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CHARACTERIZATION OF A UNIQUE SUBSET OF ANTIBODY, J606-GAC, WHICH COMPRISES A SIGNIFICANT PORTION OF THE MOUSE ANTIBODY REPERTOIRE

by

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Title CHARACTERIZATION OF A UNIQUE SUBSET OF ANTIBODY, J606-GAC, WHICH COMPRISES A SIGNIFICANT PORTION OF THE MOUSE ANTIBODY REPERTOIRE

To cope with the potentially unlimited number of antigens, vertebrates have developed an extremely heterogeneous antibody response, appropriately regulated to avoid autotoxis. These controls are secondary to the fact that antigen directed clonal selection is superimposed on the available repertoire. The repertoire itself is derived from the expression of germline genes which may become altered by somatic mutation and recombination. Prior to analyzing the relative contribution regulatory controls make in the antibody response, it is important to define the starting point (i.e., expressed repertoire). Antibody repertoires have primarily been defined by 1) fine specificity antigen binding analysis, 2) serologically defined idiotypes and 3) structural studies of monoclonal antibodies. However, using any of these methods alone is likely to yield a skewed perspective. A slightly different approach involves the use of antisera which recognize cross-reactive variable region markers on either heavy (V_H) or light (V_L) chains. This approach has the advantage of analyzing at one time, antibodies with different individual idiotypes and binding specificities. Antisera recognizing V_H or V_L subsets have previously been described and have been used to characterize the antibody repertoire in mice and

humans. Larger panels of such antisera, however, are essential for a more complete description of antibody repertoires.

With this objective in mind, an anti-V_H serum, 0-1, was prepared. It identifies V_H determinants on a distinct subset, J606-GAC, of murine heavy chain group III antibodies. This subset appears to represent an important part of the antibody repertoire since it constitutes 5-15% of splenic B cells. Partial amino acid sequences of random J606-GAC antibodies indicate that their heavy chains may be encoded for by one or a small number of variable gene segments. This together with the fact that the J606-GAC marker fails to show isotype restriction, indicates that there are probably no V_H-C_H joining constraints placed on the emerging antibody repertoire. We have also observed a high frequency of J606-GAC B cells in neonatal mice, germfree mice, and among the B cells of three additional species. These findings suggest that J606-GAC antibodies may be particularly important, possibly for protection against pathogens.

Abstract Approved by: Committee Chairman David E. Briles

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I. INTRODUCTION

Scope of Thesis

The mechanism by which an individual is able to make so many different antibody molecules, even to antigens which may never be encountered in nature, has been one of the most intriguing aspects of Immunology. The general aim of the studies described in this thesis is to add some additional insight into the continuing controversies surrounding the topic of antibody diversity. The past 30 years of experimentation have provided a detailed description of the mechanisms involved in the generation of antibody diversity. Even though the main debate as to whether diversity is generated mainly by multiple germline genes as opposed to a few genes which are diversified by somatic mutations is being brought to a close, there are still a number of questions which remain unanswered. Recent molecular studies of the immunoglobulin genes have not been able to answer such questions as what the driving force of diversity may be, and when during B cell differentiation diversity is generated, and how and/or if diversity is regulated. This latter question has attracted a great deal of interest; however, a complete understanding of this point has still not been reached.

One particular aspect of regulation of diversity which has not been completely discerned is how the response to some antigens can become highly restricted with respect to clonotype or isotype even though the potential response to that antigen is known to be extremely heterogeneous. Another aspect of this question which has generated a great

deal of discussion is whether or not there exists preferential recombination of particular V_H gene segments to particular C_H gene segments, and whether or not this type of restriction might account in part for the preferential expression of different specificities by different B cell subsets. And, finally on a larger scale it would be interesting to determine what specificities particular germline genes have evolved to recognize and/or if these genes are maintained through phylogeny.

The majority of data in this thesis deal on a basic level with the topic of antibody diversity and specifically with the latter two questions. The specific aims were to 1) determine the extent to which a particular V_H gene segment could encode antibodies of multiple different binding abilities; 2) determine whether particular V_H gene segments are restricted with respect to isotype or B cell subset; and 3) determine the extent a V_H gene segment is maintained through phylogeny. To accomplish these goals the preparation of an anti-V_H serum specific for a mouse V_H framework determinant has been attempted. This anti-V_H serum was then used to analyze mouse antibodies and B cells in detail, through the use of immunofluorescence and radioimmunoassays. B cells from rats, cats, chickens and humans were also analyzed.

In the following chapters, background information on the topics discussed in this introduction, data and a discussion covering the pertinence of the findings to the present understanding of antibody diversity will be presented. Therefore, chapter I will consist of a historical review of the topic of antibody diversity. In chapter II, the preparation and characterization of antiserum 0-1 which has been the basis

for these studies is presented. This information is presented as the manuscript form of a paper recently published in the Journal of Immunology 130:2423, 1983. In chapter III, the dilemma posed by the observations suggesting that there is preferential V_HC_H pairing although no appropriate mechanism has been seen at the molecular level is addressed. These data are also presented in manuscript form and has been submitted to the Journal of Immunology. Chapter IV deals with the possible evolutionary significance of particular variable region genes. In this chapter the ability of our anti-mouse V_H sera to detect this variable region gene marker in four different species is analyzed. In chapter V, the reader will find a more detailed discussion and some structural data on the subset of mouse antibodies identified by our anti-V_H serum and in the final chapter the implications of this work and its potential significance are discussed.

Origin of Antibody Diversity: Background

Our current understanding of antibodies and the mechanisms behind their extreme diversity had its birth in Paul Ehrlich's 1900 Croonian Lecture "On immunity with special reference to cell life" (1). In this lecture Ehrlich proposed his now famous side chain theory where he suggested that certain cells in the body have side chains to which toxin (antigen) reacts. This reaction, he suggested, then caused regeneration of the side chains to such an extent that they are eventually pushed out or released into the blood plasma. The two basic concepts of this theory, which have shaped our current understanding of humoral immunity, are 1) the possibility that there existed on cells preformed receptors and 2) the proposition of a chemical basis for what we now

term antigen-antibody reactions. This theory, however, received a great deal of criticism, particularly when Landsteiner demonstrated that antibodies could be formed against synthetic haptens which never occurred in nature (2). However, an opposing theory, the instructive theory of antibody formation was not proposed until 1930 by Brienl and Haurowitz (3). They suggested that the diversity of antibodies is produced by using the antigen as a template which can induce conformational modification of the antibody molecule (4). The instructive theory was further refined by L. Pauling who in 1940 stated that all antibody molecules contain the same amino acid sequence, and that they acquire specificity as a result of contact with antigen (5). The instructive theory was gradually disproved as molecular studies firmly established that the three dimensional structure of proteins is determined by their primary structure and that the primary structure of proteins is fully coded in DNA. Ehrlich's side chain theory was revived as the natural selection theory by Niels Jerne in 1955 (6). Jerne stated:

> that the role of the antigen is neither that of the template nor that of an enzyme modifier. The antigen is solely a selective carrier of spontaneously circulating antibody to a system of cells which can reproduce this antibody. Globulin molecules are continuously being synthesized in an enormous variety of different configurations. Among the population of circulating globulin there will spontaneously be fractions possessing affinity toward any antigen to which the animal can respond.

One discrepancy between Jerne's theory of natural selection of antibody and current thinking is that he attributes memory to serum and not to cells. However, Burnet eliminated this objection by localizing selection to cells (7). The essence of Burnet's clonal selection theory is that one cell produces one type of antibody and that extensive production of this specific antibody is accomplished by a growth of a clone

of cells each producing the same antibody.

The main question that arose from the clonal selection theory was how to explain the presence, before contact with antigen, of cells with receptors against all possible antigens to be encountered in nature, even synthetic antigens which might never be encountered. In what follows, this historical survey will deal with the theories which have formed our current understanding of the mechanisms behind the generation of antibody diversity.

The 1960's and 70's were the years during which the mechanisms behind the generation of antibody diversity were hotly debated, and the two main theories proposed were the germline theory and the somatic mutation theory. The germline theory states that the V genes for all antibodies exist in the genome of every cell and that these genes were generated during evolutionary time, and are transmitted in a Mendelian fashion. One of the first proponents of the germline theory was Szilard (8) although it was further developed by a number of other investigators (9,10).

The two major arguments in support of the germline theory of antibody diversity came about from 1) extensive amino acid sequence studies and 2) studies of idiotypic inheritance. From the analysis of the N-terminal amino acid residues of 41 kappa chains and 23 lambda chains, Hood and Talmage found that differences between the V-region of light chains of the same V region subclass are similar in character to those between members of families of proteins with a common evolutionary origin (9). This idea was supported by the finding of Capra and Kunkel that the N-terminal amino acid sequences of two anti-gammaglobulin antibody light chains from two genetically distinct humans were identical

(11). The fact that two identical variable regions were found in two different individuals supports the idea that the genes controlling these proteins arose from a common inherited precursor gene and thus lended further support to the germline theory of antibody diversity. The second major argument at this time in support of the germline theory was that of inheritance of idiotypes. This was first demonstrated by Eichmann and Kindt in the rabbit (12). Since this initial description over a dozen more such inherited idiotypes have been demonstrated in the mouse (13).

The other major theory used to explain the generation of antibody diversity, the somatic mutation theory, postulates that only a few genes are transmitted by heredity and that during cellular development, somatic mutations give rise to antibody heterogeneity. This theory was initially proposed by a number of investigators (14-16). As might be expected the major arguments against the germline theory were the same ones proposed to support somatic mutation. Therefore, in the 1970's the two major arguments in support of somatic mutation theory were the presence of phylogenetic specific residues (17), and V region allotypes in rabbits (18). The existence of both V region allotypes and phylogenetically associated residues were easier to explain with a somatic mutation pauci-gene theory, than by a germline multi-gene theory, since in the latter theory complicated genetic mechanisms would be required to account for the observed traits.

The theory of intrachromosomal somatic mutation proposed by Gally and Edelman was the beginning of our present view of diversity and first brought together the two major theories of antibody diversity (19). They emphasized the fact that unequal crossing over of families

of homologous V region genes would increase or decrease the size of the V region pool.

