IMMUNOLOGIC STUDIES OF B CELLS AND THEIR ANTIBODY PRODUCTS.

by

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A DISSERTATION

Presented to the Department of Microbiology and Immunology
and
the Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
September, 1987

APPROVED:

(Professor in Charge of Thesis)

(Chairman, Graduate Council)

To Richard
and our children
Matthew, Laura and Emily
in gratitude for their endless giving and patience,
for eating gourmet macaroni and cheese,
and without whom this endeavor would have been impossible.

Acknowledgements

I would like to thank my thesis advisor, Marvin Rittenberg, for his support of this project and personal encouragement. Special thanks are given to Abigail Buenafe for her contribution to this work and for the many hours of discussing experimental results. I am grateful to McKay Nutt for helpful suggestions and to Max Joffe for his assistance in the EBV transformation and cloning of human B cells. Finally, special thanks are given to Carolynn Brault for her skillful and speedy preparation of this manuscript.

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INTRODUCTORY REMARKS

Hapten-protein conjugates have allowed immunologists in many laboratories the opportunity of asking a variety of questions regarding the mechanisms influencing the clonal expansion of hapten-specific B cells. Of great interest in our laboratory has been the determination of cellular and molecular mechanisms involved in development of diversity in the immune response to haptens in the mouse and in man. With much enthusiam, I began studies of the human anti-TNP-Brucella abortus response in vitro to investigate mechanisms of the induction of diversity in this response at both the cellular and molecular levels. A molecular analysis of a diverse population of anti-TNP specific human B cells would involve producing human monoclonal antibodies. While successful in inducing proliferation and differentiation of heterogeneous populations of TNP-specific human B cells (the focus of Part II of this thesis), numerous attempts to produce human monoclonal antibodies by hybridoma technology and by EBV transformation were unsuccessful. It was at that point that I continued studies on mechanisms involved in generating immunoglobulin diversity with work in the murine response to the organophosphate hapten Soman (the focus of Part I of this thesis).

Part I

ABSTRACT

The hapten Soman (0-1,2,2,trimethylpropylmethylphosphonofluoridate) is an organophosphate sharing spatial and structural features with the common environmental organophosphorous-containing hapten, phosphocholine (PC). While anti-PC antibodies have been well characterized, little is known about anti-Soman antibodies at the protein and molecular levels. Due to the structural similarities of the Soman and PC haptens the question of whether similarities occur as well at the protein and molecular levels of antibodies to these haptens is an intriguing one, and is the primary focus of the research described in Part I of this thesis.

Previous comparisons of the combining site specificities of eight anti-Soman monoclonal antibodies with anti-PC antibodies demonstrated very little cross-reactivity of antibodies to these two haptens (1), which was believed to be attributed to a charge difference between structurally similar portions of these two haptens, Soman having an uncharged pinacolyl group and PC having a positively charged choline group in the same relative positions in their respective haptens. The eight anti-Soman antibodies were derived from an anti-Soman secondary response to Soman-KLH and displayed two distinct binding specificities for the Soman molecule which were designated Group A and Group B. Group A antibodies were bound by dipinacolylmethylphosphonate (DPMP), p-aminophenyl-Soman (NH_2-O-So) and p-nitrophenyl-Soman (NO_2-O-So). It was suggested that these antibodies recognized the pinacolyl moiety as the dominant feature of Soman-KLH. This binding pattern is analogous to Group I anti-PC

antibodies which bind to the choline moiety of the PC molecule (1). Group B anti-Soman antibodies have a higher affinity for NO_2-O-So than Group A anti-Soman antibodies. Grcup A and Group B anti-Soman antibodies have comparable affinity for NH_2-O-So but Group B has no affinity for DPMP. The Group B anti-Soman antibodies were thought to be directed primarily at the phenylphosphate portion of Soman-KLH.

The combining site characteristics of thirty-eight additional anti-Soman monoclonal antibodies derived from primary and secondary responses to Soman-KLH were studied to determine the degree of heterogeneity displayed with respect to fine specificities. Eight additional Group A anti-Soman antibodies were identified which showed similar combining site specificities. We were interested in determining whether these Group A hybridomas, displaying similar fine specificity patterns, were utilizing common V_H gene segments. In addition, since four of these Group A anti-Soman antibodies had low affinity for NPPC we hypothesized that these hybridomas would be using V_H genes from V_H gene families used in the anti-PC-KLH response and that the remaining non-NPPC binding Group A anti-Soman antibodies would use members of other V_H gene families. In order to test this hypothesis the following objectives were undertaken:

(1) To determine the degree of heterogeneity displayed by secondary response anti-Soman antibodies by an ELISA hapten inhibition assay using Soman and PC derivatives.

(2) To determine the extent of heterogeneity in V_H gene usage of eight Group A antibodies using Southern blot analysis.

(3) To determine whether Group A anti-Soman antibodies which bind NPPC and those which do not bind NPPC use members of the S107, Q52 and/or J558 V_H gene families and whether there is preferential use of a particular family.

INTRODUCTION

Immunoglobulin structure and formation.

The humoral immune system has the capacity to recognize an enormous array of molecular structures. In response to this recognition, a B lymphocyte produces antibodies of a single specificity which bind to the molecular structure which induced their production. One of the most exciting areas in modern immunology has been that involved in determining how the information for the production of a seemingly limitless number of different antibody specificities is encoded in the genome.

Each immunoglobulin molecule is composed of two identical heavy chains and two identical light chains. The heavy chains have variable regions (2) on the amino-terminal portion of the molecule and constant regions on the carboxy-terminal end which are termed V_H and C_H, respectively. There are five major classes of heavy chains including mu, delta, gamma, alpha and epsilon. The gamma class is comprised of four subclasses. The light chains also have variable region amino-terminal ends and constant region carboxy- terminal ends which are termed V_L and C_L, respectively. There are two major classes of light chains, kappa and lambda. The variable regions of the heavy and light chains bind to complementary antigens. Within these variable regions are regions known as complementarity-determining regions (CDRs)(3,4) which come into contact with the specific antigen. Between these CDRs (also termed hypervariable regions) are stretches of amino acids that do not vary

considerably from one antibody molecule to another and are termed framework regions. There are numerous kinds of variable regions expressed by different B cells, with a single B cell producing antibody of one specificity. In contrast, the number of constant regions are limited to the number of heavy and light chain classes and subclasses. The constant regions are important in executing the effector functions such as Fc-receptor binding and the fixing of complement following antibody-antigen recognition and binding of antigen to the immunoglobulin variable region.

Since Dreyer and Bennett's suggestion in 1965 (5) that the V regions and C regions of heavy and light chains are encoded by separate sets of genes, work by many investigators (reviewed by Tonegawa (6)) has demonstrated that V and C regions of heavy and light chains are the result of somatic rearrangements of multiple gene segments encoded in germline DNA. The V region of the heavy chain is formed by somatic recombination of germline V_H (variable), D(diversity) and J_H(joining) segments and the V region of the light chain is formed by somatic recombination of germline V_L and J_L segments (6). The V regions formed by V-D-J and V-J recombination in the heavy and light chains, respectively, are combined with a constant region gene to form complete H and L chains which are transcribed into protein (7,8).

In the mouse, immunoglobulin genes are found in three unlinked families. The heavy chain genes are found on chromosome 12 (9) and are comprised of many V_H segments (10), perhaps greater than 1000 (11), at least 12 D_H segments (12), four J_H segments (13) and 8 C_H genes (14). The

kappa light chain genes, located on chromosome 6 (15) are comprised of many V kappa segments (16), 5 J kappa segments including one pseudogene and one C kappa gene (13,17). The lambda light chain genes, located on chromosome 16 (18) are comprised of 2 V lambda segments, 4 J lambda segments and 4 C lambda segments (19). The organization of the mouse immunoglobulin gene segments in germline DNA is depicted in Figure 1.

Mechanisms of generating immunoglobulin diversity.

Several mechanisms are involved in generating immunoglobulin diversity including the presence of multiple gene segments (referred to above), combinatorial joining, junctional diversity, insertional base replacement and deletions, and somatic mutation.

Combinatorial joining refers to the site-specific recombination, for example, of any V_H, D and J_H segment to form a complete heavy chain variable region. Manser and Gefter (20), in the analysis of the available V gene repertoire in the nonimmune mouse, showed that V_H genes appeared to be used at random in their association with the available pool of D, J_H, V_L and J_L gene segments. This combinatorial diversity would contribute at least 10^7 different V region structures (21). Work by other investigators has demonstrated that the association of V, D and J heavy chain gene segments and V and J light chain gene segments may not be random. Nishi et al. (22) demonstrated that recombination of V_K segments in the V_K-KpnI family do not recombine randomly to form functional K light chains. V_K-

KpnI segments recombine more frequently with the J_K1 gene than with the J_K5 gene and rarely use the J_K3 gene segment. Studies by Yancopoulos et al. (23) and Perlmutter et al. (24) have shown that pre-B cells in the BALB/c mouse use V_H segments from the most J_H-proximal V_H family preferentially during ontogeny, indicating non-randomness of V_H gene segment usage. The bias seen in V_H gene segment usage in pre-B cells, however, is not found in the mature B cell population suggesting that all the gene segments in the genome encoding V_H and V_L regions may undergo recombination during the formation of immunoglobulin molecules. The site-specific recombination appears to occur by the joining of heptamer and nonamer sequences flanking the V, D and J segments (7,13). Combinatorial joining also refers to the association of any light chain with any heavy chain (6).

Junctional diversity refers to the imprecise joining of, for example, the V, D and J segments when recombining to form the heavy chain variable region (7,17). Insertional base replacements and deletions occur at junctions with heavy chain D and J segments (12). Inserted bases, referred to as N (nucleotides) sequences, are rich in G and are thought to be the result of the action of terminal transferase (25).

Somatic mutation, the least understood mechanism of diversity, refers to mutations seen in rearranged immunoglobulin variable region genes not observed in their corresponding germline segments (6). Somatic mutations may be the result of hypermutation within V genes (6,26,27,28), or reciprocal recombination between homologous V genes (29), or gene

conversion (30,31). There is little known about why somatic mutation is specific for V genes or about when it occurs during B cell maturation. It has been proposed that in heavy chains somatic mutation occurs following V-D-J joining (32,33) and may be linked to immunoglobulin class switching (32,33,34). However, Rudikoff et al. (26) and Griffiths et al. (35) have described IgM-secreting cells with both V_H genes somatically mutated, and Gearhart et al. (33) have described alpha heavy chains containing a germline V_H gene. Rudikoff et al. (26) and Owen et al. (36) have suggested that somatic mutation occurs continuously during the maturation of the B cell and is not associated with class switching.

The four mechanismns of generating immunoglobulin diversity (multiple gene segments, combinatorial joining, insertional base sequences and somatic mutation) can potentially create an enormous repertoire of immunoglobulin molecules ($>10^7$) (6,8,37) using a limited germline genome.

VH gene families.

The majority of heavy chain variable regions contain 98 amino acids encoded by the variable gene segment V_H. Kemp et al. (10) first estimated that there were 160 V_H gene segments by counting the number of DNA restriction enzyme fragments that hybridized to 3 murine V_H probes. Later work by Brodeur and Riblet (38) estimated the total number of V_H genes to be 100 based upon studies of 18 inbred mouse strains using 24 V_H probes. The work described by Livant et al. (11) in which a J558 V_H probe

excess hybridization technique was used, suggests that there are 500 - 1000 J558 V_H gene segments bringing the estimate of the number of V_H gene segments in the mouse to greater than 1000. These V_H gene segments are located upstream from the D segments. Brodeur and Riblet divided these V_H gene segments into seven families with members within each V_H gene family sharing greater than 80% sequence homology (38). Winter et al. (39) described 2 additional novel V_H gene families. Later work by Dildrop et al. (40) confirmed the existence of at least 9 V_H gene families.

Although the number of V_H gene segments likely exceeds 1000, it has been estimated that as many as 40 - 50% of these V_H genes may be pseudogenes (32,41,42). The nucleotide sequences of many of these V_H pseudogenes (43) indicate that immunoglobulin V_H pseudogenes carry only a few deletions and have not diverged at any faster rate than functional V_H genes (44). Thus it appears that immunoglobulin V_H pseudogenes may have only recently evolved from functional V_H genes and that their homology to intact V_H genes is retained by correction repair mechanisms in the event they may reenter the pool of functional V_H genes. Such reentry may be possible following recombination between non-allelic V_H genes as suggested by Seidman et al. (45).

The immune response to many haptens arises from one or only a few members of a V_H multigene family. For example, Group I anti-PC antibodies utilize the S107 V_H family gene segment V_H1 (46). In the anti-p-azophenylarsonate response, Id^{CR+} antibodies utilize a single germline

V_H gene segment V_HId^{CR} (48). There is widespread evidence in many hapten systems, however, that the V_H genes in any particular anti-hapten response are derived from a variety of V_H gene families. In the anti-PC Group II antibody response V_H genes from the J558, Q52 , S107 and other as yet unidentified V_H gene families are used (49 and Stenzel-Poore et al., unpublished). In the anti-beta-(1,6)-galactan antibody response (47) V_H genes from the J606 and 7183 V_H gene families are used in addition to V_H genes from the X24 V_H gene family.

The number of gene segments within each V_H gene family ranges from 1 or 2 in the X24 family (47), 4 in the S107 family (46) to 500-1000 in the J558 family (11). In the mouse homologous gene segments of a V_H family are grouped together on the chromosome (10). The order of V_H gene families along the chromosome in the mouse has been determined using deletion and recombinant inbred strain mapping (10,38). While differences in restriction fragment lengths corresponding to members of each of the V_H gene families exist between inbred strains, the number of bands corresponding to the members within each V_H gene family remains similar but not identical (i.e.expansion and contraction), indicating that these V_H gene families have been retained through evolution (38).

Homogeneous and heterogeneous variable gene usage in the murine immune response to haptens and complex antigens.

Despite the small size of haptens when compared to larger immunogenic molecules, hapten-carrier conjugates may induce numerous

antibody-producing clones each showing specificity for the hapten (50). These anti-hapten antibodies, while specific for the hapten, display either homogeneous or heterogeneous patterns of binding affinities and combining site specificities (51).

Molecular studies of hapten systems demonstrating a homogeneous response have collectively demonstrated that the generation of the response involves restricted V_H or V_L gene segment usage. Thus in the anti-PC primary response, the V_HI gene of the V_HS107 family is used by most of these antibodies (46) in association with V_k22, V_k8 and V_k24 light chains. Pierce et al. (52) described the use of the V_HII gene segment from the S107 V_H family when a heavy chain containing the V_HII gene product was paired with a light chain from an anti-PC binding antibody and was shown to bind PC.

Similarly, the primary responses to 2-phenyloxazalone (53), p-azo-phenylarsonate (54) and (4-hydroxy-3-nitro-phenyl)acetyl (55) involve restricted germline V_H gene usage.

One germline V_H gene is not restricted to use in the response to a single antigen or hapten. The same germline V_HI gene used in the primary response to PC, in combination with a different light chain may bind 2-phenyloxazalone (35) and Streptococcus Group A (56). The V_HII gene of the V_HS107 family, in combination with different light chains can bind PC (52), 2-phenyloxazolone (35) and influenza hemagglutinin (28). Sikder et al. (57) reported that three anti-(1-6)-dextran hybridoma antibodies used the identical V_k-Oxl germline gene used in the light chains of the

2-phenyloxazalone response, in combination with heavy chains bearing V_H gene segments from the J558 V_H gene family.

The importance of a particular V_H gene segment in the binding to an antigen is seen in the primary response of A/J mice to p-azophenyl-arsonate. Wysocki et al.(1985) have observed that while antibodies in this restricted response use a single V_H gene segment, this V_H gene can combine with a variety of D and J_H segments and retain specificity for the p-azophenylarsonate hapten. In contrast to the restricted V_H gene usage seen in the primary responses to PC, for example, the diversity in the response to the hemagglutinin molecule of the influenza virus is extensive (59). It is estimated that the BALB/c mouse can produce at least 500 different antibodies to the influenza hemagglutinin molecule (59). In addition to extensive somatic mutation in the anti-hemagglutinin response antibodies, V_H genes from the 7183, S107, 36-60 gene families and different D and J_H genes contribute to this diversity. Diversity has also been demonstrated in the Group II anti-PC secondary response antibodies where V_H genes from the J558, (60) Q52, S107 and other V_H gene families are used(49 and Stenzel-Poore et. al., unpublished).

The reasons for homogeneity versus heterogeneity of V gene usage in an immune response to a particular antigen are unknown. The dominance of V_H gene usage in the anti-p-azophenylarsonate-KLH response does not appear to be due to increased precursor frequency of cells utilizing the dominant V_H gene (20). Studies on the role played by somatic mutation in

the immune response allow some speculation about mechanisms governing homogeneous versus heterogeneous V gene usage.

It has been suggested (61) that somatic mutation of immunoglobulin variable region genes has a greater influence upon increasing the affinity of an antibody for its antigen than in changing the specificity of the antibody. Strong evidence for this role of somatic mutation exists in the anti-p-azophenylarsonate system in which secondary response antibodies to p-azophenylarsonate, using somatic V_H gene variants of primary response germline V_H genes, display a higher affinity for p-azophenylarsonate than the primary response antibodies which bear germline V_H sequences (62).

A reasonable explanation for the restricted V_H gene usage as seen in antibodies isolated from the late primary anti-p-azophenylarsonate response would be due to an initial high affinity interaction of this hapten with antibodies using a particular V_H gene, that, in combination with various D and J_H gene segments, has a much higher affinity for p-azophenylarsonate than other V-D-J combinations binding to p-azophenylarsonate. The use by secondary response antibodies of a particular V_H gene found in the primary and late primary anti-p-azophenylarsonate response suggests that these higher affinity germline antibodies were preferentially selected by antigen for clonal expansion and maintenance in the memory pool.

Heterogeneous responses, on the other hand, may initially involve low affinity interactions between antigen and antigen-specific B cells triggering

a wide variety of clones to expand, many of which will be reactivated by antigen in the secondary response. Due to the initial low affinity interaction with antigen, no one clone will dominate. Although higher affinity clones may arise following V region somatic mutation, the chance of obtaining a higher affinity antibody most likely will be the same among the diverse clones such that clonal expansion of somatically mutated higher affinity antibodies will retain a heterogeneous collection of antibodies, i.e. no clone will dominate.

Alternatively, the maintenance of a restricted V gene usage in the secondary response may well depend on a particular germline sequence being adaptable to somatic mutation such that antibodies of the same or increased affinity for antigen are formed. If a germline V_H is unadaptable to somatic mutation in the sense that somatic mutation results in a greater number of variants that can no longer bind antigen, or have a lower affinity for antigen, the secondary response may be formed by antibodies from other previously low affinity, non-dominant clones that are highly adaptive to somatic mutation and their resultant production of higher affinity antibodies for antigen allow their preferential expansion over primary response, nonadaptive clones that no longer have high affinity for antigen. This may explain the loss of T15 dominance in the secondary anti-PC response and the expansion of Group II antibodies bearing different V_H genes than those found in the Group I primary response anti-PC antibodies.

A model for the development of a homogeneous versus a heterogeneous antibody response to hapten-conjugates or to complex antigens is

described below and incorporates observations of V_H gene family usage in the anti-PC, anti-P-phenylarsonate and anti-influenza hemagglutinin responses.

In this model, the preimmune mature B cell repertoire consists of B cells that have undergone random combinatorial association of V_H-D-J_H and V_L-J_L gene segments and random combinatorial heavy and light chain associations. B cells in this preimmune state have varying degrees of affinity and specificity for antigens which could trigger their activation and clonal expansion. Thus the S107 family V_HI gene that had recombined with a D and J_HI and associated with a V_K22 light chain, for example, would have a higher affinity for PC than other anti-PC binding B cells using a different combination of gene segments. The higher affinity anti-PC B cell would expand preferentially in the presence of antigen thus dominating the primary response, i.e. T15 dominance of the primary anti-PC response. Lower affinity anti-PC B cells, using V_H genes from non-S107 V_H gene families, would be activated and expanded but would comprise a very small and perhaps undetectable percentage of anti-PC antibodies in the primary response, i.e. Group II anti-PC antibodies. Similarly, a B cell possessing a V_HId^{CR} gene with high affinity for p-azophenylarsonate would dominate the primary and late primary anti-p-azophenylarsonate response following exposure to antigen. Lower affinity V_HId^{CR} negative clones may develop but would play a minor role in the primary response.

These dominantly expressed antigen-expanded anti-PC and anti-p-azophenylarsonate B cells now undergo somatic mutation producing V_H

gene variants whose amino acid changes result in either a reduced affinity of loss of binding to antigen (non-adaptability) or, to an increased binding to antigen (adaptability). The Group I anti-PC antibodies bearing V_H genes from the S107 V_H gene family may be less adaptable to somatic mutation and the pool of B cells triggered in the primary response would be reduced. The lower affinity Group II anti-PC clones found in low numbers in the primary response, are perhaps able to adapt well to somatic mutation resulting in anti-PC Group II B cells with equal or greater affinity for PC than the depleted pool of Group I anti-PC B cells. The Group II B cells, following secondary exposure to antigen, are then found in greater numbers in the secondary response. In contrast to the non-adaptive Group I anti-PC B cells, anti-p-azophenylarsonate, high affinity primary response clones may be well adaptive to somatic mutation increasing the population of high and higher affinity anti-p-azophenylarsonate B cells that then undergo clonal expansion and continued dominance following secondary exposure to antigen.

