obtained on day four of culture contained apparently more radiolabelled TCA precipitable molecules than on any other days (Fig. 12). Viability of the cells remained constant throughout the four and half hour labelling period.

3.1.5 Analysis of Culture Supernatants by SDS-PAGE and
Autoradiography for the Presence of L(³⁵S)-Methionine
Labelled and Secreted Igs

Aliquots of culture medium containing 5×10^5 biosynthetically labelled cells (L(³⁵S)-methionine) were removed after gentle resuspension at 0 and three hours of labelling. The cells and corresponding supernatants were separated by centrifugation. The macromolecules in the supernatants were TCA precipitated and washed in cold acetone before drying. The supernatant precipitates were solubilised in reducing or non-reducing sample buffer by boiling. Electrophoresis of solubilised samples was performed on flat vertical slab gradient resolving gels with concentrations ranging from 7.5-17.5% acrylamide using the buffer system of Laemmli, (1970).

After electrophoretic separation, the proteins were visualised by staining with Coomassie Brilliant Blue R250. The gels were dried and autoradiographed for varying lengths of time depending on the amount of radioactivity in the gels.

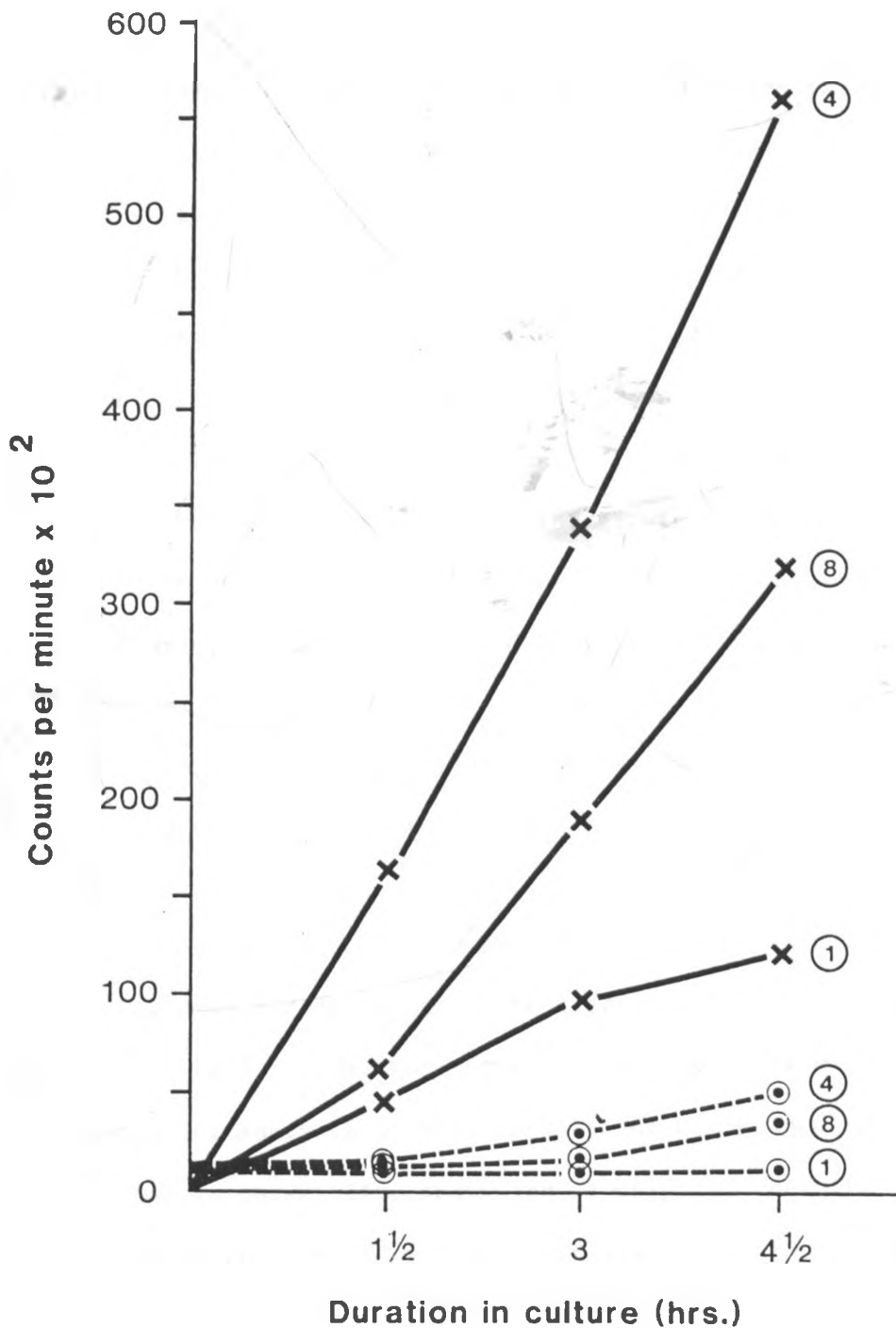


Figure 12. L(³⁵S)-Methionine Incorporation and Release into Culture Supernatants by PBM During Culture with PWM; Solid Lines Represent Incorporation by the Cells and Broken Lines Represent the Release of Radiolabelled Molecules into the Supernatant. 1, 4 and 8 are Days of Examination.

Bands of suspected IgM (μ) and IgG (γ) heavy chains (M.Wt. 75K and 55K respectively) light (L) and J-chains (M.Wt. 25K and 14K respectively) were noticed in the supernatants (Fig. 13) starting on day two or three. The bands appeared to increase in intensity until day eight (end of culture). Other radiolabelled molecules which were not identified were also visualised in the supernatants (Fig. 13). These results lent support to the findings of cIg staining, whereby bovine PBM cultured in the presence of PWM started to show cIg positive cells from about day three till day eight of culture.

3.1.6 Induction of Direct Plaque-Forming Cells to SRBC by Bovine PBM Activated with PWM

Bovine PBM (2.5×10^6 /ml) were cultured with SRBC (2.5×10^6 /ml) and different concentrations of PWM ranging from 0.040 to 2.5 ug/ml for 1-8 days. Viable cells were harvested for analysis of SRBC-specific direct plaque forming cells on days three, five and seven of culture. Results presented in Table 6, show that 0.156 and 0.625 ug/ml of PWM in combination with 2.5×10^6 SRBC/ml gave comparatively higher numbers of plaque forming cells on day five of culture. The plaques were also clearer and larger as compared to all other culture conditions (not shown). The number of plaque forming cells was still high (but lower than day five) on day seven of culture under similar conditions of culture. There was very little difference in the number of plaque forming cells in

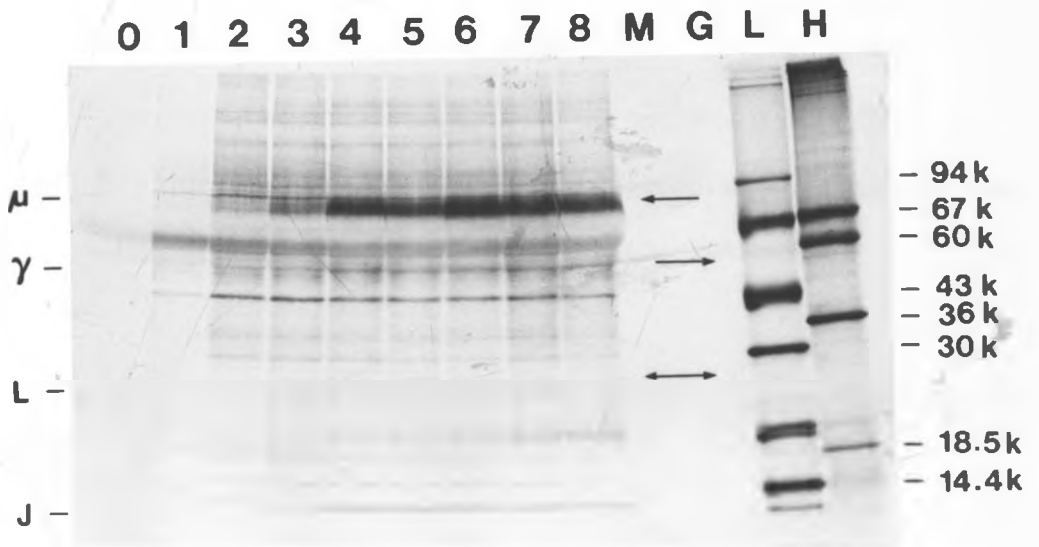


Figure 13. SDS-PAGE and Autoradiography of L(³⁵S)-Methionine Labelled and Secreted Proteins in Culture Supernatants of PBM Cultured with PWM for 0-8 Days. (M) Purified Bovine IgM; (G) Purified Bovine IgG; (L) Low Molecular Weight Standard; (H) High Molecular Weight Standard; The Numbers at the Right Hand Side Represent Approximate Molecular Weights in 10³ Daltons. Arrows Indicate the Positions of the μ and γ Heavy Chains and the L-Chains.

cultures with and without SRBC when 2.5 ug/ml of PWM was used (Table 6). No plaque forming cells were detected on days three, five and seven when the PBM were cultured with SRBC alone, and no background plaques were observed (Table 6).

TABLE 6. Comparison of Different Concentrations of PWM to Induce Direct Plaque Forming PBM to SRBC

Culture Conditions	Plaque Forming cells/5 x 10 ⁵ PBM		
	Day 3	Day 5	Day 7
0.040 ug/ml PWM + 2.5 x 10 ⁶ SRBC/ml	28	30	5
0.156 ug/ml PWM + 2.5 x 10 ⁶ SRBC/ml	34	100	60
0.625 ug/ml PWM + 2.5 x 10 ⁶ SRBC/ml	27	110	85
2.5 ug/ml PWM + 2.5 x 10 ⁶ SRBC/ml	20	60	40
2.5 ug/ml PWM; No SRBC/ml	25	45	30
No PWM; 2.5 x 10 ⁶ SRBC/ml	-	-	-
PBM alone	-	-	-

3.1.7 Induction of Specific Direct Plaque Forming Cells to SRBC by Bovine PBM After Subcutaneous and Intrasplenic Immunisation (conducted with Ms. V. Lutje)

Cattle with exteriorised spleens were immunised

against SRBC (10^{11}) by subcutaneous injection at several sites along the back. Two months later, a booster injection of the same dose of antigen was injected into the spleen.

Peripheral blood cells from the immunised bovines were isolated 3 and 9 days after the booster injection and cultured at 2.5×10^6 cells/ml in the presence of 2.5×10^6 SRBC/ml.

Peripheral blood mononuclear cells obtained on day 3 after the secondary intrasplenic immunisation gave 17 plaques on day 5 of culture with SRBC for every 5×10^5 cells. But when the PBM were isolated on day 9, stimulated in vitro with SRBC and examined on days 5, 7, 9 and 13 for plaque formation more than 200 plaque forming cells were recorded on each of the test days (Table 7). The results suggested that in vitro antibody response to SRBC could be induced only if the PBM came from bovines primed to the same antigen.

TABLE 7. Direct Plaque Forming Bovine PBM to SRBC, After Subcutaneous and Intrasplenic Immunisation, Followed by In Vitro Culture of PBM with SRBC.

Culture Conditions (cells/ml)	Plaque Forming cells/ 5×10^5 PBM			
	Day 5	Day 7	Day 9	Day 13
2.5×10^6 PBM + 2.5×10^6 SRBC	200	200	200	200

Taken together, the studies demonstrate that in vitro activation of bovine PBM with PWM or in vitro activation of bovine PBM with SRBC in combination with a low dose of PWM (0.625 ug/ml), or in vivo antigen (SRBC) primed bovine followed by in vitro boosting of spleen cells or PBM from the same bovines contained appropriately activated B cells for fusion studies.

RESULTS

SECTION 2

GENERATION OF HAT SENSITIVE BOVINE LEUKAEMIA CELL LINES FOR
FUSION TO BOVINE LYMPHOCYTESIntroduction

The plasma membrane of almost any two cell types can be made to fuse by the addition of Sendai virus inactivated by UV irradiation or β -propiolactone (Graham 1971; Tarkowski and Balakier, 1980) or by addition of polyethylene glycol (Kao and Michayluk, 1974; Eglitis, 1980), or lysolecithin (Wakahara, 1980), or polyarginine (Bennet and Mazia, 1981a, b), or by electric field induction (Richter et al., 1981; Berg et al., 1983). The fusion of plasma membranes results in the formation of heterokaryons which possess two or more nuclei. At the next division, the nuclei fuse and a hybrid cell results. When tumour cells are fused with normal cells, the hybrids which are formed can be rapidly overgrown by unfused tumour cells. It can however, be ensured that only hybrids will grow by using the strategy devised by Littlefield (1964), which depends on complementarity between the fused cells to permit DNA synthesis.

Mammalian cells have two pathways for DNA biosynthesis; the main classical or de novo pathway, and the alternative or salvage or scavenge pathway (Fig. 14). The de novo biosynthetic pathway for purines, pyrimidines, and glycine, can be blocked by the folic acid antagonist, aminopterin, (Littlefield, 1964), which inhibits dihydrofolate reductase. Cells treated with aminopterin can however still synthesize DNA via the salvage or scavenge pathway (Fig. 14), in which preformed nucleotide bases, like thymidine and hypoxanthine, are recycled via the enzymes thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl transferase (HGPRT). If one of these enzymes is absent, DNA synthesis stops. Transformed cell lines which do not have the enzyme TK or HGPRT, and hence do not have a nucleotide pathway, can be obtained by selection.

Mutant HGPRT negative cells can be obtained by growing the cells in medium containing a guanine analogue like 8-azaguanine (8AG) or 6-Thioguanine (6TG) (Beaudet et al., 1973). The drug is incorporated into the cells via the enzyme HGPRT and is converted to the ribonucleotide, which is a potent inhibitor of 5-phosphoribosyl-1-pyrophosphate transferase, an enzyme that is essential to the biosynthesis of both purine and pyrimidine nucleotides, as well as for the conversion of inosinic acid to adenylic and xanthylic acids, which are precursors of guanylic acid (Fig. 14). The net result is the prevention of DNA synthesis, which results in cell death.

Thymidine kinase (TK) negative mutant cells can also be produced by the use of toxic drugs like 5-bromo-2'-deoxyuridine (BrdU) (Kit et al., 1963). But such cells are much more difficult to select because two simultaneous rare mutational events are required.

Hybrid cells derived from the fusion of transformed cells which lack HGPRT and normal cells (PBM, spleen, lymph node etc), can be selected in medium containing hypoxanthine, aminopterin and thymidine (HAT). Although aminopterin blocks de novo DNA synthesis, the capacity to utilise hypoxanthine and thymidine via the salvage/scavenge pathway is reintroduced at fusion, with the gene encoding HGPRT, from normal cells.

Only certain HAT sensitive, transformed cell lines, can be fused to antigen or mitogen activated B cells to obtain immunoglobulin production. Fusion of mouse myeloma cells to mouse spleen cells results in Ig production but not expression of the T cell marker Thy-1 (Kohler et al., 1977). In contrast, when mouse thymoma cells are fused to mouse spleen cells, there is no Ig production but the Thy-1 marker is expressed (Hammerling, 1977). When fibroblasts, which are ontogenetically less related to the lymphoid cell lineage, are fused to thymoma ~~of~~ myeloma cells, there is no expression of Thy-1 nor any Ig production, (Hyman and Kelleher, 1975; Coffino et al., 1977). These experiments would point to a simple rule for function rescue fusions. That is, only tumor lines ontogenetically closely related to the normal cell fusion

partners are able to express the specialised function of normal cells.

Another type of restriction in cell fusion has been obtained from phylogenetic studies. In general, intraspecies specific hybrids are more successful (Kohler and Milstein, 1975; Kohler et al., 1977; Galfre et al., 1977) than interspecies hybrids, because the rate of chromosome loss is lower (Croce et al., 1980).

Two bovine lymphoblastoid cell lines have been described. These are designated BL3 (Theilen et al., 1968) and BL20 (Morzaria et al., 1982). BL3 cell line was originally isolated from the lymph node of a cow and was established in culture by Theilen et al. (1968). The authors never indicated whether the cell line was derived from a case of sporadic bovine leukosis or enzootic bovine leukosis. The cells resembled neoplastic lymphocytes and were aneuploid in constitution. Electron microscopic studies failed to reveal any viral particles (Theilen et al., 1968).

BL20 cells were adapted to culture by Morzaria et al. (1982). The cells were from the bronchial lymph node of a four month old heifer calf suffering from sporadic bovine leukosis in its multicentric form. No viral agent was detected in the cell line. Ultrastructural morphology was characteristically that of an actively multiplying lymphoblastoid cell line. The round or oval BL20 cells had several lamellipodia on their surface, and the cytoplasm contained scanty rough endoplasmic reticulum and abundant free ribosomes with a tendency to form

polysomes. The Golgi complexes were prominent and active with numerous budding vacuoles and associated lamellae. Karyotype analysis of BL20 cells showed the normal female bovine diploid chromosome complement (Morzaria et al., 1982). Surface Ig was not detected using the reagents available then, nor did the cells carry receptors for peanut agglutinin implying that they were neither B- nor T-cells. The mode of transformation in both BL3 and BL20 was not established.

To reiterate further, some of the major considerations in choosing a cell line for hybridization with immune B cells are: the presence of drug resistant markers for selection against unfused cells, lack of production of undesired Ig heavy and light chains by the fusion partner, and the stability of the hybrids formed by fusion with the chosen cell line. Other characteristics, which are not minor by any means, and which are desirable for a fusion partner are: a high hybridization frequency, a high yield of antigen-specific hybridomas, a high level of Ig secretion, a high cloning efficiency, and a short division time (Nilsson and Ponten, 1975).

This section describes the selection of 8-azaguanine and/or 6-Thioguanine resistant and aminopterin sensitive BL3 or BL20 cells and their use as bovine fusion partners. The selected cells obtained were hybridized with bovine lymphocytes derived from in vitro PWM cultures or in vivo antigen-primed (SRBC) bovine spleen cells or in vitro SRBC boosted bovine PBM from bovines previously immunised with the antigen to obtain intraspecies specific hybridomas.

Figure 14. PURINE METABOLISM IN B AND T CELLS

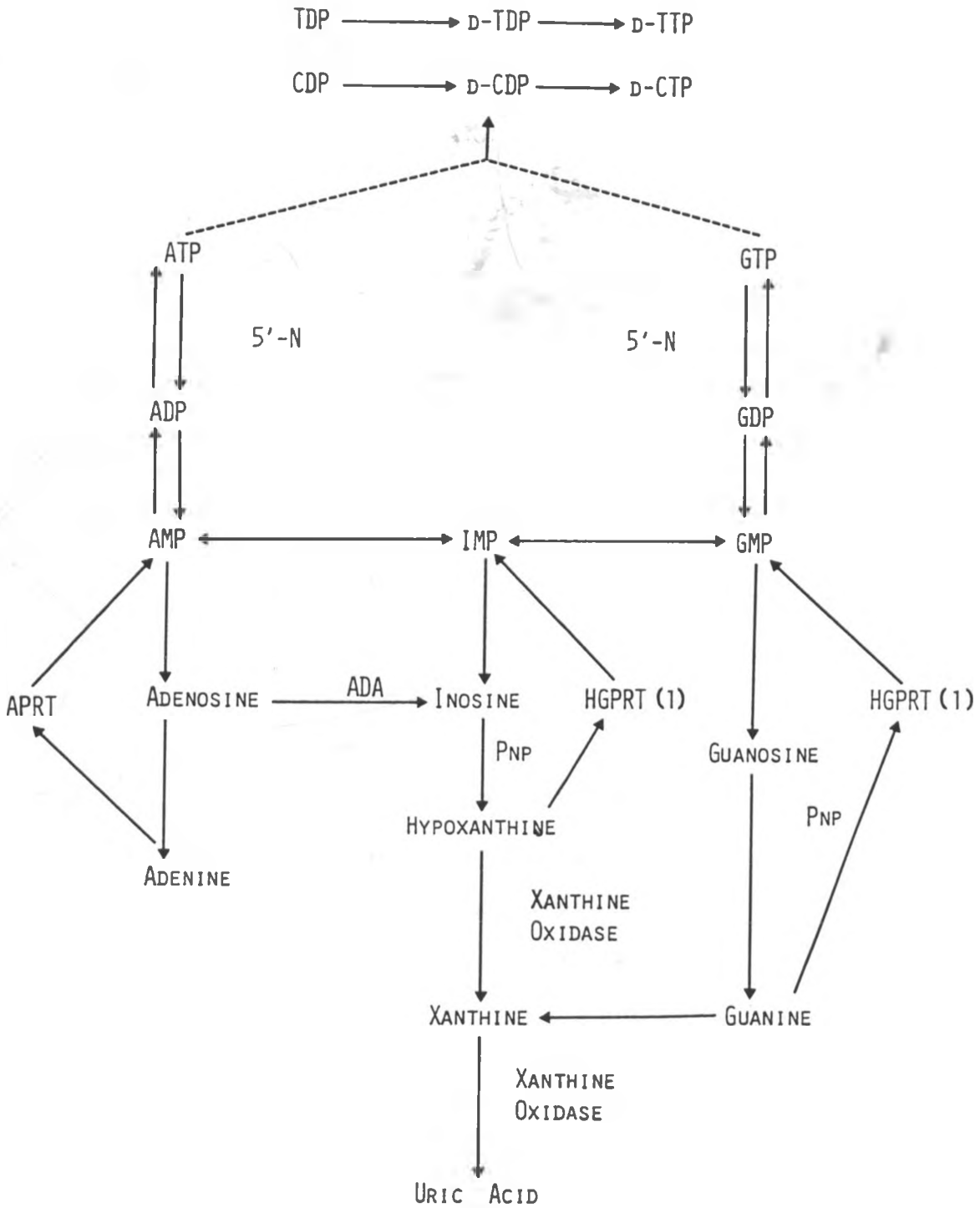


FIGURE 14 Purine Metabolism in B and T Cells, Showing (1)
the Sites of Inhibition by Agents Blocking the
Salvage Pathway of DNA Synthesis.