In the mid 1970's the advent of the technique of RNA-DNA hybridization initiated a series of investigations which led to our present understanding of the mechanisms behind the generation of antibody diversity. One of the first conclusive experiments utilizing the RNA-DNA hybridization techniques was done by Tonegawa using a light chain messenger RNA probe prepared from the M104E myeloma protein which he hybridized to mouse liver DNA (20). This experiment presented an initial piece of molecular evidence favoring somatic mutation which suggested that the number of V germline genes was too small to account for the diversity seen among the known sequences of mouse lambda chains. Further progress in the field of molecular biology eventually led immunologists to a better understanding of the organization of immunoglobulin genes and the complexity of antibody diversity.

These molecular studies described above were done by a number of different investigators (21-25). The following consists of a summary of the basic approaches used. Restriction enzymes were used to cleave DNA from embryonic cells or plasmacytomas into smaller fragments. These fragments were then separated according to size on agarose electrophoresis, then hybridized with either mRNA's, or cDNA probes which had been inserted into bacterial plasmids and then amplified. As a result of these studies, we now know that the heavy and light chain genes are found in genomic DNA on three separate chromosomes. Each of the chains is encoded by sequences of nucleotides (exons) which are interrupted by nontranslated regions (introns), and variable region genes must translocate to the constant region gene before messenger RNA is

produced. Additional experiments showed that there are other DNA segments which contribute to the complete immunoglobulin molecule the J, or joining, segment, and the D, or diversity, segment. It has subsequently been determined that the joining of the V, J and C gene segments in the light chain and the V, D, J and C gene segments in the heavy chain provides diversity in the immunoglobulin molecule by 1) the possibility of any V gene segment being able to combine with any D and or J gene segments and 2) by the inaccuracy of V-J, V-D, and VD-J joining.

The 1980's provided a new series of experiments which even further defined the mechanism of antibody diversity. Three different groups of investigators were able to prepare cDNA probes which coded for V regions of specific anti-hapten antibodies. They utilized these probes to screen and isolate germline DNA segments which cross-hybridized, and found that a large number of different antibody polypeptide chains could be encoded by one or a small number of variable region gene segments (26-28).

What the studies described above have not analyzed are the mutated products of these gene segments which do not participate in the specific antibody response in question. The following chapters describe a system which can address this issue.

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TEN PERCENT OF NORMAL B CELLS AND PLASMA CELLS SHARE A V_H DETERMINANT(S) (J606-GAC) WITH A DISTINCT SUBSET OF MURINE V_HIII PLASMACYTOMAS[1]

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RUNNING TITLE: J606-GAC V_H DETERMINANT(S) IS EXPRESSED BY 10 PERCENT OF B CELLS

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[3]Abbreviations used in this paper: BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; GAC, streptococcal group A carbohydrate; LPS, bacterial lipopolysaccharide; NP^b, the heteroclitic group of anti-(4-hydroxy-3-nitrophenyl) acetyl antibodies which bear the cross-reactive idiotype of the Igh^b hapolotype; PBS, phosphate buffered saline; PWM, pokeweed miotgen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPIT, solide phase immunoisolation technique; RIA, radioimmunoassay; RITC, rhodamine isothiocyanate.

ABSTRACT

Our findings indicate that a subset of V_HIII antibodies, which we refer to as J606-GAC, contains a determinant(s), which is present on 5-15% of normal splenic B cells and plasma cells as detected by immunofluorescence. This subpopulation is detected by purified antibody, 0-1, which was prepared against a murine anti-group A carbohydrate hybridoma antibody. The J606-GAC subset includes the β2,1 fructosan myelomas J606, EPC109, W3082, ABPC4, and UPC61, as well as 13 anti-GAC hybridomas. The 0-1 antiserum failed to react with hybridoma and myeloma immunoglobulins from murine V_H groups I and II or other V_HIII antibodies. By western blot analysis it was observed to react with isolated heavy, but not light, chains of J606-GAC bearing antibodies. Purified antibody, 0-1, failed to react with myelomas, XRPC44 and J539 which have the same J region as J606 but a very different V_H region. These observations indicate that purified antibody, 0-1, is detecting a V_H region determinant. The J606-GAC marker recognized by 0-1 was expressed as early as 4 days after birth and was expressed at similar frequencies in germfree and conventional mice. Immunoprecipitation of both surface and biosynthetically labeled proteins from spleen cells or J606-GAC positive hybridoma cell lines, respectively, confirmed that 0-1 was recognizing an immunoglobulin determinant.

INTRODUCTION

The mechanisms involved in the generation of the extensive diversity seen among antibody molecules have been a major source of study for some time. Certainly much of the heterogeneity can be accounted for by the germline genes and the combinatorial diversity generated by V_H, V_L, J_H, J_L and D segments (1,2,3,4,5). However, recent evidence suggests that somatic mutation also plays a major role in developing the heterogeneity of the antibody repertoire. Bentley and Rabbitts estimated the size of the human V_K gene pool by using the technique of Southern filter hybridization with cloned human V_K genes as probes, and concluded that a large portion of the human V_K gene pool is encoded by 15-20 genes (6). Since the kappa light chain is represented in about 60% of human antibodies, they suggest that the small number of genes detected supports the theory that somatic mutation is a major source of antibody diversity in man. Crews et al. analyzed the genes which encode anti-phosphocholine antibodies in mice. They concluded that the heavy chains of a panel of murine hybridoma antibodies with distinct idiotypes and amino acid sequences are encoded by one heavy chain variable region gene (7). Bothwell et al. examined the heteroclitic group of anti-(4-hydroxy-3-nitrophenyl) acetyl antibodies which bear the cross-reactive idiotype of the Igh^b haplotype $(NP^b)^3$ and showed that they are encoded by a small number of genes, possibly as few as one (8).

To date, the majority of the available DNA cloning and sequencing data has dealt with families of antibodies with one binding specificity. However, it is important to examine, as Bentley and Rabbitts have with human kappa genes, the possibility that antibody molecules of

different binding abilities could be encoded for by one gene segment, or by a small number of similar variable region gene segments (6).

In the present report we describe an antiserum, 0-1, that identifies a distinct subset of murine antibodies, J606-GAC. Further genetic and sequence analysis of this subset of antibodies may provide an excellent model system for determining more specifically the extent to which heavy and light chains of antibodies of different binding abilities can be encoded for by the same genes. This in turn may add to the information concerning the potential role of somatic mutation and combinatorial diversity in the development of the antibody repertoire.

Heterologous anti-V_H serum, 0-1, recognizes a subset (J606-GAC) of murine heavy chain variable region group III myeloma and hybridoma proteins. The members of this subset bind either of two structurally unrelated carbohydrate antigens, β2,1 fructosan or N-acetyl-D-glucosamine. The amino acid sequences of those members of the J606-GAC subset whose sequences are known are (except for two or three amino acids) identical through position 56 (9, 10, R. M. Perlmutter, personal communication). It seems possible that protein sequences with such homology are the products of a single V_H gene segment (6,7,8). Since the 0-1 antiserum recognizes 5-15% of splenic B cells, our data may suggest that up to 5-15% of murine immunoglobulins are the products of either one V_H gene segment or a family of very similar V_H gene segments.

MATERIALS AND METHODS

Mice. AKR/J, CBA/J, BALB/cJ, DBA/2J, and C57BL/6J mice were purchased from Jackson Laboratory, Bar Harbor, Maine. CBA/N and germ-

free BALB/c mice were obtained from Dr. J. McGhee, University of Alabama in Birmingham. NZB/BlN mice were obtained from Dr. T. Marion, University of Alabama in Birmingham.

Myeloma and hybridoma proteins and cell lines. Two BALB/c anti-GAC hybridoma antibodies were prepared in this laoratory as previously described (11). The A/J anti-group A carbohydrate (GAC) hybridoma antibodies GAC-8,9, 10, 11,39,40,46 and 50 were gifts from Drs. M. Nahm, B. Clevinger and J. Davie. Myeloma cell lines MOPC460, MOPC104, J606, EPC109, W3207 and MOPC21, and hybridoma cell lines HD1-1, FD3-6, 3-19, 16-165-2, 26-30-11, HB3-16, ACR-27-3 and 1B2-B7-H9 were provided by Dr. J. F. Kearney and members of his laboratory. Myeloma cell lines SAPC10, TEPC601, MOPC173, Y5606, ABPC4, XRPC44, J539 and UPC10 were provided by Dr. M. Potter. Hybridoma protein PC2567 was provided by Dr. M. Weigert. Hybridoma protein 22.1A4 was provided by Dr. J. L. Claflin. Hybridoma proteins JA4-6 and FC2-4 were provided by Dr. H. Kubagawa.

Preparation of anti-V_H antibody. Purified antibody, 0-1, was prepared by immunizing a goat with the affinity purified (12) BALB/c anti-GAC hybridoma antibody 51n-B7 (μ,κ) emulsified in complete Freund's adjuvant. The hyperimmune goat antiserum was absorbed extensively with a Sepharose 4B column, to which two unrelated mouse (μ,κ) hybridoma antibodies (JA4-6 and FC2-4) were coupled. After removing anti-μ and anti-κ antibodies, the absorbed antiserum was then applied onto a Sepharose 4B column coupled with 51N-B7. Bound antibodies were eluted from the column by 0.05 glycine-HCl buffer, pH 2.8. Purified antibodies were coupled to rhodamine isothiocyanate (RITC) by methods previously described (11).

Immunofluorescence. Cytoplasmic immunofluorescent staining of myeloma cells was performed on cytocentrifuge preparations which had been fixed in 5% acetic acid-95% ethanol for 20 minutes at -20°C after which cells were washed in phosphate buffered saline (PBS). Direct staining was then performed by incubating cells with isolated RITC coupled 0-1 antibody. Cells were also counterstained with fluorescein isothiocyanate conjugated (FITC) antibodies specific for mouse immunoglobulin isotypes, prepared as previously described (13). Membrane staining was also performed on viable cells, after which cytocentrifuge preparations were prepared and fixed as described above. Inhibition of cytoplasmic immunofluorescence was performed by incubating GAC-11 cell smears with RITC conjugated 0-1 in the presence of 45 μg of the test antibody.