In the anti-influenza hemagglutinin response the heterogeneity seen in the primary response would be due to the presence of numerous highly antigenic determinants on the immunogen (four major antigenic sites have been identified (63,64) when compared to the PC or p-azophenylarsonate haptens. Each of these determinants has the potential for binding to one or more anti-determinant B cells with high affinity. These determinants thus trigger a variety of B cells which codominate in the primary response. Due to the larger population of codominant clones, following somatic mutation,

the adaptability or nonadaptability of these primary response B cells is less critical, and secondary response antibodies draw equally from the codominant primary response pool resulting in a population of secondary response B cells of higher affinity than primary response B cells but demonstrating equal or greater diversity than seen in the primary response.

The anti-Soman-KLH immune response.

The murine immune response to Soman-KLH is of interest because the organophosphate hapten Soman bears spatial and structural similarities to the organophosphorous-containing hapten PC (1). The immunizing forms of these two haptens are shown in Fig. 2. Reports describing characteristics of monoclonal antibodies to Soman are limited and the number of monoclonal antibodies described are few. Lenz et al. (65) described the specificity of a monoclonal anti-Soman antibody and compared its specificity to polyclonal anti-Soman antibodies. The monoclonal antibody described by Lenz et al. (65) reacted with Soman and p-aminophenyl-Soman. Seiders et al. (66) described fine specificities of two anti-Soman antibodies, BE2 and CC1. While these anti-Soman antibodies may be representative of the anti-Soman immune response, they are too few in number to draw any conclusions regarding the degree of heterogeneity in the anti-Soman response.

A collection of 47 primary and secondary anti-Soman-KLH hybridoma antibodies in our laboratory allowed the determination of whether

homogeneous or heterogeneous combining site specificities were characteristic of this response. The combining site specificities of some of these anti-Soman antibodies have been reported (1) and, with one exception, were found to belong to two major specificity groups designated Group A and Group B. Group A antibodies in addition to having affinity for p-aminophenyl-Soman (NH_2-O-So), p-nitrophenyl-Soman (NO_2-O-So) bound dipinacolylmethylphosphonate (DPMP), and were thought be be recognizing the pinacolyl moiety of Soman-KLH. Group B antibodies, also having affinity for NH2-O-So and NO2-O-So, were unable to bind DPMP and were thought to be directed mainly at the phenylphosphate portion of Soman-KLH. The remaining anti-Soman antibodies have been characterized for their combining site specificities in an ELISA hapten inhibition assay. The results of this characterization are found in Paper I and in Appendix I. Fine specificity analysis of these anti-Soman antibodies revealed two additional major fine specificity groups of anti-Soman antibodies designated Group C and Group D. Group C antibodies are distinct in having affinity for NH_2-O-So but are unable to bind NO_2-O-So. Group D antibodies have very low affinity for Soman as shown by their ability to bind specifically to Soman-BSA but were unable to be inhibited by Soman or PC derivatives in an ELISA hapten inhibition assay.

Although the Soman molecule bears similarities to PC, antibodies to these haptens show little, if any, cross-reactivity. This finding, together with the knowledge that the Soman molecule (a neurotoxin) is not found commonly in nature would allow the prediction that the primary anti-

Soman response would be comprised of a panel of low affinity antibodies. The secondary anti-Soman response would be heterogeneous containing anti-Soman antibodies of higher affinity for the hapten Soman. This was in fact, what was determined in the initial characterization of the combining site specificities of the forty-seven anti-Soman antibodies and will be discussed with respect to the model proposed for generation of either a restricted or a heterogeneous antibody response.

Of interest, and the subject of work in Paper I, was the question of the degree of heterogeneity of V_H gene usage among selected high affinity Group A antibodies. Four of these Group A anti-Soman bind NPPC with low affinity. Since NPPC binding is one characteristic of many anti-PC antibodies and anti-PC antibodies have been demonstrated to utilize V_H genes from the S107, Q52, and J558 V_H gene families, it was hypothesized that these Group A anti-Soman antibodies would also use V_H gene segments from the S107, Q52 and/or J558 V_H gene families. The non-NPPC binding Group A anti-Soman antibodies would likely use members from other V_H gene families.

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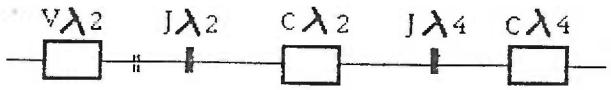
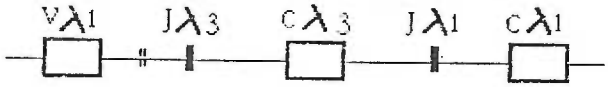
Literature Review Figure 1. Organization of germline murine immunoglobulin genes. The lambda light chain gene segments are located on chromosome 16. The kappa light chain gene segments are located on chromosome 6. The heavy chain gene segments are located on chromosome 12.

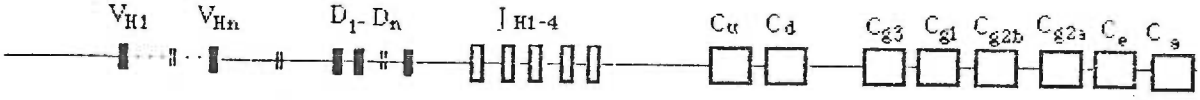
Lambda Light Chain



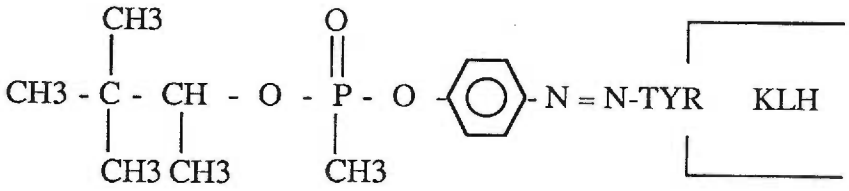


Kappa Light Chain

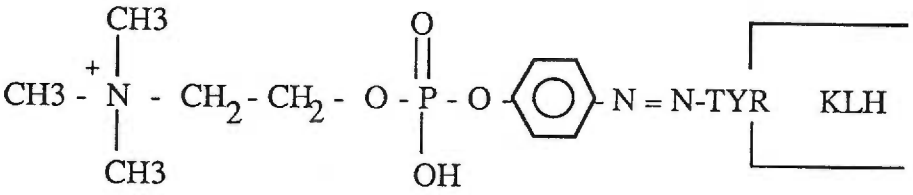
Heavy Chain



Literature Review Figure 2. Structures of the Soman-protein (So-KLH) immunizing antigen and the phosphocholine-protein (PC-KLH) immunizing antigen. The immunizing form of both proteins are shown coupled to keyhole limpet hemocyanin (KLH) via a diazotized tyrosine bridge, but may also couple to KLH via a diazotized histidine bridge.



(a) SOMAN-KEYHOLE LIMPET HEMOCYANIN (SO-KLH)



(b) PC- KEYHOLE LIMPET HEMOCYANIN (PC-KLH)

Paper 1.

COMBINING SITE SPECIFICITIES OF MONOCLONAL ANTIBODIES TO THE ORGANOPHOSPHATE HAPTEN SOMAN

II. Fine Specificities and V_H Gene Usage Within Group A Antibodies.

ABSTRACT

The combining site specificities of eight Group A anti-Soman monoclonal antibodies are described and compared to previously reported Group A anti-Soman monoclonal antibodies. Collectively the Group A hybridomas demonstrate similar fine specificity patterns by an ELISA hapten inhibition assay using structurally related haptens as inhibitors. However, Southern blot analysis revealed the use of several V_H genes and Northern blot analysis indicated that the majority (6/8) use V_H genes from the J558 V_H gene family. The V_H genes used by the other two hybridomas have not been identified but do not belong to either the S107 or the Q52 V_H families.

INTRODUCTION

Eight murine monoclonal anti-Soman antibodies from a secondary response in Soman-KLH immunized BALB/c mice have previously been described (Buenafe and Rittenberg, 1987). The specificities of these antibodies were compared to monoclonal antibodies specific for an analog of a naturally occurring organophosphorous-containing hapten, phosphocholine (PC), which shares spatial and structural properties with the organophosphate-containing hapten Soman. The anti-Soman monoclonal antibodies showed little cross-reactivity with anti-PC antibodies, attributed, in part, to a charge difference between the Soman pinacolyl moiety and the PC choline moiety.

The studies by Buenafe and Rittenberg (1987) of the combining site specificities of monoclonal anti-Soman antibodies detected two groups of hybridoma antibodies (Group A and Group B) which were differentiated by their specificity for Soman analogs. Group A antibodies were bound by dipinacolymethylphosphonate (DPMP), p-aminophenyl-Soman (NH_2-O-So) and p-nitrophenyl-Soman (NO_2-O-So) and were thought to recognize the pinacolyl moiety as the dominant feature of Soman-KLH. Group B anti-Soman antibodies had a higher affinity for phenyl-containing Soman analogs but no measurable affinity for DPMP. The Group B anti-Soman antibodies were thought to be directed primarily at the phenylphosphate portion of Soman-KLH.

Fine specificity analysis of an additional thirty-eight anti-Soman antibodies (Buenafe et al., in preparation) has identified eight additional Group A anti-Soman antibodies from a secondary response to Soman-KLH

immunization. In this paper we present data on the combining site specificities and V_H gene usage of these eight additional Group A anti-Soman hybridoma antibodies. Collectively, these Group A hybridomas show similar fine specificity patterns as determined by an ELISA hapten inhibition assay but different V_H gene usage as determined by Southern blot analysis.

Heterogeneity of V_H gene usage is seen in anti-PC-KLH antibodies. Group I anti-PC antibodies utilize the S107 V_H gene family (Crews, 1981) while some Group II anti-PC antibodies have been shown to use V_H genes from the Q52 (Stenzel-Poore et al.,1987), S107 and J558 V_H gene families (Stenzel-Poore et al., manuscript in preparation). Due to structural similarities between the Soman and PC molecules we were interested in testing whether these Group A hybridomas utilized V_H gene segments from any of the S107, Q52 and J558 V_H gene families.

MATERIALS AND METHODS

Animals:

Female BALB/c mice, age 4 to 6 weeks, were obtained from Jackson Labs, Bar Harbor, Me. Mice were first immunized at approximately 10 to 12 weeks of age.

Haptenation of protein carriers:

Soman-KLH and Soman-BSA were prepared as previously described (Buenafe and Rittenberg, 1987).

Immunizations:

Secondary response hybridomas were made from mice immunized against Soman by three injections of Soman-KLH. The first immunization of 100 ug was given intraperitoneally on day 0, followed by a second immunization of 100 ug given intraperitoneally on day 14, with a third injection of 50 ug given intravenously on day 28. The first injection was given in Complete Freund's Adjuvant, the second in Incomplete Freund's Adjuvant.

Hybridomas:

Hybridomas were made according to the method of Fazekas de St. Groth and Scheidegger (1980) as previously described (Buenafe and Rittenberg, 1987). Briefly, spleen cells were fused with the non-secreting myeloma line, F0, four days after the last injection of Soman-KLH. Fused cells were cultured in 96-well microculture plates at 5×10^5 cells per well with HAT-selective medium. Hybridomas positive in an ELISA for anti-Soman antibody were cloned twice by the single cell hanging drop technique (Rittenberg et al., 1986). Class- and subclass-specific reagents (Chang et al., 1982) (Zymed, San Francisco. CA) were used in an ELISA to determine isotypes of the resulting monoclonals. Soman specific antibodies were grown as ascites (Chesebro and Metzger, 1972) and characterized.

Fine specificity analysis of hapten-specific antibodies:

Fine specificity analysis of Soman-specific monoclonal antibodies was performed using an ELISA inhibition assay. Anti-Soman antibodies were incubated on plates coated with 10 ug/ml So-BSA in the presence of varying concentrations of inhibitor. The inhibitors included the following

Soman derivatives: p-aminophenyl-Soman (NH_2-0-So), p-nitrophenyl-Soman (NO_2-0-So), dipinacolylmethylphosphonate (DPMP) and hydroxy-Soman (OH-So) (Dr. D. Lenz, USAMRICD, Aberdeen Proving Ground, Md.). The PC derivative nitrophenylphosphocholine (NPPC) (Sigma Chem. Co., St. Louis, MO) was also used as an inhibitor. After incubation, ELISA plates were washed and incubated with alkaline phosphatase-labeled isotype-specific reagents (Chang et al., 1982) and the color reaction developed by addition of NPP. The percent inhibition was calculated by quantitation of the antibody bound in the presence of varying amounts of inhibitor using a standard curve set up with each assay. The I_{50} value for each inhibitor was calculated according to the method of Reed and Muench (1938) and is expressed as the millimolar concentration required for 50% inhibition of anti-Soman antibody binding to So-BSA.

DNA and RNA isolation:

For DNA and RNA isolation, Group A and PCG1-1 (Chang et al., 1983) hybridoma cells and FO cells were grown in tissue culture in RPMI 1640 + 10% fetal calf serum. TEPC 15 and M104E cells were grown as subcutaneous tumors in BALB/c mice. High molecular weight DNA and total RNA were extracted by the guanidinium thiocyanate cesium chloride method (Maniatis et al., 1982).

Southern blot analysis:

Isolated DNA was digested with EcoR1 or Xba1 at 37°C, electrophoresed on 0.7% agarose, tris-acetate gel for 17 hours and transferred to

Nytran membrane (Schleicher and Schuell, Inc., Keene, N.H.) according to Southern (1975). Prehybridization and hybridizations were performed at 42°C in 6X SSC (1X = 0.15 M sodium chloride, 0.015 M sodium citrate), 0.5% SDS, 5X Denhardt's solution (1X = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll), 50% formamide, 10% dextran sulfate, 0.01 M EDTA, and 100 ug/ml denatured salmon sperm DNA. J_H probes were labeled with ^{32}P-dATP to a specific activity of 5.0 X 10^8 dpm/ug using random oligonucleotide primer extension (Feinberg and Vogelstein, 1982) and hybridized at 2 X 10^6 dpm/ml. Filters were washed by the method of Brodeur and Riblet (1984) at 65°C. A 3.2-kb probe (pMJH) containing all four BALB/c J_H genes cloned into pBR322 (Calame et al., 1980) was provided by Drs. R. Perlmutter and L. Hood. An approximate 1.95 kb HpaII - HpaII fragment of pMJH containing the J_H coding sequences was used to probe both EcoRI and XbaI digested DNA. A 3' J_H HpaII - EcoRI fragment of pMJH was used to probe XbaI cut DNA.

Northern blot analysis:

RNA was electrophoresed on 1.5% agarose, formaldehyde gel for 6 hours and transferred to Nytran membrane according to Southern. Prehybridization, hybridizations, probe labeling and washing of RNA blots were performed in the same manner as with Southern blots. A .98 kb TaqI-TaqI fragment containing the coding region from the V_H of M104E was cut from a J558 probe (Brodeur and Riblet, 1984) provided by R. Riblet and cloned into pUC18 for use in detecting J558 V_H gene family members. S107 V_H gene family usage was determined using a probe provided by R.

Perlmutter containing 445 bp of the rearranged V-D-J genes from S107 (Early et al., 1980). Q52 V_H gene family usage was detected using a probe containing the V_H from PCG1-1 which uses the M141 V_H gene (Stenzel-Poore and Rittenberg, 1987).

RESULTS

Fine specificity analysis of Soman-specific monoclonal antibodies:

The fine specificities of the eight Group A monoclonal antibodies were determined by an ELISA inhibition assay in which Soman derivatives and nitrophenylphosphocholine were used as inhibitors. The chemical structures of these inhibitors are depicted in Fig. 1. The I_{50} values obtained in the ELISA hapten inhibition assay using each inhibitor with each anti-Soman antibody are summarized in Table 1.

All of these Group A antibodies are inhibited by NH_2-O-So, NO_2-O-So and DPMP which are the criteria used for their designation as Group A anti-Soman antibodies (Buenafe and Rittenberg, 1987). Their ability to bind both phenyl derivatives of Soman as well as their binding to DPMP indicates these antibodies may primarily recognize the pinacolyl moiety of the immunizing form of Soman, Soman-KLH, but contribution by phenyl-phosphate recognizing subsites cannot be ruled out. Four of these Group A anti-Soman secondary response antibodies, So-G2a-3, So-G2a-4, So-G2a-5 and So-G2a-6, are of the $IgG_{2a}k$ isotype and bind with low affinity to OH-So and NPPC. The IgG_1k antibodies, So-G1-2, So-G1-3, So-G1-4 and So-G1-5 also have low affinity for OH-So, but were not inhibitable by NPPC at the highest concentration used (10^{-2} mM).

Representative hapten inhibition curves from each isotype for these Group A anti-Soman antibodies are shown in Fig.2 and indicate that they differ very little in their overall fine specificities for the Soman and phosphocholine derivatives used.

V_H rearrangements in Group A hybridomas:

We were interested in whether these Group A anti-Soman hybridomas, displaying similar combining site specificities, shared common V_H gene segments. Utilization of common V_H gene segments has been demonstrated by hybridization of J_H probes to rearranged, identically sized, DNA restriction fragments (Siekevitz et al., 1983; Clarke et al., 1985). DNA from these Group A hybridomas was cut with EcoRI, blotted to Nytran membrane and hybridized to a probe containing all four J_H gene segments (see Fig.3). This probe hybridizes to a single germline 6.3 kb fragment containing J_H sequences (Calame et al., 1980) and to a 5.6 kb fragment containing a rearranged J_H segment from the FO fusion partner. Interestingly, the results from a Southern blot presented in Fig. 4 show that So-G2a-3, So-G2a-4, So-G2a-5 and So-G2a-6 share a 7.2 kb band and a 4.6 kb band suggesting shared V_H gene segment usage. So-Gl-2, So-Gl-3, So-Gl-4 and So-Gl-5 are heterogeneous each displaying unique sized fragments which suggests different V_H gene segment usage. So-Gl-2 has an approximately 20 kb band; So-Gl-3 has 4.3 kb and 2.5 kb bands; So-Gl-4 has 4.8 kb and 3.3 kb bands; and So-Gl-5 has 7.3 kb and 3.6 kb bands.

The results of probing XbaI cut DNA with a 3' J_H probe (see Fig.3) are shown in Fig. 5. This probe hybridizes to a single germline 3.6 kb fragment and to a 4.8 kb fragment containing a rearranged J_H segment from the FO fusion partner. So-G2a-4, So-G2a-5 and So-G2a-6 share a 9.0 kb band. So-G2a-3 is missing this band but has instead a 1.2 kb band not found in the other IgG_{2a} hybridomas. Reprobing of these XbaI cut IgG_{2a} antibodies with a J_H coding region probe (Fig. 6) revealed a 7.8 kb fragment in So-G2a-3 which if added to the 1.2 kb fragment detected with both the J_H coding region and the 3' J_H probes would yield a 9.0 kb fragment comparable to the 9.0 kb band in So-G2a-4, So-G2a-5, and So-G2a-6; this suggests that So-G2a-3 may have gained an XbaI site through mutation. Double digestion of So-G2a-3 DNA with EcoRI and XbaI and probing with the coding region J_H probe detected a 2.7 kb band instead of a 3.8 kb band (as would be expected to be seen if no mutation had occurred, see Fig. 3), supporting this conclusion (data not shown). These Southern blot data provide substantial evidence that So-G2a-4, So-G2a-5 and So-G2a-6 are using identical V_H gene segments and suggests that So-G2a-3 may be using a similar V_H.

As would be predicted from results shown in Fig. 4 with the Group A IgG_1 antibodies, the fragment sizes of XbaI cut DNAs from these antibodies probed with a 3' J_H probe are heterogeneous. Although So-G1-2 shares a 2.3 kb XbaI cut fragment with So-G2a-6 and So-G2a-3, it does not share EcoRI cut fragment sizes with any of the Group A IgG2a antibodies shown in Fig. 6. However, So-G1-2 may bear a V_H gene segment highly homologous to that used by So-G2a-3, So-G2a-4, So-G2a-5 and So-G2a-6

since Northern blot data indicate So-Gl-2 utilizes a V_H gene from the J558 V_H gene family (seen in Fig. 8), as do all the Group A IgG_{2a} antibodies described in this paper.

V_H gene family usage in Group A hybridomas:

In addition to determining the heterogeneity of V_H gene usage in these Group A anti-Soman hybridomas, we were interested in determining which V_H gene family or families could contribute to a Soman-specific combining site. To do this we analyzed Northern blots with probes specific for the J558, S107 and Q52 V_H gene families all of which have been shown to encode antibodies in the PC-KLH response.