TDP	-	thymidine diphosphate;
CDP	-	cytidine diphosphate;
d-TDP	-	deoxythymidine diphosphate;
d-CDP	-	deoxycytidine diphosphate;
d-TTP	-	deoxythymidine triphosphate;
d-CTP	-	deoxycytidine triphosphate;
ATP	-	adenosine triphosphate;
ADP	-	adenosine diphosphate;
GTP	-	guanosine triphosphate;
GDP	-	guanosine diphosphate;
AMP	-	adenosine monophosphate;
IMP	-	inosine monophosphate;
GMP	-	guanosine monophosphate;
5'-N	-	5'-nucleotidase;
NK	-	nucleotide kinase;
APRT	-	adenosine phosphoribosyl transferase;
ADA	-	adenosine deaminase;
HGPRT	-	hypoxanthine-guanine phosphoribosyl transferase;
PNP	-	purine nucleoside phosphorylase.

3.2 Growth of BL3 and BL20 Cell Lines

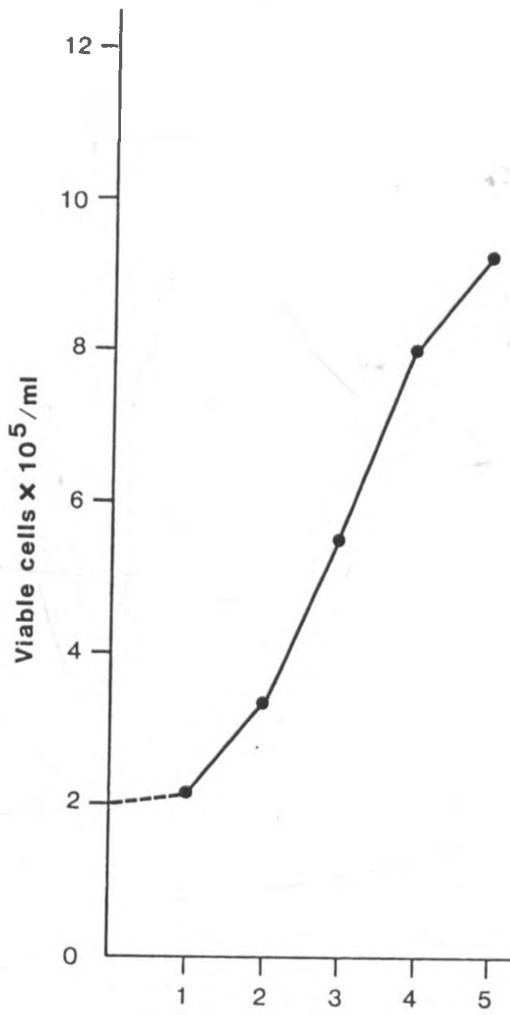
Bovine leukaemia cell lines BL3 and BL20 were seeded at an initial density of 2×10^5 cells/ml of either L15 or RPMI-1640 medium respectively supplemented with 10-15% FBS. The cell suspensions in L15 medium were incubated at 37°C without CO₂ gas while those in RPMI-1640 were incubated at the same temperature in the presence of a 7-10% CO₂ gas in air atmosphere. BL3 and BL20 cell lines were observed to multiply vigorously and had doubling times of between 18-24 hours. A maximum density of between $8 \times 10^5 - 1 \times 10^6$ cells/ml was achieved by BL20 cells in about 4-5 days of culture (Fig. 15) while BL3 cells obtained a peak density of between 1×10^6 to 1.2×10^6 cells/ml at about the same time of culture (Figure 16). Viability of these cell lines was always above 90% as determined by trypan blue dye staining. BL20 cells were observed to grow as single cell suspensions while BL3 cells tended to grow in clumps.

3.2.1 Expression of Different Surface Antigens on Parental BL3 and BL20 Cell Lines

BL3 and BL20 cells were collected at the logarithmic phase of growth. The cell suspensions were dispensed in U or V shaped wells of the 96-well plate at about 2.5×10^5 cells/well and incubated in RPMI-1640 medium containing 10% NRS

at 37°C for 1 hour to block non-specific or Fc mediated binding of antibodies.

The cells were stained in an indirect immunofluorescence assay as described in Section 2.8.2 using a panel of antibodies listed in Table 2. Stained cells were examined using a FACS II, immediately after staining or after storage in 2% formalin solution at 4°C overnight. Monoclonal antibody B5/4 which detects an epitope on bovine IgM stained over 95% of BL20 cells (Fig. 17; Table, 8) and none of BL3 cells (Fig. 18; Table 8). The antibody A₂ which reacts with an epitope on bovine IgG₁ and IgG₂ did not stain either of the two cell lines (Fig. 17 and Fig. 18 Table 8). A bovine T-cell marker MAb P5, was expressed on about 70% of BL20 cells (Fig. 19 Table 8), but none of the BL3 cells reacted with this antibody (Table 8). Other T-cell markers, such as IL-A12, which detects the BoT4 chain on class II restricted T-cells, and IL-A17 which detects the BoT8 chain on class I restricted T-cells, did not stain either BL20 or BL3 cell lines (Fig. 20 representative histogram; Table 8). The presence of bovine histocompatibility antigens on BL20 and BL3 cell lines was shown by staining with MAbs IL-A3 and IL-A7. These antibodies detect different polymorphic epitopes on bovine leucocyte antigens (BoLA) class I. Monoclonal antibody IL-A3, stained over 90% of BL20 cells (Fig. 21; Table 8) and none of BL3 cells (Fig. 23; Table 8). But MAb IL-A7 failed to stain either the BL20 or BL3 cells (Figs. 22 and 23 respectively; Table 8).



Duration in culture (days)

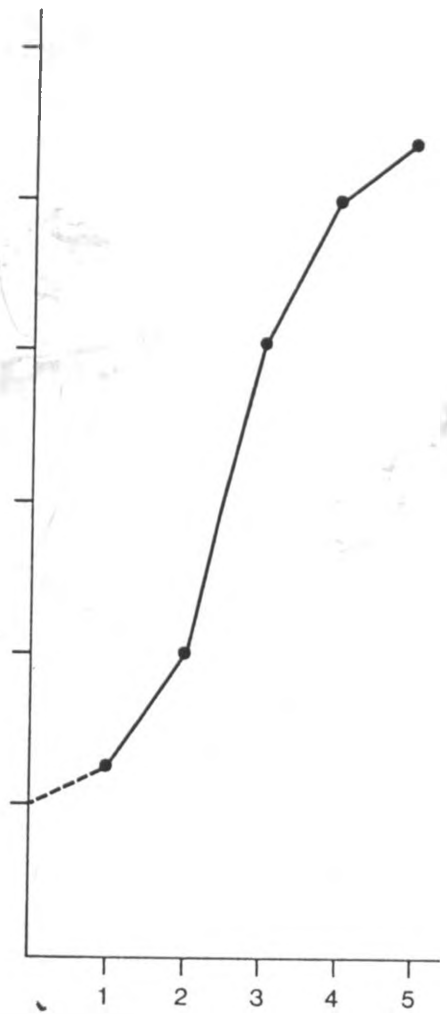


Figure 16

Figure 15

Growth of BL20 Cells in Complete RPMI 1640 Medium (Figure 15) and BL3 Cells in Complete L15 Medium (Figure 16).

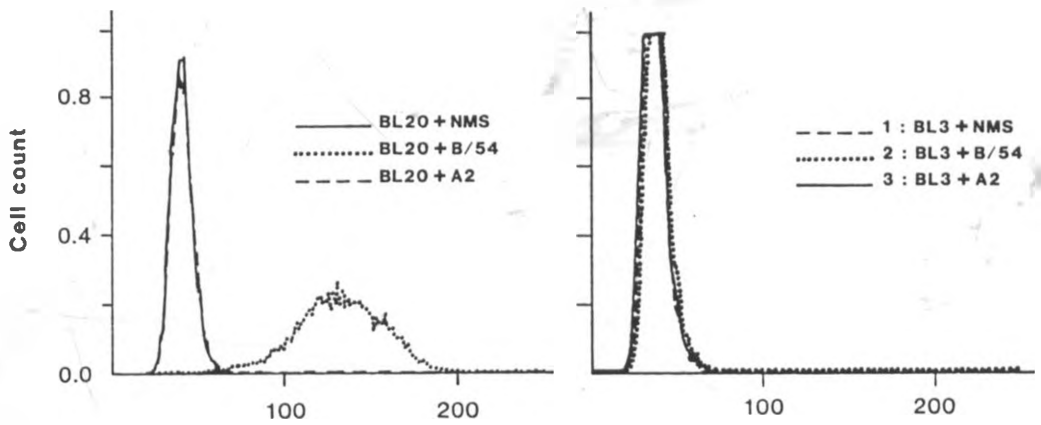


Figure 17

Relative intensity

Figure 18

Histograms of BL20 Cells Stained with Antibody B5/4 or A₂ (Figure 17) and BL3 Cells Stained with Antibody B5/4 or A₂ (Figure 18). In Each Case the Cells Were Stained with Normal Mouse Serum (NMS) as Negative Control.

Table 8 Expression of Different Surface
Antigens on Parental BL3 or BL20
Cell Lines (% +ve Cells)

Antibody Designation	BL3	BL20
B5/4	0	95%
A2	0	0
P5	0	70
IL-A12	0	0
IL-A17	0	0
IL-A3	0	90
IL-A7	0	0

3.2.2 Expression of Cytoplasmic Igs by BL3 and BL20 Cell Lines

The cells to be stained i.e. BL3 and BL20 were resuspended in 100% NRS. Thin smears of the cell suspension were made on coverslips and allowed to dry at room temperature. After fixation in a solution of ethanol/acetone, the fixative was allowed to dry and the cells were stained with monoclonal antibodies B5/4 and A₂, as described in Section 2.8.3, to reveal the presence of bovine IgM or IgG respectively.

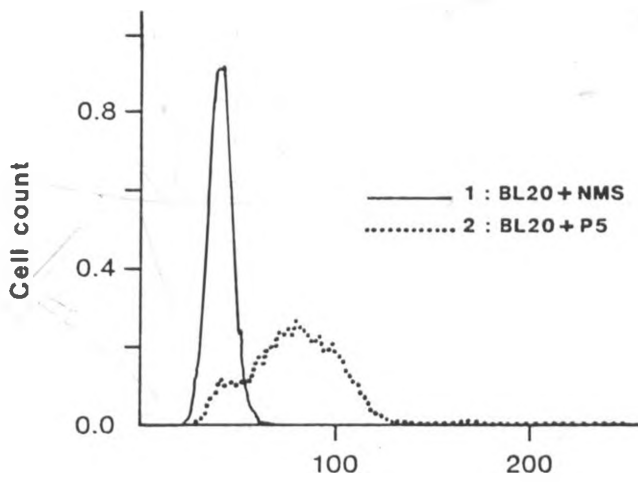


Figure 19

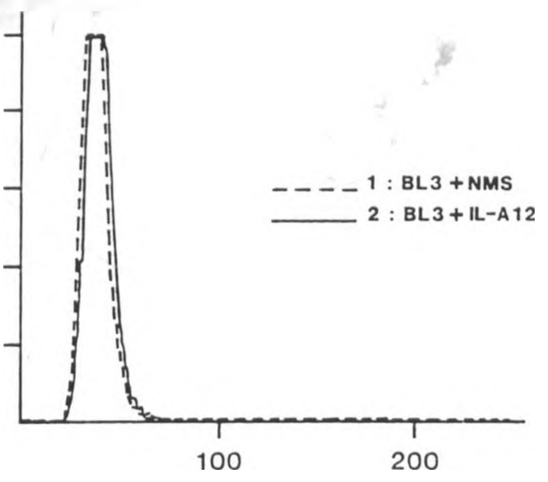
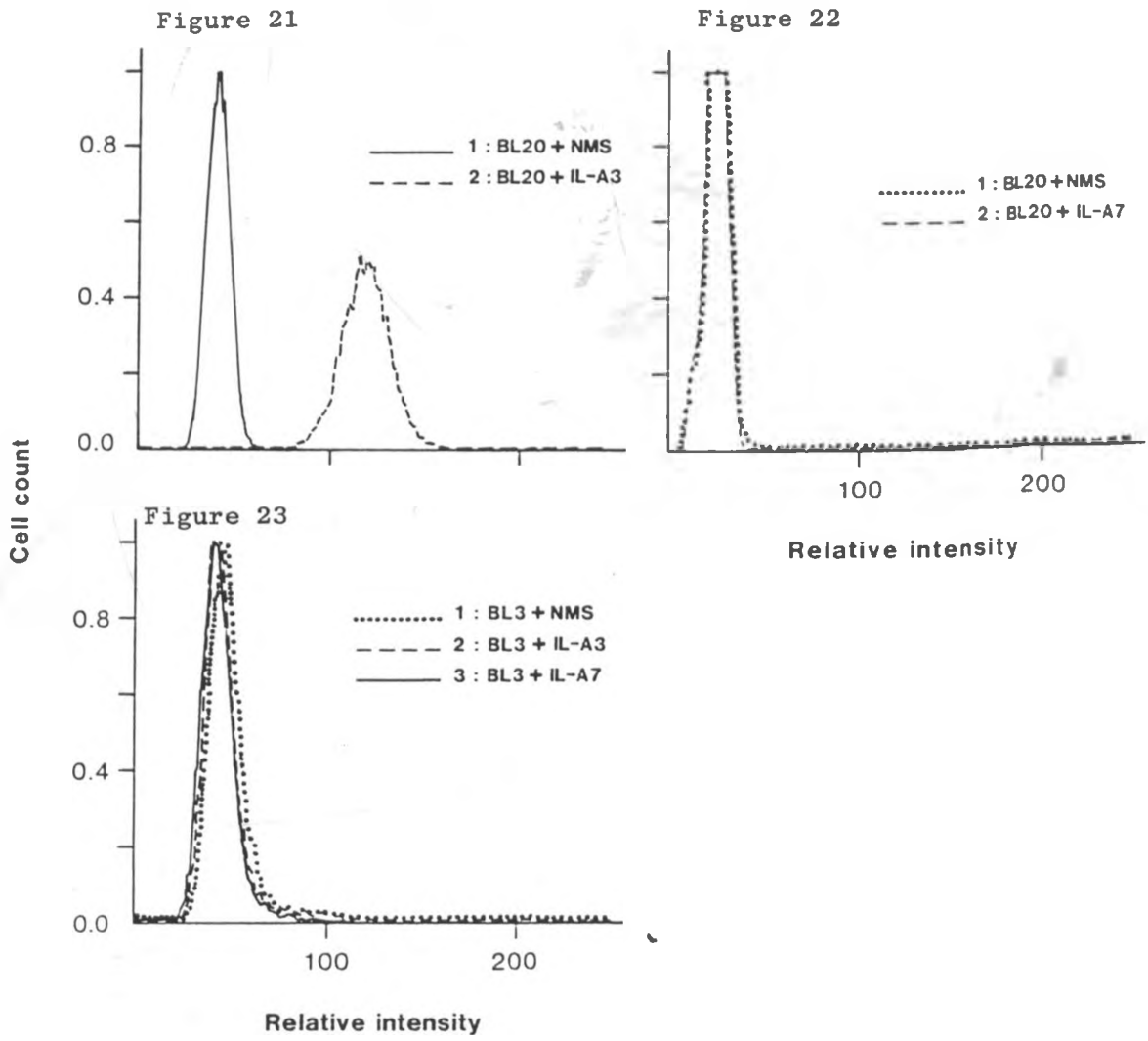


Figure 20

Histograms of BL20 Cells Stained with Antibody P5 (Figure 19) and BL3 Cells Stained with Antibody IL-A12 (Figure 20) NMS was Used in Either Case as a Negative Control.



Representative Histograms of BL20 Cells Stained with Antibody IL-A3 (Figure 21); or Antibody IL-A7 (Figure 22) and BL3 Cells Stained with Antibody IL-A3 or IL-A7 (Figure 23) NMS Represents Negative Controls.

Stained cells were viewed under a Leitz fluorescence microscope, and the percentage of cells expressing cytoplasmic bovine IgM or IgG was determined after counting a total of between 50-100 cells. None of BL3 or BL20 cells was found to contain either cytoplasmic IgM or IgG (Figure not shown).

3.2.3 Karyotype Analysis of BL3 and BL20 Cell Lines

Metaphase derived chromosome spreads of BL3 and BL20 cell lines were made as described earlier (Section 2.7) from logarithmic phase derived cells. Individual chromosome spreads from the cell lines were examined using a high power objective microscope and enlarged photographic records were prepared to facilitate examination of the chromosomes. A total of between 50-100 individual chromosome spreads were counted before determining the modal chromosome number of each cell line. Results showed that parental BL20 cells had a stable modal chromosome number of 60 (Fig. 24). BL3 cells had an aneuploid chromosome number ranging between 41-61 (Fig. 25). No efforts were made to identify the individual chromosomes in either cell type. The percentage of BL3 cells containing 41 chromosomes was about 10% and about 30% of the cells had 60 chromosomes. The rest of the cells had chromosomes ranging between 56-58. The clones derived from BL3 cells (15 examined) were aneuploid, with a modal chromosome number of 41 to 61, indicating that the cell line was unstable.



Figure 24. Representative Metaphase Chromosome Spreads
of BL20 Cells.

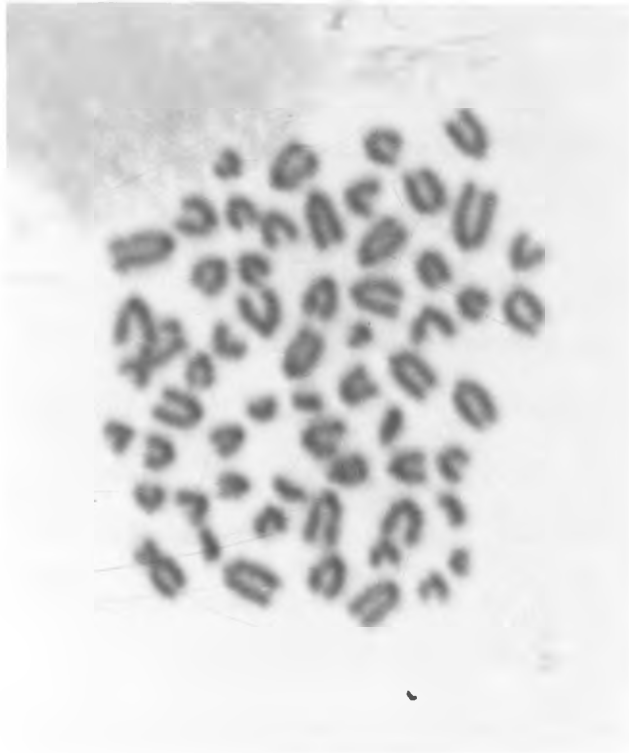


Figure 25. Representative Chromosome Spreads of BL3 Cells.

3.2.4. Limiting Dilution Cloning of BL20 Cells in the Presence or Absence of Feeder Cells or Supernatants Containing Growth Factors

Logarithmic phase derived BL20 cells were resuspended in RPMI-1640 medium containing 15% FBS. The cell suspensions were delivered in 100 ul volumes into individual wells of a flat-bottomed 96-well microtitre plate, in concentrations ranging from 1 cell to 10,000 cells/well (Section 2.6.3), with or without feeder cells or supernatants containing growth factors, to test their ability to improve the cloning efficiency. The feeder cells included irradiated bovine PBM, mouse thymocytes, and bovine thymocytes. The growth factors tested were: 20% Con-A derived T-cell growth factors (gift from Bruno Goddeeris and Kathleen Logan), and 40% culture supernatant from PWM cultures. As shown in Table 9, only bovine thymus cells improved the cloning efficiency of the BL20 cells, and in the presence of bovine thymus cells multiplication was observed even at 1 cell/well. However, the presence of other contaminating thymus cells in culture confused interpretation of the results. In addition to the BL20 cells, two types of bovine thymus cells were observed in the cultures. The first type formed a confluent monolayer at the bottom of the culture dishes while a second type remained in suspension. The latter cells were observed to multiply, but at a slower rate as compared to BL20 cells. The thymus cells in suspension appeared to be larger than BL20 cells.

The multiplication of BL20 cells on other feeder cells or in the presence of growth factor containing supernatants was similar to the control cultures. In these cultures, the cells were observed to multiply only if they were at a density of 10^4 cells/well or above (Table 9).

3.2.5 Establishment of Bovine Thymus Fibroblast-like Cells as a Feeder Layer

Bovine thymus biopsy was performed as described in materials and methods (Section 2.2.5). Isolated bovine thymus cells were seeded at 10^6 /ml in RPMI-1640 medium and dispensed in tissue culture flasks. The cultures were maintained at 37°C in a 7-10% CO₂ in air atmosphere. Two types of cells were observed in the cultures, an adherent population which formed a monolayer at the bottom of the flasks between 7-14 days of incubation and a round or oval population of cells which remained in suspension and appeared to multiply slowly. This latter population of cells was discarded and fresh RPMI-1640 medium was added onto the confluent cells and incubated further. The adherent thymus fibroblast-like cells were passaged weekly or used as feeder layer after detaching the cells using trypsinisation medium. Excess cells were resuspended in a solution of 10% DMSO in FBS and frozen in liquid nitrogen. Cells recovered after freezing had a viability of over 80%.