Mitogen stimulation. Spleen cells at 8×10^5 cells/ml were stimulated with bacterial lipopolysaccharide (LPS), (Sigma 2630), 50 μg/ml in RPMI-1640, 15% fetal calf serum, 2×10^{-5}M β-2 mercaptoethanol and 2mM glutamine plus antibiotics. After five days in culture, cytocentrifuge slides were made and frozen unfixed at -70°C for future cytoplasmic staining. Pokeweed mitogen (PWM) stimulated cells were obtained from the spleens of mice which were injected intraperitoneally (i.p.) seven days earlier with 0.25 μg PWM (Sigma). Cytocentrifuge slides were prepared and treated as above.

Immunoprecipitation. Cells were internally labeled with ^{35}S cysteine and methionine for 6 hours. Immunoprecipitation with 0-1 was then performed on the internally labeled cell lysates with the addition of S. aureus (14). Immunoprecipitates were then analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Solid phase immunoisolation technique (SPIT). Cell surface components labeled according to Goding (15) were analyzed by SPIT (16). Briefly, labeled cell lysates were added to polyvinyl microtiter wells coated with either 0-1, goat anti-mouse immunoglobulin, or bovine serum albumin. After thorough washing of the plates, bound lysate was eluted from the wells with an SDS buffer containing 2-mercaptoethanol and analyzed by SDS-PAGE.

Electrophoresis. SDS-PAGE analysis was performed according to Laemmli (17). In experiments where Western blots were performed, proteins from SDS-PAGE gels were electrophoresed onto nitrocellulose paper (18). Unreacted sites on the paper were blocked with 1% bovine serum albumin (BSA), and the nitrocellulose paper was overlayed with ^{125}I-0-1 antibody. Following extensive washing of the blots, autoradiography was performed.

RIA. Affinity purified antibodies were titrated in 5-fold dilutions on polyvinyl microtiter plates in PBS, starting with 30-60 µg of protein in the first well. Plates were incubated overnight at 4°C, blocked with 1% BSA in PBS, and then 100 µl of ^{125}I-labeled 0-1 containing 100,000 cpm was added to each well. In order to make a numerical comparison among the results of this assay we have chosen the 50% maximum binding of ^{125}I-0-1 to J606 coated wells as a reference point. This level of binding is referred to as the 50% cross-reactive value. The number of micorgrams of each antibody that had to be added to the wells to obtain this amount of ^{125}I-0-1 binding was determined. Data are expressed as the ratio obtained by dividing the number of micrograms of each antibody required to give the 50% cross-reactive value with the number of micrograms of J606 required to give the same level of binding.

RESULTS

Antiserum specificity. The specificity of antiserum 0-1 was determined by cytoplasmic immunofluorescence and radioimmune direct binding. Table I lists the results obtained from the cytoplasmic immunofluorescent staining of a panel of 13 myeloma and 19 hybridoma antibody cell lines. 0-1 reacted with 15 out of 32 cell lines examined. It appeared not to react with isotypic determinants since the hybridoma cell lines FD3-6, HB3-16, 16-165-2 and 26-30-11, which are all of the same heavy and light chain class as the immunizing antibody, showed no reactivity with 0-1. The 32 cell lines examined represented antibodies from three mouse heavy chain variable region groups. Although at least 12 different antigen binding specificities were represented among these cell lines, antibodies from the 0-1 positive cell lines bound either β2,1 fructosan or N-acetyl-D-glucosamine. Of the 0-1 positive antibodies, complete amino acid sequences are known for EPC109, W3082, J606, UPC61, ABPC4 (19,20,21) and partial sequences for GAC-9 and GAC-11 (R. M. Perlmutter, personal communication). All 7 of these antibodies are classified as members of the murine heavy chain variable region group III (22). Since the panel of 0-1 positive antibodies included the myeloma protein J606 and nine GAC-binding hybridoma proteins, we have termed this 0-1 reactive subset of antibodies J606-GAC. To facilitate the screening of additional antibody V regions we have used two assays that allow analysis of secreted antibody, inhibition of cytoplasmic immunofluorescence and a direct binding assay. In the direct binding assay it is apparent that a much higher fraction of the ^{125}I-0-1 binds to 51N-B7 coated plates than to

plates coated with J606 or GAC-8 (Fig. 1). This would be expected since the 0-1 antibody preparation would be expected to contain antibodies specific for the 51N-B7 idiotype as well as antibodies reactive with the J606-GAC bearing antibodies as illustrated by the binding of ^{125}I-0-1 to J606 and GAC-8 coated wells. MOPC460 and MOPC104 show little or no binding of the labeled 0-1 antibody as in the case with other antibodies lacking the J606-GAC marker(s).

In the inhibition of cytoplasmic immunofluorescence assay we chose to inhibit the immunofluorescence of the 0-1 antisera directed against GAC-11. Thus the inhibition assay would be specific only for antibodies bearing the J606-GAC determinant(s) and would not be affected by 51N-B7 specific anti-idiotypic antibodies in the preparation of the 0-1 antibody.

As can be seen by comparing the data in Table II and Fig. 1, both of the secreted antibody assays gave identical results to cytoplasmic immunofluorescence. These new detection techniques identified another J606-GAC bearing antibody, PC2567, an NZB anti-β2,1 fructosan myeloma protein, and increased the total number of antibodies recognized by 0-1 to 16 of 40 examined (Table II). Of the 40 monoclonal antibodies we have examined, complete or partial heavy chain sequence information is known for 22. Five of these 22 antibodies bind β2,1 fructosan and are among those that we have shown are positive for the J606-GAC V_H marker(s). All five of these antibodies have identical amino acid sequences for the first 20 residues (22). Complete sequences of these five has shown them to differ at no more than 2 amino acids in the first 95 residues (19,20,21). The other group of antibodies bearing the J606-GAC marker are those binding GAC. Data from three different

published anti-GAC sequences indicate that these antibodies are all identical to J606 in the first 20 residues (10,25). More extensive sequence analysis of GAC-9 and GAC-11 indicates that they differ from J606 in only 2-3 amino acids in the first 56 residues (R. M. Perlmutter, personal communication). The 0-1 antiserum failed to react with myelomas XRPC44 and J539 which have the same J region as J606 (26) but a very different V_H region (22). In Figure 2 we have reproduced in a modified form, the heavy chain relationship tree of Hood et al. (23). In this figure we have listed all of the different antibodies we have tested for the presence of the J606-GAC determinant(s). Based on this analysis it is apparent that the J606-GAC bearing antibodies form a distinct subset of V_HIII antibodies.

It is known that the β2,1 fructosan binding myeloma proteins and the N-acetyl-D-glucosamine binding hybridoma proteins we examined contain different light chains (10,24). It was therefore of interest to determine whether the J606-GAC marker(s) could be identified on isolated heavy chains or whether the idiotype also depended on residues contributed by a light chain. To answer this question, an 0-1 positive hybridoma protein, GAC-11 (γ_3,κ), an 0-1 non-reactive hybridoma protein, GB4-10 (γ_1,κ), and the immunizing hybridoma protein, 51n-B7 (μ,κ), were run under both reducing and non-reducing conditions on an SDS-PAGE. A western blot was then made on nitrocellulose paper and overlayed with ^{125}I-0-1. The autoradiographic results shown in Fig. 3 illustrate that both the intact molecule and the isolated heavy chain of GAC-11 reacted with 0-1, as did the isolated heavy chain of 51N-B7. This is illustrated by the three high molecular weight bands in lanes 5,2 and 1, respectively, GB4-10 was non-reactive in all

instances. Since the immunizing antibody 51n-B7 is of the (μ,κ) isotype, it did not enter the gel under non-reducing conditions. In lanes 1 and 4 a low molecular weight band was also recognized by 0-1. We do not believe these bands represent light chain products because of their apparent molecular weights. We are assuming these proteins may be heavy chain breakdown products, especially since the band in lane 1 represents a protein with a molecular weight similar to an Fd fragment. In summary, we conclude that 0-1 is recognizing framework determinants on certain sets of closely related heavy chain variable regions, which do not rely on conformational determinants contributed by associated light chains.

Ten to 15 percent of murine antibodies bear determinant(s) in common with the J606-GAC subset. In order to estimate what portion of the normally expressed antibody repertoire is made up of J606-GAC determinant(s) bearing antibodies, we used cytoplasmic immunofluorescence to determine the frequency of 0-1 positive cells in the unstimulated splenic plasma cell population. Table III illustrates such an analysis in five separate mouse strains. The data indicate that between 5-15% of splenic plasma cells are synthesizing immunoglobulin that bears the J606-GAC marker(s).

In addition to the examination of plasma cells by cytoplasmic staining we examined the percentage of B cells bearing J606-GAC positive immunoglobulin on their surface. There were no significant differences between the percentage of J606-GAC positive B cells and plasma cells, 16 and 15 percent positive cells were seen, respectively.

To verify that the plasma and B cell determinants detected by immunofluorescence were expressed by immunoglobulin molecules, immuno-

precipitation and immunofluorescent co-capping studies were performed on appropriate cells. Biosynthetic labeling of two anti-GAC hybridoma lines and immunoprecipitation with both an anti-mouse immunoglobulin serum and 0-1, followed by SDS-PAGE under reducing conditions indicated that both antisera precipitated only two bands, one corresponding to heavy chain and one to light chain (data not shown). Therefore, in high rate secreting cells 0-1 appeared to recognize only immunoglobulin. Solid phase immunoisolation (SPIT) of lactoperoxidase ^{125}I surface labeled murine spleen cells showed that 0-1 isolated bands of the same molecular weight as a goat anti-mouse immunoglobulin antiserum (Figure 4). To obtain additional evidence we performed immunofluorescent co-capping experiments. When cells were capped with goat FITC anti-mouse immunoglobulin and then counterstained with RITC 0-1 antibody, both fluorochromes were located in the cap.