Probing of Northern blots of RNA from these IgG_{2a} and IgG_1 Group A antibodies indicated that while no V_H gene segments from the S107 and Q52 V_H gene families were used (Fig. 7), 6/8 of these antibodies use V_H gene segments from the J558 V_H gene family (Fig. 8).

DISCUSSION

Few anti-Soman monoclonal antibodies have been described to date. Lenz et al.(1983) described in vivo studies and binding characteristics of a single anti-Soman monoclonal antibody. Seiders et al. (1986) described the fine specificity of two anti-Soman antibodies, BE2 and CC1. We currently have forty-seven anti-Soman hybridomas that we have characterized for combining site specificities (Buenafe and Rittenberg, 1987; Makowski, Buenafe and Rittenberg, this paper; Buenafe, Makowski and Rittenberg, manuscript in progress). This characterization identified four major fine specificity groups designated Group A, Group B, Group C and Group D which

represent primary and secondary response antibodies following Soman-KLH immunization. Further studies of Group A antibodies indicate that some display low affinities for nitrophenylphosphocholine and hydroxy-Soman (Table I).

Fine specificity data from the additional Group A antibodies we have described in this paper indicate that the Group A secondary response antibodies appear to recognize the pinacolyl moiety of the Soman molecule, although in most instances they have appreciably lower I_{50} values for NH_2-O-So than for DPMP indicating that they also recognize the associated phenyl structure which would be present in the diazophenyl-linked hapten used for immunization.

While no binding to PC-BSA in the initial screening of these antibodies was detected by these Group A anti-Soman antibodies they are analogous to Group I anti-PC antibodies in their recognition of the trimethyl-bearing endgroup (pinacolyl and choline moieties, respectively). The inability of these anti-Soman antibodies to bind to the choline moiety of PC may be due to the presence of a positively charged nitrogen in its choline structure. However, V_H gene usage by the Group A anti-Soman antibodies described here is distinct from that of the Group I anti-PC antibodies. Group A anti-Soman hybridomas preferentially use members of the J558 V_H gene family, whereas Group I anti-PC hybridomas are highly restricted to usage of V_HI from the S107 family.

Hapten systems demonstrating restricted fine specificity patterns and variable gene usage have been described. In the murine response to PC,

Claflin et al. (1974) found the anti-PC PFC response to be predominantly of the IgM class and that it exhibited no affinity maturation during the immune response, indicating restricted affinity heterogeneity. In the analysis of nineteen anti-PC myeloma and hybridoma antibodies, Crews et al. (1981) found by amino acid sequencing that all the V_H regions of these antibodies were derived from the T15 germline V_H segment, a member of the S107 V_H gene family. In the anti-p-azophenylarsonate (Ars) system, idiotypic analysis of anti-Ars antibodies in A/J mice (Pawlak and Nisonoff, 1973) demonstrated restricted V gene usage. Demonstration by Capra and Nisonoff (1979) of a homogeneous sequence throughout the variable regions of heavy chains of anti-Ars cross-reactive idiotypic antibodies also gave evidence for restricted V gene usage. Griffiths et al. (1984) found that maturation of the primary response to 2-phenyl-5-oxazolone is characterized by somatic variants of restricted germline-encoded sequences used earlier in the primary response.

In contrast to these systems where V gene usage is restricted, we have found four major fine specificity Groups distributed among 47 anti-Soman hybridomas. The Group A antibodies are inhibited by p-aminophenyl-Soman, p-nitrophenyl-Soman and dipinacolylmethylphosphonate. Within these Group A antibodies, as judged by I_{50} values, four IgG_{2a} antibodies also bind with low affinity to hydroxy-Soman and nitrophenylphosphocholine, and appear to be using identical V_H genes from the J558 V_H gene family. Four IgG_1 Group A antibodies bind with low affinity to hydroxy-Soman but are not inhibitable by nitrophenylphosphocholine at

10 mM hapten which was the highest concentration used. These Group A IgG_1 antibodies appear to be using different V_H genes, although two of them utilize V_H genes from the J558 V_H gene family.

In summary, although these Group A antibodies collectively utilize different V_H gene segments and V_H gene families, the V regions formed display generally similar combining site specificities which could imply strong selection by antigen. This is analogous to the heterogeneity of V_H gene usage seen in the anti-PC-KLH Group II response where V_H gene segments from the S107, J558, Q52 and other, as yet to be determined, V_H gene families have been used. The use of J558 V_H family genes by 6/8 of these Group A antibodies suggests that sequences within this gene family may be particularly efficient in binding to Soman-KLH.

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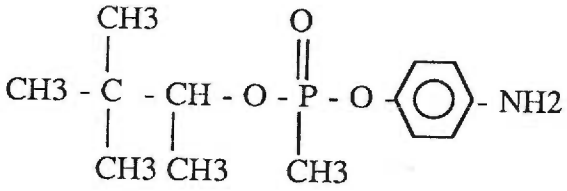
Table I. RANGE OF I50 VALUES (mM) FOR GROUP A SOMAN-SPECIFIC ANTIBODIES.

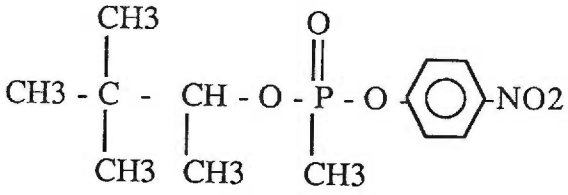
Antibody	Inhibitors				
	NH2-0-So	N02-0-So	DPMP	0H-So	NPPC
So-G2a-3	0.001	0.015	0.9	1.0	1.0
So-G2a-4	0.004	0.02	0.24	0.9	5.2
So-G2a-6	0.00086	0.026	0.6	0.48	2.29
So-G2a-5	0.00014	0.0067	0.096	2.2	10
So-Gl-2	0.01	0.12	0.08	0.28	>10*
So-Gl-3	0.0009	0.26	0.004	3.8	>10
So-Gl-4	0.0005	0.93	0.86	0.81	>10
So-Gl-5	0.0003	0.002	1.0	8.0	>10

* uninhibitable at highest concentration of hapten used (10 mM)

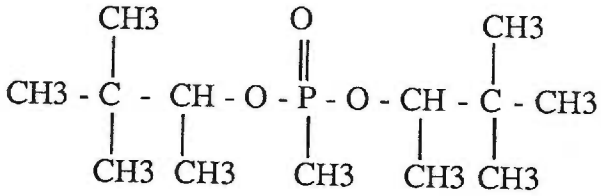
Figure 1. Chemical structures of Soman and phosphocholine derivatives. NH_2-O-So, NO_2-O-So, DPMP, OH-So and NPPC were used to inhibit binding of anti-Soman antibodies to Soman-protein in an ELISA assay.



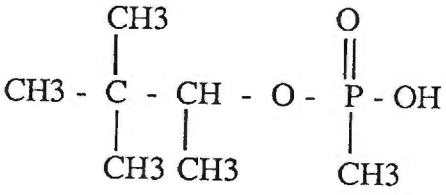
(a) p-AMINOPHENYL-SOMAN (NH_2-Ø-SO)



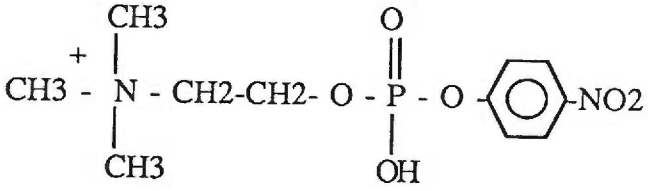
(b) p-NITROPHENYL-SOMAN (NO_2-Ø-SO)



(c) DIPINACOLYLMETHYLPHOSPHONATE (DPMP)



(d) HYDROXY-SOMAN (OH-SO)



(e) p-NITROPHENYLPHOSPHOCHOLINE (NPPC)

Figure 2. Hapten inhibition profiles of Group A anti-Soman antibodies. So-G2a-6 (a) is representative of the Group A IgG_{2a} antibodies. So-G1-2 is representative of Group A IgG_1 antibodies. Binding of purified hybridoma antibodies to Soman-BSA was measured in an ELISA in the presence of varying concentrations of the haptens as shown.

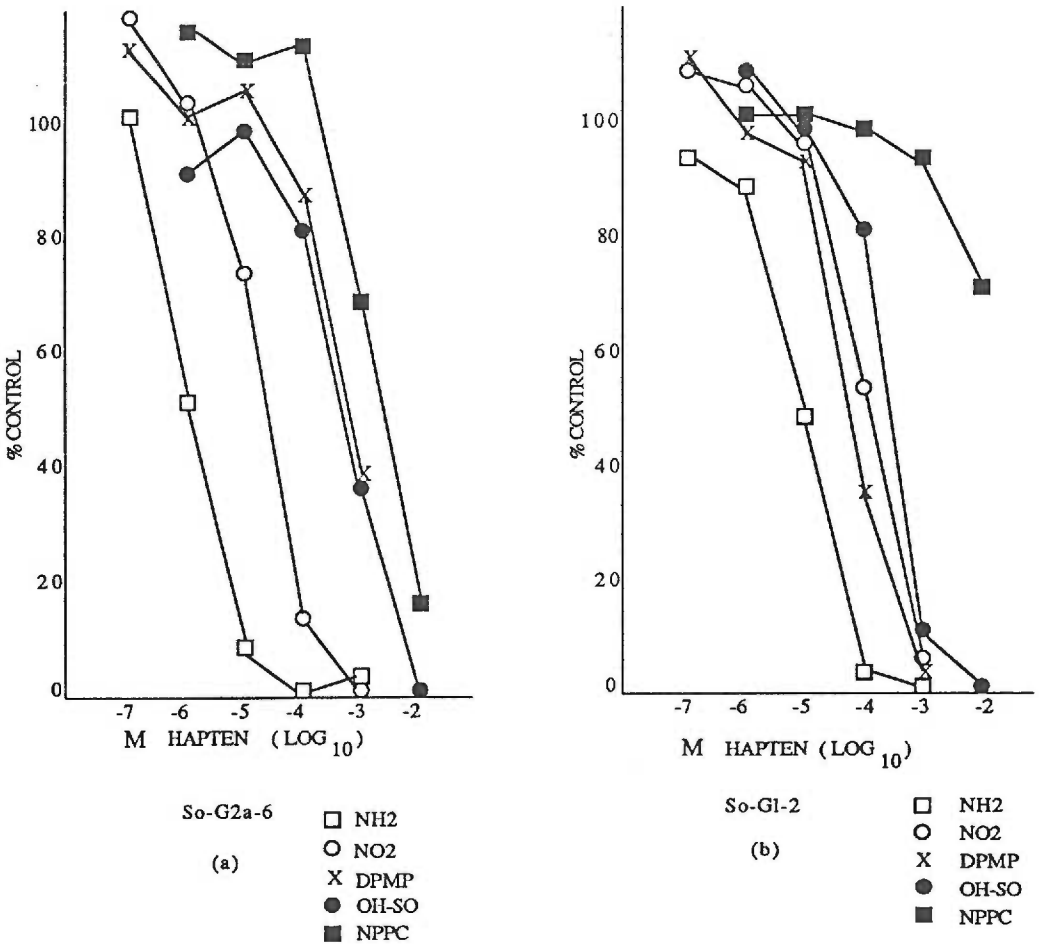

% CONTROL
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
M HAPTEN (LOG 10)
So-G2a-6
(a)
NH2
NO2
DPMP
OH-SO
NPPC
% CONTROL
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
M HAPTEN (LOG 10)
So-GI-2
(b)
NH2
NO2
DPMP
OH-SO
NPPC

Figure 3. Diagram of 1.95 kb J_H coding region probe and 1.2 kb 3' J_H probe used in Southern blot analyses of EcoRI and XbaI digested DNA from Group A anti-Soman antibodies. Predicted XbaI-site-forming mutation in So-G2a-3 shown by arrow.

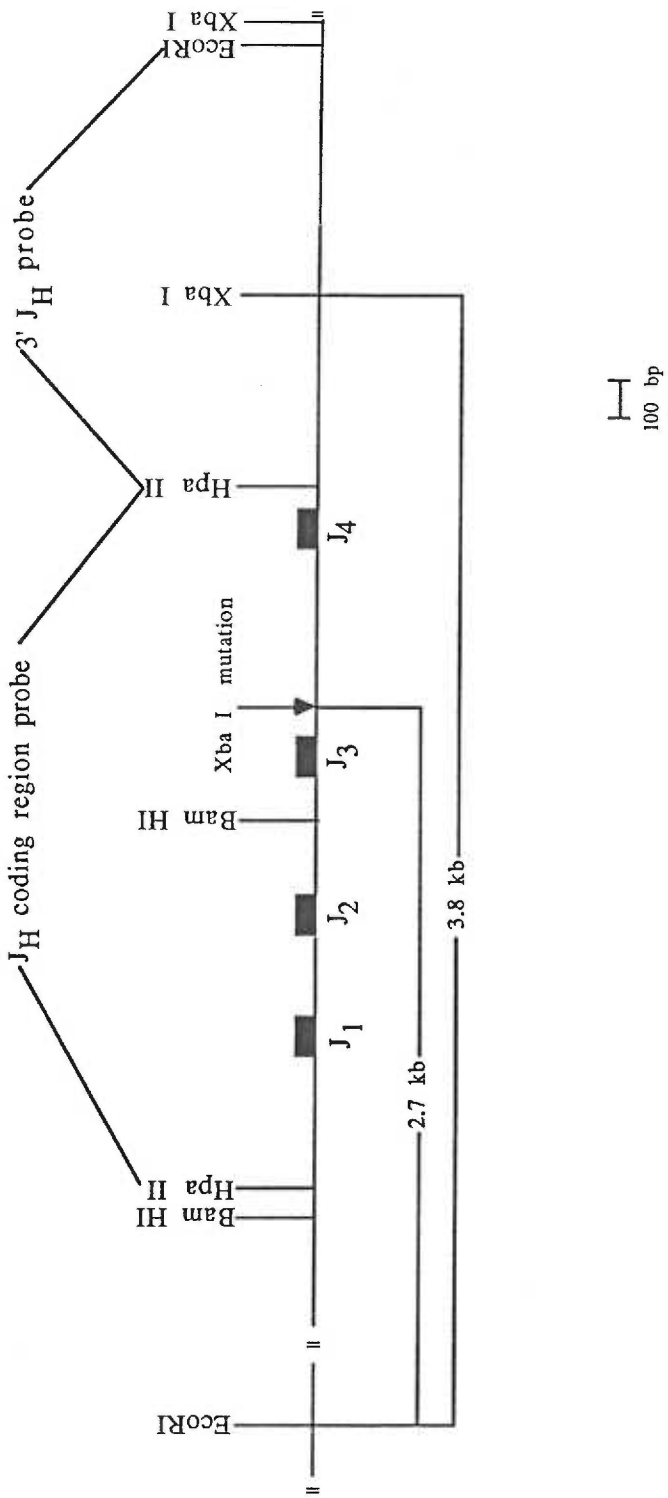

EcoRI
Bam HI
Hpa II
J1
J2
Bam HI
Xba I mutation
J3
J4
Hpa II
Xba I
EcoRI
Xba I
2.7 kb
3.8 kb
JH coding region probe
3' JH probe
100 bp

Figure 4. Southern blot of 10 ug of EcoRI-digested DNA were hybridized to a 1.95 kb probe containing the J_H coding region. Sources of DNA are indicated above each lane. The positions of DNA size markers (kb) are indicated at the left of the figure.

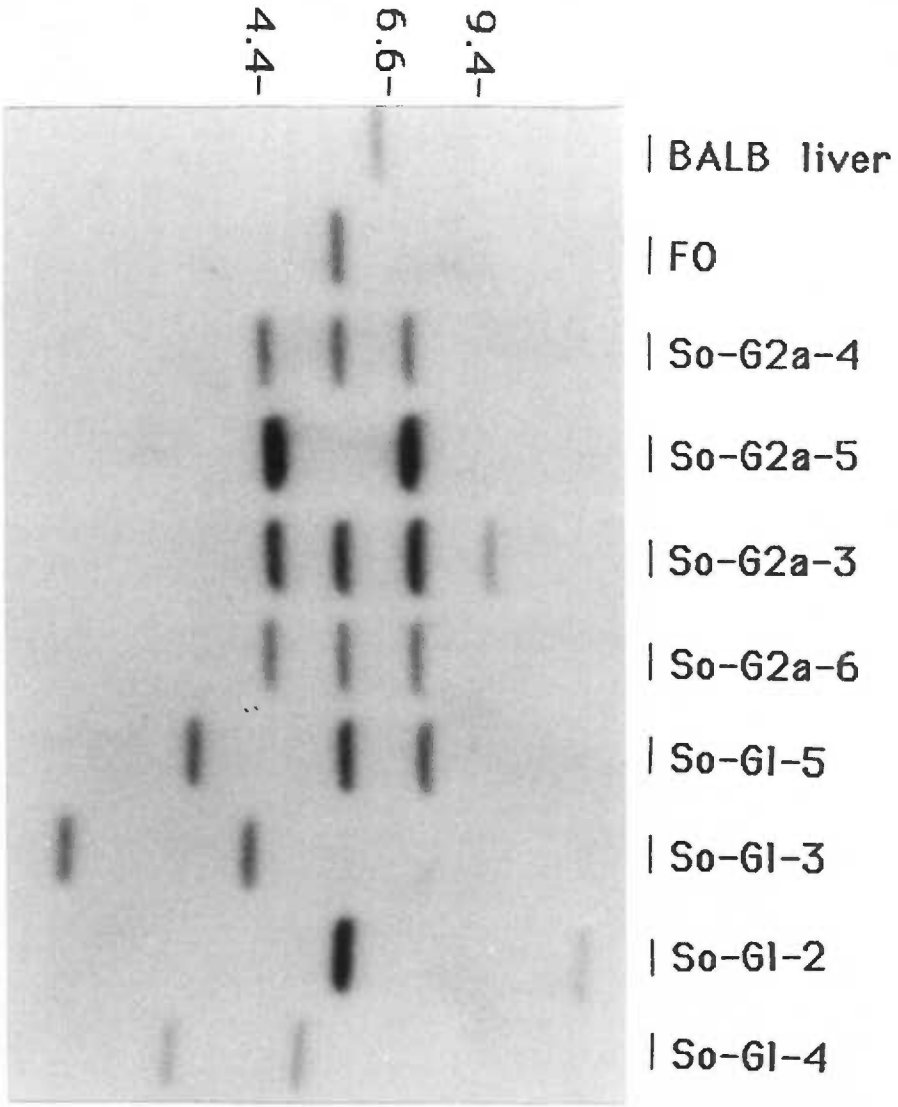

9.4-
6.6-
4.4-
BALB liver
FO
So-G2a-4
So-G2a-5
So-G2a-3
So-G2a-6
So-Gl-5
So-Gl-3
So-Gl-2
So-Gl-4

Figure 5. Southern blot of 10 ug of XbaI-digested DNA were hybridized to a 1.2 kb probe containing sequences 3' of the J_H coding region. Sources of DNA are indicated above each lane. The positions of DNA size markers (kb) are indicated at the left of the figure.