3.2.6 Expression of Different Surface Antigens on Bovine Thymus Fibroblast-Like Cells

Bovine thymus fibroblast-like cells obtained by trypsinisation were washed in RPMI-1640 medium and resuspended in the same medium at 10^4 cells/ml. The cells were dispensed in 200 μ l volumes into each chamber of Lab-Tek tissue culture chamber slides and allowed to adhere at 37°C in a 7-10% CO₂ incubator for 4-48 hours. The monolayer of cells formed was stained in direct and indirect immunofluorescence assay (Section 2.8.4) using a panel of monoclonal antibodies

Table 9 Ability of Different Cell Types or Supernatants Containing Growth Factors to Improve Cloning Efficiency of BL20 Cells (% +ve wells)

Cell Type or Supernatant	% +ve wells obtained at Cells/Well:				
	1	10^1	10^2	10^3	10^4
Irradiated Bovine PBM	0	0	0	0	100
Mouse Thymocytes	0	0	0	0	100
Bovine Thymus Cells	100	100	100	100	100
TCGF (20%)	0	0	0	0	100
PWM Supernatant (40%)	0	0	0	0	100
BL20 Alone	0	0	0	0	100

described in Section 2.8.1. Monoclonal antibody P5, which reacts with bovine T cells and monocytes, stained about 100% of the cells, but staining was weak. Monoclonal antibody P8 (monocytic marker) on the other hand did not stain any of the cells. The BoLA class II specific MAb R1 also did not stain the cells.

3.2.7 Survival of Spleen Cells or PBM on Bovine Thymus Fibroblast-Like Cell Monolayer

Bovine thymus fibroblast-like cells were obtained from culture by mild trypsinisation and resuspended in RPMI-1640 medium at 10^5 cells/ml before being dispensed in tissue culture flasks. The cells were allowed to form a monolayer by incubating the flasks at 37°C in a CO_2 incubator for 4-48 hours. Spleen cells from normal cattle with exteriorised spleens were obtained by splenic puncture, and contaminating red cells were separated over Ficoll-Paque, and PBM were obtained over Ficoll-Paque. The cells were resuspended in RPMI-1640 medium containing FBS and seeded onto the thymus fibroblast-like cell monolayer at 10^6 cells/ml and maintained at 37°C in a 7-10% CO_2 incubator. The culture medium was changed every week by centrifuging out the spleen cells or PBM in suspension and re-introducing them onto new feeder layer cells. The spleen cells or PBM were observed to survive on the bovine thymus fibroblast-like cells for about three weeks. The percentage of viable cells as determined by trypan blue dye

staining kept on dropping until no viable cells were noticed after that time. No multiplication of the spleen cells or PBM was seen to occur in the cultures upto 8 weeks after initiation.

3.2.8 Limiting Dilution Cloning of BL20 Cells on Bovine Thymus Fibroblast-Like Cell Feeder Layers

In order to test the ability of long term cultured bovine thymus fibroblast-like cells to support cloning of BL20 cells, monolayers of the feeder cells were prepared as described in Section 2.2.5. BL20 cells to be cloned were derived at logarithmic phase of growth and cloned by limiting dilution as in Section 2.6.3, with cell densities varying from 1 cell/well to 10,000 cells/well of the 96 well plate. The cloned cells were maintained at 37°C in the presence of a 7-10% CO₂ in air atmosphere. The wells were inspected for cell multiplication once a week. The percentages of wells with cell growth are shown in Table 9. All the wells seeded with cell densities varying from 1 cell to 10,000 cells/well exhibited growth.

3.2.9 Selection of BL20 Cells That Could Clone in the Absence of Bovine Thymus Fibroblast-Like Cell Feeder Layer

Results from the previous section (Section 3.2.8) had shown that BL20 cells could not grow at a density of below

10^4 cells/well in the absence of bovine thymus fibroblast-like cells. Further limiting dilution experiments were initiated to try to isolate a population of BL20 cells that could be cloned at lower densities (below 10^4 cells/well) without the requirements for a feeder layer. BL20 cells were obtained at logarithmic phase of growth, cloned by limiting dilution at 10^4 cells/well (96-well plate) followed by incubation as previously described (Section 2.6.3). All the wells on the plate showed positive cell growth and were ready for expansion to larger cultures after about five days of incubation. The cells which grew up were pooled and expanded. At the next logarithmic phase of growth, the cells were seeded at 5×10^3 /well and allowed to expand. About 90% of the wells exhibited growth at this density and the positive wells were again pooled, expanded and seeded again at a half the previous density i.e. at 2.5×10^3 cells/well. This protocol was continued until a population of BL20 cells was obtained that could clone at a frequency of about 20% without feeder cells or growth factors. The exercise was conducted to reduce the dependence of the BL20 cells on the feeder layer.

3.2.10 Generation of 8-Azaguanine Resistant BL20 Cell Line

Preliminary studies were conducted to establish whether or not any of the parental BL20 cells possessed spontaneous resistance to 8AG. Logarithmic phase derived cells

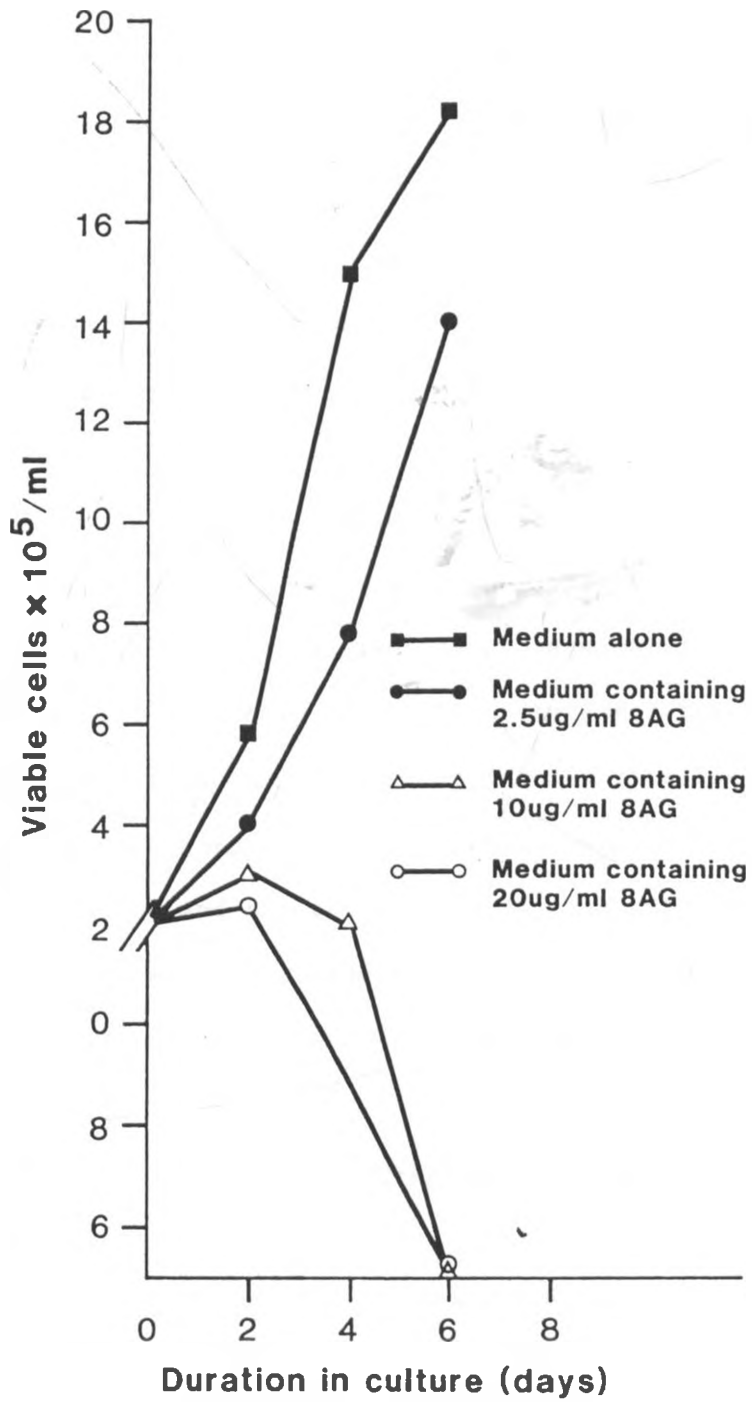


Figure 26. The Effect of Varying Concentrations of 8AG on Growth of Parental BL20 Cells.

were cultured at 2×10^5 cells/ml in RPMI-1640 medium containing 10% dialysed FBS and varying concentrations of 8AG ranging from 2.5 ug/ml to 40 ug/ml. The cells were incubated at 37°C in a 7-10% CO₂ in air atmosphere, and viability was determined by staining with trypan blue dye. The growth of BL20 cells in medium containing the different concentrations of 8AG is shown in Fig. 26. Cells incubated in medium without 8AG and in medium containing 2.5 ug/ml 8AG apparently multiplied normally and reached a peak density of between $1-1.5 \times 10^6$ cells/ml in 4-5 days (Fig. 26). When the concentration of 8AG in the medium exceeded 10 ug/ml, cell multiplication was not normal. At this concentration and above, the cells appeared not to multiply, and all cells were killed by day six of culture. The cells were observed to die in a shorter time when the concentration of 8AG was increased to 20 ug/ml (Fig. 26).

3.2.11 Irradiation of BL20 Cells to Induce Mutant Cells

Logarithmic growth phase derived BL20 cells which had been selected to clone at low cell numbers in the absence of the Thymus Fibroblast-like feeder layer cells were seeded at 5×10^6 cells/ml of RPMI-1640 medium and dispensed into Costar flasks. The cells were placed on ice and irradiated with gamma (γ) rays from a ¹³⁷Caesium source at a total dose of 600R. Irradiated cells were centrifuged and resuspended at 2×10^5 cells/ml of RPMI-1640 medium containing 15% FBS, and given 2-3 population doublings to recover by incubating at 37°C in a

7-10% CO₂ incubator. After subjecting the cells to 600R about 90% of BL20 cells died. Irradiation doses of over 600R killed 100% of the cells after about three days in culture.

3.2.12 Selection of 8AG Resistant BL20 Cells After Irradiation

Preliminary studies had shown that few if any of the BL20 cells tested had spontaneous resistance to 8AG concentrations above 10 ug/ml (Section 3.2.11). The cells were irradiated to try to induce mutations and were selected in increasing concentrations of 8AG (Figure 3; Section 2.10.1). Irradiated BL20 cells which had undergone 2-5 population doublings in RPMI-1640 medium supplemented with 15% FBS were collected and reseeded in the same medium containing 10% dialysed FBS and an initial concentration of 2.5 ug/ml of 8AG. The cells were incubated at 37°C in a CO₂ incubator. After three days of incubation surviving cells were obtained, washed in drug free medium, and seeded at 2×10^5 cells/ml in RPMI-1640 medium containing 10% FBS and 10 ug/ml of 8AG and incubated for a further three days. In this concentration of 8AG about 60% of the cells were lost by day three of culture. Viable cells were obtained, washed and expanded in drug free medium as previously described. After allowing the cells to undergo 2-3 population doublings, they were next seeded at 2×10^5 cells/ml of medium containing 20 ug/ml of AG and cultured at 37°C in a CO₂ incubator for another three days. About 95% of the cells were killed during the treatment. Further

expansion of the cells was conducted as before, giving them 2-3 population doublings in 8AG free medium before seeding the cells in 40 ug/ml of the drug in RPMI-1640 medium. At this concentration the viability of the cells was apparently not affected. Surviving cells in 40 ug/ml of 8-azaguanine were expanded and tested for sensitivity to HAT containing medium.

3.2.13. Sensitivity of 8-Azaguanine Resistant BL20 Cells to HAT Supplemented Medium

BL20 cells which were resistant to 40 ug/ml of 8AG were obtained from the logarithmic phase of growth and suspended at 2×10^5 cell/ml of RPMI-1640 medium containing 15% FBS and concentrations of aminopterin ranging from 4×10^{-7} M to 1×10^{-6} M. The concentrations of thymidine (3×10^{-5} M) and hypoxanthine (10^{-4} M) were kept constant. The cells were incubated at 37°C in a CO₂ incubator for 14 days. Many of the cells died during the first three days of culture. However, some cell multiplication was noticed starting on day three of culture despite extensive cell death in all the culture flasks. The 8-azaguanine resistant BL20 cells were cloned by the limiting dilution method on a bovine thymus fibroblast-like monolayer. The clones obtained were tested further for sensitivity to aminopterin. Out of the 41 clones tested, three were found to be completely resistant to aminopterin while the others gave rise to cells which were sensitive to aminopterin.

3.2.14. Further Selection of 8AG Resistant BL20 Cells in
Medium Containing 6-Thioguanine (6TG)

Results shown in Section 3.2.13 indicated that some 8AG resistant BL20 cells could survive in the presence of aminopterin. The 8AG resistant BL20 cell line was selected further in medium containing 6TG using a similar protocol shown in Fig. 3. The cells to be selected were obtained from the logarithmic phase of growth and seeded at 2×10^5 cells/ml in RPMI-1640 medium containing 10% dialysed FBS and an initial concentration of 2.5 ug/ml of 6TG (Ref. Fig. 3; Section 2.10.1). After three days of culture at 37°C in a CO₂ incubator the cells appeared to multiply normally. On this day, the cells were isolated and washed in drug free RPMI-1640 and seeded at 2×10^5 /ml in the same medium containing 20 ug/ml of 6TG and 10% dialysed FBS. The cells were incubated as before, and viability was examined daily using trypan blue dye stain. On the third day of culture, about 10% of the cells were dead. The surviving cells were adjusted to a density of 2×10^5 /ml of medium containing 20 ug/ml of 6TG, and allowed to expand by incubation at 37°C in a 7-10% CO₂ in air atmosphere. The selected BL20 cells which were resistant to 40 ug/ml of 8AG and 20 ug/ml of 6TG (Fig. 27) were routinely maintained in medium containing 20 ug/ml of 6TG to kill any possible "revertants", and were thereafter referred to as ATS/BL20 cells.

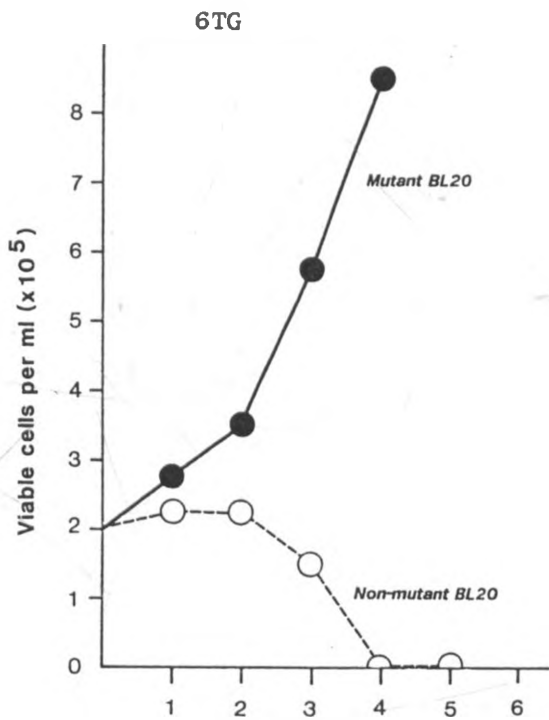


Figure 27

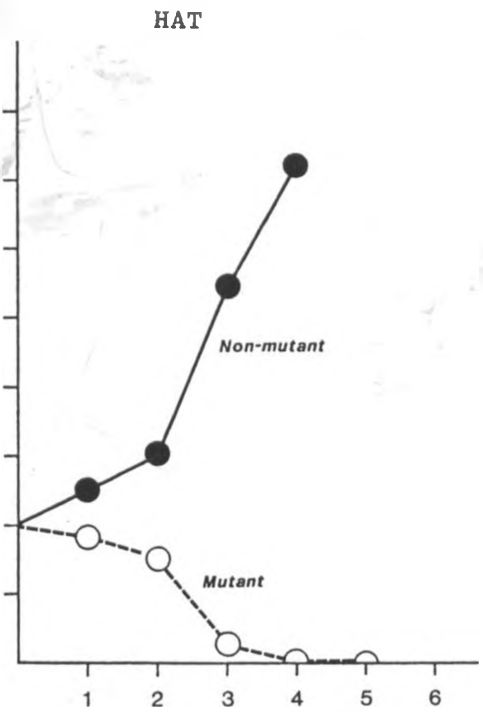


Figure 28

Duration in culture (days)

Differential Growth of BL20 Cells and ATS/BL20 Cells in Medium Containing 6-Thioguanine; (Fig. 27) or HAT (Fig. 28).

3.2.15 Sensitivity of 8AG and 6TG Resistant ATS/BL20 Cells to HAT Medium Treatment

ATS/BL20 cells which were grown in RPMI-1640 medium containing 20 ug/ml of 6TG, and ATS/BL20 cells which were grown for 10 passages in medium without the drug to allow any possible revertants to expand, were obtained at logarithmic phase of growth. The cells were tested for sensitivity to HAT containing medium as before (Section 2.10.2). In both cases, the ATS/BL20 cells were killed by aminopterin within six days (Fig. 28). Parental BL20 cells (non-selected) were apparently not very much affected. They kept on multiplying until a peak growth was attained at about day six of culture (Fig. 28).

3.2.16 Fusion of HAT Sensitive ATS/BL20 Cells with PWM Activated Bovine PBM, or In Vivo Derived Spleen Cells or SRBC Activated Bovine PBM

Logarithmic growth phase ATS/BL20 cells were obtained and washed twice in serum and HEPES free RPMI-1640 prior to fusion. Peripheral blood mononuclear cells were stimulated with PWM for six days before use. Lymphocytes isolated on this day had been shown in preliminary studies to generate some hybrid cells in three out of 96 wells when compared to lymphocytes isolated on either day four or day eight, where no hybrids were detected from a similar number of wells after fusion to the commonly used mouse myeloma fusion partners NS1.

Spleen cells were isolated from cattle with exteriorised spleens four days after the last booster immunisation with SRBC (Section 2.4.3). In vitro antigen (SRBC) activated PBM from cattle previously immunised with the same antigen (Section 2.4.5) were obtained on day 3 of culture. By this time, the in vivo derived spleen cells or in vitro antigen activated PBM were known in preliminary studies to have only few Ig producing cells, as compared to those cells isolated later after immunisation. This indicated that the cells were not yet at the peak of Ig production and were probably suitable fusion partners. The cells to be fused, ATS/BL20 with PBM or spleen cells, were mixed at a 1:1 ratio and pelleted by centrifugation. The cells were fused as previously described using PEG 4000 (section 2.10.3; Appendix 1), and seeded in 100 μ l and one ml volumes in individual wells of 96 or 24 well plates at 10^5 or 10^6 cells/well respectively. Bovine thymus fibroblast-like cells were included in some wells to promote hybrid cell growth. The cells were incubated at 37°C in a 7-10% CO₂ incubator and selection of fused cells in HAT and HT containing medium was carried out as previously described (Section 2.10.3). No growing hybrids arose in cultures performed without a feeder layer. In contrast, in the presence of feeder layer cells, 17 out of 72 wells seeded with 10^6 PBM x ATS/BL20 cells, 3 out of 48 wells seeded with 10^5 spleen x ATS/BL20 cells, and 24 out of 24 wells seeded with 10^6 spleen x ATS/BL20 cells, gave rise to growing cell lines (Table 10). Cultures which gave rise to growing hybrids arose,

and remained dormant for between 4 and 8 weeks after seeding ie., no cell growth was observed until 4 to 8 weeks post fusion. In control cultures where bovine lymphocytes were cultured with ATS/BL20 cells in the presence of feeder cells, then subjected to HAT selection without PEG mediated fusion, no growing cells were noticed even after 8 weeks of culture.

3.2.17 The Effect of Supernatants Containing Growth Factors on the Promotion of Hybrid Growth Between ATS/BL20 Cells and Bovine PBM

To try to promote hybridoma cell growth soon after fusion, fused cells between ATS/BL20 and bovine PBM were immediately resuspended in fusion medium supplemented with 40% growth factor containing culture supernatant from PWM cultures or 20% Con-A derived culture supernatants. The cell suspensions were distributed at 10^5 , 2×10^5 and 5×10^5 cells/well in 100 ul volumes in wells of a 96-well plate with bovine thymus fibroblast-like cells and incubated as previously described. Fused cells were selected in HAT medium supplemented with 40% growth factor containing culture supernatant from PWM cultures or HAT medium supplemented with 20% TCGF or HAT medium alone. The selection medium was added 24 hours after fusion and was replaced every two days. Selection was continued for 10 days. Thereafter, the cells were cultured in HT medium without growth factors for another seven days prior to culture in fusion medium alone. The

inclusion of growth factor containing culture supernatants in the initial selection medium had no influence on the promotion of hybrid growth. Results were similar to those presented in Section 3.2.16 and Table 10.

Table 10 Growth of ATS/BL20 X Bovine PBM or Spleen Hybrid Cells in the Presence of Bovine Thymus Fibroblast-Like Cells

Fused Cells	Culture conditions*	
	96-well plate 10 ⁵ cells/well	24-well plate 10 ⁶ cells/well
ATS/BL20 x PBM (PWM activated)	0/288	17/72
ATS/BL20 x PBM (SRBC activated)	0/96	10/24
ATS/BL20 x spleen cells (<u>in vivo</u> derived)	3/48	24/24

* Wells with growth per total number of wells seeded with fused cells.

3.2.18 Expression of Cytoplasmic Immunoglobulins (cIgs) by Hybrid Cells Between ATS/BL20 and Bovine Lymphocytes

Hybrid cells formed by fusion between ATS/BL20 and PBM or spleen cells were examined for the presence of cytoplasmic IgM or IgG using monoclonal antibodies B5/4 or A₂ (Section 2.8.1) respectively. The stained cells were viewed with a Leitz fluorescence microscope and the percentage of cells expressing IgM (B5/4) or IgG (A₂) was determined after counting a total of between 50 and 100 cells.

Out of a total of 17 positive wells of hybrids between ATS/BL20 and bovine PBM examined, only one showed hybrid cells containing cytoplasmic IgM (Fig. 29). No cytoplasmic IgG positive cells were detected. Of the 27 wells positive for hybrid growth between ATS/BL20 and spleen cells, none was found to contain either cytoplasmic IgM or cytoplasmic IgG positive cells (not shown). None of the wells positive for hybrid growth between ATS/BL20 and in vitro antigen activated PBM also stained for cIg.