The above data indicate that the determinant(s) recognized on 5-15% of B cells and plasma cells by purified antibody 0-1 are specific for a distinct subset of mouse V_H regions. This high frequency of J606-GAC bearing B lymphocytes and plasma cells is probably either a natural result of B cell generation in the mouse or a consequence of amplification due to antigenic stimulation. Because of the rapid turnover within the B cell pool (27) we would have expected differences between the frequencies of B cells and plasma cells expressing the J606-GAC marker(s) if antigen stimulation was affecting the expression of B cells synthesizing J606-GAC bearing molecules. However, since we observed the same high frequency of J606-GAC cells among both splenic B cells and plasma cells it seemed unlikely that the high frequency of this marker was due to antigen-induced amplification. The possibility

remained, however, that differences in the cytoplasmic and surface staining procedures might have allowed the purified antibody, 0-1, to cross-react differently in the two assays. To rule this out, we used mitogens to non-specifically stimulate B cells to differentiate into plasma cells, which could then be cytoplasmically stained with 0-1. We found essentially the same percentages of J606-GAC positive cells among unstimulated plasma cells, and among plasma cells induced in culture with LPS or *in vivo* with pokeweed mitogen (Table IV). Because of the ease of detection of positive cells by cytoplasmic staining we have used mitogen stimulated cells in all subsequent experiments.

The data in Table V show that the percentage of J606-GAC positive plasma cells in LPS stimulated 4 or 12 day old spleen cells, or adult bone marrow cells, was within the same range as that seen in adult spleen. Similar results were seen with surface staining where 12% of 4 day spleen cells were observed to be J606-GAC positive. These results suggest that B cells at very early stages in development already express J606-GAC bearing immunoglobulin in the same frequency as the adult, and that J606-GAC expression is unlikely to be the result of antigen induced maturation. This conclusion is supported by the observation that J606-GAC bearing plasma cells also make up similar percentages, 16 and 12 respectively, of LPS stimulated spleen cells from germ-free mice and conventionally reared BALB/c mice.

DISCUSSION

In this paper we have described a heterologous antiserum, 0-1, which recognizes immunoglobulin determinants on between 5-15% of murine splenic B cells and on a distinct subset, J606-GAC, of murine V_HIII

myeloma and hybridoma antibodies. Purified antibody, 0-1, appears to recognize framework determinants on certain heavy chain variable regions. Immunoglobulins making up this subset differ in light chain sequence but all have V_H sequences that are virtually identical, with no more than 3 amino acid differences in the first 56 amino acids (10, 19,20,21,24, R. M. Perlmutter, personal communication). This finding suggests that perhaps only one or a small number of genes code for the J606-GAC variable region chains, a hypothesis which can only be confirmed through DNA cloning and hybridization studies. Amino acid sequencing and molecular studies of anti-inulin binding hybridomas including J606 already indicate that they are the products of a saml1 number of genes (19,20,21). However, the fact that one V gene may encode 5-15% of murine immunoglobulin heavy chains is an intriguing possibility which would be consistent with other molecular and idiotypic evidence suggesting that somatic mutation and combinatorial diversity can play a major role in developing the B cell repertoire.

Antisera recognizing other V_H subsets have been described previously (28,29,30,31,32), as has a V_L marker located on some of the anti-GAC hybridoma proteins discussed here (33). Bosma et al. described an antiserum which identifies a subset of murine antibodies termed U10-173 (32). Although our antiserum recognizes a subset of antibodies distinct from the U10-173 subset, many similarities do exist: 1) Both antisera detect markers on carbohydrate binding antibodies of more than one specificity; 2) the percentage of normal mouse immunoglobulin which is recognized by these sera is larger than one would expect with a typical cross-reactive anti-idiotypic serum; 3) both antisera recognize determinants on isolated heavy chain. Although

the significance of the J606-GAC and U10-173 subsets is not clearly understood, the J606-GAC subset may be particularly important to the mouse since it is expressed in 5-15% of B cells.

The finding that the J606-GAC marker(s) is associated only with immunoglobulins of a particular V_H region subgroup makes it likely that the marker(s) is associated with that portion of the immunoglobulin molecule coded for by V_H gene segments rather than J or D region gene segments. This supposition is supported by the observation that hybridoma antibodies XRPC44 and J539, which have the same J region as J606 but a different V region (22,26), fail to express the J606-GAC specificity. We cannot rule out the possibility that 0-1 reacts with a D region encoded determinant, especially since the few J606-GAC bearing antibodies with known D region sequences, all of which are anti-inulin, have identical D regions (19).

If, however, as we suggest, 0-1 is identifying a marker coded for by a single V_H gene, our data suggest that 5-15% of mouse immunoglobulin utilizes that same V_H gene. It seems unlikely that all antibodies bearing the J606-GAC marker bind either GAC or β2,1-fructosan since, in normal mouse sera, antibody binding either of these antigens is either non-detectable or detectable in very small amounts (34,35). Also, anti-β2,1-fructosan reactive B cells are known to appear late in ontogeny, while we were able to see J606-GAC positive cells in mice less than a week old (34). It is not known, however, if the specificities of other J606-GAC antibodies are also directed against carbohydrates. The high frequency of the J606-GAC antibodies might be an indication that some of them are particularly important in protection against pathogens. An analogous situation has been proposed for

naturally occurring anti-PC antibodies which bind pneumococcal C-polysaccharide and appear to play an important role in the protection against pneumococci (36).

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TABLE I

0-1 reactivity profile of a panel of murine myeloma and hybridoma cell lines

Cell Lines	Isotype	V_H Group	Specificity	Positive fluorescence[a] staining with FITC 0-1
Myelomas				
MOPC460	(α,κ)	I	DNP	-
MOPC104	(μ,λ)	II	LAX	-
SAPC10	(α,κ)	III	GAL	-
TEPC601	(α,κ)	III	GAL	-
X3PC44	(α,κ)	III	GAL.	-
J539	(α,κ)	III	GAL.	-
MOPC173	(γ_{2a},κ)	III	?	-
Y5606	(γ_3,λ)	III	?	-
EPC109	(α,κ)	III	Fruct.	+
W3082	(α,κ)	III	Fruct.	+
UPC51	(α,κ)	III	Fruct.	+
A3PC4	(α,κ)	III	Fruct.	+
J606	(γ_3,κ)	III	Fruct.	+
Hybridomas				
51n-37	(μ,κ)	?	GAC	+
41N	(μ,κ)	?	GAC	+
GAC[b]-8,9,10,11,39,40,46,50	(γ_3,κ)	III[b]	GAC	+
ACR-27-3	(γ_3,κ)	?	ACR	-
G34-10	(γ_1,κ)	?	α-Id	-
HD1-1	(γ_3,κ)	?	α-Id	-
13Z-37-H9	(μ,κ)	?	DNA	-
FD3-6	(μ,κ)	?	CRP	-
3-19	(γ_1,λ)	?	DEX	-
16-165-2	(μ,λ)	?	?	-
26-30-11	(μ,κ)	?	?	-
HB3-16	(μ,κ)	?	?	-

[a]Affinity purified 0-1 (see Material and Methods) was conjugated to rhodamine isothiocyanate and used at a concentration of 0.05 µg/ml to stain fixed cytocentrifuge preps of the respective myeloma cell lines.

[b]Placed in V_HIII based on the amino acid sequence of GAC-9 and GAC-11 through position 56 (R. M. Perlmutter, personal communication).

TABLE II

Comparison of J606-GAC detection assays

Myelomas and hybridomas[a]	Positive fluorescence with 0-1 Rhodamine	Inhibition of 0-1-Rhodamine's fluorescent staining of GAC-11 with the listed test antibodies[b]	Relative Binding[c]
51N-87	+	+	11
GAC-11	+	+	0.75
J606	+	+	1
EPC109	+	+	0.63
MOPC104	-	-	<.0006
MOPC460	-	-	<.0006
S117	ND	ND	<.0006
G34-10	-	ND	ND
UPC10	ND	-	ND
W3207	ND	-	<.0006
22.1A4	ND	-	<.0006
PC2567[d]	ND	ND	3
Y5444	ND	ND	<.0006
MOPC21	ND	ND	<.0006
MOPC315	ND	ND	<.0006

[a]The following myeloma and hybridoma cell lines were not listed in Table I: S117 (α,κ), anti-GAC, G34-10 (γ_1,κ) anti-idiotype, W3207 (α,κ) anti-PC, 22.1A4 (μ,κ) anti-PC, PC2567 (γ_3,κ) anti-fruct., Y5444 (γ_{2a},λ), MOPC21 (γ_1,κ), MOPC315 (α,κ) anti-DNP.

[b]Inhibition of cytoplasmic immunofluorescence was performed by adding a mixture of 75 µg of test antibody and 5 µl of RITC 0-1 (0.05 µg/ml) to a GAC-11 cell preparation. Inhibition of fluorescence was then scored as a plus.

[c]Radioimmune direct binding was performed by titrating out the test antibodies (1/5 dilutions) in PBS on microtiter plates beginning with 30-50 µg in the first well. Plates were then overlayered with ^{125}0-1. Relative binding refers to the relative ability of 0-1 to bind the indicated antibody as compared to J606. The method of calculation is given in the methods section.

[d]Myeloma antibodies PC2567, Y5444 and MOPC21 were 47% saturated $(NH_4)_2SO_4$ precipitation of ascitic fluid; all other antibodies were affinity purified.

TABLE III

Percentage of J606-GAC positive splenic plasma cells in five strains of mice

Strain	H-2	IgH-C	% of Plasma Cells[a] Staining with 0-1
BALB/c	d	a	40/330 (12%)
AKR	k	d	25/210 (12%)
NZB	d	e	23/157 (15%)
C57BL/6	b	b	23/225 (10%)
SWR	g	g*	8/152 (5%)

[a]The percentage of J606-GAC positive plasma cells in mouse spleen was determined by 2 layer cytoplasmic immunofluorescence. The first layer consists of 0-1 coupled to rhodamine and the second layer consists of goat anti-mouse immunoglobulin coupled to fluorescein. Less than 1% of non-immunoglobulin positive cells stained with 0-1.