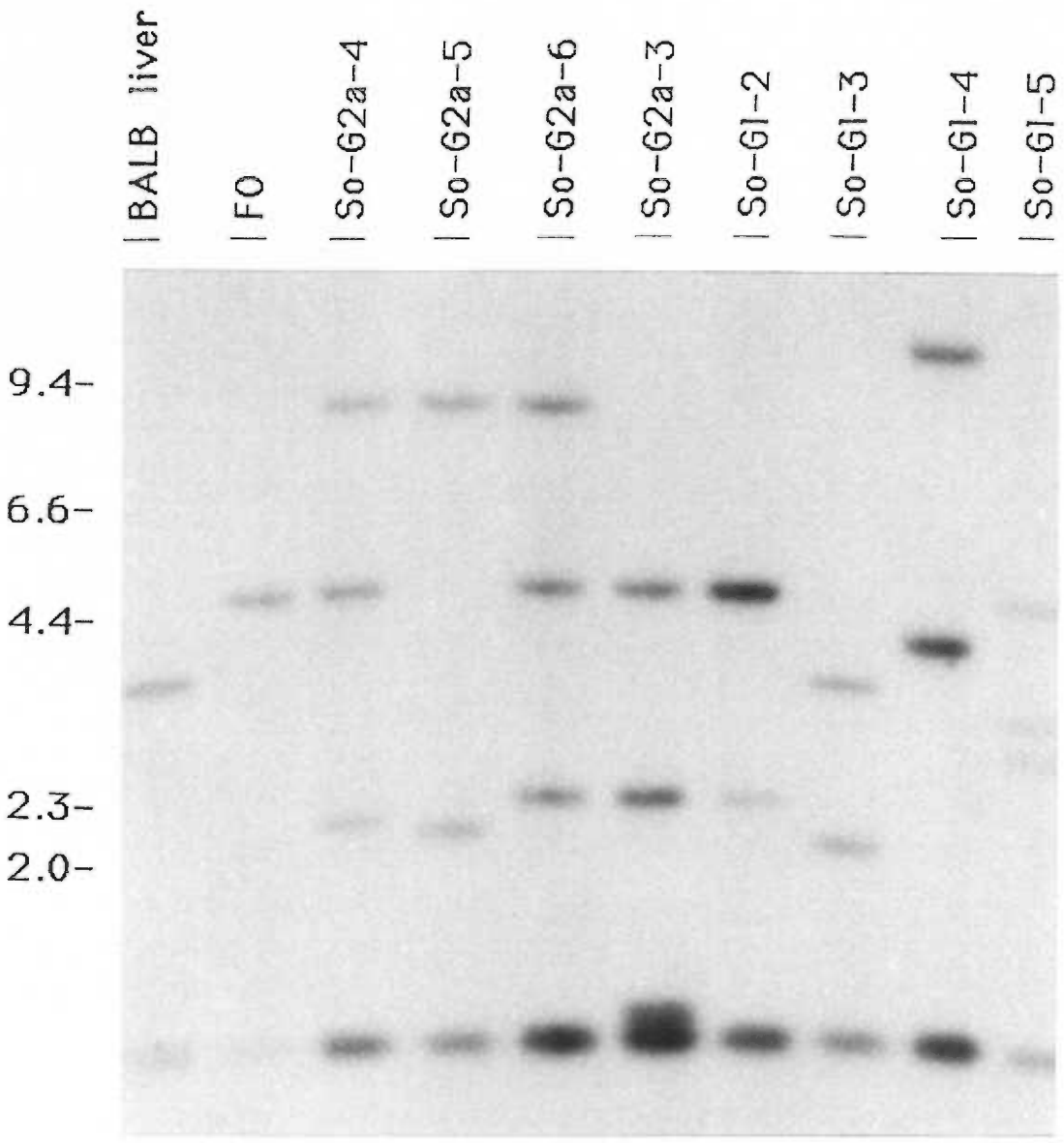

BALB liver
FO
So-G2a-4
So-G2a-5
So-G2a-6
So-G2a-3
So-G1-2
So-G1-3
So-G1-4
So-G1-5
9.4-
6.6-
4.4-
2.3-
2.0-

Figure 6. Southern blot of 10 ug of XbaI-digested DNA were hybridized to a 1.95 kb probe containing the J_H coding region. Sources of DNA are indicated above each lane. The positions of DNA size markers (kb) are indicated at the left of the figure.

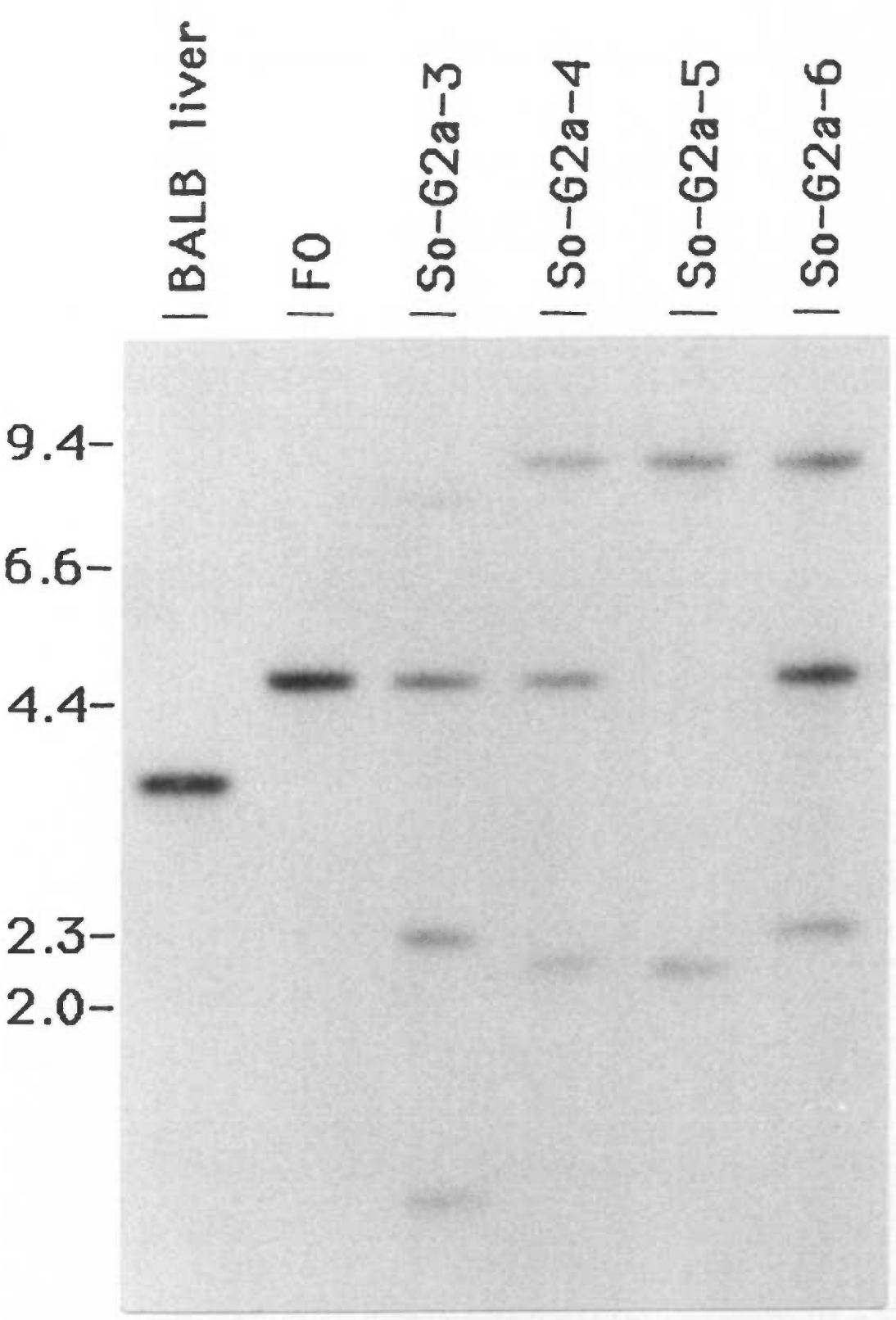

BALB liver
F0
So-G2a-3
So-G2a-4
So-G2a-5
So-G2a-6
9.4
6.6
4.4
2.3
2.0

Figure 7. Northern blots of 15 ug of total RNA were hybridized to a Q52 V_H family probe (shown in the upper figure) and to an S107 V_H family probe (shown in the lower figure). Sources of RNA are indicated above each figure.

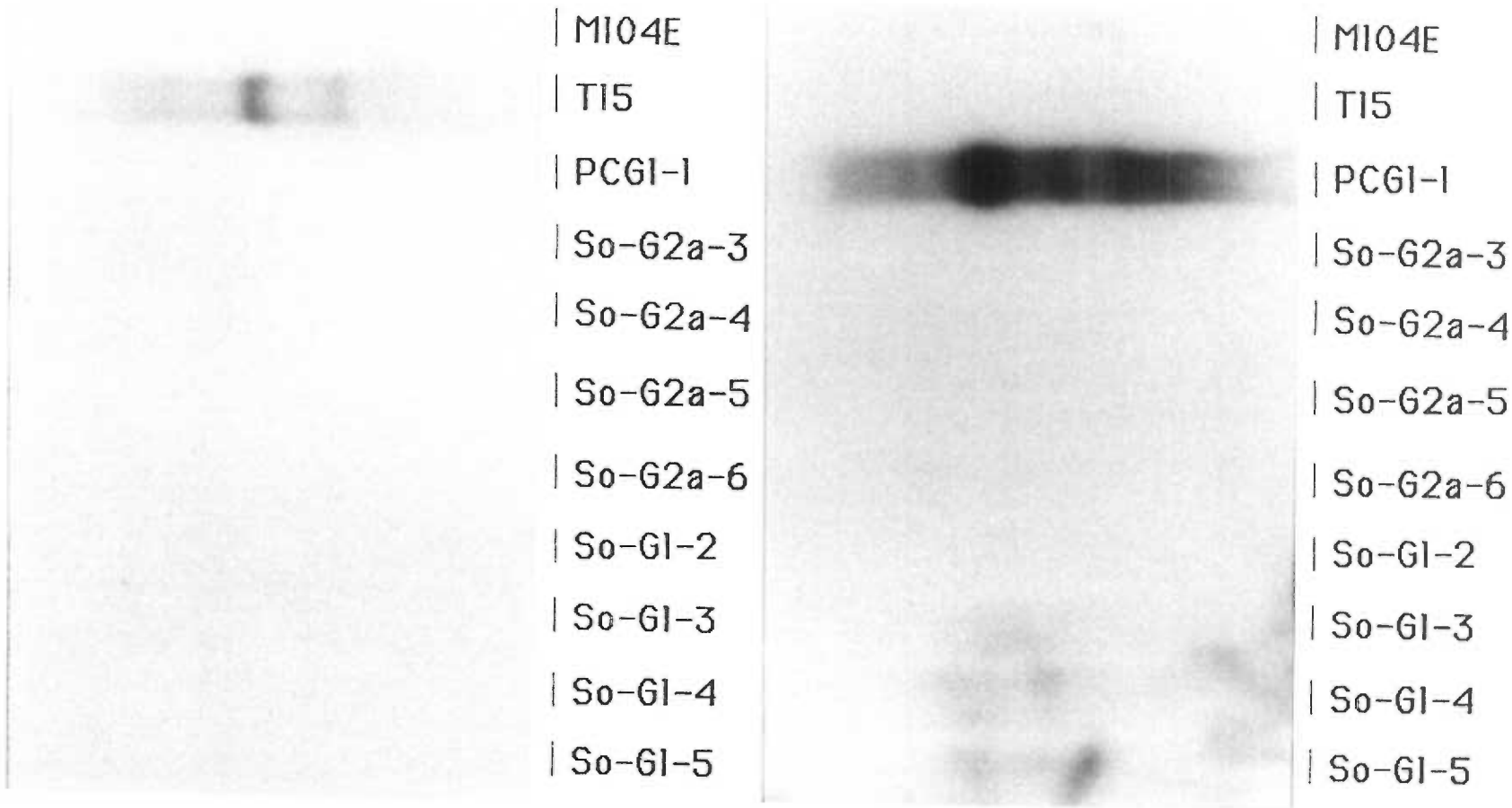
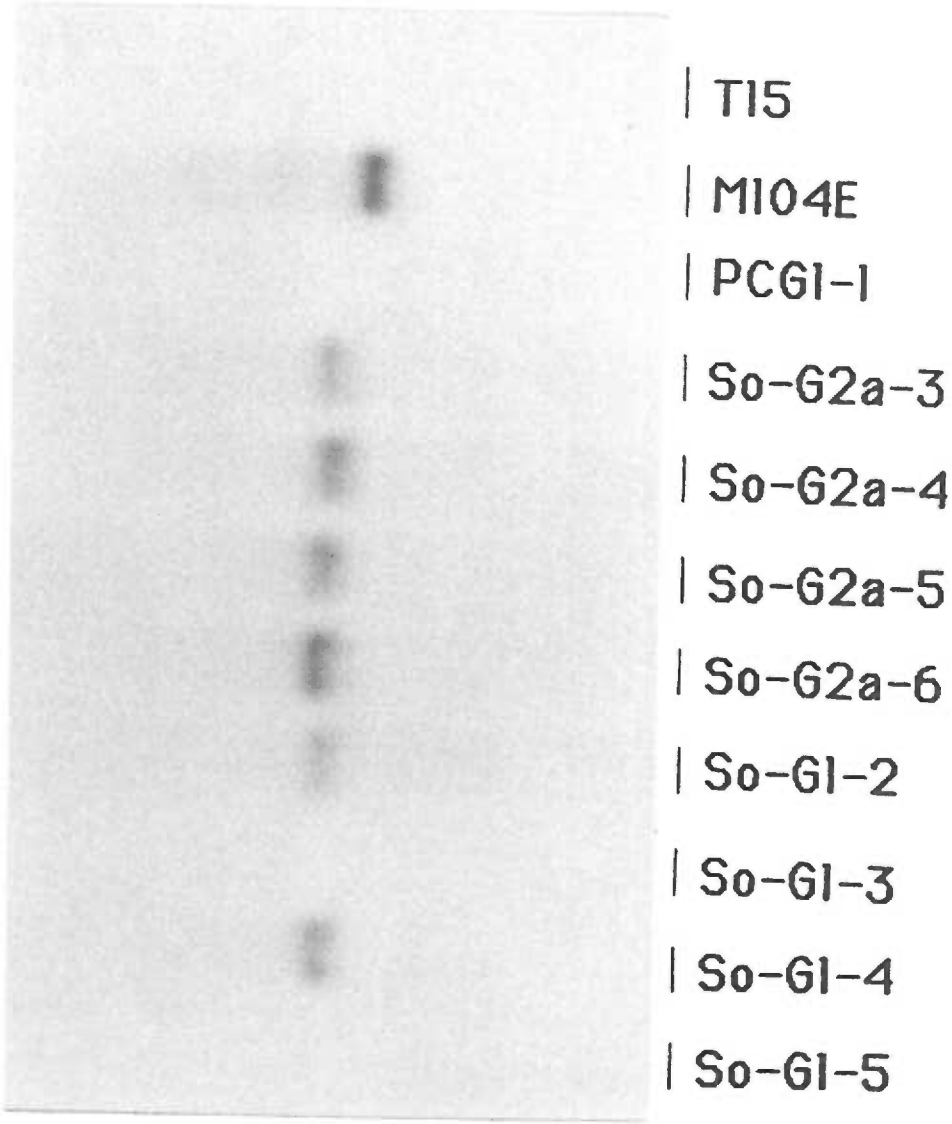

M104E
T15
PCG1-1
So-G2a-3
So-G2a-4
So-G2a-5
So-G2a-6
So-G1-2
So-G1-3
So-G1-4
So-G1-5
M104E
T15
PCG1-1
So-G2a-3
So-G2a-4
So-G2a-5
So-G2a-6
So-G1-2
So-G1-3
So-G1-4
So-G1-5

Figure 8. Northern blots of 15 ug of total RNA were hybridized to a J558 V_H family probe. Sources of RNA are indicated above the figure.


T15
M104E
PCG1-1
So-G2a-3
So-G2a-4
So-G2a-5
So-G2a-6
So-G1-2
So-G1-3
So-G1-4
So-G1-5

SUMMARY AND DISCUSSION

The primary BALB/c response to the organophosphate hapten Soman following Soman-KLH immunization consists of antibodies having a low affinity for Soman (1). Preliminary DNA analysis of these primary response antibodies, all of which are IgM isotype, indicates heterogeneity in V_H gene usage and predominance of J558 V_H gene family usage (Buenafe and Rittenberg, unpublished). The secondary anti-Soman KLH response consists of a population of higher affinity antibodies that display heterogeneity in their combining site specificities. It was possible, following ELISA hapten inhibition analysis using Soman and PC phenyl analogs, to assign these primary and secondary anti-Soman antibodies to one of four major distinct fine specificity groups designated Group A, Group B, Group C and Group D. The relative abilities of these subgroups to be inhibited by hapten are summarized in Table I of Appendix I and indicate the diversity seen among this collection of 47 hybridomas.

We have begun molecular analyses of many of these antibodies to determine the degree of heterogeneity of V_H gene usage within each of the four major groups of anti-Soman antibodies. In an analysis of eight Group A antibodies which display similar combining site specificities, we have found heterogeneity of V_H gene usage and heterogeneity of V_H gene family usage. Among this selection of Group A anti-Soman antibodies were four antibodies that bind NPPC with low affinity. These NPPC binding anti-Soman antibodies display homogeneity of V_H gene usage and V_H gene family usage by both Southern blot and Northern blot analysis. It is

possible that these two NPPC binding antibodies represent a pair of sister clones but this would have to be confirmed by sequence analysis.

The non-PC binding Group A antibodies analyzed demonstrate heterogeneous V_H gene usage by Southern blot analysis. Northern blot analysis indicates two of these Group A antibodies use V_H genes from the J558 V_H gene family. The remaining non-NPPC binding Group A antibodies do not use V_H genes from either the S107 or Q52 V_H gene families. Future work will determine which of the remaining V_H genes from the known V_H gene families (2) contribute to the anti-Soman response.

The majority of the anti-Soman antibodies that have been screened (25/35) utilize J558 V_H family genes (Buenafe and Rittenberg, unpublished and Paper I), suggesting that sequences common to J558 family members are important in the formation of the combining site of anti-Soman antibodies. The J558 V_H gene family is the largest V_H gene family (3) and the presence of 500-1000 V_H genes in this family (4) would allow the generation of a very large pool of V regions, following V_H-D-J_H recombination, from which to draw heavy chains with affinity for Soman. The Soman molecule is a potent neurotoxic molecule and one would not expect that the immune system had been exposed to this molecule prior to artificial immunization. Although the general similaritiies of structure between Soman and phosphocholine initially suggested that there might be immunologic cross-reactivity this has not been born out experimentally since only a small number of anti-Soman hybridomas cross-reacted with NPPC. Thus it is not unreasonable to view the anti-Soman response as a

true primary response and to find that it consists primarily of antibodies with low affinity for Soman. Nor is it surprising that the pattern of V_H gene usage would be heterogeneous. Such a pattern in the primary response to Soman-KLH would be what the model proposed in the Introduction to this work would predict for antibodies having low affinity for antigen. This would be analogous to the predicted low affinity Group II antibodies in the primary response to PC. The adaptive ability following somatic mutation of these primary anti-Soman antibodies would generate a pool of higher affinity anti-Soman antibodies that undergo clonal expansion upon secondary immunization. Thus one would also see a heterogeneous population of higher affinity secondary response antibodies (as we have seen) whose V_H genes would be somatic variants of the V_H genes used in the primary response. We have yet to test this hypothesis by comparing sequences of genes from primary and secondary response antibodies. Based upon the number of primary and secondary response antibodies using J558 V_H gene family members it seems likely that such an analysis would prove fruitful.

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Part II

ABSTRACT

The Mishell and Dutton in vitro microculturing system (1) has been an important technique for delineating mechanisms involved in the activation, proliferation and differentiation of murine B and T lymphocytes. Adaptation of this microculturing system for human in vitro studies has allowed similar analyses in human cells. The adaptation for human studies of the hemolytic plaque forming cell assay originally developed by Jerne and Nordin (2) has been of equal importance for determining the role of numerous substances in human B cell function. Both of these techniques were instrumental in previous characterizations in our laboratory of the human B cell response to trinitrophenyl-Brucella abortus(TNP-Ba) in vitro. This response was shown to be a T cell independent response (3) which was modulated in the presence of Concanavalin A (4) and which displayed anti-TNP antibody populations with both high and low affinity anti-TNP PFC (5).

The objectives of this work were:

(1) To determine the influence of B cell inducing factors (BIF) on the human anti-TNP antibody response in TNP-Ba-stimulated microcultured cells.

(2) To determine the optimum concentration of BIF necessary for maximum TNP-Ba stimulated response.

(3) To determine the state of activation necessary for BIF enhancement of the TNP-Ba-stimulated anti-TNP PFC response by altering the time of BIF addition to cultures.

(4) To determine the effect of T lymphocytes on the TNP-Ba plus BIF stimulated response by removing T cells from in vitro cultures.

(5) To determine the heterogeneity of the anti-TNP antibodies in TNP-Ba plus BIF stimulated cultures, with respect to antibody avidity and specificity, by using TNP hapten analogs to inhibit anti-TNP plaque-forming cells.

INTRODUCTION

In vitro B cell culturing and analysis systems.

Investigation of the regulation of the human immune response has been and continues to be an area of intense interest. Adaptation of the Mishell and Dutton in vitro murine culture technique (1) for inducing and measuring human T and B cell functions was a major advance towards understanding this regulation.

Early in vitro studies of human B cell function measured immunoglobulin production after stimulation of cultured lymphocytes using a radioimmunoassay (6), or by fluorescent anti-immunoglobulin techniques to stain intracytoplasmic immunoglobulin (7).

The adaptation of the hemolytic plaque forming cell (PFC) assay (2) to analyze single cell antibody production following in vitro stimulation was also of great significance in the evaluation of in vitro induction of B cells. Several PFC systems for human B cells have since been developed (8), and are used to detect both polyclonal and antigen-specific B cell responses.

Many in vitro PFC studies involved analysis of non-antigen specific regulation following polyclonal activation (9,10,11,12, 13), and demonstrated several characteristics of mitogenic agents such as the time dependency of the response (14), optimal conditions for stimulating tissues from various sources (15,16), the importance of T cells in induction of the response (8), serum requirements (17), or antigen dose dependency (14). The ability to induce and measure antigen-specific antibody responses in vitro (17,18) was a critical step in understanding regulatory aspects of the

human response.