3.2.19 Cloning and Stability of Bovine x Bovine Hybridomas Producing Cytoplasmic IgM

Cells from the well which showed a proportion (15%) of the cells with cytoplasmic IgM were cloned by the limiting dilution method at 1, 3, 5, 10 and 20 cells/well in individual

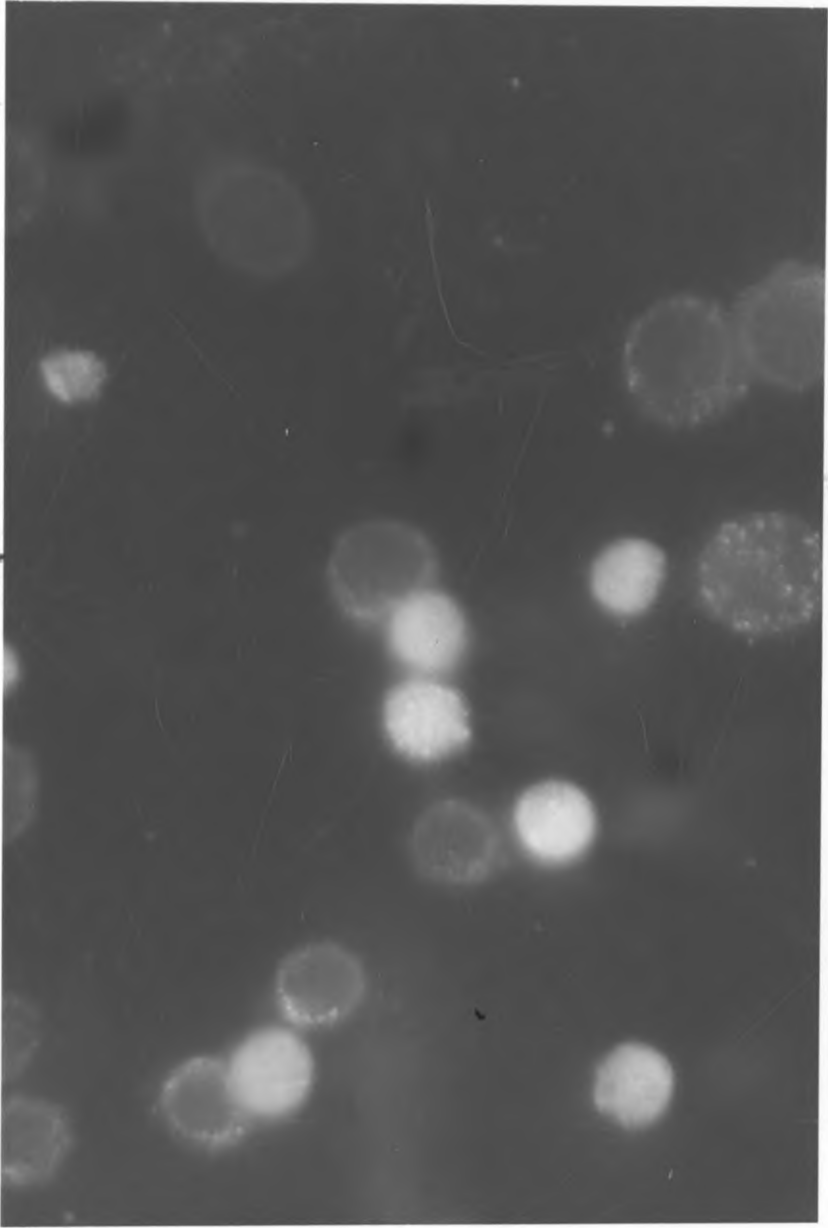


Figure 29. Expression of cIgM by ATS/BL20 x Bovine
PBM Hybrid Cells.

wells (100 ul volumes) of a 96-well microtitre plate containing bovine thymus fibroblast-like cells as feeder layer. The cultures were maintained at 37°C in atmosphere of a 7-10% CO₂ in air, and the wells were inspected for cell growth once a week for the next four weeks. None of the wells with 1 and 3 cells/well showed any growth. However, at 5, 10 and 20 cells/well 1%, 47% and 100% of the wells showed cell growth respectively. When cells from positive wells were analysed for the presence of cIgM, all were found to be negative, indicating they had lost Ig production.

3.2.20 Expression of Surface Antigens on ATS/BL20 Cells, Donor Bovine Lymphocytes and Hybrid Cells Between ATS/BL20 and Bovine PBM

Bovine lymphocytes were freshly isolated over Ficoll-Paque, and ATS/BL20 cells, and hybrid cells between ATS/BL20 cells and bovine lymphocytes, were collected at the logarithmic phase of growth. The cells were stained in suspension in U-or V-shaped wells of a 96 well microtitre plate by indirect immunofluorescence assay (Section 2.8.2), using a panel of monoclonal antibodies listed in Section 2.8.1. Stained cells were examined using a FACS II. The IgM epitope detected by monoclonal antibody B5/4 was expressed on about 75% of the ATS/BL20 cells (Figs. 30; Table 11). This percentage was somewhat lower as compared to the original BL20 cells (refer Fig. 17; Table 8). Monoclonal antibody A₂ did not

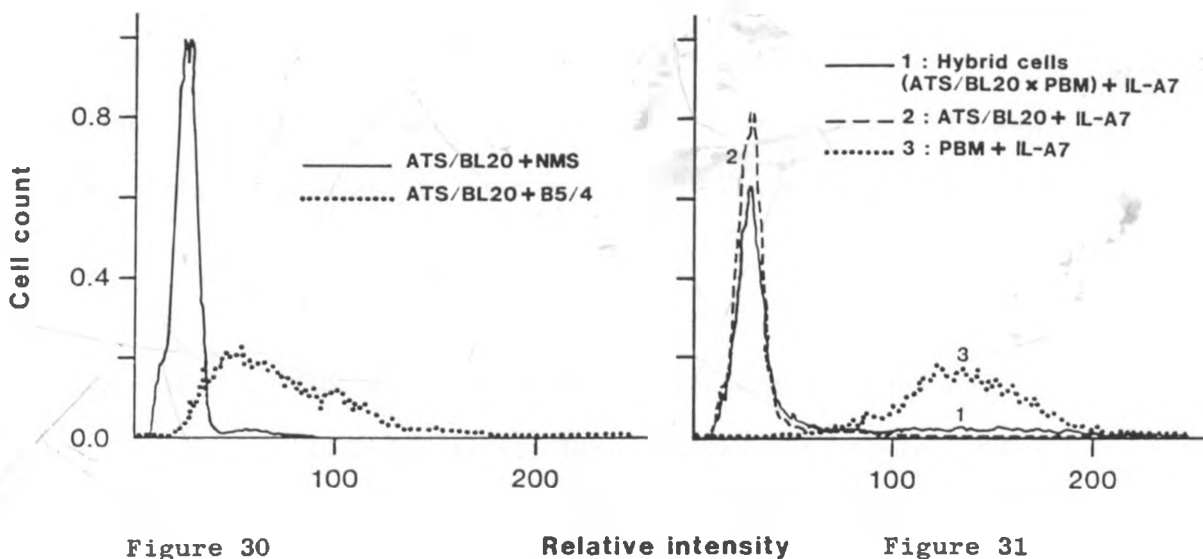


Figure 30

Relative intensity

Figure 31

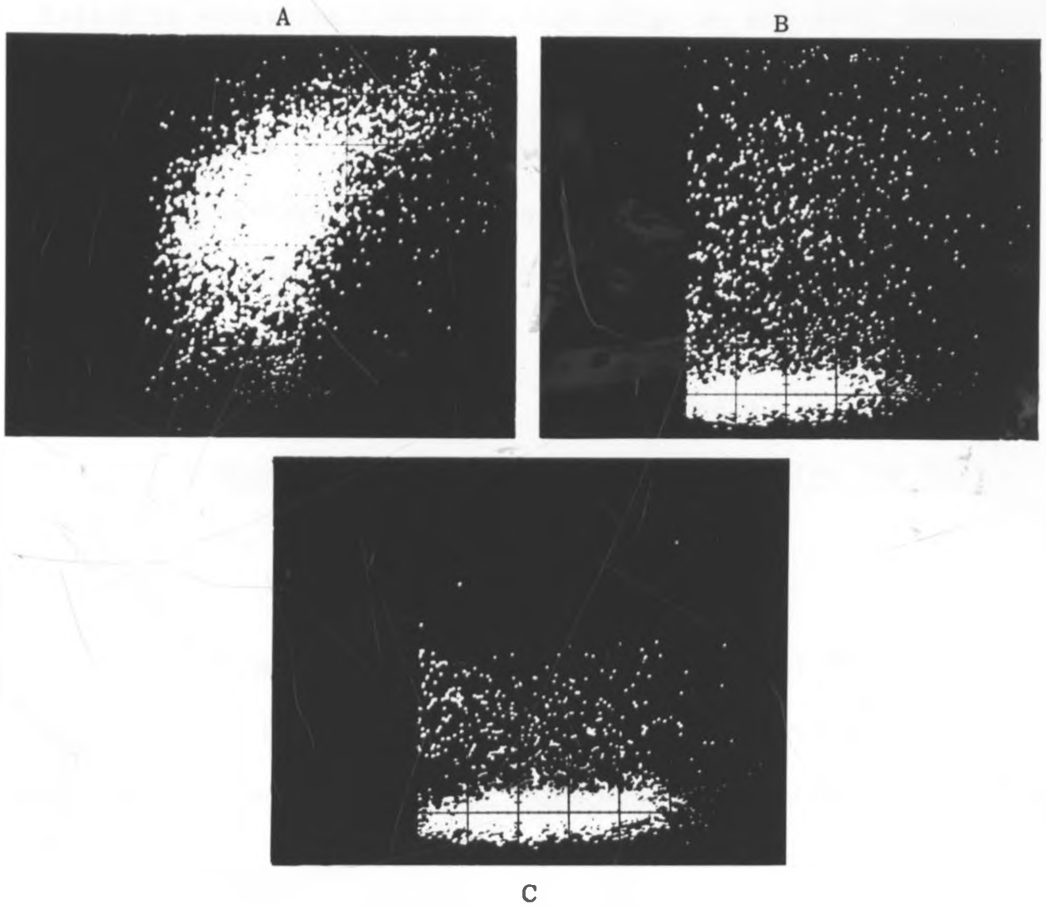
Representative Histograms of ATS/BL20 Cells Stained with Antibody B5/4 (Figure 30); and Hybrid Cells Between ATS/BL20 x PBM; ATS/BL20 Cells, or Normal Bovine PBM Stained with Antibody IL-A7 (Figure 31). NMS was used as a Negative Control.

stain any of the BL20 or the ATS/BL20 cells (not shown). Between 20-25% and 3-5% of PBM from the donor animals were positive for surface IgM as stained by MAb B5/4 (Table 11) and surface IgG as stained by antibody A₂ (Table 11), respectively. After fusion, the hybrids stained with MAb B5/4 to varying degrees ranging from 30-70% (Table 11), and none of the hybrids was stained with A₂ antibody (not shown). The presence of bovine histocompatibility antigens on the cells was demonstrated by staining with MAb IL-A7, which detects a

Table 11 Expression of Different Surface Antigens on ATS/BL20 Cells; Donor Animal Lymphocytes and Hybrid Cells between the Two Cell Types (% +ve Cells)

Antibody Designation	Donor Animal PBM	Mutant BL20	Hybrid ATS/BL20 with Bovine PBM
B5/4	20-25	70	30-70
A ₂	2-5	0	0
P5	45	70	NT
IL-A3	25	90	NT
IL-A7	95	0	20-25
IL-A12	0	0	0
IL-A17	0	0	0

Fluorescence Intensity



Forward Light Scatter

Figure 32 (A,B,C). Expression of the Molecule Detected by Antibody IL-A7 on Normal Bovine PBM (A); Hybrid Cells Between Normal Bovine PBM x ATS/BL20 (B); or ATS/BL20 only (C).

polymorphic epitope on BoLA class 1. This marker (IL-A7) failed to stain the ATS/BL20 cells (Fig. 31 and 32C; Table 11), but it was found to stain over 80% of donor animal PBM (Fig. 31 and 32A; Table 11). After hybridization of ATS/BL20 cells with bovine cells, between 20-25% of the hybrid cells stained with MAb IL-A7 (Fig. 31 and 32B; Table 11).

3.2.21 Karyotype Analysis of ATS/BL20 Cells, Normal Donor Bovine Cells and Hybrid Cells Between the Two Cell Types

Metaphase derived chromosome spreads of ATS/BL20 cells, normal bovine cells, and hybrid cells between ATS/BL20 and normal bovine leucocytes, were made as described earlier (Section 2.7). Individual chromosome spreads were examined using a high power objective microscope and enlarged photographic records were made to facilitate enumeration of the chromosomes. A total of between 50 and 100 individual chromosome spreads were counted before determining the average chromosome number of each cell line. Results showed that ATS/BL20 cells and normal bovine cells had a stable chromosome number of 60 each (refer Figs. 24 and 34). However, the hybrid cells between the two cell lines had between 56 and 60 chromosomes (Fig. 33 representative). In rare instances some hybrid cells were noticed which contained 80 chromosomes. These results suggested that hybridization might have occurred between ATS/BL20 cells and bovine lymphocytes.

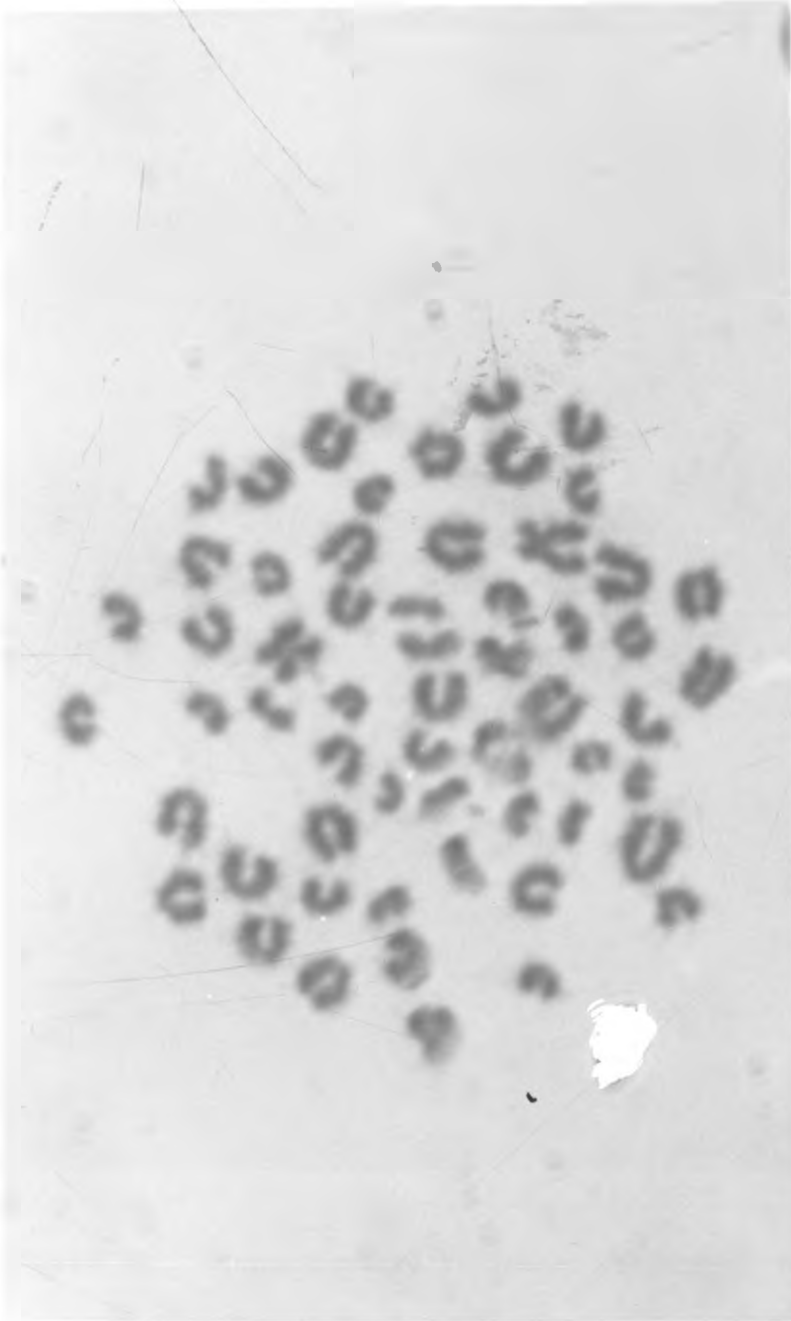


Figure 33. Representative Metaphase Chromosome Spreads
of a Hybrid Cell between ATS/BL20 x Normal
Bovine PBM.

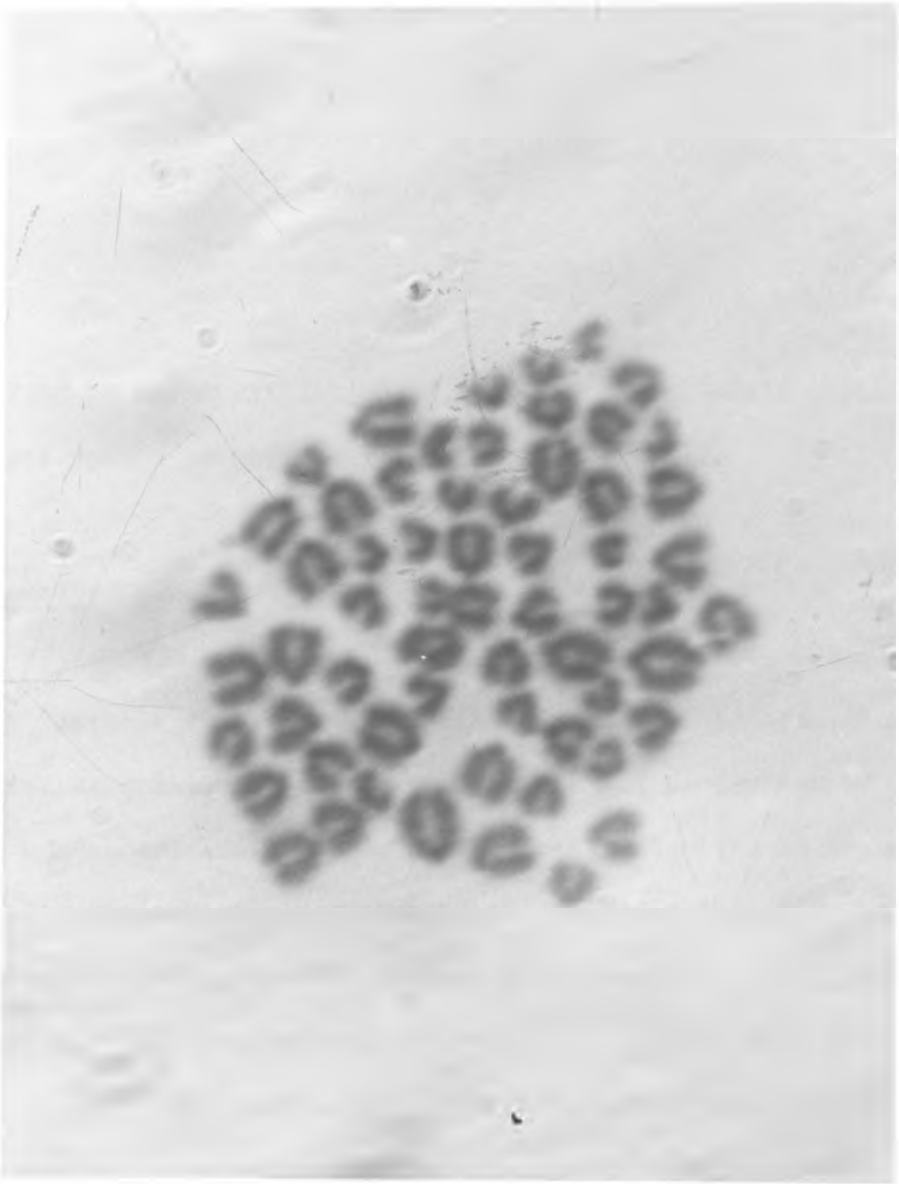


Figure 34. Representative Metaphase Chromosome Spreads of
a Normal Bovine Lymphocyte

RESULTS

SECTION 3

GENERATION OF HAT SENSITIVE MOUSE X BOVINE HETEROHYBRID CELL LINES FOR FUSION TO BOVINE LYMPHOCYTES.

Introduction

Aminopterin sensitive ATS/BL20 cells proved unsuitable as an intraspecies specific fusion partner for production of bovine monoclonal antibodies (Results Section 2) because hybrid generation was a rare event, hybrid cells took a long time to establish, and only few hybrids retained the capacity to produce Ig. Attempts were therefore made to generate mouse x bovine heterohybrid fusion partners for bovine B lymphocytes. These were compared with mouse parental cell lines in fusion studies using in vitro derived bovine lymphocytes.

Interspecies hybrid formation has been reported in the literature to be more unstable than intraspecies hybrids due to increased chromosome loss (Croce et al., 1980). Teng et al. (1983), Fong et al., (1984), have however, reported combining the favourable characteristics of the commonly used mouse myeloma cell line fusion partners and human B lymphocytes to

generate mouse x human heterohybrid cell line fusion partners which gave fairly stable hybrid cell lines in secondary fusions. The resultant mouse x human fusion partners were apparently superior to the parental mouse myeloma fusion partners, presumably as a result of increased chromosome retention.

This section outlines results obtained in attempts to generate mouse x bovine fusion partners utilising the information gained from studies in the human hybridoma system by interspecies hybrid formation between mouse myeloma cells and PWM activated bovine cells. The resultant rare hybrid cells between mouse x bovine were selected for resistance to 8-azaguanine and sensitivity to aminopterin. The mutant mouse x bovine hybrid cells were then fused back to bovine PBM to try to obtain mouse x bovine x bovine heterohybridomas. This strategy was aimed at retaining the outstanding fusion characteristics of the HAT-sensitive mouse myeloma cells, and it was hoped that the hybrid cells could retain some bovine chromosomes induced by fusion. It was also hoped that in at least some of the heterohybridomas thus constructed, there would be a significant increase in the retention of bovine chromosomes induced during the secondary fusion with normal bovine B cells, thus providing a higher yield of stable mouse x bovine x bovine hybridomas for bovine Ig production.