TABLE IV

Percentage of J606-GAC positive plasma cells among normal and mitogen stimulated spleen cells

Strain	% of Plasma Cells which also Stain with 0-1		
	Stimulated	LPS[a]	PWM[b]
CBA	ND	73/582 (13%)	64/470 (14%)[c]
BALB/c	40/330 (12%)	164/992 (17%)	ND
AKR	25/210 (12%)	39/287 (14%)	ND
NZB	23/157 (15%)	78/614 (13%)	ND
C57BL/6	23/225 (10%)	78/583 (13%)	ND
SWR	8/152 (5%)	59/623 (9%)	ND

[a]Lipopolysaccharide was added to $8x10^5$ cells/ml at 50 µg/ml in complete RPMI. Cytoplasmic immunofluorescence was examined on day 7.

[b]Pokeweed mitogen was injected into mice i.p. at a dose of 0.25 mg. Spleens were removed from mice on day 7 and spleen cells were examined by cytoplasmic immunofluorescence.

[c]CBA is the only strain know to be stimulated by PWM (J. Pasley personal communication).

TABLE V

Percentage of J606-GAC positive plasma cells in 4 day, 12 day and adult mice

Mice	% of Plasma Cells which also Stain with 0-1
4 day-spleen[a]	$\frac{32}{151}$ (21%)[b]
12 day-spleen	$\frac{128}{1010}$ (13%)
Adult-spleen	$\frac{37}{201}$ (18%)

[a]Spleens from 5, 4-day-old neonates were pooled. Spleens from 2, 12-day-old neonates were pooled. The adult spleen was obtained from the mother of the 12 day old neonates. Cells were incubated at a concentration of 8×10^5 cell/ml and stimulated with LPS at 50 μg/ml.

[b]Percentages were significantly different from adult values with the P value being <0.05.

Figure 1. Binding of ^{125}I-O-1 to monoclonal antibodies. Microtiter wells were coated with the indicated concentration of each monoclonal antibody in PBS. Wells were blocked with 1% BSA and 50,000 cpm of ^{125}I-O-1 was added to each well. Cpm of bound O-1 is indicated on the vertical axis.

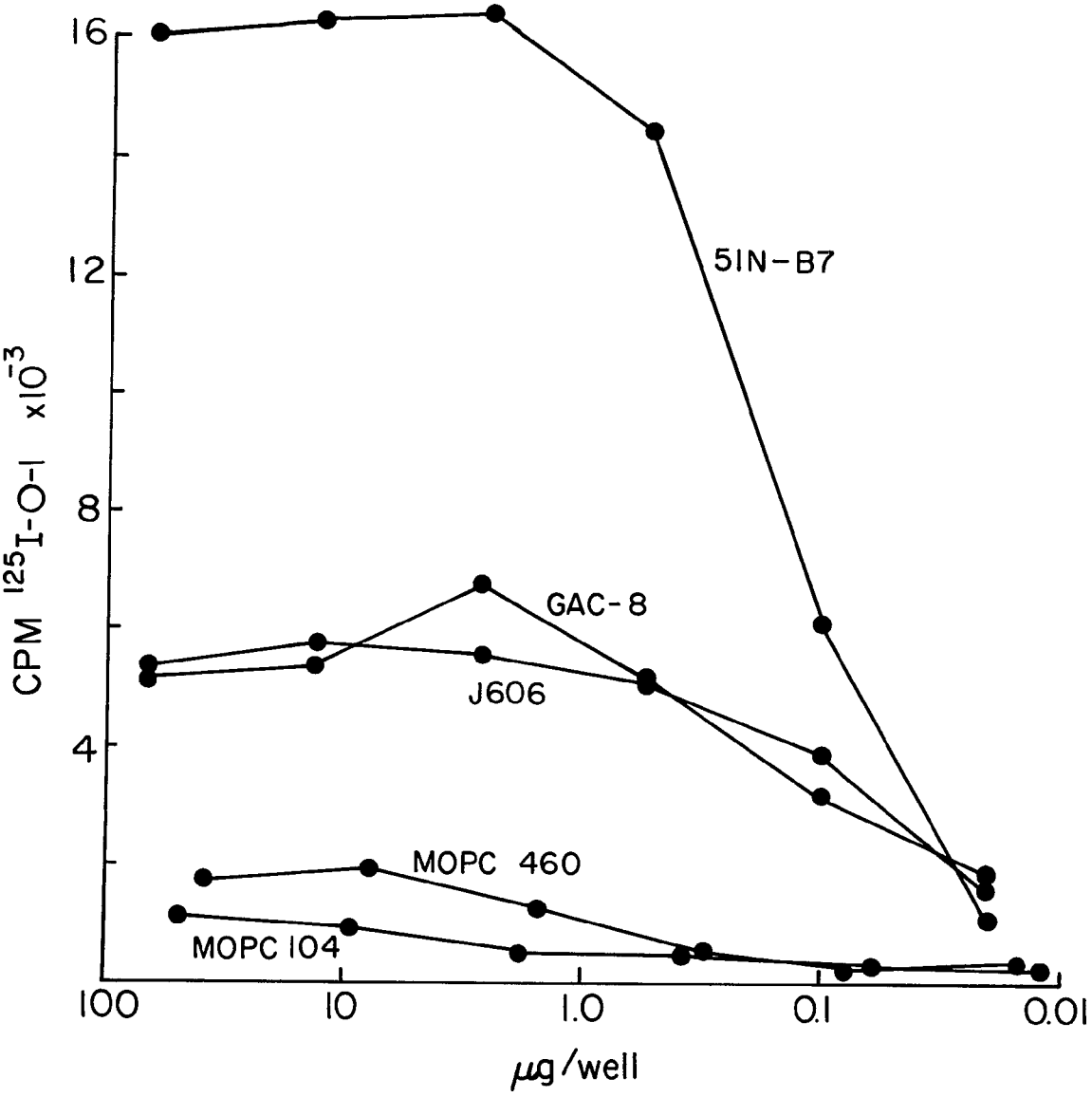
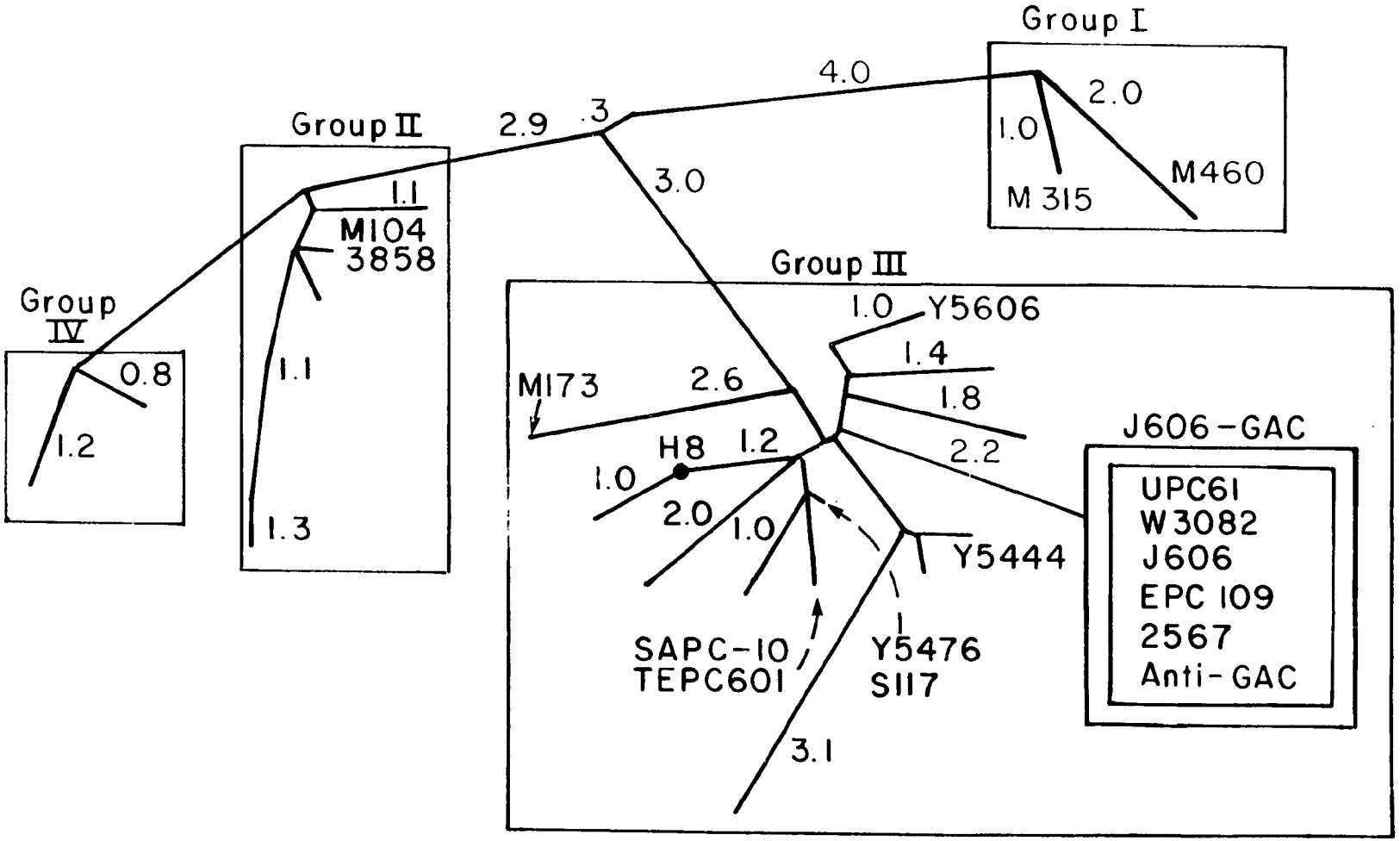

16
12
8
4
CPM ^{125}I-O-I x10^{-3}
5IN-B7
GAC-8
J606
MOPC 460
MOPC 104
100
10
1.0
0.1
0.01
μg/well

Figure 2. Immunoglobulin relationship tree, adapted from Hood et al. (23). The statistically most likely number of mutational events between branch points is indicated (37). All myeloma antibodies listed in this tree have been examined for reactivity with 0-1. Those myeloma antibodies, plus all anti-GAC hybridoma antibodies, were 0-1 positive and are enclosed here within the double box.