The PFC assay is a sensitive, reproducible method of analyzing B cell function at the single cell level. Formation of antigen-specific PFC is the result of actively synthesized and secreted antibody binding to an antigen-coated red blood cell resulting in lysis of the erythrocyte when complement is added. Antigen-coated red blood cells surrounding antigen-specific antibody-secreting cells are lysed leaving an area devoid of intact red blood cells with a plasma cell in the middle (i.e. a plaque)(2). The usefulness of the PFC assay as a tool for studying regulatory aspects of antigen-specific B cell activity depends on the demonstration that the PFC response is in fact a reflection of a specific antibody response.

The demonstration of antigen specificity has been accomplished in a number of ways. Ballieux et al. (14) added relevant antigen to antigen-coated sheep erythrocytes just before adding complement to the assay mixture, thereby inhibiting the formation of plaques. Excess antigen present in the culture binds to the secreted antibodies effectively blocking the binding of antibody to antigen-coated sheep erythrocytes preventing complement mediated lysis. Delfraissey et al. (19) demonstrated specificity in the anti-TNP response of human peripheral blood lymphocytes (PBL) induced by TNP-polyacrylamide beads (TNP-PAA), through the inhibition of PFC with a hapten-protein conjugate. Schrader and Nossal (20) similarly demonstrated inhibition of the production of anti-hapten PFC in murine PFC assays by hapten-protein conjugates.

Inhibition of hapten-carrier-stimulated PFC by varying concentrations of hapten is a method of demonstrating the presence of both high and low avidity antibodies in both murine (21) and human (5) anti-hapten responses. In this method PFC producing high avidity antibody are inhibited at low concentrations of free hapten, while PFC producing low avidity antibody are inhibited at high concentrations of hapten. Furthermore, when haptens closely related to the stimulating hapten and conjugated to amino acid carriers are used for inhibition of the PFC response, it allows for the detection of fine specificity differences within the antibody populations (5,22).

In the analysis of the effects of B cell inducing factor (BIF) on the human TNP-Ba-stimulated response, we have employed the PFC assay system for detection of hapten-specific antibody-producing cells. We have also performed hapten inhibition of the PFC assays to demonstrate hapten specificity, avidity of anti-TNP populations induced, as well as fine specificity characteristics of the anti- TNP in vitro response.

The B cell cycle.

The triggering of B cells to proliferate and differentiate into plasma cells is a complex process. Dutton (23) and Schimpl et al. (24) proposed a three stage model for this process (see Fig. 1) in which the resting B cell is first activated by antigen, then proliferatively expands before finally undergoing a differentiation phase in which the cells of the expanded population become antibody-secreting cells. More recent work by

numerous investigators suggests that B cell regulation is far more complicated than in this early model.

Indeed, the first step occurring in antigen stimulated B cell responses is the binding of antigen to the surface receptor (immunoglobulin). The binding of antigen to the variable regions of heavy and light chains of immunoglobulin on the cell surface is a necessary but insufficient first step in the activation of resting B cells. Polyclonal B cell activators such as LPS (25), lipid A (26) and lipoprotein (27) can activate the B cell without binding to surface immunoglobulin. Activation by these B cell mitogens was an indication that other signals besides antigen binding to immunoglobulin were needed to activate the resting B cell. Cooperation by helper T lymphocytes (28) accessory cells such as macrophages (29) and B cells (30) was also shown to be necessary for B cells to respond to most antigens. This cooperation by helper T cells, accessory cells and B cells was shown to be restricted to cells bearing the same class II major histocompatibility complex (MHC) molecules (31). The current concept that antigen interaction with B cells (via the imunoglobulin receptor), T cells (via the T cell receptor) and accessory cells in the context of the MHC is necessary for B cell activation has been demonstrated by Andersson et al. (32). Following B cell binding of specific antigen, it was later demonstrated that antigen non-specific growth and differentiation factors derived from T cells are essential for B cells to undergo proliferation and differentiation into plasma cells (immunoglobulin secreting) (33,34).

B cell activation may be followed by cell division without differentiation into immunoglobulin secreting cells (35). Alternatively, B cells can be driven to secrete immunoglobulin without undergoing cell division (36). A T cell- derived B cell growth factor that has been well characterized is B cell stimulatory factor I (BSF-1)(37). Extensive work on the physiologic role of BSF-1, formerly termed B cell growth factor-I (BCGF-1) and thought to be a B cell factor exclusively, has demonstrated that it not only acts as a B cell growth factor (38) but also as an inducer of resting B cells (39). Vitetta et al. (40) have also demonstrated identity of BSF-I with a factor isolated in their lab called B cell differentiation factor for IgG_1 (BCDF-gamma 1), which causes differentiation of LPS-activated B cells into plasma cells secreting IgG_1 antibodies. Honjo (41) and Arai (42) and their colleagues independently reported cDNA clones for BSF-1 and in light of its multiple activities have suggested it be designated interleukin-4 (IL-4), thus BSF-1 is currently referred to as BSF-1/IL-4. The original model proposed by Dutton (23) and Schimpl et al. (24) of a three stage progression of B cells from the resting state to the plasma cell state is obviously not sufficient to explain these observations. In particular, the model cannot account for the multiple activities nor the time of action of factors such as BSF-1/IL-4 (43).

Morphological and molecular analyses of cycling B cells have proven invaluable in delineating the mode and time of action of various factors

during the cell cycle (a diagram of this cycle is depicted in Fig. 2). Stimulation of the resting G_0 cell by antigen or mitogen to enter early G_1 is accompanied by an increase in cell volume (44,45), an increase in Ca^{++} uptake and depolarization of surface membranes (46), an increase in RNA synthesis (44,45,47), an increase in the amount of class II major histocompatibility complex molecules on their membranes (48) and changed phosphoinositol metabolism (49). Following this entry into G_1, B cells acquire the ability to respond to other signals such as interleukin-1 (IL-1), secreted by macrophages (50), allowing them to progress into late G_1. Further signals including T helper cell derived products (50) are required for progression into S where rapid DNA synthesis occurs followed by cell division (47,51,52). In the absence of continued antigen binding to the immunoglobulin receptor the cell no longer divides, while continued presence of antigen, or antibody to surface immunoglobulin, will allow the B cell to enter its cell cycle again (50).

Factors affecting B cell activation, proliferation and differentiation.

A major effort in the delineation of the signals involved in the complex events in the response of B cells to antigen has been focused on the identification of various biologically active substances which influence lymphocyte functions in in vitro systems.

In the mouse and human there are numerous soluble factors which have been described that are produced by macrophages, or other antigen presenting cells (also termed accessory cells), T cells and B cells. Those

factors which have been shown to be involved in B cell progression through the cell cycle include BSF-I/IL-4, IL-1, BCGF-II, BCDF-I, BCDF-II, IL-2 and gamma-IFN and will be discussed.

BSF-I/IL-4, formerly called BCGF-I, is a T cell derived lymphokine that, when purified by affinity chromatography using a monoclonal antibody to BSF-I/IL-4, isolates as two molecular species of 14 kd and 18-20 kd (53). While there is no controversy regarding the fact that BSF-I/IL-4 acts on B cells, there are several reports of BSF-I/IL-4 having a variety of effects on the resting and activated cell. BSF-I/IL-4 was first shown to be stimulatory to anti-immunoglobulin activated B cells causing them to progress from late G_1 to S phase of the cell cycle (37). As mentioned previously, BSF-I/IL-4 was also shown to act on resting B cells to make them more responsive to subsequent stimuli (39,54). BSF-I/IL-4 has since been demonstrated to stimulate resting B cells to express increased amounts of Ia antigens (55,56), to increase their cell volume (57,58) and to induce low affinity receptors for the Fc portion of IgE on resting B cells (59). Snapper and Paul (60) found that resting B cells preincubated with BSF-I/IL-4 secrete enhanced amounts of IgG_1 upon subsequent stimulation with LPS. Snapper and Paul (61) also demonstrated that BSF-I/IL-4 increased switching of LPS-activated B cells to express IgG_1 and IgE while markedly inhibiting their expression of IgM, IgG_3, IgG_{2a} and IgG_{2b}. Yokota et al. (62) have cloned DNA from a human T cell c-DNA library that encodes a molecule having about 50% homology

with mouse BSF-1/IL-4. The expressed molecule stimulated progression of anti-immunoglobulin treated human B cells into S phase, suggesting that it may be the human equivalent of murine BSF-1/IL-4. It will be of interest to see if the effects of this human T cell derived factor parallel the effects seen with murine BSF-1/IL-4.

Interleukin-1 (IL-1) is one of the best studied of the macrophage-derived soluble factors found to be involved in the antibody response and active on a wide variety of tissues (reviewed by Oppenheim et al. (63)). The production of IL-1 by B cells has also been reported (64). Studies using IL-1 and BSF-1/IL-4 with anti- immunoglobulin-activated murine B cells suggest that IL-1 functions as a cofactor with BSF-I/IL-4 promoting the progression of the B cell into S phase (65). Other studies could not observe any requirements for IL-1 in B cell proliferation (66), although these experiments employed serum which can be expected to have IL-1 (67). Others have demonstrated a role for IL-1 in B cell activation (68). In human PWM stimulated cultures, IL-1 was shown to be important in proliferation and differentiation at an early stage of the cell cycle because antibody to IL-1 caused inhibition of proliferation and differentiation when added during the first 24 hours of the culture (69). While IL-1 has been shown to augment proliferation and differentiation of human B cells following stimulation with Staphylococcus aureus and BSF-I/IL-4 (70,71), there have been no reports of an absolute requirement for IL-1 in B cell proliferation and/or differentiation. The cloning of murine IL-1 by

Lomedico et al. (72) and of human IL-1 by March et al. (73) has provided evidence for a 30 kd polypeptide precursor and a 15 kd active protein in both species.

Murine BCGF-II is a 55 kd protein and thus a higher molecular weight factor than BSF-1/IL-4. BCGF-II differs as well from BSF-1/ IL-4 in having the capacity to induce proliferation and differentiation of B cells (74). BCGF-II has also been shown to differ functionally from BSF-1/IL-4 in its ability to induce proliferation of dextran sulfate-stimulated B cells (75). Two distinct molecular weight forms of BCGF have been found in man, a 50 kd protein, BCGF-I, described by Ambrus and Fauci (76) and a 60 kd protein, BCGF-II, described by Shimuzu, et al. (77). While both human BCGF-I and BCGF-II support proliferation of activated human B cells (62,78), only BCGF-II has been demonstrated to induce B cell differentiation (77). Thus the bioactivity of BCGF-I appears analogous to murine BSF-1/IL-4, while that of human BCGF-II is analogous to murine BCGF-II.

The term BCDF (B cell differentiation factor) was first used to describe those factors that did not support proliferation of activated B cells but were involved in their differentiation into antibody-producing plasma cells (79). Like BSF-1/IL-4 and BCGF-II, two molecular weight forms of BCDF have been described (78). BCDF-I has a molecular weight of 20 kd while BCDF-II has a molecular weight of 30-35 kd. Both BCDF-I and BCDF-II are required in human cultures for maximum responsiveness (80). Work by Jelinek and

Lipsky (34,81) demonstrating that B cells are responsive to differentiation factors before entering S phase, most likely during the G_1 phase of the cycle, is in agreement with earier work of Kishimoto et al. (82) in which B cells were demonstrated to form or expose receptors for differentiation factors in the absence of cell division.

Traditionally, IL-2 has been described as being responsible for maintaining the growth of activated T cells (83). Human IL-2, with a molecular weight of 15-18 kd, can be obtained in pure form by recombinant technology using the cloned genes (84) or by affinity chromatography using monoclonal antibodies to IL-2 (85). Activated B cells have been shown to express receptors for IL-2 (86). IL-2 produced by recombinant DNA techniques or purified by affinity chromatography has since been demonstrated to bind to activated human B cells (87,88). This binding to B cells is also inhibited by IL-2 receptor-specific antibodies (89,90). Additionally, human B cells have both high and low avidity receptors for IL-2 (91,92) as do T cells. Recombinant IL-2 (r-IL-2) has been shown to induce human B cells to proliferate (88,93), to proliferate and differentiate into plasma cells (81,90), or to differentiate only (94,95). The varied effect of IL-2 in these studies may be due to T cells contaminating the B cell preparations and secreting proliferation and differentiation inducing factors as shown by Miedema et al. (96). Alternatively, the different modes of B cell activation reported in these studies, such as phorbol ester (88), SAC (90) and antigen (94) may

determine whether IL-2 induces proliferation and/ or differentiation. Lowenthal et al. (97) showed that B cells activated with LPS alone or with anti-immunoglobulin alone expressed fewer IL-2 receptors and binding sites for IL-2 when compared to B cells activated with LPS and anti-immunoglobulin. Furthermore, only the LPS plus anti-immunoglobulin-activated cells internalized IL-2. Thus if proliferation and differentiation of B cells by IL-2 are dependent on the concentration of IL-2 binding sites and subsequent IL-2 internalization, the mode of activation of these cells may be critical to subsequent events.

Vasquez et al. (98) have demonstrated that anti-immunoglobulin activated human B cells which express the IL-2 receptor are induced to proliferate in the presence of either recombinant IL-2 or BCGF-I, but not BCGF-II. Anti-immunoglobulin-activated B cells lacking IL-2 receptors were nonresponsive to recombinant IL-2 but proliferated in the presence of BCGF-I and BCGF-II. This work supports the suggestion that the concentration or presence of IL-2 receptors on the activated B cell determines its ability to respond to IL-2, and that the presence of other T cell derived factors such as BCGF-I or BCGF-II secreted by contaminating T cells can interfere with assays for IL-2 activity on activated B cells (regardless of the presence of IL-2 receptors on their surface). Although B cells have been shown to express IL-2 receptors following activation and to respond by proliferating and differentiating, the physiological role IL-2 may play in the in vivo response remains controversial. The magnitude of

proliferation and antibody production in cultures containing IL-2 alone is small when compared to the response in the presence of other lymphokines (93), and IL-2 in much higher concentrations is required to support B cell growth than is required for T cell growth (99). Thus the responses to IL-2 observed in vitro indicate that while IL-2 has an effect on activated B cells, it appears to play a minor role in in vivo B cell proliferation and differentiation.

The role of gamma interferon (IFN) in B cell proliferation and differentiation into plasma cells is also controversial. Use of recombinant gamma-IFN (100) has not proven to be particularly helpful in resolving this controversy. Nakagawa et al. (93) have shown that gamma-IFN was unable to induce B cell diferentiation by itself, but recombinant gamma-IFN induced tonsillar B cells activated with mitogen to secrete antibody if IL-2 was present. Romagnani et al. (101) have shown that gamma-IFN induces anti-immunoglobulin-stimulated human B cells to enter S phase of the cell cycle. Other investigators could not demonstrate this induction with gamma IFN and B cells (102). In work by Mond et al. (103) and Rabin et al. (104), gamma-IFN was shown to inhibit the stimulatory effects of BSF-1 on resting and activated cells. Snapper and Paul (61) demonstrated that gamma-IFN inhibited the production of IgG_3, IgG_1, IgG_{2b} and IgE isotypes, however, they found that the same gamma-IFN stimulated the expression of IgG_{2a}; in both instances the B cells had been activated by LPS. Thus the

same lymphokines may have alternative effects depending on the isotype of the secreted antibody.

Based upon information derived from investigators cited, I propose a model of B cell activation, proliferation and differentiation which is depicted in Fig. 3. This model will be referred to in the discussion of the human anti-TNP-Ba response in the presence of B cell inducing factor (BIF). The model incorporates functions of IL-1 reported by Hoffman (65) and Lipsky (69), functions of BSF-1/IL-4 by Howard (51), Oliver et al. (39), Rabin et al. (57), Roehm (55), and Noelle et al. (56), functions of BCGF-II by Yokota et al. (62), Kishimoto (78), and Shimuzu et al. (77), functions of BCGF-I and BCGF-II by Jelinek and Lipsky (34,81) and Kishimoto (78) and functions of IL-2 by Ando et al. (88), Nakagawa (93), Pike et al. (94), Mittler et al. (90), Kishi et al. (95), Lowenthal et al. (97) and Snapper and Paul (60,61), and functions of gamma-IFN by Nakagawa et al. (93) and Romagnani et al. (101).

The role of BSF-1/IL-4 is based upon studies using anti-immunoglobulin as surrogate antigen for activation. In the absence of antigen, BSF-1/IL-4 acts on resting B cells to increase Ia molecule expression even before the signal to progress into G_1 (i.e. antigen binding) is given. This increased Ia expression accompanies an increase in the B cell's responsiveness to anti-immunoglobulin and subsequent entry into S phase (pathway 1). Alternatively, anti-immunoglobulin alone stimulates B cells to enter early G_1. Progression into S phase depends on the presence of BSF-1/IL-4

with IL-1 as a cofactor. The B cell can also respond following anti-immunoglobulin activation in response to BCGF-II or to high concentrations of IL-2 resulting in entry into S phase and subsequent proliferation (pathway 2). The proliferating B cell may then undergo differentiation into a plasma cell in the presence of BCDF-I, BCDF-II, IL-2, gamma-IFN with IL-2 as a cofactor, or BCGF-II. Alternatively, the activated B cell may differentiate in the absence of cell division into a plasma cell in the presence of IL-1, BCDF-I, BCDF-II, IL-2 or BCGF-II (pathway 3).

The state of activation of the resting B cell may determine the pathway subsequently taken. Jelinek et al. (105) demonstrated that IgD^- B cells underwent more proliferation and differentiation in response to recombinant IL-2 than IgD^+ B cells. B cells from different lymphoid organs may vary in their activation and/or lymphokine requirements and thus may follow different pathways. For example, Suzuki and Cooper (87) found that IL-2 supported the proliferation of anti-immunoglobulin-activated human peripheral blood lymphocytes, while Mond et al. (106,107) found that IL-2 had no effect on anti-immunoglobulin-activated human tonsillar or splenic B cells. Finally, how the resting B cell is activated may dictate its subsequent responsiveness to lymphokines. Thus IL-2 was able to enhance the proliferation of human peripheral blood lymphocytes (93), tonsillar (91) or splenic B cells (85) if they had been activated by Staphylococcus aureus.

The rapid expansion of knowledge of the biochemistry and biologic activity of factors acting on B cells has been accompanied by efforts to understand the mechanisms by which these agents transduce their signals across the cell membrane controlling the internal biochemical regulation of proliferation and differentiation. The demonstration by Monroe et al. (108) that phorbol diesters, analogs of diacylglycerol, induced membrane depolarization and increased expression of Class II antigen by B cells implicated the phosphatidylinositol pathway in activation of the B cell by anti-immunoglobulin. The details of the phosphatidylinositol system are reviewed by Nishuzuka (109). Studies by Grupp and Harmony (49) demonstrated induction of the phosphatidylinositol response in murine B cells with IgM but not with LPS. Subsequent experiments were performed by Gold and De Franco (110) in a human B lymphoma cell line and in normal human B cells by Mizuguchi et al. (111) in which they used phorbol esters as blocking agents of anti-immunoglobulin-stimulated phosphoinositide hydrolysis. These studies added evidence that the phosphoinositate pathway was one mechanism of B cell activation. Studies of intracellular events triggered by LPS indicate that LPS activates the B cell by inducing the translocation and activation of protein kinase C (112,113). Furthermore, the studies by Bjisterbosch et al. (114) of the effect of LPS on B cells indicated that activation by this agent occurs without inositol lipid metabolism or Ca^{++} mobilization and thus differs from anti-immunoglobulin activation of B cells. Recent work by Roifman et al. (115) inves-

tigated the effects of the phorbol ester, 12-O-tetradecanoyl-phorbol 13-acetate (TPA), and the Ca^{++} ionophore, ionomycin, on human B cell differentiation. Roifman et al. found that TPA and ionomycin were comitogenic on tonsillar and peripheral blood B cells and induced B cell differentiation into immunoglobulin secreting cells.

The production of pure lymphokines, monoclonal antibodies to these lymphokines and monoclonal populations of B cells, T cells and accessory cells may open the way for delineating precisely the mode and time of action with which these factors affect the activation and progression of the B cell through the cell cycle when used alone or in combination with other lymphokines and/or cells.

Thymus-independent and thymus-dependent antigens.

Antigens that induce B cell responses are classified as thymus-independent (T-I)(116) and thymus-dependent (T-D)(28). This classification is determined by the requirement for T cells to induce a B cell response. T-I antigens can induce proliferation and differentiation of B cells without T cells. It is speculated that T-I antigens containing repeated determinants are able to crosslink surface B cell immunoglobulin thus triggering activation of the B cell (117,118).

Galanaud (119) speculated that the thymus independency or dependency of an antigen may depend on several parameters such as the structure of the antigen, or the in vitro culture conditions, or the type of tissue used as a source of B cells. Trump (120) showed that thymus

independency may be a function of the structure of the antigen which may depend on the manner in which a hapten is bound to the same carrier. While Scott et al. (121) demonstrated that the conditions of the culture may influence thymus independency in that thymus independency may be lost when cells are cultured at limiting dilution. Galanaud et al. (119) showed that B cells from different organs differed in their stage of differentiation and/or their mode of activation.

The human anti-TNP response in vitro.