3.3 Fusion of PWM Activated Bovine PBM to Mouse Myeloma Cells NS1, X63 or Sp2/0

Ficoll-Paque isolated bovine PBM were cultured in an optimal concentration of PWM for six days (Section 2.4.1). Mouse myeloma cell lines NS1, X63 or Sp2/0 were obtained during their logarithmic growth phase. The NS1, X63 or Sp2/0 cells were fused to PBM with PEG and distributed in 100 ul volumes at 2×10^5 cells/well of a 96-well microtitre plate. Some wells contained 10^6 irradiated mouse thymocytes as feeder cells, while others did not have feeder cells.

The selection of fused cells in HAT medium commenced 24 hrs after seeding, and was continued every two days for the next 7-10 days, in some experiments, and 14 days in others. Thereafter the cells were expanded in HT containing medium for seven days and in fusion medium. In experiments where the cells were seeded at 2×10^5 /well with or without mouse thymocytes, no hybrid cells were noticed to grow between the mouse myeloma NS1 or X63 and bovine PBM even after eight weeks of culture. In the case of PWM cells fused to Sp2/0, growing hybrid cells were visible within two weeks in three out of 96 wells seeded with 2×10^5 cells/well and containing mouse thymocytes, and the cells continued to grow without any apparent crisis. In contrast, control cultures, which consisted of NS1, X63 or Sp2/0 cell lines fused to in vivo derived spleen cells from mice immunised with SRBC and seeded

culture and resuspended in 100% NRS. The suspension was smeared onto clean coverslips, dried, fixed in ethanol/acetone and the fixative was allowed to evaporate at RT. The fixed cells were stained in an indirect immunofluorescence assay, using MAb B5/4 for detection of bovine IgM, and MAb A₂ for detection of bovine IgG₁ and IgG₂. Fixed cells were also stained in a direct immunofluorescence assay, with FITC-conjugated rabbit anti-bovine IgA and FITC-conjugated rabbit anti-mouse Ig. About 10% of the hybrid cells between NS1 and PWM activated bovine PBM showed strong fluorescence for cytoplasmic bovine IgM, and between 3-5% of the cells were stained for cytoplasmic bovine IgG. None of the hybrids between Sp2/0 and PBM were positive for either cytoplasmic bovine IgM or IgG and none of the mouse x bovine hybrid cells had detectable levels of cytoplasmic bovine IgA or mouse Ig.

3.3.2 Limiting Dilution Cloning of Mouse x Bovine Hybrid Cells

Limiting dilution cloning of mouse x bovine (NS1 x bovine or Sp2/0 x bovine) hybrid cells was performed after obtaining the cells from the logarithmic phase of growth. The cells were resuspended at 50 and 100 cells/ml in RPMI-1640 medium supplemented with 15% FBS and delivered in 100 ul volumes in the individual wells of a flat-bottomed 96-well microtitre plate to make five and ten cells/well respectively.

Irradiated mouse thymocytes (10^6 cells/well) were seeded together with some of the cells to be cloned to improve the cloning efficiency. The cells were incubated at 37°C in an atmosphere of 7-10% CO₂ in air and inspected for cell growth twice a week by light microscopic examination.

At five and 10 cells/well, 22% and 56% of the wells seeded with NS1 x bovine cells showed growth respectively (Table 12). The same hybrid cells cloned at the same densities but without mouse thymocyte feeder cells exhibited growth in only 5% of wells at 10 cells/well (Table 12). On the other hand when Sp2/0 x bovine hybrid cells were cloned in the presence of mouse thymocytes, 100% of the well seeded with 10 and 5 cells/well and about 72% of the wells seeded with 1 cell/well showed growth. No growth was noticed in wells without mouse thymocytes. It was clear from these results that the presence of mouse thymocytes was important to obtain a higher cloning efficiency of the hybrid cells.

3.3.3 Expression of Surface Antigens on Mouse x Bovine Hybrid Cells

Actively growing mouse x bovine hybrids, NS1 or Sp2/0 myeloma cells and fresh PBM isolated over ficoll-paque were stained with a panel of MAbs listed in Table 2. Before staining, the cells were incubated in RPMI-1640 medium

containing 10% NRS for one hour to reduce non-specific or Fc-mediated binding of antibodies.

For staining, an average of $2-5 \times 10^5$ cells/well were used. In direct immunofluorescence assays, optimally diluted FITC-conjugated antibody was added to the cells and incubated at 4°C for 30-45 minutes before washing and analysing the cells. For indirect immunofluorescence assays, similar staining procedures were followed as for the direct method except that the cells were first incubated with an optimally diluted antibody, and washed, before incubating further with a FITC-conjugated second antibody. Stained cells were fixed for

TABLE 12. Cloning Frequency of NS1 x Bovine or Sp2/0 x Bovine Hybrid Cell Lines in the Presence or Absence of Mouse Thymocyte Feeder Cells

Cell Line	Cells/well	Cloning Frequency(%)	
		Cells With Mouse Thymocyte Feeders	Cells Without Mouse Thymocyte Feeders
Mouse x Bovine Hybrids (NS1 x PBM)	10	56	5
	5	22	0
	1	0	0
Mouse x Bovine Hybrids (Sp2/0 x PBM)	10	100	0
	5	100	0
	1	72	0

TABLE 13. Expression of Different Surface Antigens on Donor Animal PBM; NS1 or Sp2/0 Cell Lines, or Hybrid Cells Between Bovine PBM and the Two Mouse Myeloma Cell Lines (% +ve cells)

Antibody designation	Donor PBM	NS1 Cells	Sp2/0 Cells	NS1 x Bovine Hybrid Cells	Sp2/0 x Bovine Hybrid Cells
B5/4	25	0	0	10	19%
A ₂	2-3	0	0	0	0
Anti-mouse Ig	NT	+	0	0	0
P5	44	0	0	8	0
K1	23	NT	NT	0	NT
IL-A3	22	0	0	0	0
IL-A4	51	0	0	0	0
IL-A7	80	NT	0	20	NT
H ₂ D ^b	0	90	NT	80	NT

NT - not tested; 0 - negative

a minimum of 30 minutes and sometimes overnight in 2% formalin before FACS analysis.

Results are presented in Table 13. Approximately 25% of the PBM from the donor animal and no NS1 or Sp2/0 mouse myeloma cells expressed surface bovine IgM as detected by

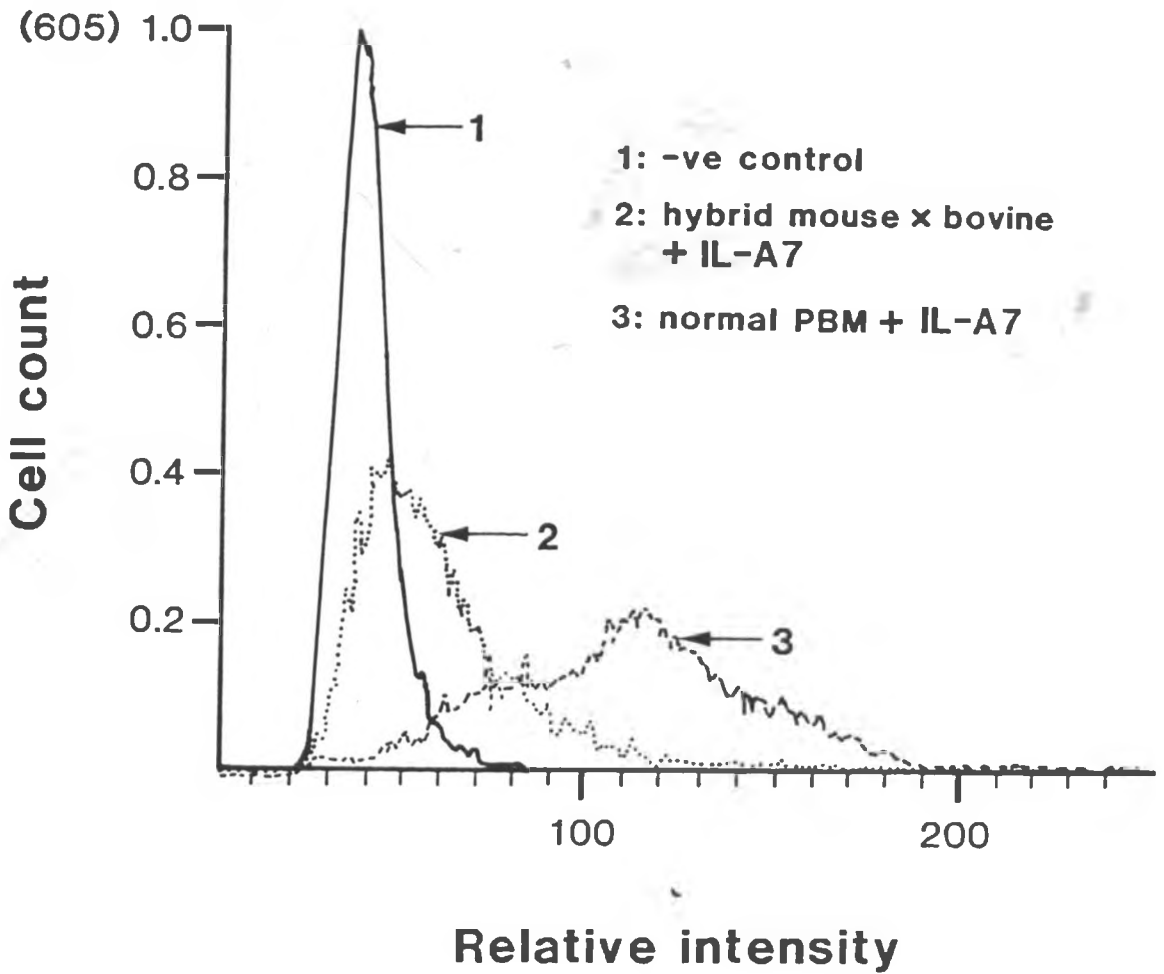


Figure 35. Representative Histogram of NS1 x Bovine Hybrid Cells and Normal Bovine PBM Stained with Antibody IL-A7 and Normal Mouse Serum as the Negative Control (-ve).

monoclonal antibody B5/4. After fusion, only about 10% of the NS1 x bovine hybrid cells and 19% of Sp2/0 x bovine cells reacted with the same antibody. The bovine T cell marker P5 was found to be expressed on 44% of PBM from the donor animal, but the percentage of the cells with the antigen was reduced to eight after hybrid formation between NS1 x bovine PBM and none of the hybrid cells between Sp2/0 x bovine PBM, expressed the P5 marker on the surface (Table 13). The bovine histocompatibility antigen (BoLA), as detected by antibody IL-A7, was present on 80% of PBM of the donor animal, and was expressed on 20% of (NS1 x bovine) hybrid cells (Fig. 35; Table 13). The NS1 or Sp2/0 myeloma cells were not stained by either P5 or IL-A7 antibody (Table 13). The polymorphic epitope on mouse H-2 complex was found to be expressed on over 90% of NS1 cells, on none of normal bovine PBM, and on 80% of NS1 x bovine hybrids, as detected by mouse anti-H₂D^b antiserum (Table 13).

3.3.4 Karyotype Analysis of Mouse x Bovine Hybrid Cells

Chromosome spreads were prepared from continuously growing NS1 x bovine, Sp2/0 x bovine hybrid cells, from the mouse myeloma cell lines NS1 and Sp2/0, and from PWM activated bovine PBM. Individual chromosome spreads of cells were prepared, photographed, and enlarged photographic prints were made to determine the number of chromosomes per cell. A total of between 50-100 individual spreads were examined before

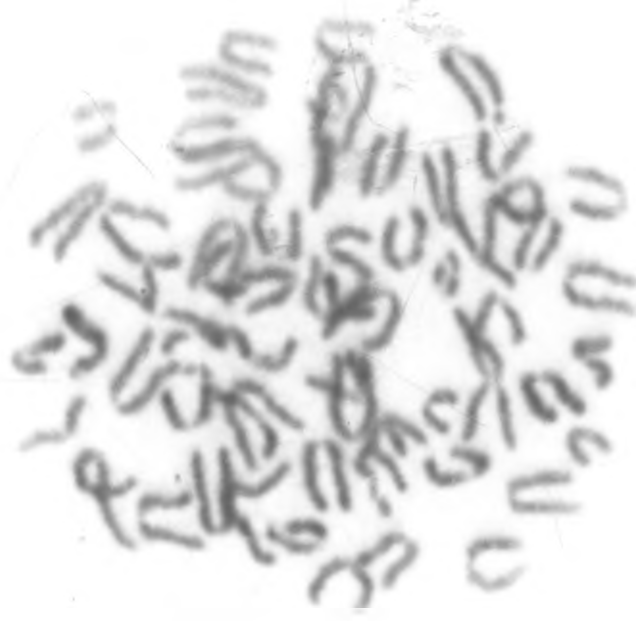


Figure 36. Metaphase Chromosome Spreads of NS1 Mouse Myeloma Cell

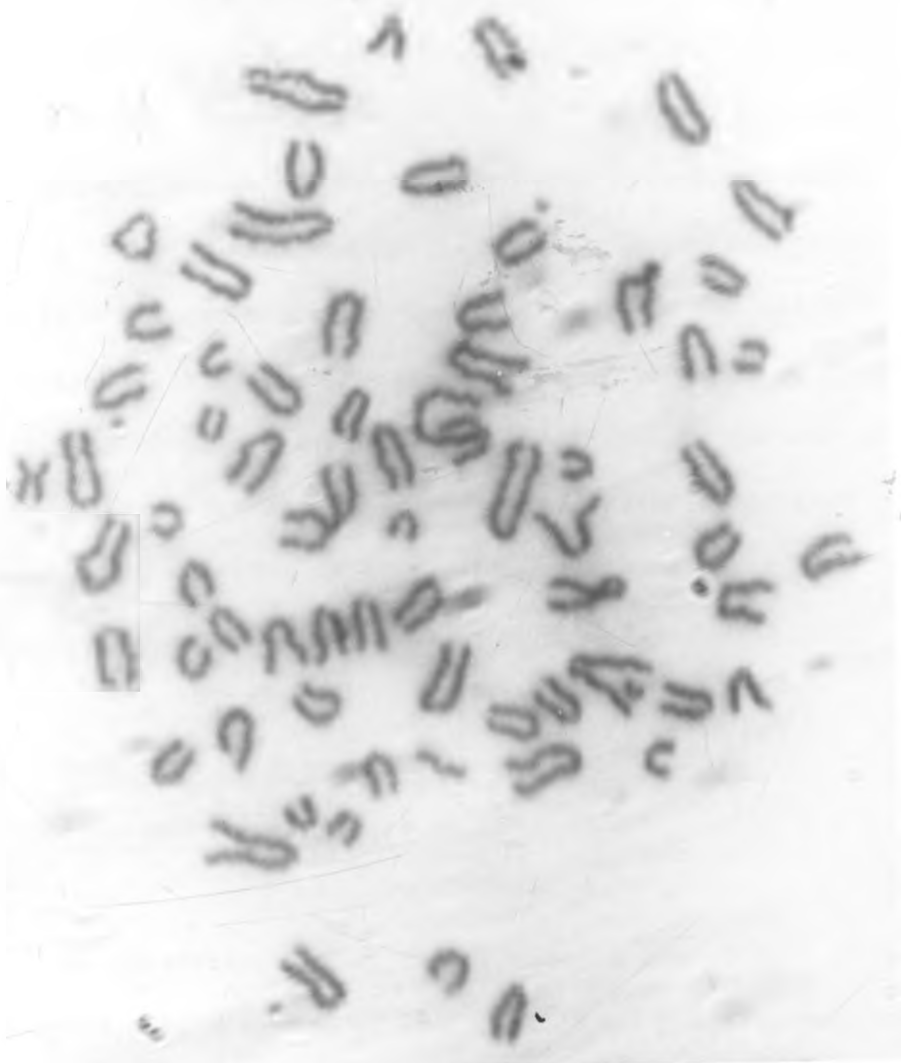


Figure 37. Representative Metaphase Derived Chromosomes
of an NS1 x Bovine Hybrid Cell.

determination of a modal chromosome number per cell line. The mouse myeloma cell lines NS1 and Sp2/0 had 50-52 and 70-72 chromosomes respectively (Fig. 36), while the bovine PBM had a stable chromosome number of 60 (refer Fig. 34). Hybrid cells between NS1 and bovine PBM revealed a modal chromosome number of 62 - 68 (Fig. 37) and the hybrids between Sp2/0 and bovine PBM had 72 - 110 chromosomes. When NS1 x bovine and Sp2/0 x bovine hybrid cells were karyotyped after 40 passages in vitro, the number of chromosomes in the hybrid cells were noticed to have decreased to between 56-58 and 70-98 respectively (not shown). It was not clear whether the chromosomes lost were of mouse or bovine origin, or whether the loss was random.

3.3.5 Selection of Mouse x Bovine Hybrid Cells for Spontaneous Resistance to 8AG

To determine whether or not some of the mouse x bovine hybrid cells (NS1 x bovine or Sp2/0 x bovine) had spontaneous resistance to 8AG, the cells were obtained at logarithmic phase of growth and seeded at 2×10^5 cells/ml in RPMI-1640 medium, containing 10% dialysed FBS, and 8AG ranging in concentrations from 2.5 ug/ml to 40 ug/ml. The cells were incubated at 37°C in an atmosphere of 7-10% CO₂ in air for five days and viability was determined daily by trypan blue dye exclusion. Hybrid cells cultured in 8AG concentrations below 20 ug/ml appeared to multiply normally. About 60% of NS1 x bovine and

75% of Sp2/0 x bovine cells in concentrations of 8AG above 20 ug/ml were dead by day two of culture (not shown). The surviving cells kept on multiplying, after their density was adjusted to 2×10^5 /ml, in the same medium, until a peak was achieved around day 10 of incubation. The 8-azaguanine resistant hybrid cells were expanded and routinely grown in medium containing 40 ug/ml of 8AG (Fig. 38, representative) to kill any possible "revertants". The 8AG resistant hybrid cells presumably arose as a result of loss of the chromosome with the HGPRT gene.

3.3.6 Growth of 8-Azaguanine Resistant Mouse x Bovine Hybrid Cells in Medium Containing Hypoxanthine, Aminopterin, and Thymidine (HAT)

The two 8-azaguanine resistant hybrid mouse x bovine (NS1 x bovine and Sp2/0 x bovine) cells were seeded at 5×10^5 cells/ml of medium containing 15% FBS and aminopterin concentrations ranging from 1×10^{-6} to 4×10^{-7} M. The concentrations of thymidine and hypoxanthine were kept constant at 3×10^{-5} and 10^{-4} M respectively. The cells were incubated at 37°C in an atmosphere of 7-10% CO₂ in air for two weeks. Cell numbers and viability were assayed every two days by staining with trypan blue dye and counting in a haemocytometer, and the cell density was adjusted to the original seeding density whenever it dropped below that.

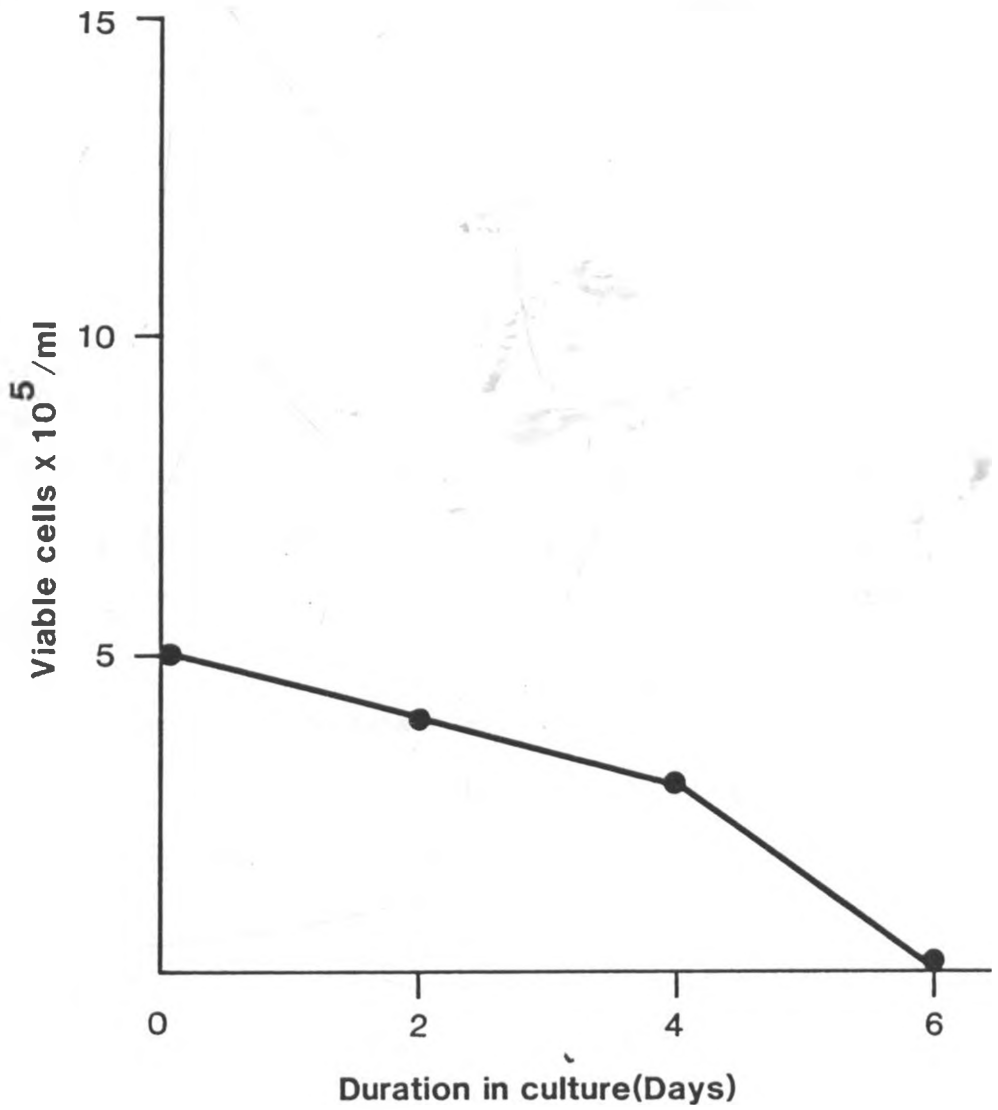


Figure 39. Representative Growth Curve of 8AG Selected Mouse x Bovine Hybrid Cells in HAT Containing Medium.