Adapted from Hood, etal. CSHS XLI: 817, 1976

Figure 3. O-1 recognized determinants on both intact and isolated heavy chains of O-1 positive antibody. Both reduced and non-reduced samples of test antibodies were run on a discontinuous SDS gel. Proteins were then electrophoresed onto nitrocellulose paper by the Western blot procedure, and overlayed with 50 µl of ^{125}I-O-1 10^5 cpm/ml in 3% BSA-Tris-Cl pH 7.4. Lanes 1 and 6 contained the immunizing antibody 51N-B7 (μ,κ); lanes 2 and 7 contained GAC-11 (γ_3,κ) which is O-1 cross-reactive by fluorescence. Lanes 3 and 8 contained GB4-10 (γ_1,κ) which is not detected by fluorescent O-1.

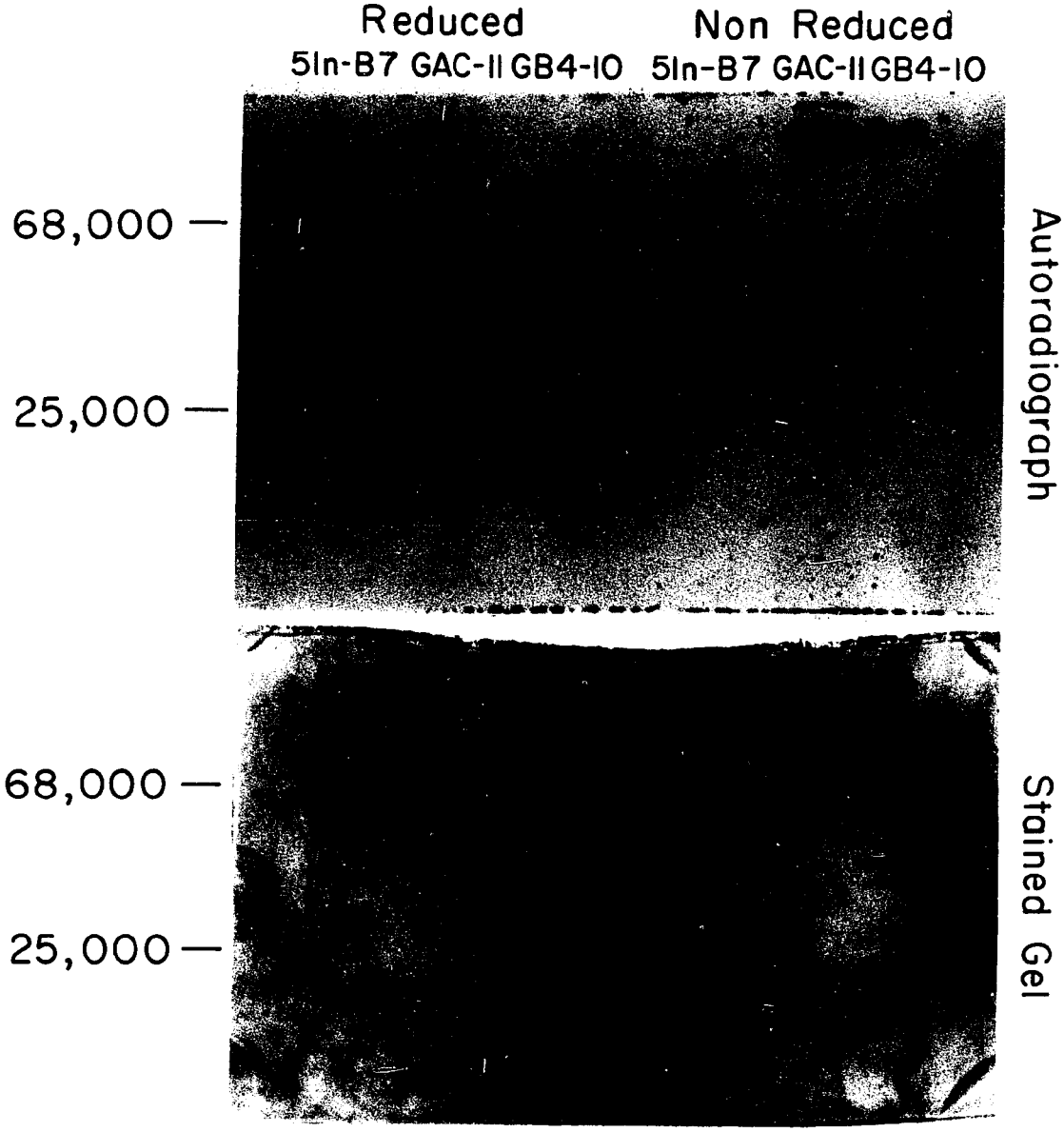
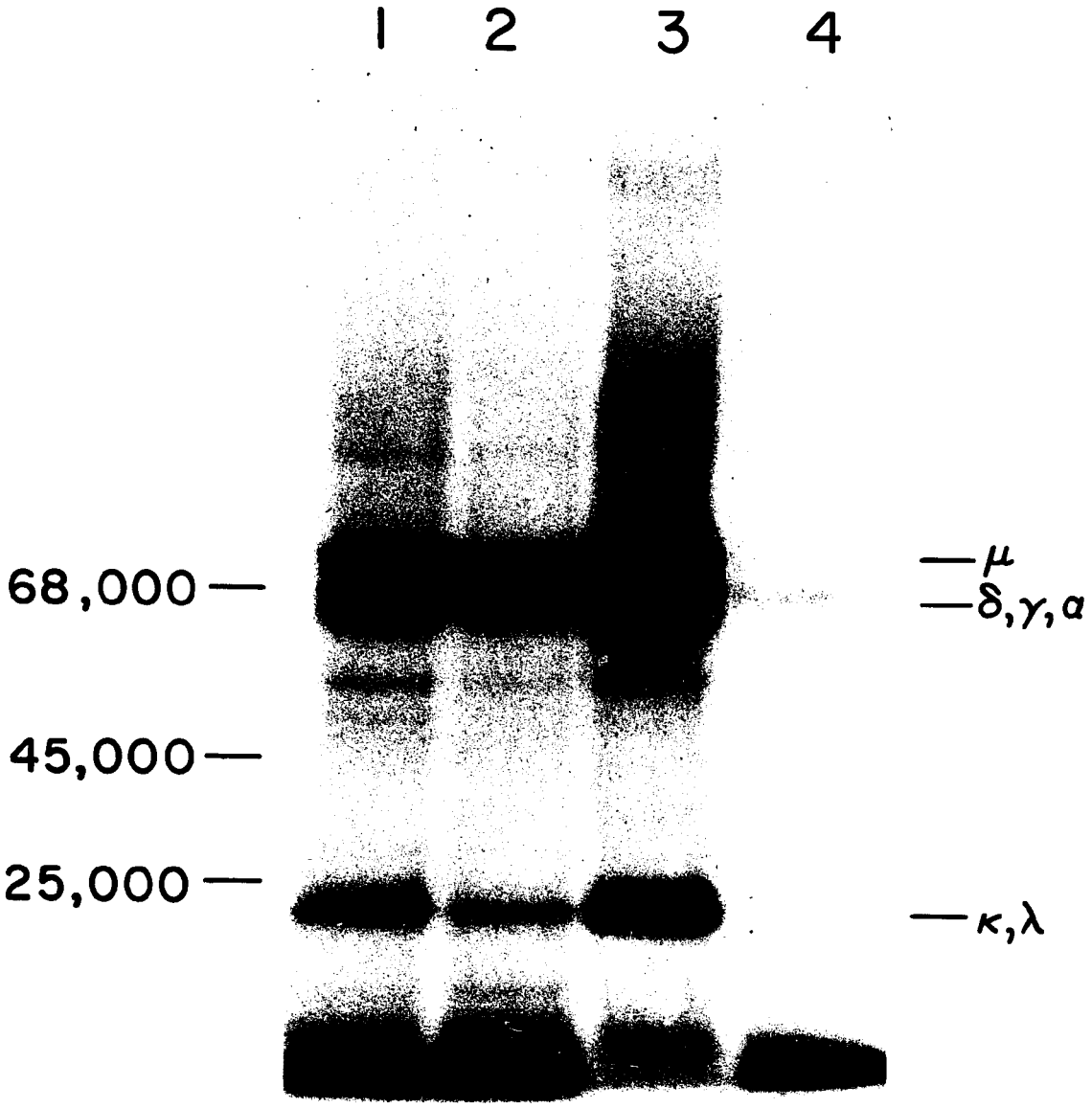

Reduced
5In-B7 GAC-II GB4-10
Non Reduced
5In-B7 GAC-II GB4-10
68,000
25,000
Autoradiograph
68,000
25,000
Stained Gel

Figure 4. The recognition of subsets of plasma and B cells by 0-1 appears to be totally dependent on its reactivity with immunoglobulin. Spleen cells were surface labeled with ^{125}I and lysed with NP40 according to Goding. Molecules reactive with 0-1 were obtained by solid phase immunoisolation and run on a discontinuous SDS-PAGE. Lane 1 is the isolated material eluted from a polyvinyl microtiter well coated with 100 µg/ml of 0-1 (100 µl of labeled cell lysate was added). Lane 2 contains the isolated material eluted from a well coated with 1 µg/ml of 0-1 (200 µl of labeled cell lysate was added to this well). Lane 3 contains the material eluted from a well coated with 1 µg/ml goat anti-mouse immunoglobulin (25 µl labled cell lysate was added to this well). Lane 4 contains the material eluted from a well coated with 1% BSA PBS (100 µl of labeled cell lysate was added to this well).


1
2
3
4
68,000
45,000
25,000
μ
δ,γ,α
κ,λ

THE MOUSE HEAVY CHAIN VARIABLE REGION MARKER, J606-GAC, IS NOT RESTRICTED TO PARTICULAR B CELL ISOTYPES OR SUBSETS[1]

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FOOTNOTES

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[3]Abbreviations used: Group A carbohydrate, GAC; lipopolysaccharide, LPS; rhodamine isothiocyanate, RITC; T-dependent, TD; T-dependent type 2, TD-2; T-independent type 1, TI-1; T-independent type 2, TI-2; heavy chain variable region, V_H; X-linked immunodeficiency, xid.