TNP-specific PFC responses were induced in human blood lymphocytes with TNP-polyacrylamide beads (TNP-PAA)(19). In this system TNP-PAA was shown to be a T cell dependent response. In our laboratory, Golding et al. (3) described the response of human lymphocytes to thymus-independent and thymus-dependent antigens. Human lymphocytes from peripheral blood, tonsils and adenoids were stimulated with TNP-Brucella abortus (TNP-Ba) to produce an anti-TNP specific response. This response to TNP-Ba could be stimulated in the absence of T cells and appeared to be T-independent. TNP-keyhole limpet hemocyanin (TNP-KLH) also induced a specific anti-TNP response which was not stimulated in the absence of T cells and thus was determined to be a T-dependent response. While the anti-TNP-Ba human response appeared to be T-independent, additional studies by Golding et al. (4) had shown that this response was also positively or negatively modulated when the plant lectin Concanavalin A (Con A) was added to cultures. The modulation may well have depended on the

state of differentiation of the B cell since the direction of modulation was dependent on the time lectin was added to TNP-Ba stimulated cultures. Late addition of Con A led to enhancement of the anti-TNP PFC response while early addtion of Con A led to suppression.

In an attempt to maximize the human in vitro anti-TNP-Ba response we supplemented cultures with B cell inducing factor (BIF). BIF had been reported by Ralph et al. (122,123) to stimulate activated B cells to proliferate and differentiate into plasma cells. Pilot experiments using BIF in TNP-Ba stimulated human in vitro microtiter cultures demonstrated that BIF had an enhancing effect on the production of anti-TNP PFC.

The purpose of this part of my work was to study the effect of BIF on the human TNP-Ba stimulated in vitro response. As previously mentioned, the hemolytic plaque assay was used to make these determinations. We were also interested in optimizing in vitro microtiter culture conditions, in the presence of BIF, determining variables such as number of cells per culture, concentration of antigen, concentration of BIF, time of addition of BIF and time of assay. We also analyzed the effect of T cell depletion on this response, the relative affinities and the fine specificity of anti-TNP antibodies in this response using hapten analogs as inhibitors of the PFC response.

In the TNP-Ba plus BIF stimulated cultures we were able to determine the state of activation of B cells necessary for BIF enhancement as well as the time when BIF would induce maximum response.

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Figure 1. Model of B cell activation, proliferation and differentiation.

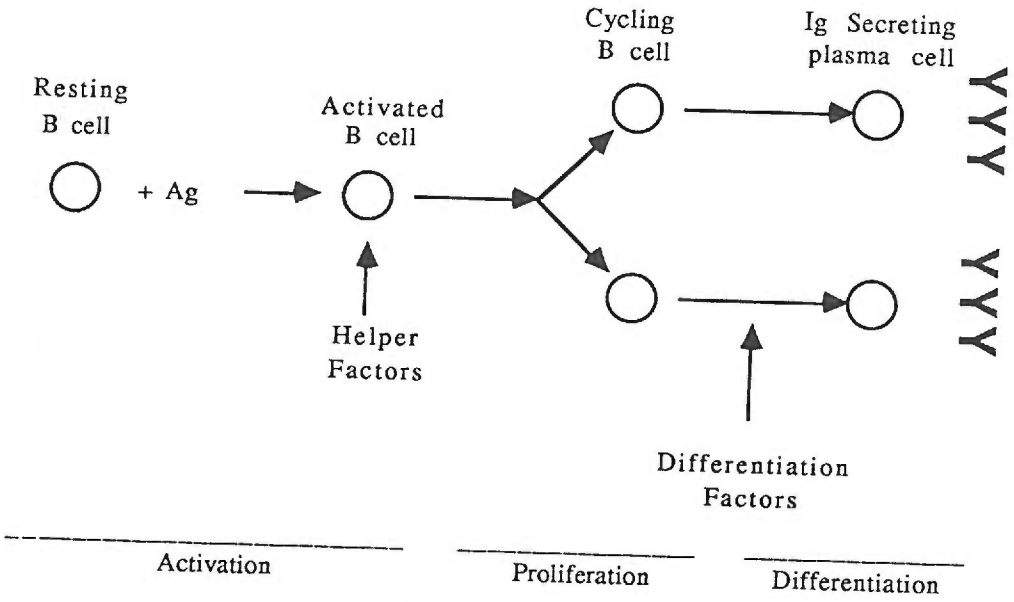

Resting
B cell
+ Ag
Activated
B cell
Helper
Factors
Cycling
B cell
Ig Secreting
plasma cell
Differentiation
Factors
Activation
Proliferation
Differentiation

Figure 2. Diagram of the cycling B cell.

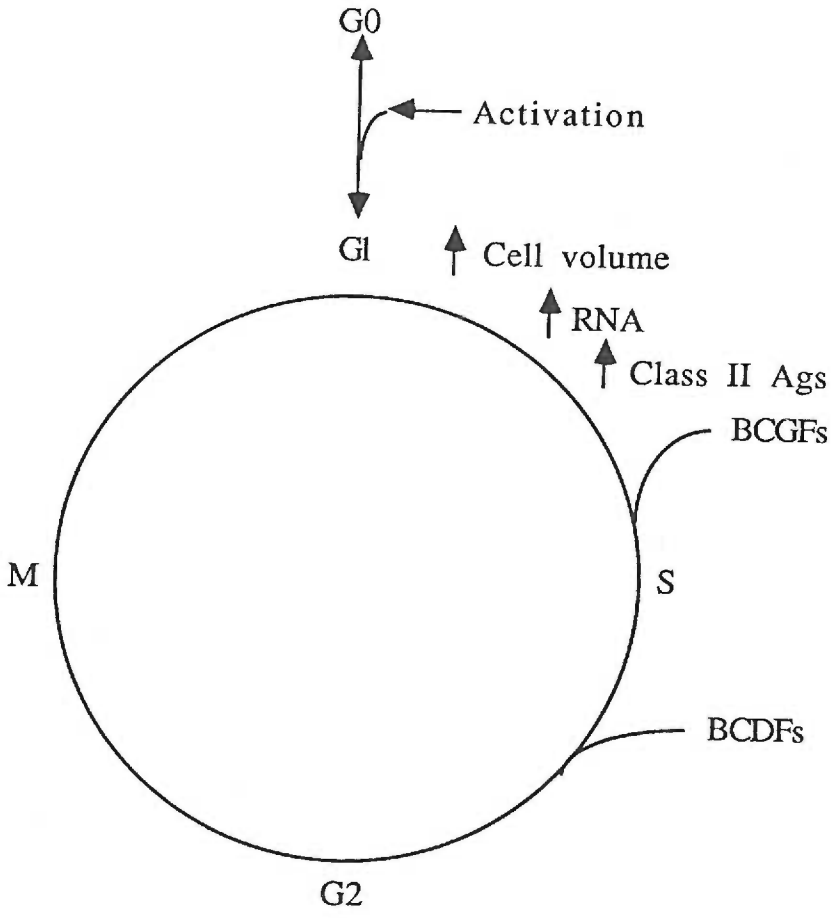
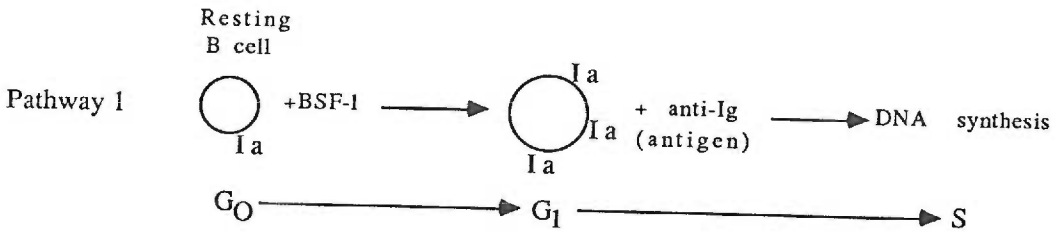

G0
Activation
Gl
Cell volume
RNA
Class II Ags
BCGFs
M
S
BCDFs
G2

Figure 3. Proposed model of B cell activation, proliferation, and differentiation.


Pathway 1
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+BSF-1
Ia
Ia
Ia
+ anti-Ig
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DNA synthesis
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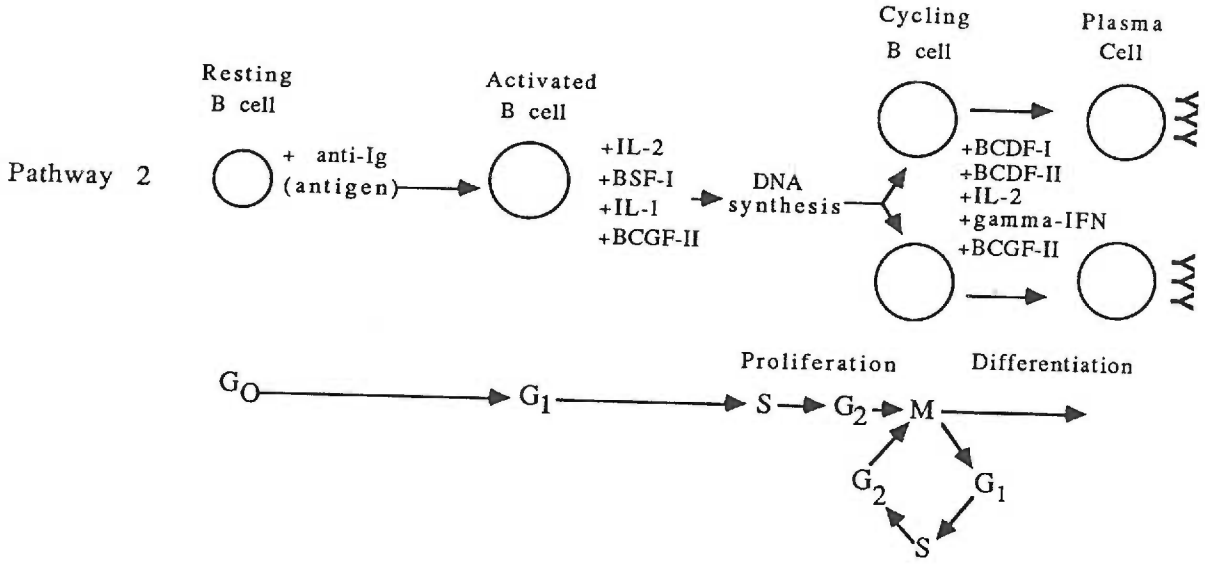
G_1
S


Pathway 2
Resting
B cell
+ anti-Ig
(antigen)
Activated
B cell
+IL-2
+BSF-I
+IL-1
+BCGF-II
DNA
synthesis
Cycling
B cell
Plasma
Cell
+BCDF-I
+BCDF-II
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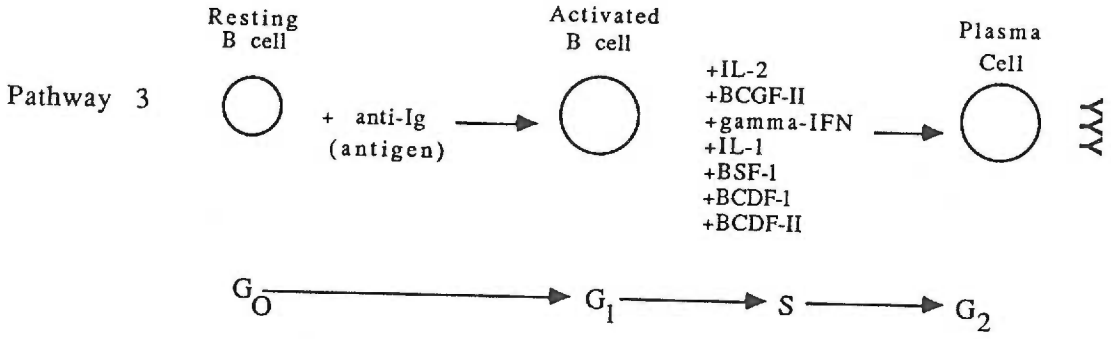
Pathway 3
Resting
B cell
+ anti-Ig
(antigen)
Activated
B cell
+IL-2
+BCGF-II
+gamma-IFN
+IL-1
+BSF-1
+BCDF-I
+BCDF-II
Plasma
Cell
G_O
G_1
S
G_2

PAPER 2.

HUMAN ANTI-TRINITROPHENYL ANTIBODY RESPONSE IN VITRO

I. Modulation of the TNP-Ba Stimulated Plaque-forming Cell Response by B Cell Inducing Factor

ABSTRACT

The in vitro plaque forming cell (PFC) response of human lymphocytes to trinitrophenyl (TNP) conjugated to Brucella abortus (TNP-Ba) is shown to be influenced by B cell inducing factors (BIF). We show that BIF, isolated from PHA stimulated peripheral blood cells, markedly enhances the human PFC reponse to TNP-Ba. TNP-Ba stimulated cells plus BIF produced antibodies to TNP, but antibodies to sheep RBC or phosphocholine could not be detected in the same cultures, indicating that the expansion and differentiation of B cells in the presence of BIF was antigen specific. Maximal responses were obtained when BIF was added on day 0 or day 2 of 7 day cultures. Optimal BIF concentration was between 5.0% and 7.5% v/v. The anti-TNP PFC response to TNP-Ba plus BIF in T cell depleted cultures was increased in a manner similar to cultures containing T cells. Hapten inhibition profiles of the anti-TNP PFC stimulated by TNP-Ba and BIF indicate the presence of a heterogeneous population of anti-TNP-antibody producing cells among donors.

INTRODUCTION

The differentiation of resting B cells into immunoglobulin secreting cells has been shown to consist of multiple processes in the mouse and in man (1,2). The resting B cell is activated by a variety of signals including antigen (3,4), mitogen (4,5), and anti-immunoglobulin (6,7). Following activation, the B cell is responsive to what are collectively termed B cell stimulatory or B cell inducing factors (BIF) (8).

The human B cell response to TNP-Brucella abortus (TNP-Ba) in vitro was previously described (9). This human B cell response to TNP-Ba appeared to be thymus independent but was also shown to be positively or negatively modulated when the T cell lectin Concanavalin A (Con A) was added to cultures; the direction of modulation was dependent on the time at which lectin was added with late addition leading to enhancement while early addition led to suppression (10). The active principle(s) causing enhancement of these cultures was not identified but may be similar to those present in BIF.

In the present study we have examined the ability of BIF to promote the development of human hapten specific plaque-forming cells in vitro in microtiter plate cultures. Optimal conditions are described for such variables as concentration of BIF and the time of its addition. The effect of T cell depletion on the anti-trinitrophenyl (TNP) response to TNP-Ba and BIF is also described. Hapten inhibition profiles of anti-TNP stimulated PFC in the presence of TNP-Ba and BIF indicate that heterogeneity of the in vitro response is exhibited among donors with respect to antibody affinities and to recognition of hapten analogs.

MATERIALS AND METHODS

Reagents. Brucella abortus ring test antigen was obtained from the U.S. Department of Agriculture, Ames, IA and was trinitrophenylated according to Mond et al. (11). B cell inducing factor (BIF) was obtained from Electronucleonics, Inc., Silver Springs, MD. It had been prepared by isolation from PHA stimulated human peripheral blood leucocytes by precipitation with 80% saturated ammonium sulfate followed by DEAE cellulose ion exchange chromatography and by size fractionation on an AcA54 Ultrogel column; these steps were followed by separation on blue agarose and Procion-red agarose columns as described by Ralph et al. (8). Recombinant human IL-2 was obtained from Amgen, Thousand Oaks, CA. The monovalent TNP and DNP haptens used for inhibition were as follows: TNP-epsilon-aminocaproic acid (TNP-EACA) was prepared according to Benacerraf and Levine (12); epsilon-TNP-Lysine (TNP-lysine) and TNP-glycine were obtained from ICN Pharmaceuticals Inc., Life Sciences Group, Cleveland, OH; and epsilon N-2, 4-DNP-lysine (DNP-lysine) and alpha-N-2,4-DNP glycine (DNP-glycine) were obtained from Sigma Chemical Co., St. Louis, MO.

Tonsil Cell Cultures. Human tonsils from juvenile patients with chronic tonsillitis were obtained following surgical removal. The culture system was used as described in detail previously (9) with the following modifications: Cells were grown in 96-well flat bottom microtitier plates (Falcon No. 3072) containing 0.2 ml of cells (5 x 10^6 cells/ml). Cells were stimulated on day 0 with TNP-Ba at a concentration of 2 x 10^7 or 2 x 10^8 TNP-Ba organisms per well. BIF was added at various concentrations as indicated.

For T cell depleted cultures, T cells were removed by rosetting with sheep red blood cells (13). The non-rosetting cells were then treated with OKT3 antibody plus guinea pig complement to remove residual T cells. Efficiency of T cell depletion was determined by cytofluorograf analysis (Ortho, Raritan, NJ) or the Quantigen Assay (Biorad, Richmond, CA); less than 5% T cells remained after depletion.

PFC Assay. Cells cultured in microtiter plates were centrifuged in situ, at 1000 RPM in a Sorvall RC-3, for 7.5 min. at 22° C. Supernatant was removed and the cells washed twice with 100 ul serum-free RPMI-1640. Cells were harvested from individual wells and assayed for direct PFC as described previously (9,10) against TNP-SRBC (14), phosphocholine (PC)-SRBC (15), or unhaptenated SRBC using the Cunningham-Szenberg technique (16).

Hapten Inhibition of PFC. Hapten inhibition of PFC was accomplished by adding different concentrations of soluble TNP or DNP haptens to the plaquing mixtures. The fraction of the PFC population that was present at each concentration of hapten was determined and plotted by the method of Goidl et. al. (17).

Statistical Analysis. Statistical significance of the data was evaluated by an analysis of variance (18).

RESULTS

Effect of BIF on the TNP-Ba stiumlated anti-TNP PFC response of human tonsillar cells.

Table 1 shows one of three separate experiments which yielded comparable results in which BIF was added to TNP-Ba stimulated tonsil cells at varying concentrations. BIF was added on day 0 of culture; the cells were then assayed on days 3, 5, 7, and 9. The human PFC response to TNP-Ba was markedly enhanced. At each concentration of BIF added with antigen, the PFC response was far greater than with either antigen or BIF alone. The effect of BIF was only minimally detectable on day 3, but by day 5, enhancement of the PFC response over the response to antigen alone was 10-fold. By day 7, the number of PFC had increased dramatically to greater than 100-fold and continued to show an increase on day 9 of culture. The optimal dose of BIF ranged from 5.0% v/v to 7.5% v/v. The reduced mean PFC response at 10% BIF observed on days 5 and 7 when compared to the response at 7.5% may reflect continued proliferation of activated cells without commitment to differentiation; by day 9 differentiation had occurred.

The increased number of anti-TNP antibody-producing cells in the presence of both TNP-Ba and BIF were specific for TNP as indicated in Figure 1 by inhibition of PFC with 10^{-4}M TNP-EACA. Failure to develop PFC to either sheep red blood cells or to the hapten phosphocholine following stimulation by TNP-Ba and BIF (Talbe 1, legend) also indicates that the response was specific and demonstrates as well that polyclonal stimulation was not occurring to a significant extent.

Data collected from experiments on 24 separate tonsils are shown in Figure 2. Each dot represents a separate culture well assayed individually and demonstrates the overall variability in tonsil cell cultures. It can be seen that some cultures responded well to antigen alone but the median of all such cultures was only 9. In contrast, addition of BIF to TNP-Ba-exposed tonsil cells increased the median anti-TNP PFC to 932 per culture.

Effect of time of addition of BIF on TNP-Ba induced PFC production.

To determine whether there is a particular time during culture when BIF is needed or whether it could be added at any time during the culture period, BIF was added on day 0, 2, or 4 following initiation of culture; the cells were then assayed on days 5, 7, and 9.

As shown in Figure 3, adding BIF on either day 0 or day 2 increased the response significantly compared to adding BIF on day 4 ($p<0.05$) regardless of the day of assay. Addition of BIF on day 0 or on day 2 did not significantly alter the maximum response ($p>0.05$). Importantly, addition of BIF at later times (day 4) showed diminshed responses, implying that a finite period of responsiveness to BIF-enhanced proliferation and/or differentiation may exist following antigen activation.

Recombinant human IL-2 does not enhance TNP-Ba induced PFC production.

Since the BIF preparation used in this study contained IL-2 in addition to B cell growth and differentiation factors, tonsil cells were cultured with TNP-Ba in the presence of recombinant IL-2 at concentrations ranging from 10 u/ml to 1000 U/ml to determine if enhancement by BIF could be replaced by IL-2. No enhancement of the TNP-Ba response in the presence

of recombinant IL-2 could be demonstrated in two experiements using a wide range of IL-2 concentrations in various combinations with antigen (data not shown). Thus, it is unlikely that the enhancement observed upon addition of BIF is due to the presence of IL-2 alone.

Hapten inhibition profiles of anti-TNP PFC from cultures stimulated with TNP-Ba and BIF.