Results obtained with cells cultured in 4×10^{-7} M aminopterin are shown in Fig. 39 (representative Figure). The two mutant hybrid cells were all killed in aminopterin containing medium in about six days. These results confirmed that the hybrid cells were deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), and were therefore potential fusion partners.

3.3.7 Selection of 8AG Resistant and HAT Sensitive Surface Ig Negative Mouse x Bovine (NS1 x bovine) Hybrid Clones for Back Fusion with Bovine PBM

Clones of 8AG resistant and HAT sensitive NS1 x bovine hybrid cells were obtained from limiting dilution studies and expanded before staining in an indirect immunofluorescence assay (Section 2.8.2) using MAb B5/4 (for surface IgM). The stained cells were analysed on the FACS 11 (Section 2.8.5) to identify surface Ig positive and negative clones. Four out of 13 clones expressed surface IgM as revealed by MAb B5/4 (Fig. 40). A fast growing surface IgM positive clone (A11), and a surface IgM negative clone (A1), were expanded for fusion with PWM activated bovine PBM.

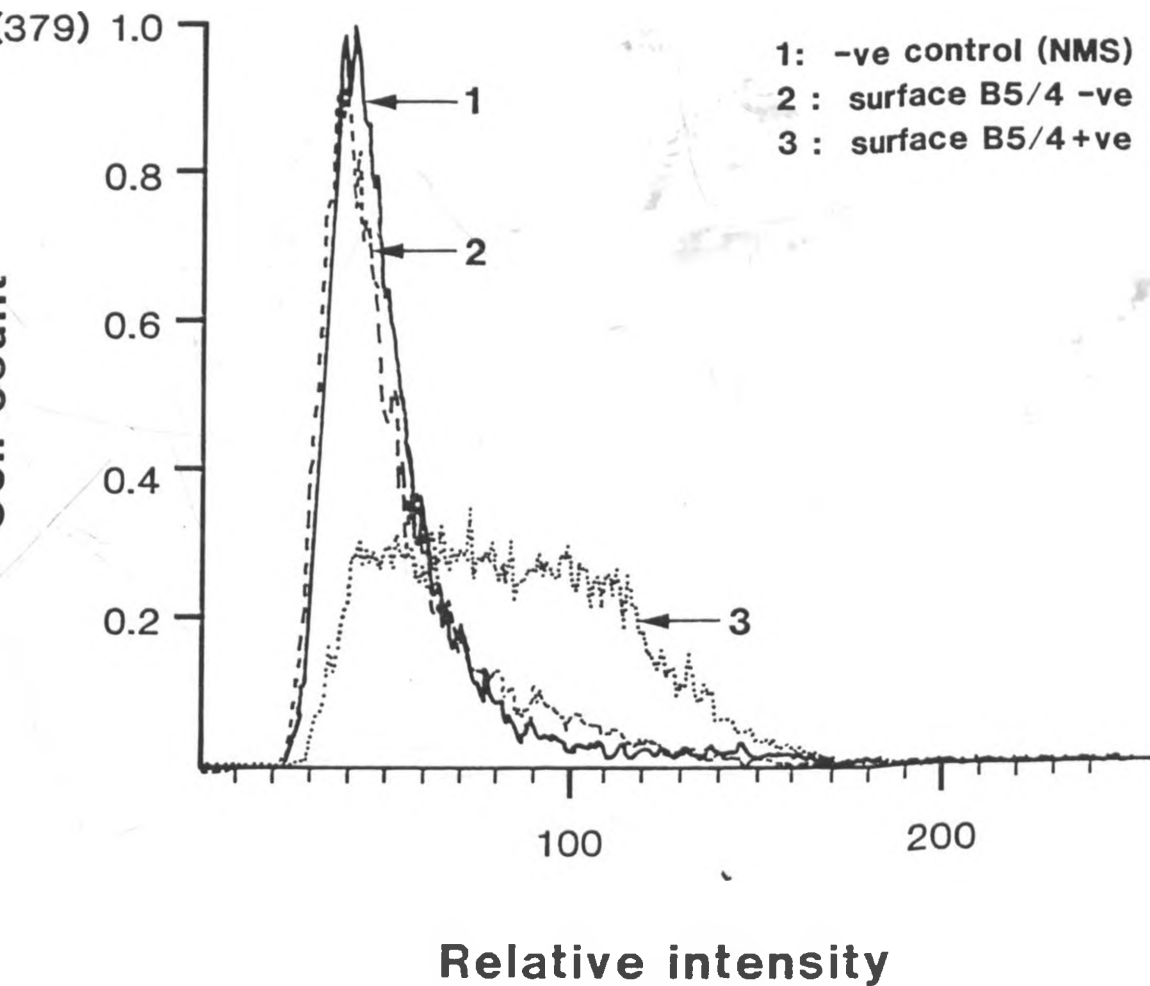


Figure 40. Histograms of Mouse x Bovine Hybrid Clones Showing a Surface IgM Negative Clone (2) and a Surface IgM Positive Clone (3) as Stained with MAb B5/4; (1) Represents the Hybrid Cells Stained with Normal Mouse Serum as a Negative Control (-ve).

3.3.8 Fusion of HAT Sensitive, Surface Ig Negative (A1), or Surface Ig Positive (All), NS1 x Bovine Hybrid Clones to PWM Activated Bovine PBM

Surface IgM positive (All) and surface IgM negative (A1) NS1 x bovine hybrid clones which were sensitive to HAT were obtained from the logarithmic phase of growth. Donor animal PBM were cultured in the presence of PWM for six days before fusion. The fusion partners were washed in HEPES and serum free RPMI-1640 and fused at a 1:1 ratio using PEG 1550 as

TABLE 14. Fusion of PWM Activated Bovine PBM with HAT Sensitive Mouse x Bovine Heterohybrid Cell Clones

Fusion Partners	Putative Hybrid Growth at 9-12 Days*		
	Fusion 1	Fusion 2	Fusion 3
Mouse x Bovine Clone A1 (surface IgM -ve)	3/96	5/96	4/96
Mouse x Bovine Clone All (surface IgM +ve)	2/96	3/96	3/96

* Wells initially seeded with 2×10^5 cells from the fusion mixture and displaying signs of growth 9 to 12 days later. Results expressed as number of wells with growth per total number of wells seeded with fused cells.

described in Section 2.10.3. The fused cells were resuspended in fusion medium and seeded in 100 ul volumes at 2×10^5 cells/well. Each well of the 96 well microtitre plate also contained 10^6 mouse thymocyte feeder cells. Subsequent incubation and selection for mouse x bovine x bovine hybrid cells was conducted as described earlier (Section 2.10.3). The results of three fusion attempts between each of the two mouse x bovine hybrid clones (A1 and A11) and PWM activated PBM are shown in Table, 14. Visible cell colonies in wells were noticed between day 9 to 12 after fusion in all experiments where the two hybrid clones were used as fusion partners. But the colonies of cells which were initially detected died consistently after about 3 weeks from fusion time. Efforts to rescue the hybrid cells by adding new feeder cells were unsuccessful. This rapid death was attributed to possible chromosome loss and/or to incompatibility between the products of the fusion partner and bovine cell genomes. It was concluded from these results that bovine PBM derived from in vitro culture with PWM gave unstable hybridomas when fused to NS1 x bovine heterohybrid cell lines.

3.3.9 Comparison of Cloning, Viability, and Fusion Frequency of Parental Mouse Myeloma Cell Lines NS1, or Sp2/0, versus Mouse x Bovine (NS1 x Bovine or Sp2/0 x Bovine) Cells Lines, Fused to Antigen Activated Bovine PBM.

A HAT sensitive NS1 x bovine cell clone, A1, which

was negative for surface Ig, and a HAT sensitive Sp2/0 x bovine cell line were obtained for fusion at logarithmic phase of growth. Donor lymphocytes were obtained from animals which had previously been primed with 10^{11} SRBC in saline and were boosted with SRBC in vitro for three days. Antigen activated lymphocytes were used in these studies because results from the previous experiments (Section 3.3.8), where PWM activated lymphocytes were used as donor lymphocytes had resulted in very few viable hybrids.

The cell lines were fused as described earlier (Section 2.10.3). Fused cells were seeded in 1 ml volumes at varying cell densities ranging from 2.5×10^5 cells (PBM)/ml to 10^6 cells/ml in the presence of mouse thymocyte feeder cells. Selection of fused cells in aminopterin containing medium was conducted as before (Section, 2.10.3).

The results are shown in Table 15. When NS1 x bovine (A1) cells were fused back to antigen activated PBM, 2 out of 24 wells, 5 out of 39 wells and 7 out of 34 wells seeded with 2.5×10^5 , 5×10^5 and 10^6 cells/ml respectively were positive for hybrid cell growth at 14 days after fusion. However, with continued culture to expand the cells, most of the putative clones of hybrid cells died except for two wells seeded with 10^6 cells/ml, which reached a stage when they could be tested. Results with the Sp2/0 x bovine fusion partner were similar to those obtained with the NS1 x bovine clone. From a total of 48 wells, with one-half of the total

wells seeded with 2×10^5 and 5×10^5 cells/ml respectively and 35 wells seeded with 10^6 cells/ml, cells from only 1 well at 10^6 cells/ml continued growing until they were ready for examination (Table 15).

Table 15. Comparison of Cloning, Viability and Fusion Frequency of Parental Mouse Myeloma Cell Lines NS1 or Sp2/0 Versus Mouse x Bovine (NS1 x Bovine or Sp2/0 x Bovine) Cell Lines Fused to Antigen Activated Bovine PBM.

Fusion Partners	No. of Cells Seeded/ml/well*		
	10^6	5×10^5	2.5×10^5
NS1	4/24	5/48	0/24
NS1 x Bovine (A1)	2/24	0/39	0/34
Sp2/0	1/36	0/24	0/24
Sp2/0 x Bovine (Line)	1/35	0/24	0/24

*Wells (1 ml cultures) with growth per total number of wells seeded with the fusion mixture.

In primary fusion experiments, i.e., NS1 cells fused to bovine PBM, 0 out of 24 wells, 5 out of 48 wells and 4 out of 24 wells seeded with 2.5×10^5 , 5×10^5 and 10^6 cells/ml respectively exhibited colony growth until testing time (Table 15).

Results from Sp2/0 cells fused to bovine PBM gave only one well with hybrid cell growth out of a total of 36 wells seeded with 10^6 cells/ml. In all other wells, i.e., 24 of each seeded with 2×10^5 and 5×10^5 cells/ml, no hybrid cell growth was seen (Table 15).

DISCUSSION4.1 Generation of Activated Bovine Lymphocytes

The aim of this study was to generate cell lines which could be used as fusion partners for production of bovine monoclonal antibodies. In the first instance, activated bovine B cells en route to Ig synthesis and secretion were generated for use as fusion partners. Secondly, a bovine lymphoblastoid cell line designated BL20 (Morzaria et al., 1982) was selected for aminopterin-sensitivity, and thereafter referred to as ATS/BL20, and was fused with bovine lymphocyte populations containing activated B cells, and the hybrids were selected in aminopterin-containing medium. When this strategy failed to generate fast growing hybrids, a different strategy was followed. Lymphocyte populations containing activated B cells were fused to aminopterin-sensitive mouse myeloma cells (Primary fusions), and resultant hybrids were selected in aminopterin-containing medium. The hybrids were reselected for aminopterin-sensitivity, and were used in back fusion (Secondary fusions) studies with populations of bovine lymphocytes containing activated B-cells.

4.1.1 Cell Types Present in Bovine PBM

Bovine peripheral blood was used as a source of lymphocytes for most of these studies. The cells were isolated over Ficoll-Paque (Boyum, 1968). This method of lymphocyte isolation gave variable yields of total PBM from bovine to bovine and from the same bovine on different days of isolation. The composition of the different cell types in PBM was also noticed to vary from bovine to bovine. The cells were identified by staining with the appropriate antibodies followed by examination for low forward angle light scattering properties on a FACS II. The B-cell population ranged between 7% to 30%, T-cells were found to range between 25% to 56%, and the macrophage composition was between 1% to 4%. The remainder of the cells on each occasion might constitute a "null" cell (non T, non B, non macrophage) subpopulation. The method of isolation of PBM excluded polymorphonuclear leucocytes, which by Giemsa staining constituted less than 1% of the total lymphocyte population.

B cells expressing IgM were identified by monoclonal antibody B5/4 (Pinder et al., 1980), which had been shown to bind to pentameric bovine IgM from serum and to the surface of B-cells. Studies by Baldwin et al. (in press) confirmed that a commercially available anti-bovine IgM antibody stained a comparable number of B-cells as did B5/4. IgG expressing B-cells were identified by monoclonal antibody A₂, which had

been shown to bind to a very small population of lymphocytes expressing either IgG₁ or IgG₂ molecules (Wells and Karr, unpublished). Using a polyvalent goat antibovine-Ig antibody, Pearson et al. (1979) identified a similar population to that described above (35%), of bovine PBM, to be surface Ig positive cells.

T cells were identified by staining with MA b P5. Studies by Lalor (1983) using P5 had revealed that about 50% of normal bovine PBM was composed of T cells. The T-cell population was divided into the so-called P5^{hi} and P5^{low} subsets. These findings were confirmed when the same antibody (P5) was used. Previous investigators had employed lectin binding as a marker for bovine T cells. Using PNA binding, different workers were able to confirm that the lectin bound to surface Ig negative cells, indicating that PNA may bind exclusively to bovine T lymphocytes (Pearson et al., 1979; Outteridge and Dufy, 1981). The method of sheep erythrocyte rosette formation has also been used as a marker for bovine T-lymphocytes (Reeves and Renshaw, 1978; Grewel et al., 1976; Wardley, 1977; Fruchmann et al., 1977). The results obtained in this study were comparable to those reported by other investigators who used the above two methods for enumeration of bovine T cells (Pearson et al., 1979; Fruchtmann et al., 1977), (although in this study results were not confirmed by PNA binding or SRBC rosette formation).

Schalm et al. (1975) showed that the cell type composition in different bovine organs was influenced by age, sex, breed, stress, nutritional and health status. It was possible that some of these factors could have been responsible for the variation in the total cell numbers and cell types in PBM observed in this study.

4.1.2 Mitogen and/or Antigen Activation of Bovine PBM

In order to establish reproducible conditions for obtaining activated bovine B cells, different densities of PBM were cultured in the presence of varying concentrations of PWM. To achieve maximum proliferation of PBM a combination of 2.5×10^6 cells/ml with 2.5 ug/ml of the mitogen was found to be optimum. The degree of stimulation under these conditions was variable between PBM from different bovines, but the peak of proliferation was always on day four of culture. Similarly, a critical cell density and concentration of mitogen had previously been reported to allow maximum proliferation of bovine PBM (Pearson et al., 1979; Muscoplat et al., 1974), and leucocytes of other species (Levitt et al., 1981). It was concluded from these studies that the conditions for optimal stimulation of proliferation in bovine PBM by PWM parallels those found in the murine system (Andersson et al., 1972; Coutinho and Moller, 1973), and in the human system (Janosy et al., 1977; Montazeri et al., 1980).

Several agents have been studied to determine their capacity to function as polyclonal activators of normal B cells in other species. Some of these agents include; dextran sulfate; polyvinylpyrrolidone (PVP); purified protein derivative (PPD); pneumococcal polysaccharide type 111 antibodies to β_2 -microglobulin (reviewed by Waldmann and Broder, 1982); lipopolysaccharide (LPS; Andersson et al., 1972); Bactostreptolysin O reagent (SLO); staphylococcal phage lysate (SPL) types 1 and 111 (Dean et al., 1975); wheat germ agglutinin (WGA; Greene et al., 1981); Staphylococcus aureus Cowan strain 1 cells (Kim et al., 1979; Forsgren and Sjoquist, 1966); and Epstein-Barr virus line B95-8 supernatant (Bird and Britton, 1979). Many of these agents may act as polyclonal activators of B cells from one species and may fail to stimulate cells from others. A number of factors could be responsible for these differences. One such factor is a possible difference in the organ distribution of cells that could respond to these agents (Finkelman and Lipsky, 1979; Gronowicz et al., 1976). The organ differences may reflect differences in the distribution of subsets of B-cells, considered in terms of their receptors for different activators or in terms of their stage of maturation. Alternatively, the organ distribution differences may reflect differences in the proportion of cells that could be activated to become suppressor cells by the activators used (Waldmann and Broder, 1982). In this study, PWM was chosen as a polyclonal B cell

activator because it has been widely used in the murine and human systems as a stimulator for B cells (reviewed by Waldmann and Broder, 1982) and was found to work similarly in the bovine system described here.

When bovine PBM were cultured in the presence of PWM and aminopterin, there was a reduction in the proliferative response of the cells to about 25% as compared to non-treated cultures, although the peak of proliferation of cells was still seen on day four of culture. Olsson et al. (1983) had earlier made similar observations when they cultured human PBM with PWM and aminopterin, in order to adapt the cells to the DNA synthetic rescue pathway that is used by hybridomas to overcome the aminopterin blockage during selection in HAT medium. The treatment resulted in a marked decrease in the proliferative response of the cultured lymphocytes. Reports by Evans and Vijayalaxim (1981) showed evidence of some human PBM which were resistant to 8-azaguanine i.e., synthesized DNA by the classical pathway only. Such cells are killed in aminopterin containing medium leaving only those with both pathways for DNA synthesis. The results obtained in this study suggested that a similar population of cells existed in bovine PBM. Another possible explanation for the reduced PWM induced proliferative response of PBM in the presence of aminopterin was that, after aminopterin blockage of the classical DNA-synthetic pathway, the cells were left with only the alternative pathway for DNA

synthesis. This could have resulted in a reduction in the rate of DNA synthesis.

The conditions established for optimum proliferation of bovine lymphocytes in the presence of PWM alone, or PWM plus aminopterin, were also found to drive the B cells to the terminal differentiation stages of Ig synthesis and secretion. There was little synthesis and release of Ig by lymphocytes during the first three or four days of culture with PWM. However, after this time, the concentration of detectable IgM, and to a lesser extent IgG, increased rapidly, until day eight (end of culture), indicating a maturation of B cells into immunoglobulin synthesizing and secreting cells. The amount of Ig synthesized and subsequently released by the B-cells increased soon after the total lymphocyte population had achieved the maximum peak of DNA synthesis. The requirement for DNA synthesis in the induction of Ig secretion by B-lymphocytes in human PBM was shown by Jelinek and Lipsky (1983), using several activators (PWM, LPS, Staphylococcus aureus Cowan 1 and Salmonella typhimurium cells). Similar findings have not been reported in bovine lymphocytes. From these preliminary studies, it may be that DNA synthesis is also a pre-requisite for Ig production by bovine B-cells stimulated with PWM, although more conclusive evidence is still required to confirm this observation.

The use of SRBC as targets for the measurement of antibody synthesis after polyclonal B-cell activation is well

established in the mouse (Coutinho and Moller, 1973), and human systems (Fauci and Pratt, 1976). This technique is based on the premise that polyclonal B-cell activators stimulate lymphocytes to secrete antibodies with wide ranges of specificities, including those that react with determinants on SRBC. It was clear from the present studies that this principle was also applicable to bovine PBM stimulated with PWM and tested for plaque forming cells to SRBC. It was concluded from these findings that PWM was likely to be a polyclonal activator of bovine B cells.

Some investigators have shown that the presence of non-specific lymphocyte activators (eg. staphylococcal protein A (SpA); phytohaemagglutinin (PHA), pokeweed mitogen (PWM), concanavalin A (Con A), lipopolysaccharide (LPS), supernatant from a mixed lymphocyte culture, or T-cell growth factor (TCGF), Lipsky, 1980; Lagace and Brodeur, 1985), could amplify the production in vitro of specific antibodies to an antigen. To try to establish whether this could be reproduced for the bovine system, a lower concentration of PWM (lower than that used to stimulate bovine PBM alone i.e., 0.156 and 0.625 ug/ml of PWM) was used to stimulate bovine PBM, in combination with an optimal concentration of SRBC in vitro. A higher number (twice as many i.e. 110 plaques) of anti-SRBC plaque forming cells was generated when compared to the controls, which showed only about 45 plaques for every 5×10^5 PBM. This result

confirmed that PWM could augment SRBC to generate anti-SRBC forming cells in bovine PBM.

To examine the effects of repeated in vivo immunisations on specific antibody responses to antigen (SRBC) in vitro, cattle were primed by subcutaneous injections of SRBC followed by booster injections of SRBC into the spleen. After incubating PBM from such bovines in vitro in the presence of SRBC, the cells were examined for plaque formation at different time points. More than 200 plaque forming cells to SRBC for every 5×10^5 PBM were obtained each time as compared to less than 20 PFC in controls. These results confirmed the reports of Fillion et al., (1984) who had earlier shown that a specific in vitro antibody response could be induced by culturing bovine PBM in the presence of KLH only if the cells came from bovines previously primed to the antigen.

The observation, that PBM from different bovines responded by varying degrees of Ig production after stimulation with PWM or antigen could have several meanings. As mentioned earlier, initiation of B-lymphocyte differentiation by PWM is a complex event involving interactions between helper and suppressor T-cells and accessory cells (reviewed by Waldmann and Broder, 1982). The activation of many prosuppressor T-cells into effectors of suppression, or the presence of fewer helper cells than required, could have inhibited the maturation of B cells observed in the system. Preliminary studies indicate that the presence of bovine T-cells is necessary for

Ig production by PWM activated PBM (Black, unpublished). Other as yet unidentified mechanisms could have also contributed to the variability of responses observed.