ABSTRACT

The anti-heavy chain variable region (V_H) marker J606-GAC, which is expressed on a subset of mouse heavy chain variable region group III antibodies, is expressed at similar frequencies on antibodies with μ, γ_3, γ_1, γ_2, and α heavy chains. We have previously shown the J606-GAC determinant to be present on all anti-inulin and on the majority of anti-group-A-carbohydrate (GAC) antibodies examined. The responses to these two antigens are designated thymus-independent type 2 (TI-2) and thymus-dependent type 2 (TD-2), respectively, and have previously been shown to be largely restricted to the μ and γ_3 heavy chain classes. TI-2 and TD-2 antigens are distinguished from other antigens such as T-independent type 1 (TI-1) and other thymus-dependent (TD) antigens, in part because they are virtually not immunogenic in CBA/N mice which express the x-linked immunodefiency (xid) allele. Surprisingly, we found no difference in the percentage of J606-GAC determinant-bearing plasma cells in the spleens of xid versus normal mice.

INTRODUCTION

It has previously been shown that antibody responses to many carbohydrate antigens are preferentially restricted to certain heavy chain classes in mice, rats and humans (1-6). In particular, the antibody responses to certain TI-2 and TD-2 antigens in mice have been shown to be predominately of the μ and γ_3 heavy chain classes (1). The responses to antigens other than carbohydrates have also been shown to be isotype restricted (4,6-9). Results of one of these studies have indicated that certain idiotypes associate preferentially with certain isotypes (9). One implication of these studies is that there may be preferential pairing of V_H and C_H gene segments (1-3,9,10). However, since no direct evidence has been given for such a mechanism at the molecular level, it has been suggested that the preferential association seen is due to other regulatory mechanisms of B cell development (9). Many of these studies have analyzed this question through the use of anti-idiotypic sera, which most likely identified only a small subset of the products of particular V_H gene segments. We have chosen to reanalyze this question with an anti-V_H serum which appears to recognize many of the products of one or a small number of similar V_H gene segments. We feel that analysis with this type of antiserum may more accurately address the potential for preferential V_H-C_H association.

We have used anti-V_H antiserum 0-1, which recognizes a subset of mouse antibodies (J606-GAC), to probe for preferential V_H-C_H association. This antiserum is highly specific since it only detects immunoglobulins whose heavy chains are virtually identical to that of

J606 (11). J606-GAC-positive antibodies account for virtually all anti-streptococcal group A carbohydrate (GAC) and anti-inulin antibodies (11). They probably express a number of other specificities as well, since they constitute about 10% of the antibodies produced by normal mice. We have also looked for the presence of the J606-GAC determinant in CBA/N mice, since anti-carbohydrate responses have been shown to be virtually absent in these mice (2). We have observed similar frequencies of association of the J606-GAC V_H marker with all isotypes and have found normal expression of the marker in CBA/N mice. Our results provide additional evidence for the idea that the preferential idiotype-isotype association seen in many immune responses is not due to restricted V_H-C_H pairing, but is more likely the result of regulatory mechanisms. In addition, the equal presence of J606-GAC determinant-bearing plasma cells in CBA/N and CBA/J mice contradicts the hypothesis that $Lyb5^+$ and $Lyb5^-$ B cells use different V region repertoires (12,13).

MATERIALS AND METHODS

Mice. BALB/cJ, CBA/J, SWR/J and C57BL/6J mice were purchased from Jackson Laboratory, Bar Harbor, Maine. CBA/N mice were obtained from Dr. J. McGhee, University of Alabama in Birmingham.

Preparation of anti-V_H antibody. Purified antibody, 0-1, was prepared as previously described (11). Briefly, a goat was immunized with the affinity purified (14) BALB/c anti-GAC hybridoma antibody 51N-B7 (μ,κ) emulsified in complete Freund's adjuvant. The hyperimmune antiserum was then absorbed extensively with a Sepharose 4B column to which two unrelated mouse μ,κ hybridoma antibodies (JA4-6 and FC2-4)

were coupled. The absorbed antiserum was then affinity purified from a 51N-B7 Sepharose 4B column. Purified antibodies were coupled to rhodamine isothiocyanate (RITC) by methods previously described (15).

Cell preparation. Spleens were removed from mice and teased into 5% fetal calf serum in Dulbecco's phosphate buffered saline (PBS). A portion of the spleen cells were then cytocentrifuged onto slides and frozen unfixed at -70°C until needed for cytoplasmic fluorescent staining. Another portion of the spleen cells was cultured at $8x10^5$ cells/ml and was stimulated with bacterial lipopolysaccharide (LPS), (Sigma 2630), 50 µg/ml in RPMI-1640, 15% FCS, $2x10^{-5}$ M β-2-mercaptoethanol and 2 mM glutamine plus antibodies. After five days in culture, cytocentrifuge slides were made and frozen unfixed at -70°C for future cytoplasmic staining.

Immunofluorescence. Cytoplasmic immunofluorescent staining of unstimulated or LPS stimulated mouse spleen cells was performed on cytocentrifuge preparations which had been fixed in 5% acetic acid - 95% ethanol for 20 minutes at -20°C after which cells were washed in PBS. Direct staining was then performed by incubating cells with RITC coupled 0-1 antibody. Cells were also counterstained with fluorescein isothiocyanate conjugated antibodies specific for mouse immunoglobulin isotypes prepared as previously described (14).

Radioimmunoassay. Purified antibody 0-1 was coated on polyvinyl microtiter plates in PBS at a concentration of 1 µg/ml. Plates were incubated overnight at 4°C, then blocked with 1% bovine serum albumin in PBS. The plates were washed with PBS and 150 µl of test normal mouse serum was added to well one and titrated in three-fold dilutions. Twenty µl of ^{125}I-labeled anti-group A carbohydrate hybridoma

antibody GAC-54 (provided to us by J. M. Davie, Washington University, St. Louis, MO) was then added to each well. Affinity-purified 0-1 cross-reactive antibody, GAC-11, was also titrated and used as the standard. The number of μg of antibody needed to inhibit 50% binding of ^{125}I-GAC-54 was then calculated. The amount of kappa-bearing antibody in each normal mouse serum was previously determined by radioimmunoassay.

RESULTS AND DISCUSSION

An abundance of data has accumulated showing that different antigens can preferentially elicit certain classes of antibody (1-3). Further analysis of this observation has revealed preferential pairing of certain idiotypes with particular isotypes in a variety of different antibody responses (5,9). A number of these studies have involved antibody responses to carbohydrate antigens such as GAC, dextran and phosphocholine (PC) on pneumococcal polysaccharide, which elicit an antibody response restricted to a few clonotypes (16-18). However, studies in which a more heterogeneous response has been examined, specifically the response to dinitrophenyl (7-9), have reached a similar conclusion. One interpretation of these results is that the variable region repertoire of different heavy chain classes, paricularly different IgG subclasses, may be distinct (1).

Exceptions to this general conclusion have been reported (19,20), however they have dealt with the PC response which is dominated by a single clone. Evidence which is more difficult to reconcile with this conclusion is that of Teale *et al.*, who using, the *in vitro* splenic

focus assay (21), has shown that the progeny of a single stimulated B cell can produce multiple isotypes, including γ_1 and γ_3.

We have chosen to readdress this question with an anti-V_H serum which appears to recognize many of the products of one or a small number of similar V_H gene segments (11), rather than with an anti-serum which would be expected to recognize only a restricted portion of the possible somatic variants of any single V_H gene segment.

Isotype distribution of J606-GAC antibodies. We have used purified anti-V_H antibody, 0-1, to screen by cytoplasmic immunofluorescence unstimulated or LPS stimulated mouse spleen cells for the presence of the J606-GAC marker on plasma cells of five different isotypes. The results obtained by staining BALB/c, CBA/J, C57BL/6J and SWR/J spleen cells are listed in Tables I and II. Examination of normally occurring (Table I) or LPS induced (Table II) plasma cells indicates that the J606-GAC determinant is not predominantly associated with any particular isotype. This finding is particularly interesting since it has been shown that the antibody responses to both inulin and GAC are restricted primarily to the μ and γ_3 heavy chain classes (1,10,22,23). In some strains a slight, but significantly higher percentage of J606-GAC bearing plasma cells of the IgG_3 subclass than the IgG_1 or IgG_{2x} subclasses is seen (Tables I and II). This finding probably means that in these strains there is a slightly higher percentage of anti-carbohydrate than anti-protein antibodies bearing the J606-GAC V_H marker. This is not surprising since the J606-GAC marker is present on virtually all anti-group A Streptococcal carbohydrate and all anti-inulin antibodies.

Thus when only a portion of the products of a particular V_H gene segment are examined (e.g., anti-GAC or anti-inulin antibodies), restricted association to particular heavy chain isotypes is observed, but when a larger fraction of the products are examined (e.g., all J606-GAC antibodies) preferential V_H-C_H pairing is found not to exist.

An alternative interpretation of our data is that J606-GAC immunoglobulins maybe the products of a number of different V_H gene segments, each of which is restricted to a different heavy chain class. This criticism can not be dismissed completely without studies at the DNA level. However, our preliminary data (24) on the N-terminal amino acid sequences of the non-GAC, non-inulin binding members of the J606-GAC subset suggest that they are encoded by the same or very similar V_H genes to those coding for the anti-inulin and anti-GAC subsets. It is difficult to reconcile how very similar V_H gene segments could show preferential V_H-C_H pairing in some instances and not in others, particularly since no molecular evidence favoring preferential V_H-C_H joining exists.

Furthermore, the above data are consistant with the in vitro results of Teale et al. (21) and the in vivo data of Claflin and Cubberly (19), which have been interpreted to indicate that it is possible for a V_H region gene segment to combine with multiple heavy chain constant region gene segments.