Hapten inhibition of PFC was performed in four separate experiments using hapten-amino acid analogs. TNP-EACA, TNP-lysine, DNP-lysine, DNP-glycine or TNP-glycine were added in the plaque assay and the results compared to controls in which only diluent was added. The results presented in Figure 4 show differences among the four donors whose cells were cultured individually. PFC from donor #1 were relatively restricted to low affinity PFC with most (75%) of the population inhibited only at 10^{-6}M or higher concentration of hapten, although approximately 20% of the PFC were inhibited by certain haptens at 10^{-7}M. In contrast, donors #2, #3, and #4 produced a response restricted to higher affinity PFC. Approximately 60 to 75% of the latter were inhibited by 10^{-8}M TNP-glycine or DNP-glycine whereas 10^{-8}M DNP-lysine, TNP-lysine and TNP-EACA varied in effectiveness for PFC from these three donors. Differences in inhibition pattern were also observed between the TNP and DNP-lysine analogs, thus, for donor #2, TNP-Lysine > DNP-lysine whereas for donor #4, TNP-lysine < DNP-lysine. Such results indicate clearly that the anti-TNP response of humans is heterogeneous although an individual response may be restricted.

Effect of T Cell depletion on the BIF enchanced anti-TNP response of human tonsil cells.

Results obtained in three experiements in which cells were cultured with anitgen and BIF with or without T cell depletion are shown in Figure 5. Mean PFC/culture was reduced in Exp. #1 from 4287 to 1258 by T cell depletion. Similarly, in experiments #2 and #3, T cell depleted cultures demonstrated markedly lower PFC responses following antigen and BIF simulation when compared to non-T-depleted cultures. Even though T-depleted, responses in cultures containing antigen and BIF (about 700-1400 PFC) were still significantly greater than in T depleted cultures to which antigen alone was added (0 to 36 PFC/culture, not shown).

DISCUSSION

The results indicate that human tonsil cells can respond to specific antigen (TNP-Ba) and human B cell inducing factors (BIF) in vitro through the production of hapten specific plaque-forming cells. The magnitude of the response is large and markedly greater than that induced by either antigen or BIF alone. Ralph and his coworkers have shown that human cells respond to BIF by proliferation and differentiation to immunoglobulin secreting cells following activation by the polyclonal activator Staphylococcus aureus Cowan strain I (8,19). This would suggest that in this study BIF has acted on cells already activated by antigen. Thus, we found that BIF alone had little, if any, effect on non-stimulated resting cells in the population evidenced by the lack of detectable PFC to SRBC or phosphocholine-coated SRBC. We did detect a low level of anti-TNP PFC which developed in cultures to which BIF alone was added; these may represent cells recently activated in vivo prior to in vitro culture since it is well known that tonsils may contain activated lymphocytes (20). However, it is also possible that BIF caused a low level of polyclonal activation that was only detectable against TNP (Figure 2) possibly because the precursor frequency may be higher for this antigen that for either PC or SRBC.

Golding et al. previously found that human tonsil cell responses to TNP-Ba were enhanced when Con A was added on day 2 of culture (10). The magnitude of the responses obtained in the present study using BIF plus TNP-Ba was much greater than those reported previously, suggesting that if similar factors were produced in the Con A stimulated cultures, they were not present at optimal concentration or that suppressive factors produced in

Con A stimulated cultures were not present in the BIF preparation thus allowing maximum proliferation and differentiation of responsive cells. The expansion of TNP-Ba responsive clones in the presence of BIF is appreciably greater than the response to antigen alone as was the case in cultures to which Con A was added (21).

A variety of B cell differentiation factors have been described (2,22-24) which are presumed to be required for differentiation of B cells into immunoglobulin secreting cells (22,23). It has been shown that BCGF-induced proliferation occurs following activation of the resting B cell by antigen or mitogen (25,26). Similarly, B cell differentiation in the presence of BCDF occurs following activation by antigen or mitogen (22,23,26). Others have reported the proliferation (27) or differention into immunoglobulin secreting cells (28) of resting B cells in the presence of BSF-1 or BCDF, respectively, without prior B-cell activation. The present results indicate that TNP-Ba stimulation was required to obtain optimal numbers of anti-TNP immunoglobulin secreting cells in the presence of BIF.

The BIF preparation used in this study was isolated from supernatants of PHA stimulanted human peripheral bood leukocytes and contained IL-2 in addition to B cell growth and differentiation factior(s), but was free of gamma-interferon, IL-1 and PHA (Ralph et al. (8); Electronucleonics, personal communication). While some investigators have reported that IL-2 does not have an effect on B cell differentiation (8,29), others have described B cell populations having receptors for IL-2 (30-32) and that IL-2 may indeed play a role in B cell proliferation (33,34) and in their development into plasma cells (35,35,37). In the experiments reported by Ralph and his

coworkers (8) it was shown that BIF could stimulate immunoglobulin secretion in the absence of IL-2. Ralph et al. later demonstrated that IL-2 is required in much higher concentrations for supporting B cell growth than is required for T cell growth (38).

Our results using recombinant IL-2 indicated that it does not substitute for BIF in cultures with TNP-Ba and tonsil cells when added at concentrations that support T cell proliferation.

The hapten inhibition profiles (Figure 4) of individual responses indicate that the anti-TNP antibody secreting cells which respond to TNP-Ba plus BIF represent a heterogeneous population. Not surprisingly, PFC from different individuals differed in their relative affinities with all but one of the four individuals shown in Fig. 4 possessing a significant proportion of higher affinity PFC (inhibitable by 10^{-7} and 10^{-8} M hapten). Additionally, differences in the degree of inhibition of PFC between hapten-amino acid complexes (TNP-lysine vs DNP-lysine) among individuals (#2,#3, and #4) were demonstrated. The heterogeneity exhibited among donors with respect to affinity is seen in the murine anti-TNP-Ba response only following multiple immunizations (39). We have previously detected both high and low affinity human anti-TNP PFC in cultures stimulated with TNP-Ba in the absence of BIF (21). The high affinity population may reflect a prior exposure to antigens that cross-react with TNP.

Depletion of T cells revealed a partial T-cell dependence in the response to TNP-Ba even in the presence of 7.5% BIF (Fig.5); BIF may lack a key element provided by T-cell help or perhaps such an element is not present in adequate concentration in BIF preparations. Alternatively, IL-2

in BIF could stimulate T cells to secrete additonal B cell stimulatory lymphokines which reach higher concentrations in non T-cell depleted cultures. Previous studies (9) indicating T independence of the human TNP-Ba response used only E rosetting to deplete T cells. Others have shown (40) that immature T cells which have not yet acquired the E receptor, may rapidly mature, proliferate, and become a significant contaminating population of T cells in E rosette minus populations. This factor may be more appreciable in cultures containing BIF (and IL-2). T cell removal may have been more rigorous in the present study since both E rosetting and OKT3 plus complement treatment were performed thus revealing partial T-cell dependence.

These results demonstrate clearly that BIF markedly enhances the human anti-TNP antibody response to TNP-Ba in vitro. In addition to providing a basis for further study of lymphokines in specific antigen-induced PFC responses by human lymphocytes, the results suggest that the use of BIF in in vitro stimulation may improve the ability to achieve maximal stimulation of antigen specific B cells which could prove particularly useful in producing human hybridomas.

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Table 1

Effect Of BIF Concentration On Induction Of Anti-TNP PFC in Human Lymphocyte Cultures[a]

ADDITIONS TO CULTURES	DIRECT PFC/CULTURE[b] Day 3	Day 5	Day 7	Day 9
Tonsil Cells[c]	1[d]	0	1	1
Tonsil Cells + TNP-Ba[e]	4 ± 3	21 ± 3	8 ± 1	2 ± 3
Tonsil Cells + BIF(10%)[f]	18 ± 2	23 ± 5	30 ± 6	10 ± 3
Tonsil Cells + TNP-Ba + BIF(2.5%)	22 ± 3	208 ± 31	1126 ± 199[g1]	2337 ± 547
Tonsil Cells + TNP-Ba + BIF(5.0%)	63 ± 10	347 ± 36	3061 ± 262[g2]	3818 ± 332
Tonsil Cells + TNP-Ba + BIF(7.5%)	42 ± 7	324 ± 24	2858 ± 199[g3]	5456 ± 254
Tonsil Cells + TNP-Ba + BIF(10.0%)	25 ± 2	292 ± 30	740 ± 94[g4]	3981 ± 198

a data from one of three experiments which gave similar results

b n=12 cultures

c 10^6 cells/culture assayed for direct PFC on the days indicated

d mean ± S.E.M.

e 2×10^7 TNP-Brucella abortus added day 0

f B cell inducing factor, 10% v/v, added day 0

g1-4 When PC-SRBC were used as targets in the plaque assay the results were 0, 0, 2, and 0 respectively. When SRBC were used as targets in the plaque assay, the results were 0, 9, 4, and 2 respectively.

FIG. 1. Hapten specificity of the in vitro-generated PFC response to TNP-Ba and BIF. B cell inducing factor was added at 7.5% v/v on day 2 to cultures containing 10^6 cells and 2 x 10^7 TNP-Brucella abortus. Cultures (n=6) were assayed for direct PFC on day 5. 10^{-4} M TNP-epsilon amino caproic acid (TNP-EACA) was added to plaquing mixtures. Horizontal bars represent mean ± S.E.M.

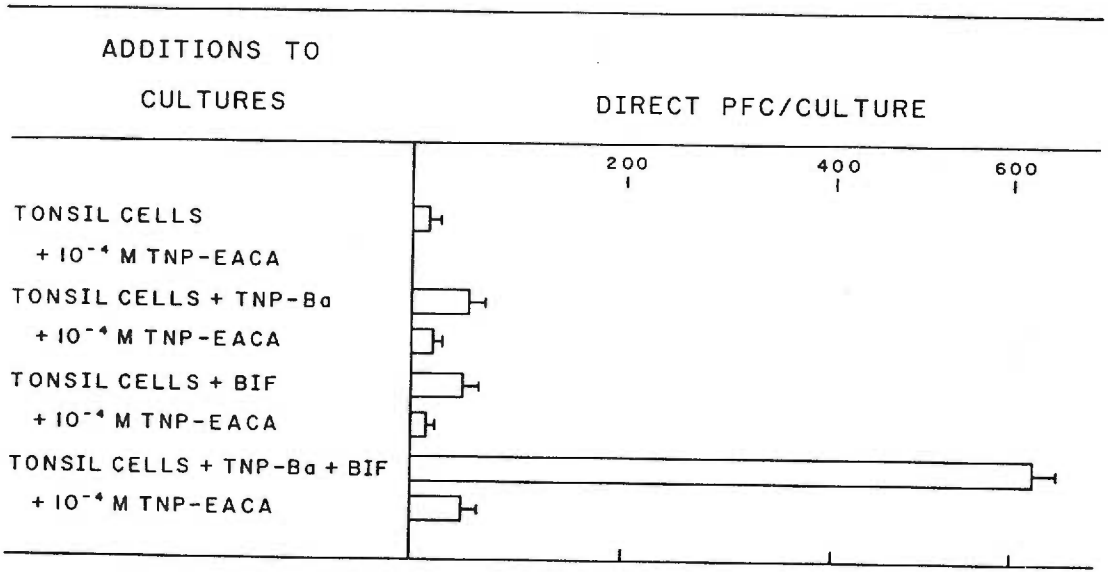

ADDITIONS TO CULTURES
DIRECT PFC/CULTURE
200
400
600
TONSIL CELLS
+ 10⁻⁴ M TNP-EACA
TONSIL CELLS + TNP-Ba
+ 10⁻⁴ M TNP-EACA
TONSIL CELLS + BIF
+ 10⁻⁴ M TNP-EACA
TONSIL CELLS + TNP-Ba + BIF
+ 10⁻⁴ M TNP-EACA

FIG. 2. Dot plot of TNP-Ba plus BIF stimulated tonsil cultures. Each dot represents PFC from a single culture well from data shown in figures and tables in this paper and from data not shown. PFC in cultures containing TNP-Ba + BIF represent PFC in a single culture less the mean of TNP-Ba stimulated cultures without BIF in their respective experiments.

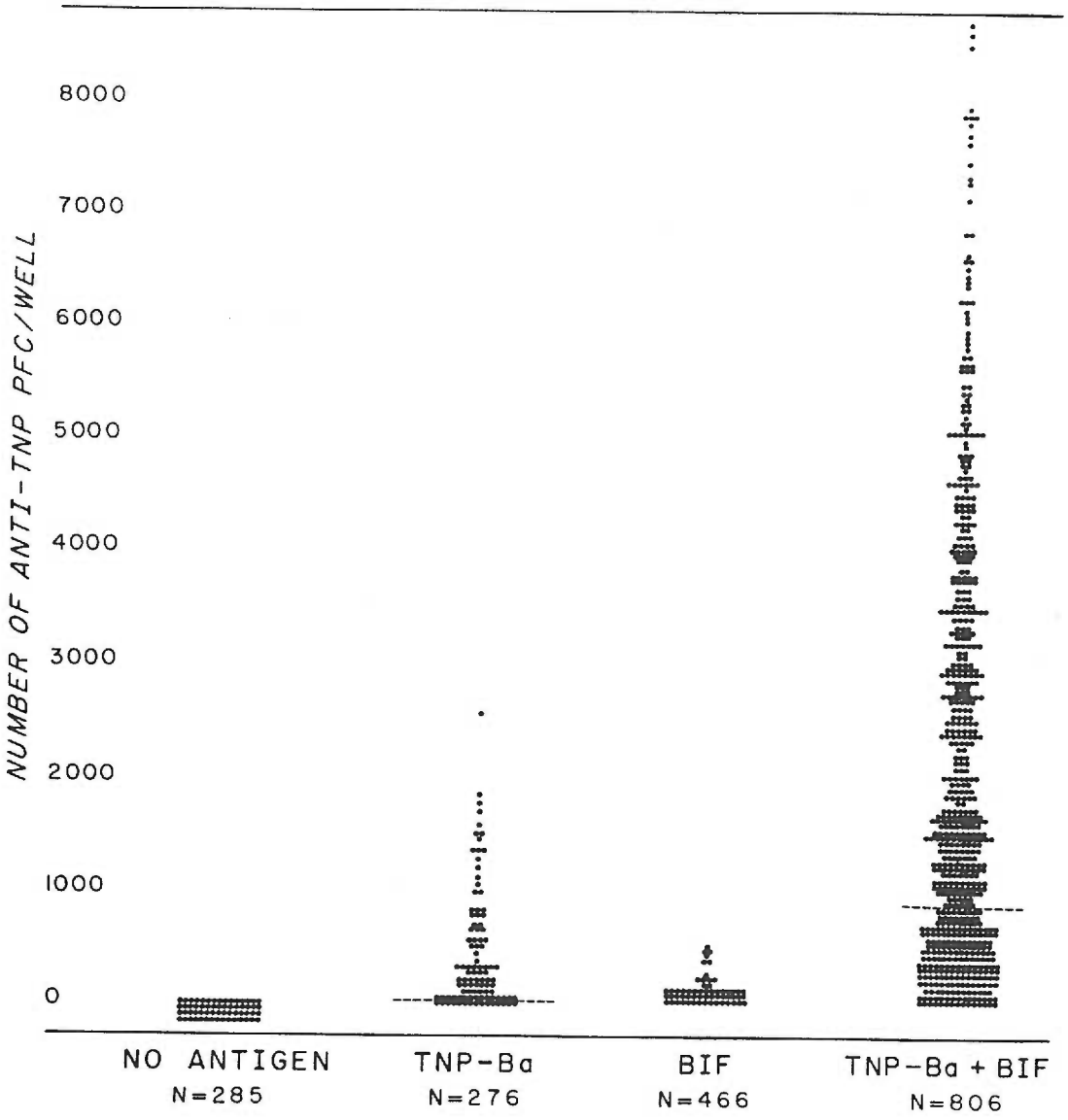

NUMBER OF ANTI-TNP PFC/WELL
8000
7000
6000
5000
4000
3000
2000
1000
0
NO ANTIGEN
N=285
TNP-Ba
N=276
BIF
N=466
TNP-Ba + BIF
N=806

FIG. 3. Effect of timing of the addition of BIF on TNP-Ba induced PFC production by tonsil cells. B cell inducing factor was added at 7.5% v/v to cultures (n=12) containing 10^6 cells and 2×10^7 TNP-Brucella abortus. Data are from one of two experiments which gave similar results. Horizontal bars represent mean $\pm$ S.E.M.

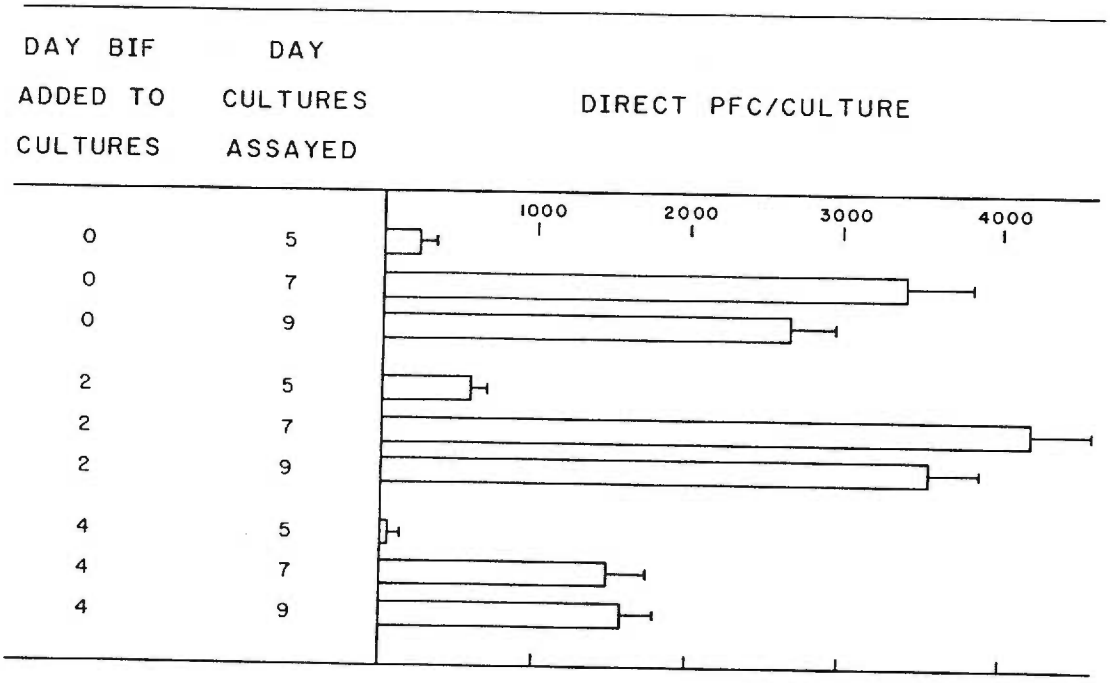

DAY BIF ADDED TO CULTURES
DAY CULTURES ASSAYED
DIRECT PFC/CULTURE
1000
2000
3000
4000
0 5
0 7
0 9
2 5
2 7
2 9
4 5
4 7
4 9

FIG. 4. Hapten inhibition profiles and fine specificity of TNP-Ba plus BIF stimulated tonsil cells. Each histogram illustrates the distribution of direct anti-TNP PFC in the presence of the haptens as shown following in vitro culturing of 10^6 human tonsillar cells with 2 x 10^8 TNP-Brucella abortus and 7.5% v/v BIF. PFC were assayed on day 7 of culture (n=6). The ordinate represents the percent of the total population of PFC present in each subpopulation. The abscissa represents the $\log_{10}$ of the inverse of the free hapten concentration used in the plaque inhibition assay. The mean $\pm$ S.E.M. PFC per uninhibited culture is given in the upper right hand corner.