Previous studies had centred on examining mitogen binding to, and inducing proliferation in, bovine PBM (Pearson et al., 1979; Lazary et al., 1974b). In these studies it was shown that bovine B cells could be driven to proliferation and Ig synthesis and secretion when cultured in vitro in the presence of PWM. Methods for the primary and secondary in vitro immunisation of bovine lymphocytes with specific antigens are not fully defined, apart from a single report in the literature on the secondary immunisation of bovine PBM to KLH (Filion et al., 1984). In these studies too, it proved possible to obtain some specific-antibody producing B-cells in vitro, by culturing bovine PBM with SRBC and PWM, or by co-culturing SRBC with PBM from bovines which had previously been primed with SRBC.

One important limitation in the bovine hybridoma system is apparently resident in the poorly developed technology for generating reproducible specific bovine antibody responses in vitro. An extension of the studies reported here could contribute to identification of the conditions for in vitro immunisation of bovine lymphocytes with antigen. This is an important goal and would greatly increase the flexibility of the bovine hybridoma system with respect to the number of antigens which could be used. These culture systems could also

be used to examine the cellular and interleukin requirements for activation of bovine B cells into Ig synthesis and secretion.

4.2 The Development of a Bovine Lymphoblastoid Cell Line Fusion Partner

In an attempt to construct intraspecific hybrid cells producing monoclonal antibody, a bovine lymphoblastoid cell line which could serve as a fusion partner for bovine B cells was generated. The line was developed from the existing bovine leukaemic cell line BL20, after examining this line and another lymphoblastoid line BL3.

Although the BL3 and BL20 cell lines were originally isolated from cases of bovine leukosis, they did not contain any viral particles (Morzaria et al., 1982; Theilen et al., 1968; Olobo, unpublished observations). The wide range of chromosome numbers seen in BL3 cells however, suggested to us that the cell line was unstable. The BL3 cell line also did not stain for either cytoplasmic or surface bovine Ig and many other bovine markers available in the laboratory, and hence was rejected as a candidate for a potential fusion partner.

In contrast, BL20 cells, had a stable chromosome number of 60, even after γ -ray irradiation induced mutation. The doubling time of the cell line was short (between 18 and 24 hrs), being about the same as for BL3 cells. BL20 cells did

not stain for cytoplasmic IgM or IgG, but nearly all the cells (over 95%) were positive for surface IgM, suggesting they were of B-cell origin. In addition, the BL20 cell line did not express surface BoT4 or BoT8 like molecules as defined by MAbs IL-A12 or IL-A17 respectively, and hence was unlikely to be a T cell line. Parental BL20 cells could only be cloned out at 100% efficiency on bovine thymus-derived fibroblast-like cells. However, with continued selection, a cell line was isolated which could clone at a frequency of 20%, without the need for any feeder cells.

The basis for selection of a lymphoblastoid or myeloma cell line for fusion with normal B-cells, for mouse monoclonal antibody production, has been well defined (Kohler and Milstein, 1975; Kearney et al., 1979). The cell line must be B in origin, and should preferably be an Ig non-producer. With such a lymphoblastoid cell line, the hybrids constructed produce only the Ig chains of the immune B-cell. When an Ig producer is used for fusion with B-cells, two sets of heavy and two sets of light chains are synthesized by the hybrid cells. The heavy and light chains combine intracellularly to form mixed Ig molecules (Cotton and Milstein, 1973; Marguelies et al., 1976a). The lymphoblastoid fusion partner should not carry any viruses, and the cells should have a stable aneuploid chromosome number and be fast growing, with good cloning frequencies. Some similar characteristics to those described

for mouse myeloma fusion partners (NS1, X63 or Sp2/0) were shown by BL20 cells.

4.2.1 Possible Mechanisms of Transformation of BL3 or BL20 Cells

Several mechanisms of cellular transformation have been described in the literature. Transformation may arise from the action of viruses (eg. BLV virus), or from anomalies on proto-oncogenes contained in eukaryotic cells, which are concerned with the control (possibly cell type specific) of growth and differentiation, or from mutagens (carcinogens, radiations) on the genes themselves.

Although BL3 and BL20 cell lines were originally from cases of bovine leukosis, the mechanisms underlying their transformation are only speculative. Two forms of leukosis have been reported in the bovine: enzootic bovine leukosis (EBL), and sporadic bovine leukosis (SBL). It was not indicated whether BL3 was from sporadic or enzootic bovine leukosis (Theilen et al., 1968), but BL20 cells were from a case of sporadic bovine leukosis (Morzaria et al., 1982). Enzootic bovine leukosis has now been shown to be associated with bovine leukaemia virus (BLV), which is an RNA virus. Sporadic bovine leukosis has not yet been shown to be associated with a virus and the aetiology is still unknown. There is no evidence that BLV plays a role in sporadic bovine

leukosis (Ferrer, 1980). Antibodies to the dominant BLV antigens, gp51 or P24, have not been detected in SBL cases, and molecular hybridization studies have so far failed to detect BLV nucleic acid sequences in tissues from animals infected with SBL (Derse and Casey, 1986).

The role of oncogenic viruses (tumour causing) in inducing cellular transformation has been widely studied (Paul, 1984; Doolittle et al., 1983; Waterfield et al., 1983; Ringertz and Savage, 1976). Oncogenic viruses have been divided into RNA and DNA groups, depending on the method of cellular transformation. There is now strong evidence that, in transformed cells, the viral genomes, or a part of them, become co-valently linked (integrated) to the host cell genome, and then replicate in synchrony with the host cell nucleus, without giving rise to infectious particles (Ringertz and Savage, 1976). The part of the viral genome that is present in transformed cells is believed to carry a transformation gene. But the exact location of the gene, the nature of the gene product, and the mechanisms by which the transformed state is induced, is presently an intense area of research.

DNA sequence studies from normal eukaryotic cells have shown some matching sequences to viral oncogenes. These genes have been referred to as proto-oncogenes. Proteins coded by proto-oncogenes are in some way concerned with growth regulation (Doolittle et al., 1983; Waterfield et al., 1983).

Several different mechanisms have been uncovered which are associated with activation of proto-oncogenes.

Quantitative or qualitative anomalies of expression of these genes can give rise to malignant transformation. One means of activation of proto-oncogenes occurs when a long terminal repeat DNA sequence, usually associated with a proviral sequence, is inserted in front of the gene causing an increased rate of transcription. Another method by which the proto-oncogene can be activated is when an enhancer, usually associated with a virus, has been inserted near the gene. Other possible ways for activation could be by a point mutation, or by a deletion, in the gene (Paul, 1984).

A number of reports have appeared in the literature concerning neoplastic transformation of cells using chemical carcinogens. Namba et al. (1978, 1981) have transformed human embryo fibroblasts by treatment with ^{60}Co gamma rays and 4-nitroquinoline 1-oxide (4NQO). Kakunaga (1978) has described the transformation of fibroblasts from an adult human using 4NQO or N-methyl-N'-nitrosoguanidine (MNNG). Dorman et al. (1983) have transformed human endometrial cells with MNNG.

Physical means, such as gamma ray treatment, have also been used to induce cellular transformation, and they induce chromosome aberrations in many more cells than do the chemical carcinogens so far examined (Glover et al., 1979; Maher et al., 1982). Gamma rays cause more exchange types of aberrations than gaps or breaks, while chemical carcinogens

induce more gaps or breaks than exchanges. Although a direct relationship between chromosome aberrations and mechanisms of carcinogenesis has not been established at present, a high frequency of exchange types of chromosome aberrations seems to be closely related to mutational events, including cell transformation (Dorman et al., 1983).

Cellular transformation could thus be achieved in several ways. It was not essential to determine the mechanisms of transformation of BL3 or BL20 cell lines before rendering them suitable for fusion, though a knowledge of the transformation mechanisms would have been helpful in interpreting results with the BL20 fusion partner.

4.2.2 Selection of Aminopterin Sensitive BL20 Cells

In an attempt to increase the frequency of BL20 cells which could be selected for resistance to 8AG and/or 6TG, the cells were first subjected to gamma irradiation. Hendrickson et al. (1981) postulated that most γ -ray-induced purine analogue resistant mutants were associated with two types of gross structural changes: those resulting in breakage of the X-chromosome proximal to the HGPRT locus, leading to the loss of the gene at mitosis; and those resulting in breakage or damage directly at the locus itself. Studies by Cox and Masson (1978) also showed that some x-ray induced mutants in fibroblasts had stable x-chromosome aberrations (eg. deletions,

translocations etc) whose locations were consistent with the mapped position of the HGPRT locus on the x-chromosome. The type of mutation brought about in BL20 cells by gamma irradiation was not however determined in these studies.

After irradiation, BL20 cells were selected in gradually increasing concentrations of 8-azaguanine to isolate the mutant cells lacking the HGPRT gene. The resultant cells were tested for sensitivity to aminopterin. Not all the 8AG resistant BL20 cells were killed by the folic acid antagonist, suggesting that some of the cells still had an intact and functional salvage pathway for DNA synthesis. These same cells were selected further in increasing concentrations of 6-Thioguanine, which has been reported to produce cell lines with complete absence of cellular HGPRT activity. When these cells were tested in aminopterin, all were killed by the drug in about six days.

Resistance to the cytotoxic purine analogues 8-AG or 6TG is known to be associated with deficiency in the x-chromosome-linked purine-salvage enzyme HGPRT. 8-azaguanine and 6-Thioguanine are guanine analogues, usually lethal to cells with normal levels of hypoxanthine guanine phosphoribosyl transferase (HGPRT). This enzyme converts both hypoxanthine and guanine, the normal physiological substrates, as well as the two synthetic analogues (8AG or 6TG), to their corresponding 5'-mononucleotides. It has been reported by several laboratories that while a single-step selection for

6-thioguanine resistance almost invariably leads to the complete absence of cellular HGPRT activity (Gillin et al., 1972; Sharp et al., 1973), resistance to 8-azaguanine can occur in cells with apparently normal levels of HGPRT activity. Consequently, cells selected only for 8AG resistance are often resistant to HAT medium as well (Gillin et al., 1972; Sharp et al., 1973). The basis of this differential sensitivity of some cells to the two purine analogues has not been fully established (Van Diggelen et al., 1979).

Some mechanisms have however been postulated for the differential sensitivity of mammalian cells to 8AG. One postulate is that 8AG might interact poorly with cellular HGPRT in some cells (Donahue et al., 1976). The metabolism of 8AG in these cells could be highly efficient eg. 8AG could be degraded rapidly after entry into the cells. Finally, mammalian cells could have more than one genetic locus which is involved in guanine analogue resistance. This last possibility is consistent with the discovery of genetic complementation between 8AG-resistant cells (Shapiro et al., 1985), and with the report that some mammalian cells have more than one HGPRT locus, which could be active under certain experimental conditions (Milman et al., 1976). In these studies, efforts were not made to establish the mechanisms of resistance of BL20 cells to 8AG treatment.

4.2.3 Construction of ATS/BL20 x Bovine Hybrid Cells

Aminopterin sensitive BL20 cells which were generated by selection in 8AG and 6TG were designated ATS/BL20. These cells were fused with PWM activated bovine lymphocytes, using essentially the method of Kohler and Milstein (1975), with some modifications. Preliminary studies using PEG for fusion showed that a short exposure time of cells to PEG was necessary in order to produce a low mortality rate. Similarly, short exposure times for fusion of mouse cells using PEG has been reported to give increased numbers of hybrid cells (Lane, 1985). PEG mediates fusion of cell membranes but not of karyons (Olsson et al., 1983). Individual batches of PEG varied considerably in their toxicity. Even the least toxic batch of PEG required a concentration quite close to the toxic concentration (Goding, 1980) in order for fusion to occur. Different fusion conditions with PEG have been reported to give variable numbers of hybrid cells. The number of hybrid colonies were for example reported to be increased when PEG was sterilized by filtering instead of autoclaving (Westerwoudt, 1985). The yield of hybrids was also reported to increase when Ca^{2+} was omitted from the medium for at least 15 minutes after fusion with PEG (Schneiderman et al., 1979), or when the pH of PEG was between 8.0-8.2 (Sharon et al., 1980).

A number of studies suggest that antibody forming hybrid cells were derived from fusion of recently activated

B-cells with aminopterin sensitive myeloma cells. Olsson et al. (1983), Chiorazzi et al. (1982), Shoenfeld et al. (1982) and Strike et al. (1984) showed that human PBM stimulated with PWM in vitro for five to seven days formed hybrids although maximal Ig production in vitro was several days later (Waldmann and Broder, 1982). Galfre and Milstein (1981) showed that peak hybrid formation using antigen stimulated mouse spleen cells preceded peak plaque forming cell responses. Preliminary studies using PWM activated bovine PBM showed that hybrid formation was most efficient after six days of in vitro mitogen activation. This was later than the peak proliferative response of the culture which occurred four days after stimulation. No attempt was made to study the kinetics of activation of bovine T-cells and B-cells with PWM hence it is possible that the peak proliferative response correlated with T-cell and not B-cell activation. In retrospect it would have been useful to directly conduct studies on the kinetics of B-cell activation.

The fusion mixture of ATS/BL20 cells and PBM activated bovine lymphocytes was seeded onto bovine thymus fibroblast-like cells. These latter cells were initially essential for ATS/BL20 cell cloning. The culture system was composed of a mixture of autologous, semi-allogeneic and allogeneic components, which would have been expected to generate cytotoxic immune responses. But this was however not determined. In the mouse system it has been reported that

allogeneic thymocytes were as efficient as autologous thymocytes, as far as feeder cells were concerned (Goding, 1980), and even rat thymocytes seemed to function well as feeder cells for mouse x mouse hybrids.

The use of feeder cells as a support for hybrid cell growth early after fusion, and in cloning procedures, has been reported previously (Brodin et al., 1983; Goding, 1980; Coffino et al., 1972; Lernhardt et al., 1978). The commonly used feeder cells which have been reported in other systems include: thymocytes (Lernhardt et al., 1978; Oi and Herzenberg, 1980); normal spleen cells (Levy et al., 1978); peritoneal cells (Hengartner et al., 1977); and peripheral blood cells (Barry, personal communication). The exact function of feeder cells in general is not known. One role which has been suggested is that the toxicity of the plastic tissue culture trays (Goding, 1980) may be reduced. Macrophages have been thought to remove dead parental cells and debris from culture, and in addition they may provide some growth factors for hybridoma cell proliferation (Mao and France, 1984). The mechanisms by which the bovine thymus fibroblast-like cells enhanced cloning of BL20 cells, and also enhanced growth of ATS/BL20 x PBM or spleen cell hybrids, was not clear.

Fibroblast-like cells from other species have been shown to have receptors for low density lipoproteins (LDL; Goldstein and Brown, 1977), and human LDL have recently been

shown to enhance hybrid cell cloning (Mao and France, 1984). It was possible that the supportive effect of bovine thymus fibroblast-like cells for the hybrid cells was secondary to the effect of bovine LDL from the bovine serum supplement. Fibroblasts have also been reported to have a low phagocytic capacity (Uchida et al., 1984). This might have helped to reduce the debris and dead cells in the cultures. A colony stimulating factor (CSF-1) of fibroblast-like cell origin was isolated by Stanley (1979) and Stanley and Guilbert (1981). It was shown to be a glycoprotein with two disulphide bonded sub-units. This factor was found to enhance the growth and maturation of macrophages. It still remains to be determined whether the supportive effects of the bovine thymus fibroblast-like cells was due to reduction in toxicity of the culture plates, phagocytosis of dead cells and debris, effects of bovine LDL on the fibroblast-like cells, production of growth enhancing factors, or by some other as yet unknown mechanisms.

4.2.4 Confirmation of ATS/BL20 x Bovine Hybrid Cell Formation

Fusions between ATS/BL20 cells and PWM stimulated bovine PBM or SRBC stimulated spleen cells resulted in the generation of some hybrid cell lines. The frequency of hybrid generation was low in each case (approx. $1/2-4 \times 10^7$ input

cells), and hybrid cells required a long adaptation period in culture before they displayed visible growth.

Hybrid cell formation between ATS/BL20 cells and donor animal lymphocytes was confirmed by the demonstration of cytoplasmic IgM, surface antigens not expressed on BL20 cells but expressed on donor animal lymphocytes, and karyotype analysis. BL20 cells or ATS/BL20 cells did not express cytoplasmic IgM, but they expressed surface IgM.

After hybrid cell formation between ATS/BL20 cells and bovine lymphocytes, a proportion of the hybrid cells which appeared expressed cytoplasmic IgM as detected by monoclonal antibody B5/4. The cells expressing IgM were unlikely to be directly derived from ATS/BL20 cells, because in preliminary studies the cells were shown not to express cytoplasmic immunoglobulin molecules when cultured alone, or on bovine thymus fibroblast like cells, or in the presence of PWM. Again in other control cultures which consisted of antigen or mitogen activated bovine lymphocytes, co-cultured with ATS/BL20 cells on bovine thymus fibroblast like cells in HAT containing medium, but without PEG mediated fusion, no continuously growing cell lines were obtained after HAT selection. This indicated that the cells obtained after PEG fusion were most probably true hybrids.

A histocompatibility antigen detected by antibody IL-A7 was not expressed by either BL20 cells or ATS/BL20 cells. However, this antigen was found to be expressed on over 95% of donor animal lymphocytes. After fusion, between 20% and

25% of the hybrid cells expressed this antigen. It was most improbable that these cells were aminopterin resistant ATS/BL20 cells or that they were unfused bovine lymphocytes since the cells were analysed several weeks post fusion. By this time, all ATS/BL20 cells had been killed by HAT treatment, and also all unfused bovine lymphocytes in control cultures died within three weeks when cultured on bovine thymus fibroblast-like cells.

Another method, used to confirm hybrid cell formation between ATS/BL20 cells and bovine lymphocytes, was karyotype analysis. Both ATS/BL20 cells and donor animal lymphocytes had 60 chromosomes. Analysis of the chromosome numbers of hybrids revealed between 58 and 60 chromosomes, showing that they were near diploid or actually diploid. In some rare cases, a few hybrid cells with more than 60 chromosomes were observed. It was possible that the cells with around 58 chromosomes were hybrid cells which had lost chromosomes since ATS/BL20 cells had a stable chromosome number of 60, even after prolonged periods in culture.

4.2.5 Cloning and Stability of ATS/BL20 x Bovine Hybrid Cells

Only one out of 54 culture wells examined post-fusion, was found to contain IgM producing cells, as detected by monoclonal antibody B5/4. These cells were cloned out at 1, 3, 5, 10 and 20 cells per well. At 5, 10 and 20 cells per well,

1%, 47% and 100% of the wells seeded with the hybrid cells respectively showed growth. There was no growth at 1 and 3 cells/well, and the cells from positive wells were ready for analysis four weeks after cloning. On further analysis, none of the wells showed any cells expressing cytoplasmic IgM, suggesting that the cells had lost Ig production. Since loss of chromosomes may lead to loss of function, it was possible that the hybrids had lost the chromosomes responsible for Ig production during the prolonged culture period. Similar observations were made by Olsson et al. (1983), for human x human hybridomas. Out of 100 fusions, they were able to obtain only three hybrids producing antibody of interest. In their system, they obtained 21 out of 100 wells with hybrids producing antibody against antigens. Almost one-half of the 21 cultures died out during cloning, and of the others a few lost Ig production (Olsson et al., 1983).

4.2.6 Enhancement of the Rate of Growth of ATS/BL20 x Bovine Hybrid Cells by Exogenous Growth Factors

It was surprising that the hybrid cells remained quiescent for about 4 to 8 weeks after fusion, before displaying visible growth, when the doubling time of ATS/BL20 lymphoblastoid fusion partner was between 18 and 24 hours. This suggested that the transforming phenotype of ATS/BL20 was suppressed after fusion to normal bovine cells. Similarly

prolonged periods in culture prior to hybrid cell growth have been reported in several fusion systems (Irigoyen et al., 1981; Mayer et al., 1982; Foug et al., 1982).

Attempts were made to enhance the rate of growth of hybrid cells between ATS/BL20 and bovine lymphocytes, by adding exogenous growth promoters (Con A or PWM derived bovine leucocyte culture supernatants). But this proved unsuccessful. One shortcoming with the PWM derived bovine leucocyte culture supernatant was that it was not established in preliminary studies that the supernatant could support proliferation of a population of bovine B-cells. PBM from different animals stimulated with Con A have been shown to vary in the amount of TCGF produced (Goddeeris, personal communication). A similar picture might also be true for PWM derived culture supernatants. It was also possible that the time of isolation of the supernatant from PWM cultures was not optimal since only day three derived culture supernatants were used. The failure of Con A derived culture supernatant to enhance proliferation of the hybrids was also intriguing. The batch of Con A derived culture supernatants used had previously been shown to enhance proliferation of cloned bovine T-cells.

B-cell specific growth factors (BCGF; Maizel et al., 1983), T-cell specific factors e.g. interleukin-2 (IL-2; Watson, 1979), and a macrophage derived factor, (IL-1; Watson, 1979) are among the several growth factors that have been described. The effect of exogenous growth factors on the

maintainance of proliferation and clonal expansion of normal human or mouse B-cells has previously been reported by several workers (Maizel, 1983; Sredni et al., 1981; Butler et al., 1984). Supernatants from PWM or Con A stimulated cultures have been used as a source of growth factors. Such supernatants have been shown to contain a mixture of BCGF, IL-2, TRF and perhaps IL-1 as well as several other lymphoid cell products (Watson et al., 1979; Parker, 1980). Interleukin-2 is known to support the growth of certain types of T-lymphocytes. It has also been suggested that it was critical for the proliferation of B-cells (Watson, 1979; Swain et al., 1981). Swain et al. (1981) suggested that IL-2 acted directly on the B cell as a B-cell growth factor. Their data however did not exclude the possibility that the IL-2 could have been acting through residual T-cells. It was possible that no effect of added growth factors was seen because all the cultures were started with mixed lymphocyte cultures which was a good source of these factors. It was also possible that the use of purified growth factors could have promoted the hybrid growth (although this was not done).