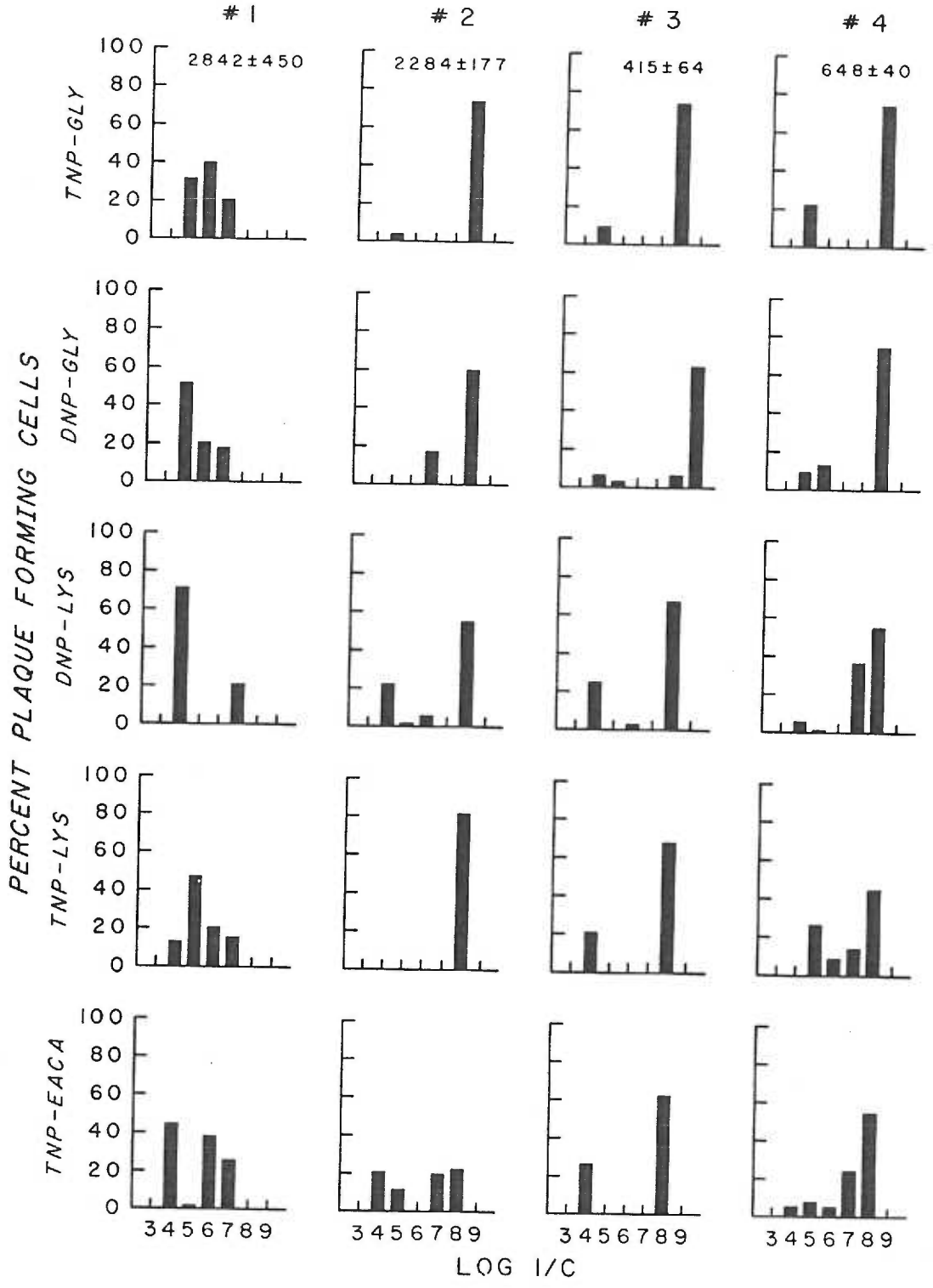
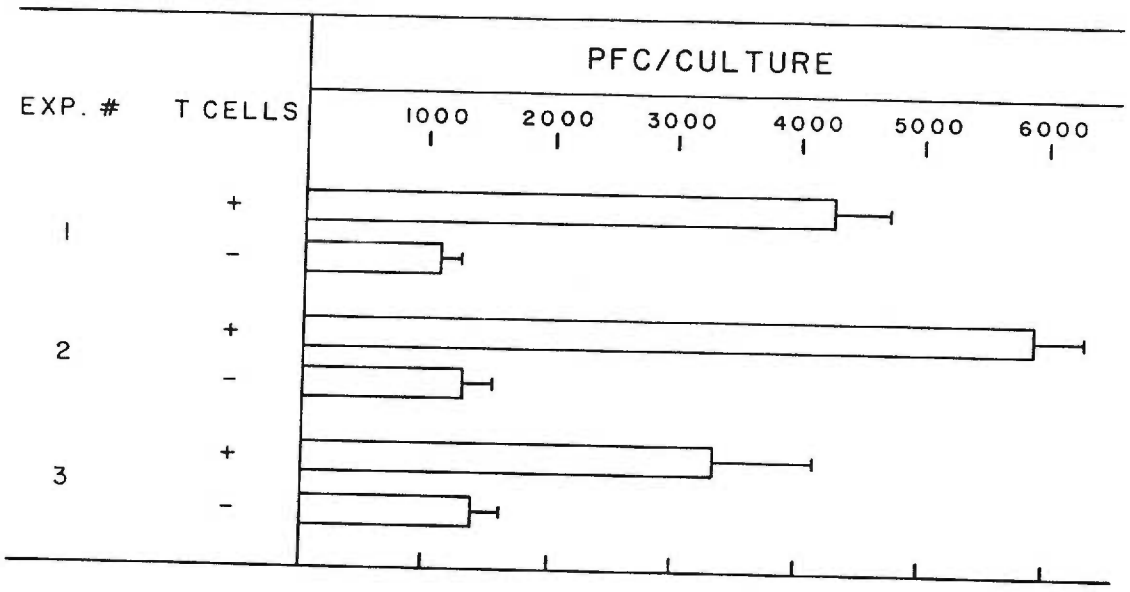

#1
#2
#3
#4
2842±450
2284±177
415±64
648±40
TNP-GLY
DNP-GLY
DNP-LYS
TNP-LYS
TNP-EACA
PERCENT PLAQUE FORMING CELLS
100
80
60
40
20
0
3456789
LOG 1/C

FIG. 5. Effect of T-cell depletion on TNP-Ba plus BIF induced PFC production by tonsil cells. B-cell inducing factor was added at 7.5% v/v on day 2 to cultures containing 10^6 cells and 2 x 10^7 Brucella abortus. Cultures (n=12) were assayed for direct PFC on day 7. Horizontal bars represent mean $\pm$ S.E.M.


PFC/CULTURE
EXP. #
T CELLS
1000
2000
3000
4000
5000
6000
1
2
3
+
-
+
-
+
-

SUMMARY AND DISCUSSION

The humoral response to antigen results in the expansion of antigen-specific B cells and their differentiation into immunoglobulin secreting plasma cells (1). B cells can be activated to undergo expansion without differentiating into plasma cells (2) or they can differentiate into plasma cells without dividing (3). The in vitro culturing of murine and human lymphoid cells has allowed investigators to analyze the various aspects of control of B cell activation, proliferation and differentiation into mature immunoglobulin-secreting cells.

The human immune response to TNP-Ba in vitro is a thymus independent response (4). This response has been shown to be positively or negatively modulated by the plant lectin Concanavalin A (Con A) (5). The effect of Con A was a function of the time of Con A addition to TNP-Ba-stimulated cultures. These studies were an indication that the state of activation of anti-TNP specific B cells determined their ability to respond to monokines and/or lymphokines by proliferating and/or differentiating into immunoglobulin secreting cells. Work by Golding et al. (5) demonstrating modulation of the anti-TNP-Ba response by Con A (a T cell stimulatory lectin) indicated that T cells elaborate factors which can induce or suppress proliferation and/or differentiation of B cells. Many investigators have described factors which influence B cell functions including IL-1 (6,7,8,9), IL-2 (10,11,12,13,14,15), BSF-1/IL-4 (16,17,18,19, 20,21,22,23), BCGF-II (24,25,26,27,28), BCDF-I and BCDF-II (29). A major focus of

research on activation, growth and differentiation agents has been the determination of the time of action of these agents during the B cell cycle, the effect these agents have upon the growth and differentiation of the B cell and the intracellular mechanisms of signal transduction and control of B cell events. Ralph et al. described in vitro studies using B cell inducing factors (BIF) with human B cell lines (30) and with SAC-activated human peripheral blood lymphocytes (31). In these studies it was demonstrated that BIF induced immunoglobulin secretion in a human B cell line and in SAC-activated B cells. These studies prompted our investigation of the action of BIF in the antigen(TNP-Ba)-activated human in vitro culture system.

The objective of this work was to expand our understanding of the human in vitro response to TNP-Ba by analyzing this response in the presence of BIF. The principal findings of these studies were:

(1) BIF induced a remarkable increase in the number of anti-TNP specific plaque forming cells when added to microcultured human cells in the presence of the T-independent antigen TNP-Ba. This increase was far greater than that seen in TNP-Ba plus Con A stimulated cultures (5) where the maximum response of antigen-specific B cells to TNP-Ba in the presence of Con A and T cells was in the range of 600 PFC per million cultured cells. In contrast to TNP-Ba plus Con A stimulated cultures, the median anti-TNP PFC response to TNP-Ba in the presence of BIF, in 24 separate experiments was 932, with maximum responses reaching 8000 PFC per

million cultured cells. This induction of TNP-specific B cell proliferation and differentiation was dependent upon antigen activation and thus was an indication that G_0 resting antigen-specific B cells were driven by antigen to a stage in the B cell cycle during which they were responsive to BIF.

(2) The increase in anti-TNP-specific PFC following TNP-Ba stimulation in the presence of BIF is antigen-specific and not the result of polyclonal activation as indicated by the absence of detection of anti-phosphocholine PFC or anti-SRBC PFC in TNP-Ba plus BIF stimulated cultures.

(3) The time of BIF addition as well as BIF concentration in micro-cultures were critical in obtaining a maximum response to TNP-Ba. Optimum anti-TNP plaque-forming cell induction occurred when BIF was added before the fourth day of culture at concentrations of 5.0 % - 7.5 %. A decrease in the anti-TNP PFC response when BIF was aded on day 4 following antigen stimulation indicated that TNP-Ba induces in antigen-specific B cells a state of responsiveness to BIF that appears to diminish with time. This may be due to G_1 cells reverting to the resting G_0 state during which time they are no longer responsive to BIF. If antigen-activated B cells were suspended in G_1, waiting to proliferate and differentiate in the presence of BIF, one would expect to detect by day 9 of cultures to which BIF was added on day 4 a PFC response comparable to that seen by day 7 in cultures to which BIF was added on day 0 or day 2, but this was not observed. Alternatively, TNP-Ba activated B cells may

have progressed to a point in the B cell cycle where they respond preferentially to differentiation signals rather than proliferation and differentiation signals. Another explanation for this observation may be that there are two different subsets of TNP-Ba responsive B cells, one subset that can undergo proliferation and differentiation and another subset that can only undergo differentiation. Thus, in the absence of B cell growth factors, following activation and entry into G_1, the latter subset of B cells (perhaps having a short-lived time frame of B cell growth factor responsiveness) may revert to G_0. The B cell differentiation factor responsive subset of activated B cells (perhaps having a long-lived responsiveness to B cell differentiation factor) differentiate into anti-TNP antibody secretion.

(4) The response in humans to TNP-Ba plus BIF is heterogeneous displaying differences in affinity and in specificity of anti-TNP antibodies. These differences are seen in the anti-TNP response of an individual and in the anti-TNP responses between different individuals. We have previously detected both high and low affinity human anti-TNP PFC in cultures stimulated with TNP-Ba plus Con A (32). In the hapten inhibition studies of cultures stimulated with TNP-Ba plus BIF, the majority of individuals (4/5) had a greater proportion of higher affinity PFC. This may be a reflection of previous exposure to antigens cross-reacting with TNP. This observation is analogous to that seen in murine studies by Andersson (33) in which animals immunized with TNP-conjugates displayed heterogeneity

in the affinities of the resultant anti-TNP antibodies. An alternative explanation for the presence of greater numbers of higher affinity antibodies would be the occurence of a more rigorous expansion of anti-TNP specific B cells having higher affinity interactions with TNP-Ba than B cells having lower affinity interactions with this activating antigen.

(5) While BIF markedly stimulated proliferation and differentiation of TNP-Ba activated B cells in the absence of T cells, the response was greater when T cells were present. This is likely due to the elaboration of additional lymphokines by T cells preactivated in vivo. It is well known that tonsillar cells may contain activated lymphocytes (34). These activated T lymphocytes would secrete B cell factors to which the TNP-BA activated B cells would respond.

(6) The model proposed in the introduction to these human studies (Fig. 3, page 115) reflects findings of the action of several factors acting on resting and activated B cells. Pathways 2 and 3, pertaining to antigen activated B cells, propose that cells may either proliferate and differentiate into immunoglobulin secreting cells (pathway 2) or differentiate without proliferation (pathway 3). In these studies the magnitude of the PFC response in the presence of TNP-Ba plus BIF indicated that anti-TNP human B cells can follow pathway 2. Experiments demonstrating different effects of altering the time of BIF addition following activation suggest that TNP-Ba-activated cells may also follow pathway 3 and differentiate in the absence of proliferation. Both pathways are consistent with BIF acting on B

cells no longer in the G_0 phase of the cycle. The period of time following TNP-BA activation and the addition of BIF to cultures may dictate which pathway is taken. The possibility that different subsets of activated B cells may respond to BIF, one proliferating and differentiating into plasma cells and the other differentiating with little or no proliferation, cannot be excluded.

The mechanisms involved in the triggering, expression of function and immunoregulation of human B cells are complex. It is apparent from these studies and studies by other investigators that several molecules with their corresponding receptors may regulate the same or different subsets of B cells in a variety of different ways. These different manifestations may be governed by differences in activation mechanisms, differences in activation states over time, differences in susceptibilities to individual B cell factors or perhaps to various factors in combination with one another. The answers to these and other questions of B cell functioning will be determined when long term B cell culture techniques for antigen-specific B cells have been perfected and when antigen-specific clonal B cell populations have been established.

The TNP-Ba plus BIF stimulated in vitro culture system has provided a means of activating and expanding TNP-specific B cells differing in affinity and specificity for this stimulating antigen. This culturing system may be useful in the production of hybridomas or in transforming B cells with anti-TNP specificity, or in delineating the mechanisms in human pathological

conditions in which B cells are defective in activation, proliferation and/or differentiation.

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Appendix 1

Table 1. HAPTEN INHIBITABILITY OF SOMAN-SPECIFIC ANTIBODIES.

Group:	Inhibitors:					#Antibodies (Isotypes):
	NH_2-0-So	NO_2-0-So	DPMP	0H-So	NPPC	
A1	+*	+	+	+	+	4(G2a,k)
A2	+	+	+	+	-	4(Gl,k)
A3	+	+	+	-	-	1(M,k) 2(Gl,k) 2(G2a,k) 4(G3,K)
B1	+	+	-	+	+	1(M,k) 2(Gl,k)
B2	+	+	-	+	-	2(Gl,k) 1(G2a,k)
B3	+	+	-	-	-	2(Gl,k) 1(G2b,k)
C1	+	-	-	+	+	1(M,k) 1(Gl,k)
C2	+	-	-	+	-	1(Gl,k)
C3	+	-	-	-	-	4(Gl,k) 2(G2b,k) 1(G3,k
D	-	-	-	-	-	10(M,k)

* + = measurable inhibition in ELISA

- = no inhibition at highest hapten concentration used in ELISA

Appendix 2

Paper 3.

SINGLE CELL CLONING OF EBV TRANSFORMED CELLS
IN 20 ul HANGING DROPS

A simple method for cloning EBV transformants from single cells in 20 ul hanging drops is described. The hanging-drop method allows direct verification prior to the addition of irradiated feeder cells that clones will be established from single cells. The average efficiency of cloning from wells containing a single transformed cell was 74%, ranging form 60-95% in eight separate experiments.

Keywords: EBV transformed cells - single cell cloning - hanging drop

INTRODUCTION

It is generally observed that EBV transformed cells do not grow well when plated as single cells, even when accompanied by a variety of filler cells (Hammerling et al., 1984). Winger et al., (1983) reported successful cloning under limiting dilution conditions where 0.5 cells per well were plated in the presence of feeder cells. Under these conditions, outgrowth of clones from single cells is determined statistically by Poisson analysis. The limitation of this procedure is that one cannot be assured that outgrowth of clones arises from a single cell and it is necessary to perform multiple clonings in order to achieve a high degree of statistical probability that single cell cloning has been achieved.

We have developed a simple and rapid method for cloning EBV transformed human B cells using an adaptation of the hanging-drop method previously used for studying antibody production and for cloning hybridomas (Bell et al., 1983, and Rittenberg et al., in press). The hanging-drop method for cloning EBV transformants allows microscopic verification

that limit dilution has been achieved after which feeder cells are added to allow clonal development.

MATERIALS AND METHODS

EBV Transformed Cells

Peripheral bood or tonsillar B lymphocytes (10^6) were incubated for 2 hours with 1 ml of mycoplasma-free supernatant from the EBV-producing cell line B95-8. Infected cells were then cultured at 37° C in 5% CO_2 in 96-well plates (Corning, #25850, Corning, NY) at a density of either $2x10^5$ per well, for peripheral bood lymphocytes, or $1x10^5$ per well, for tonsillar B lymphocytes. Cells were plated in 0.2 ml RMPI-1640 medium containing 20% heat-inactivated fetal calf serum (Hyclone, Logan, Utah) with $1x10^4$ irradiated unseparated tonsillar lymphocytes as feeders. At 14 days of culture, wells containing antibody of interest as determined by ELISA were cloned by hanging drop.

Feeder Cells

Cells used as feeders for hanging drop cloning were obtained from human cadaver spleen or peripheral blood. Isolated cell populations were irradiated with a Cs source at a rate of 203 rads/min. for 10 minutes.

Cloning of Transformants

One day prior to cloning, transfromed cells from ELISA-positive cultures were fed with medium containing 30% fetal calf serum. The following day the cells were harvested and viability determined by trypan blue dye exclusion. Cells were diluted in medium containing 30% serum and dispensed into Terasaki plates (Nunc, Gibco Labs, Grand Island, NY) at 1 and

10 cells per 20 ul drop using a Hamilton repeating dispenser (Robbins Scientific, Mt. View, CA) fitted with a 1 ml disposable 21 gauge needle. Following cell dispensing, the lids were replaced and the plates inverted and incubated in a 5% CO_2, humidified, 37°C incubator for two hours to allow cells to settle onto the meniscus. The Terasaki plates were then examined microscopically, while inverted, at 100X magnification and the number of cells present in each drop was recorded. The culture plates were turned over and $1x10^4$ irradiated feeder cells were added to each well. Feeder cells were added in 1 ul droplets using a Hamilton repeating dispenser as above. The lids were replaced and the plates inverted and returned to the incubator. One week later the hanging drops were re-examined microscopically for growth of transformed clones. Those which had arisen from a drop originally containing no more than a single lymphocyte were selected for further propagation. Clones to be propagated further were recovered by inverting the plate and removing the entire contents of a well using a sterile plastic-tipped pipette (Pipetman P20, Rainin Instruments, Woburn, MA). The cells were transferred to a Terasaki monoclonal cluster plate (Costar No. 3560, Costar, Cambridge, MA) containing 50 ul of culture medium (20% fetal calf serum) for expansion after which the cells were diluted appropriately and recloned by hanging drop as described.

RESULTS

Hanging drop cultures provide a simple method for cloning EBV transformed cell lines from single cells. Drop cultures have the advantage over limiting dilution cloning by conventional methods (Winger et al., 1983) in that drops can be examined microscopically immediately after distribution

to determine the number of cells initially contained in each well. Thus, only those wells which contained a single cell at the start of culture need be followed.

The results of eight separate clonings, using as feeder cells a mixed population of lymphocytes obtained from either human spleen or peripheral blood, are summarized in Table I. From a total of 408 drops plated, 139 or 34% contained a single cell. Between 60 and 95% of drops containing a single cell gave rise to clones when cultured with $1x10^4$ irradiated lymphocytes. In contrast, 0/9 and 1/17 (Footnote C experiments #2 and #3) cells cultured as single cells in hanging drops without feeder cells resulted in clonal growth. The level of error of clones growing in drops originally scored as cell-free with cells delivered in 20 ul drops was 3.2% (data not shown) which is consistent with but higher than observed previously with hybridoma cells (Rittenberg et al., in press).

DISCUSSION

This simple method for cloning EBV transformed cells in hanging drop cultures is a modification of a system described previously for the cloning of B cell hybridomas (Bell et al., 1983 and Rittenberg et al., in press). The results indicate that EBV transformed cells also can be successfully cloned from single cells in the presence of irradiated feeder cells.

Numerous methods have been published regarding cloning of EBV transformed cells. A few of these include cloning by cluster picking (Hammerling et al., 1984), cloning on soft agar (Sasaki, et al., 1984) and cloning by limiting dilution (Winger et al., 1983, Paulie et al., 1984 and Doyle et al., 1985). All are accepted methods but have limitations of time or

reliance on statistical probability to ensure that a monoclonal culture has been achieved unless some form of clonal analysis on the product has been performed. The hanging drop method which provides for direct microscopic observation obviates the need for statistical arguments. Although it is possible to overlook a second cell at the edge of the meniscus the probability of this is less than 4% as judged by the number of wells originally scored negative but which eventually developed growth. Thus, monoclonality is likely to be achieved by a single round of hanging drop cloning although in practice a second round is employed to assure that such is the case.

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Table 1

Cloning Efficiency of EBV Transformed Cells in Hanging Drops

Experiment #[a]	Growth/single cell[b]	Cloning Efficiency (%)
1	17/23	74
2[c]	9/11	82
3[c]	20/24	83
4	15/24	63
5	12/17	71
6	20/21	95
7	9/14	64
8	3/5	60
TOTAL	105/139	MEAN ± S.E.M. 74 ±4

a EBV transformed cells obtained following infection (see Materials and Methods).

b The number of wells that formed clones/number of wells that were intitially scored as containing a single cell.

c Corresponding hanging-drop cultures without feeder cells resulted in 0/9 (#2) and 1/17 (#3) wells forming clones.