Several reasons have been advanced for delay in growth of hybrid cells and these include: thymidine inhibition of cell growth; suppression of tumorigenicity; diploidy of the fusion partners; the presence or absence of growth controlling molecules and other unidentified factors in in vitro derived donor lymphocytes (refer Section 4.3.2).

Growth inhibition by thymidine has been reported to occur in some fusion systems where HGPRT deficient cell lines were used as fusion partners and subsequently selected in HAT containing medium (Schachtschabel et al., 1966; Fox et al., 1981; Fong et al., 1982; Mayer et al., 1982; Taggart and Samloff, 1983). The possibility of thymidine inhibition in this bovine fusion system is unlikely to have occurred since preliminary studies have shown that hybrids selected in azaserine had similarly prolonged initial growth periods (Olobo, unpublished).

Although the molecular basis of suppression of tumourigenicity has not been fully elucidated for many systems, earlier studies demonstrated partial and complete suppression of tumourigenicity in intraspecies mouse cell hybrids (Harris et al., 1969; Harris, 1979; 1980-1981). Perhaps the best evidence so far for complete and stable suppression of tumourigenicity is from studies with intraspecies hybrids of diploid human x tumourigenic cells (Stanbridge, 1976).

All such hybrids were initially suppressed, and nearly all remained suppressed for prolonged periods of time (Stanbridge, 1976; Klinger et al., 1978; Stanbridge et al., 1981). It was not until some chromosomes were lost that the tumour hybrids started to grow again (Harris, 1979). On the contrary, very few diploid human x tumourigenic mouse cell hybrids were suppressed (Harris, 1980-1981; Guerts et al., 1981). Further studies of hamster x human and mouse x human

hybrid cells showed that no single chromosome was consistently associated with suppression in hybrids, and a number of chromosomes were implicated in the suppression of tumourigenicity. This observation may explain why tumourigenic segregants were rarely recovered from hybrids with a large number of the implicated suppressor chromosomes, since the loss of some of these chromosomes would still leave other combinations that could suppress growth (Klinger et al., 1978; Guerts et al., 1981; Klinger and Shows, 1983). Some of the same human chromosomes were observed to follow a similar pattern of suppression in studies with other rodent cell hybrids (Klinger et al., 1978; Guerts et al., 1981). An exception to the above reports was however found in two studies with intraspecies human hybrids, in which only chromosome 11 was implicated in suppressor activity (Stanbridge et al., 1981). Almost all of the implicated suppressor chromosomes were frequently found to be involved in histopathologic tumour-type specific abnormalities in human neoplasias (Rowley, 1980; Mitelman and Levan 1981). Proto-oncogenes (c-onc genes) were also mapped on many of these same chromosomes, and sometimes at the same sites as the structural alterations (Rowley, 1982).

The bovine leukaemia cell line used as a fusion partner (ATS/BL20) had a diploid chromosome number. The hybrid cells obtained after fusion with diploid normal bovine cells also had chromosome numbers which were actually diploid or near

diploid. Some reports have appeared indicating that when the karyotype of the malignant parental population was close to diploid it was not possible to obtain long-term hybrid cell populations from fusions between these cells and normal human cells. Clones derived from such fusions did arise in selective medium, but they apparently underwent a limited number of population doublings, and then apparently entered a senescent phase (Bunn and Tarrant, 1980; Stanbridge et al., 1982). This may have occurred in this bovine fusion system, although there was no evidence to support that contention. In other instances where the tumorigenic parental fusion partners contained at least a tetraploid, and often a hexaploid chromosomal complement, successful hybrid cells were formed with normal diploid cells (Stanbridge et al., 1982). From these results it was postulated that diploid tumorigenic cell lines could not give rise to continuously proliferating hybrids when fused with normal cells, unless an increase in ploidy occurred (Stanbridge et al., 1982). A partial explanation which was ascribed to the phenomenon was that of gene dosage effect (Evans et al., 1982). In brief, when there was a diploid complement of both tumorigenic fusion partner and normal fibroblast chromosomes, hybrid clones were capable of only a limited proliferative capacity, perhaps dictated by the proliferative capacity of the normal parent cells. However, when there was a change in the balance of chromosomes, then the hybrid cell lines were both

transformed and tumourigenic. This possibility still awaits further exploration.

Retarded hybrid cell growth observed in the bovine system could indicate that the normal bovine cells contributed something, probably chromosomes, to the hybrids, which suppressed the highly malignant character of the ATS/BL20 cells. It was only after this contribution was lost that the hybrid cells started to expand rapidly again. Investigations into the genetic and biochemical basis of the regulatory control of transformation and tumourigenicity are rapidly increasing. Recent technological advances in gene transfer with metaphase chromosomes (McBride and Ozer, 1973), microcells and DNA (Hidaka et al., 1985), should complement somatic cell hybridization techniques and accelerate the quest for the identification of the genetic basis of suppression.

Other recent studies have also shown that for growth controls not involving factors transmitted through the extracellular medium, junctional communication between cells may play a role (Loewenstein, 1979). Mehta et al. (1986), Schwartzman et al. (1981) confirmed that the interiors of adjacent cells were directly inter-connected by specialised membrane channels, and that growth inhibition of the transformed cells were dependent on the junctional communication with their normal-cell partners. These specialised membrane channels were wide enough to conduct many kinds of low molecular weight cytoplasmic molecules.

Growth-controlling molecules which were either inhibitory or stimulatory in character, were conducted through these channels (Mehta et al., 1986; Schwartzman et al., 1981). Neoplastic cell transformation is probably a multistep process that may alter several genes and pathways. The changes leading to malignant transformation need not occur sequentially, or affect steps in a single pathway, so that control or blockage of some of these changes, by molecules similar to those outlined above, could suppress or enhance cell growth.

The merits of application of ATS/BL20 cell line as a fusion partner for production of bovine monoclonal antibodies should be conducted using in vivo derived bovine lymphocytes. The low fusion frequency, the low cloning frequency of the hybrid cells, the low stability, the loss of antibody production, and inhibition of hybrid cell growth by unexplained mechanisms (such as suppression or some other unknown factors), observed when ATS/BL20 cells were fused to in vitro activated bovine lymphocytes would be an unfair assessment for the cell line as a suitable fusion partner.

Over 10 human fusion partners have so far been generated for the production of intraspecies specific human hybridomas (reviewed by Kozbor and Roder, 1983). A detailed comparison of hybridization frequencies, yield of antigen specific hybridomas, immunoglobulin secretion levels, cloning frequencies, division times, and stability, has not revealed any line which is superior to the others and which would be

widely acceptable as a fusion partner (Kozbor and Roder, 1983; Carson and Freimark, 1985). This is the first report on a bovine cell line fusion partner. More bovine myelomas or lymphoblastoid cell lines need to be isolated and adapted in culture. These lines could then be rendered suitable for fusion, for possible production of bovine monoclonal antibodies, and for comparison with ATS/BL20 cell line described above.

4.3 Generation of Mouse x Bovine Heterohybridoma Fusion Partners

Following the observation that ATS/BL20 cells were not suitable for use as a fusion partner for generation of bovine monoclonal antibodies, attempts were made to use aminopterin sensitive mouse myeloma cells to produce mouse x bovine cells as fusion partners. Few reports have appeared in the literature on attempts to generate bovine monoclonal antibodies, by fusing the mouse myeloma cells NS1, X63 or Sp-2/0 to in vivo derived bovine lymphocytes (Srikumaran et al., 1983/1984; Davidson et al., 1982). The drawbacks in the above system included low antibody production, and lack of stability of the hybrid cells. The low stability of the hybrids could most probably have been due to chromosome loss which has been reported to be a common phenomenon in interspecies hybrids (Croce et al., 1980b).

A strategy was therefore devised aimed at generating mouse x bovine fusion partners to compare with parental mouse myeloma cell fusion partners. A similar strategy was reported previously to be successful in the human system. While interspecies mouse x human hybrid cell formation was unstable, mouse myeloma x human B-cell heterohybridoma fusion partners formed more stable hybrids in secondary fusions with in vivo derived human B lymphocytes, due presumably to retention of greater numbers of human chromosomes in the hybrid cells (Teng et al., 1983; Fong et al., 1984).

4.3.1. Fusion of Mouse Myeloma or Mouse x Bovine Cells to PWM Activated PBM

Mouse myeloma fusion partners NS1, X63 and Sp 2/0 or NS1 x bovine cells which were sensitive to aminopterin, were fused in initial experiments with bovine PBM obtained after in vitro activation with PWM (refer Discussion 4.2.3). Hybrid cells were then selected in aminopterin containing medium. The growth of the hybrid cells between NS1 or X63 and bovine PBM was slow. After about seven weeks of fusion, a few hybrids between NS1 and bovine PBM started to multiply vigorously, while the hybrid cells between X63 and bovine PBM continued dying and were all dead by the 9th week after fusion. On the other hand, hybrid cells between Sp 2/0 and PWM activated bovine PBM in one experiment grew normally without any apparent

crisis. Fusion of NS1 x bovine cell clones (A1 or A11) back to PWM activated bovine PBM yielded an initial fast growing hybrid cells. But the putative hybrid cells eventually died after a few weeks in culture. This confirmed that the fusion system was unstable. The fusion efficiency in each case was low.

4.3.2 Comparison of Fusion Efficiency Between Mouse Myeloma Cells Versus Mouse x Bovine Heterohybrids

In experiments designed to compare viability, fusion frequency and possible antibody production in primary fusions (mouse myeloma cells fused to bovine lymphocytes), versus secondary fusions (mouse x bovine heterohybridomas fused back to bovine lymphocytes), NS1 x bovine or Sp2/0 x bovine (mouse x bovine) fusion partners were generated, by selecting the hybrid cells, in increasing concentrations of 8-azaguanine. It was established in preliminary studies that between 25 and 40% of the two heterohybrids (Sp2/0 x bovine and NS1 x bovine respectively) had spontaneous resistance to the purine analogue. These populations of cells were also found to be sensitive to aminopterin treatment. This indicated that the hybrid cells had lost the gene coding for the enzyme HGPRT, which mediates the uptake of preformed nucleotide bases by cells through the salvage pathway, and were therefore potential fusion partners.

Fusion of antigen activated bovine PBM to NS1, Sp2/0, NS1 x bovine (A1), or Sp2/0 x bovine cell lines generated some fast growing hybrid cells in each case from about two weeks of fusion, and no crisis in hybrid growth was apparently noticed. Comparison of viability and fusion frequency of primary fusions, i.e. NS1, or Sp2/0 fused to bovine lymphocytes, as opposed to secondary fusions i.e. NS1 x bovine or Sp2/0 x bovine fused to bovine lymphocytes, revealed some inconsistent results which were unreliable because of poor reproducibility of the fusions. In secondary fusion systems, there was apparently no difference between NS1 x bovine or Sp2/0 x bovine cell lines as far as hybrid viability and fusion frequency was concerned when compared to primary fusions involving the respective parental mouse myeloma cell lines.

It was expected that secondary fusions would result in an increased retention of bovine chromosomes and the retention of hybrid viability. These expectations were not fulfilled even when in vitro antigen activated PBM were used as donor lymphocytes. This was another indication that in vitro antigen activated lymphocytes were not suitable as fusion partners.

Relatively little is known about factors which encourage successful hybrid cell formation. It has been shown that to obtain a maximum yield of hybrid cells, the donor animal cells must be fused to the myeloma fusion partner at narrowly defined times of the cell cycle (i.e. at an active mitotic phase). At present, the appropriate right stage of B

cell differentiation for function rescue fusions is unknown (Westerwoudt, 1985). Isolation of activated B cells at the right stage allows the hybrid cells to achieve synchronisation of DNA synthesis, which is essential for the survival of the hybridomas. If one fusion partner is not at an active mitotic phase, the resultant hybrids appear to be very unstable and to die in the next mitosis (Johnson and Rao, 1970) (refer Discussion 4.2.3).

In cattle, the availability of antigen specific B-cells from in vitro activation is not fully explored, although the expansion of rare specific B cells from PBM was a critical step for production of bovine monoclonal antibody with defined specificity. In addition, the fact that only about 20 per cent of bovine peripheral blood B lymphocytes could be activated after mitogen or antigen treatment was no small barrier.

Pokeweed mitogen was used initially (before the system for in vitro activation of PBM with antigen became available) to provide a continuous source of activated bovine B cells for fusion. Lymphocytes obtained after PWM activation did not however support successful primary or secondary hybrid formation between parental mouse myeloma cells NS1, X63 or NS1 x bovine fusion partners respectively.

Although the in vitro immunisation of PBM with antigen was not primary but secondary in response, it was effective to increase the viability of the lymphocytes as compared to

lymphocytes derived from PWM activation. About 80% of the lymphocytes were found to be viable after antigen activation, while only between 50 to 60% of the lymphocytes were viable after PWM activation. These differences could have also resulted in a marginally better viability of hybrids which was observed when lymphocytes were obtained from in vitro antigen versus the PWM systems.

Failure to obtain reproducible growing hybrids using the in vitro activated bovine PBM could also possibly have been due to some adverse effects caused to the cells during culture, which made them unable to withstand the drastic process of fusion, resulting in poor hybrid viability and growth.

Many other factors have been reported to affect the fusion frequency and hybrid cell growth. These include: species differences; different batches and concentrations of PEG (reviews by Goding, 1980; Westerwoudt, 1985); the pH of PEG used (Sharon et al., 1980); temperature of fusion mixture and method of PEG sterilization (Westerwoudt, 1985); feeder layer cells, (Goding, 1980), some cellular selection processes by factors operating in the in vitro environment, etc. (refer Discussion 4.2.3).

In this study attempts were made to reproduce successful fusion methodologies using the in vitro mitogen or antigen activated PBM as donor lymphocytes, and it is probable that the appropriate conditions for in vitro activation of bovine PBM for fusion studies have not yet been fully defined.

4.3.3 Confirmation of Hybrid Formation

The fusion mixtures in all experiments were initially selected in HAT containing medium (Littlefield, 1964). This strategy ensured that only hybrid cells survived in culture because they could synthesize DNA via the salvage pathway after acquiring the HGPRT genes from normal lymphocytes at fusion.

Further evidence for hybridization between mouse myeloma cells and bovine lymphocytes was obtained from karyotype analysis of the putative hybrid cells. The mouse NS1 or Sp2/0 myeloma cell line had about 52 and 72 chromosomes respectively, while bovine lymphocytes had 60 chromosomes. After fusion, NS1 x bovine hybrid cells had 62-68 chromosomes and Sp2/0 x bovine hybrid cells contained 72-110 chromosomes. The increase in the number of chromosomes in the hybrid cells was presumably acquired through fusion. With prolonged culture, the hybrid cells showed a reduction in the number of chromosomes.

Another screening assay which confirmed hybrid formation was the demonstration of bovine cytoplasmic IgM molecules in the hybrid cells. Mouse NS1 and Sp2/0 cells were not stained by anti-bovine antibodies for cIgM, while some donor bovine cells and putative hybrid cells between NS1 x bovine stained for cytoplasmic bovine IgM molecules.

Additional evidence for successful hybridization resulted from analysis of surface antigens. The bovine IgM

molecule detected by monoclonal antibody B5/4 was shown to be present on some of the donor animal cells, but absent on NS1 or Sp2/0 myeloma cells. After fusion, some of the hybrid cells expressed the bovine surface IgM molecule. When another bovine surface antigen marker, MAb P5, was used, it was found that NS1 or Sp2/0 myeloma fusion partners did not express the target antigen, but some of the donor animal cells and the hybrid cells between NS1 x bovine did express the antigen.

Furthermore, a bovine histocompatibility antigen detected by MAb IL-A7, was found to be present on about 80% of the donor animal cells, and absent on mouse NS1 or Sp2/0 cells, but was expressed on approximately 20% of the hybrid mouse NS1 x bovine cells. When NS1 or Sp2/0 x bovine hybrid cells were tested for resistance to 8-azaguanine, some of the cells (60% and 75% respectively) were killed by this drug, indicating that the cells had acquired the genes coding for the enzyme HGPRT after fusion with normal bovine cells. Mouse myeloma cells NS1 or Sp2/0 are known to be resistant to 8-azaguanine treatment. All the above screening assays were done after several weeks of fusion, at which time the possibility of survival of normal bovine B cells, mouse NS1 or Sp2/0 cells was highly unlikely. Thus, the growing cells examined most likely represented hybrid cells between mouse myeloma cells and bovine B cells. It was inferred that some genes from the normal bovine cells coding for the properties cited above were acquired at fusion.

4.3.4 Perspectives

To reiterate further, the primary goal of this study was to generate cell lines which could serve as fusion partners for production of bovine monoclonal antibody. This goal was achieved when 8-azaguanine and or 6-Thioguanine resistant and HAT sensitive ATS/BL20, NS1 x bovine and Sp2/0 x bovine cell lines were generated. It was not possible however to compare the viability, fusion frequency, and the stability of antibody producing hybrids in this study using the above cell lines, since the in vitro activated PBM from mitogen and antigen systems did not give reproducible fusion experiments. More studies are therefore required to evaluate the applicability of these potential fusion partners using donor bovine lymphocytes from many sources (e.g. in vivo activated lymph node, spleen cells etc). The hybridoma system established here may yet provide materials for: serological standards, immunotherapy, idiotypic studies, and above all, it should be possible to analyse the bovine immune response to predefined antigens and infections like trypanosomiasis and theileriosis at the B cell clonal level.

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APPENDIX 1

Acrylamide-Bis Mixture:30-0.8

Purified acrylamide	30.0 g
Purified bisacrylamide	0.8 g
H ₂ O	69.2 ml

Filter through 0.45 um filter

Wrap foil round bottle

Store at 4°C.

Alsever's Solution

Citric acid	0.55 g
Sodium citrate	8.0 g
NaCl	4.2 g
Dextrose	20.5 g
H ₂ O	1.0 l

Adjust to pH 7.2

Autoclave 10 psi, 10 minutes

Store aliquoted at 4°C

Aminopterin - 1000 x solution

Aminopterin	9.5 mg
NaOH (0.01M)	50.0 ml

Filter through 0.22 um

Aliquot

Store at -20°C

Buffer 30

Tris-HCl	0.625 M (pH 6.8)
TEMED	0.125%
SDS	0.5%

Store at Room Temperature

Buffer 40

Tris-HCl	1.875 M
TEMED	0.125%
SDS	0.5%

Store at Room Temperature

Dulbecco's Phosphate Buffered Saline (PBS) pH 7.4

Na_2HPO_4	11.5 g
NaCl	80.0 g
KCl	2.0 g
KH_2PO_4	2.0 g
H_2O	7.5 l

Adjust to pH 7.4

Bring volume to 8.0 litres with H_2O

Autoclave 15 psi, 30 minutes

Gel staining solution

Methanol	225.0 ml
Glacial acetic acid	50.0 ml
H_2O	500.0 ml
Coomassie Brilliant Blue	1.25 g

Stir for 30 minutes

Filter through Whatman No 1

Store at RT.

Gel destaining solution

Methanol	250.0 ml
Glacial acetic acid	75.0 ml
H_2O to	1.0 l

Hank's BSS Washing Medium

Hank's BSS	485.0 ml
4% EDTA Solution	5.0 ml
1M HEPES	5.0 ml
Gentamycin (100 x)	5.0 ml

HAT and HT Medium (1X)

Fusion medium	99.0 ml
HT (100 x)	1.0 ml
Aminopterin (1000x)	0.1 ml

Store at 4°C

Keep no longer than 14 days

Hypoxanthine Thymidine (HT)-100 x solution

Hypoxanthine	130.0 mg
Thymidine	39.0 mg
NaOH (0.01 M)	100.0 ml

Filter through 0.22 um filter

Aliquot

Store at -20°C

Isotonic Saline

NaCl	9.0 g
H ₂ O	1.0 l

Autoclave 15 psi, 15 minutes

Store at 4°C.

Mounting Buffer

Glycerol	9.0 ml
PBS	1.0 ml
pH	7.2 to 7.4

Polyethylene Glycol Solution 1 (PEG 1)

Polyethylene glycol (1500 or 4000)	5.0 g
RPMI-1640 (serum and HEPES free)	7.0 ml
DMSO	1.5 ml

Autoclave PEG until liquefied

Add medium and DMSO when at 60°C

Incubate at 37°C before use

Polyethylene Glycol Solution 2 (PEG 2)

Polyethylene glycol (1550 or 4000)	5.0 g
RPMI-1640 (Serum and HEPES free)	15.0 ml

Autoclave PEG until liquefied

Add medium when at 60°C

Incubate at 37°C before use

Resolving Gel Mixture (1 gel) 7.5-17.5%

	<u>7.5%</u>	<u>17.5%</u>
H ₂ O	8.10 mls	1.65 mls
30-0.8 acrylamide bis	3.75 mls	8.75 mls
Buffer 40	3.0 mls	3.0 mls
Glycerol	-	1.5 mls
	VORTEX	VORTEX
5% ammonium persulphate	150 ul	150 ul
	VORTEX	VORTEX
	DE-GASS	DE-GASS

Running Buffer

Tris base	6.0 g
Glycine	28.0 g
10% SDS	10.0 ml
H ₂ O	1.0 l

Stacking Gel Mixture (3% acrylamide)

30-0.8 acrylamide bis	1.0 ml
Buffer 30	2.0 ml
H ₂ O	6.9 ml
	VORTEX
5% Ammonium persulphate	100 ul

Trypsinization Medium

Hanks BSS (10x) Ca ^{2a} , Mg ²⁺ free	10.0 ml
Trypsin EDTA (10x)	10.0 ml
1M HEPES	2.0 ml
7.5-8% NaHCO ₃	1.0 ml
Gentamycin (5mg/ml)	1.0 ml
Reagent H ₂ O	76.0 ml

Filter sterile on 0.45 μ M filter

Store at 4°C.