Expression of J606-GAC antibodies in xid mice. The question remains open, however, as to whether restriction is maintained at the level of B cell subsets. In particular it has been suggested that the Lyb5 positive and negative B cell subsets utilize different variable

region repertoires (2). In addition, CBA/N mice, which are lacking in the Lyb5$^+$ subset of B cells, have also been shown not to respond to a number of different TI-2 antigens (2,10). In particular CBA/N mice generally make very low responses to PC. However, Chang et al. (13) and Wicker et al. (12), in analyzing the memory response to PC in CBA/N mice, have shown that CBA/N mice can produce anti-PC antibodies but that they utilize heavy chains with very different variable regions. CBA/N mice also give abnormally low responses to GAC and inulin (22,23). One interpretation of this type of data is that the Lyb5$^-$ subset of B cells uses a different variable region repertoire from the Lyb5$^+$ B cell subset. We used our purified anti-V_H antibody 0-1 to determine whether or not CBA/N mice show normal expression of the J606-GAC subset.

Results of cytoplasmic immunofluorescent staining of spleen cells from CBA/N mice are listed in Tables I and II. The results show that, at least in this case, there does not appear to be a difference in the V_H repertoire used by Lyb5$^+$ and Lyb5$^-$ B cells, since the percentage of J606-GAC positive plasma cells is similar in CBA/N versus four other mouse strains including CBA/J.

Percentage of J606-GAC bearing antibodies in serum. The concentration of J606-GAC immunoglobulin in normal serum was determined by measuring the ability of normal serum to inhibit the binding of ^{125}I-GAC-54 to 0-1 antibody. To quantitate this assay in micrograms, affinity purified GAC-11 was used as a standard. Using this procedure we observed (Table III) that individual BALB/c mouse sera contains from 7-24% J606-GAC antibody while CBA/N sera contains from 3-5%. Although these results are not identical with those seen by fluorescence they do demonstrate that a high percentage of secreted immunoglobulin of both strains is J606-GAC-positive.

Implications of these data. Taken together our findings suggest, as do those of others (9), that the preferential idiotype-isotype associations seen in the response to a variety of different antigens in mice, as well as in other species, are probably manifestations of regulatory mechanisms which act on the B cells rather than by preferential V_H-C_H pairing. Our data also reaffirm the suggestion of Kishimoto et al. that the lack of response in CBA/N mice to a number of TI-2 and TD-2 antigens is not because these mice lack the appropriate variable region genes (25). Therefore the antibody response (or lack of response, as in the case of the CBA/N mouse) to different type antigens may phenotypically appear restricted to particular isotypes or B cell subsets, but these restrictions are probably due to regulatory interactions and not to restriction at the level of immunoglobulin genes.

A number of important observations must be considered in the understanding of this phenomenon. First, it has been well documented that there are different populations of B cells (26-30), which in turn require different activation signals (31,32). This, in combination with the fact that different antigens may be able to provide or elicit some but not others of these signals, may contribute to the idiotype restriction often seen. For instance, idiotype specific T cell help may be needed for the expression of certain idiotypes, but this help may not necessarily be stimulated by certain TI antigens (9). Another consideration may be the antigenic history of the host, which can greatly influence an antibody response (33). This may become particularly important when one considers the CBA/N mouse who possesses only the $Lyb5^-$ subset of B cells which has been shown to be as easily

tolerized (34) by TD antigens as neonatal B cells. In the case of TI-2 antigens, it is clear that although they are able to induce proliferation, they fail to induce differentiation in neonate (35). Therefore, in CBA/N mice, $Lyb5^-$ anti-polysaccharide B cells, which are continuously being stimulated by the presence of common environmental polysaccharide antigens, may become in effect tolerized if they are unable to terminally differentiate. We have suggested that anti-carbohydrate antibody may be encoded by germline genes (11). If this is the case then only clones in which somatic mutations have eliminated the anti-polysaccharide specificities may avoid this functional differentiation arrest. This hypothesis is consistent with the fact that xid mice fail to respond to most polysaccharides but make relatively normal anti-protein responses (28).

In conclusion, it appears that external stimuli must be able to direct isotype restriction.

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TABLE I

Percentage of J606-positive plasma cells from unstimulated mouse spleen

	Percent of J606-GAC bearing plasma cells which costain with anti-isotype					
	Ig	μ	γ_3	γ_1	γ_{2x}	α
BALB/c	69/531 (13%)	27/324 (8%)	30/234 (13%)	22/120 (13%)	39/560 (7%)**[b]	26/221 (12%)
CBA/J	62/501 (12%)	40/219 (18%)	7/67 (10%)	7/91 (8%)	3/42 (7%)	1/29 (3%)
CBA/N	78/709 (11%)	28/156 (18%)*	16/81 (20%)	4/20 (20%)	2/17 (12%)	17/266 (6%)***
C57BL/6	23/225 (10%)	5/120 (4%)	5/83 (6%)	3/101 (3%)	2/59 (3%)	4/122 (3%)
SWR/J	13/240 (5%)	40/334 (12%)	20/239 (8%)	3/225 (1)***	2/322 0.6%)	9/222 (4%)*

[a]The percentage of J606-GAC positive plasma cells in mouse spleen was determined by two-layer cytoplasmic immunofluorescence. The first layer consists of 0-1 coupled to rhodamine, and the second layer consists of goat anti-mouse isotype or anti-immunoglobulin specific reagents coupled to fluorescein. Data is calculated as the $\frac{\text{number of J606-GAc bearing plasma cells}}{\text{number of isotype specific plasma cells}} \times 100$.

[b]*, ** and *** indicate significance from γ_3 at $p<0.05$, 0.02, and 0.001 using Yates-corrected X^2 analysis.

TABLE II

Percentage of J606-GAC positive LPS stimulated plasma cells analyzed by isotype

	Percent of J606-GAC bearing plasma cells which costain with anti-isotype[a]					
	Ig	μ	γ_3	γ_1	γ_{2x}	α
BALB/c	330/2369 (14%)*[b]	125/773 (16%)**	59/554 (11%)	140/1337 (11%)	24/217 (11%)	17/102 (17%)
CBA/J	103/882 (13%)	11/85 (13%)	18/228 (8%)	7/102 (7%)	11/219 (5%)	1/12 (8%)
CBA/N	64/608 (11%)	89/998 (9%)	26/320 (8%)	1/71 (1%)	8/304 (3%)*	2/125 (2%)*
C57BL/6	78/582 (13%)	119/803 (15%)	70/575 (12%)	16/203 (8%)	27/378 (7%)**	9/100 (9%)
SWR/J	59/623 (9%)	82/1064 (8%)	9/134 (7%)	16/170 (9%)	11/262 (4%)	7/163 (4%)

[a]See Table I.

[b]* and ** indicate significant from γ_3 at $p<0.05$ and $p<0.02$ using Yates-corrected X^2 analysis.

TABLE III

Percentage of J606-GAC bearing immunoglobulin in normal mouse serum

Antibody	µg of antibody needed to inhibit 50% binding[a]	$\frac{\text{Amount of J606-GAC antibody}}{\text{Amount of kappa bearing antibody}} \times 100$	
GAC-11	0.007	100	
UPC-10	>15.0	0	
BALB/c NMS #1	0.035	19	
" #2	0.061	11	
" #3	0.028	24	Avg. 12
" #4	0.098	6	
" #5	0.123	6	
" #6	0.101	7	
CBA/N NMS #1	0.129	5	
" #2	0.260	3	
" #3	0.248	3	Avg. 3.6
" #4	0.340	4	
" #5	0.260	5	

[a]Inhibition of the binding of 0-1 to ^{125}I-GAC-54.

IV. SPECIES CROSSREACTIVITY OF THE V_H MARKER J606-GAC AND ITS POTENTIAL SIGNIFIGANCE

The hypothesis, first proposed by Singer and Dolittle (1), that all immunoglobulin structural genes have evolved by gene duplication from a common primordial gene, is now generally accepted. Based on the structural homology of immunoglobulin domains of light and heavy chains from both higher and lower vertebrates, it is assumed that a primordial gene of approximately 110 amino acids first split into two genes one corresponding to the primordial V gene and one corresponding to the primordial C gene. These two genes are assumed to have then evolved independently. This hypothesis is based on the similarities in length, disulfide bridges and tertiary structure of V regions and C region domains, even though their sequences exhibit very few homologies. Again based on sequence homology of light and heavy chain constant regions the next evolutionary step must have been the separation of the ancestral constant region gene into three separate genes corresponding to the constant region genes of heavy, kappa and lambda chains. And finally, the separation of the heavy chain constant region genes into genes for the various subclasses probably occurred most recently. It then must be assumed that the V region genes were under selective pressure to diversify and that the C region gene was under selective pressure to maintain the basic structures necessary to perform functions essential for a successful immune response. As is generally postulated, the main reason for the evolution of an adaptive immune system is to provide an

organism with the ability to distinquish between self and nonself, and in so doing to provide a mechanism for the elimination of harmful invasions of the host. It could be assumed then that the primordial V region genes coded for specificities against common environmental pathogens. An alternative hypothesis and one proposed by Jerne (2) implies that the germline V genes code for specificities against transplantation antigens of the body which then must somatically mutate, to avoid harmful autoimmune reactions, and thus become able to recognize foreign antigens. The recognition of foreign antigens in this case is totally random.

The data presented in the two previous chapters lend support to the former hypothesis. The J606-GAC V_H marker seems to represent an important gene to the mouse based on its high frequency in the B cell pool. Going one step further it is likely that the J606-GAC subset of antibodies must be very useful to the mouse. We wanted to determine how important the J606-GAC subset of antibodies might have been to the evolution of the repertoire of V region genes in general. We attempted to address this question by looking for the presence of the J606-GAC V_H marker in a number of other vertebrate species. The results of cytoplasmic immunofluorescent staining are shown in Table I. These data indicate that the J606-GAC V_H marker has been maintained through the evolution of vertebrate variable region genes. The chicken, being the phylogenetically most distinct species examined, had the smallest percentage of J606-GAC bearing plasma cells, while humans and cats had intermediate levels as compared to mice and rats. These results are what we would have expected. However, we feel that the percentages of J606-GAC seen in these other species poses a strong argument for the

hypothesis that the J606-GAC marker could identify a primitive gene. Also, since a significant portion of the J606-GAC bearing antibodies in the mouse are specific for bacterial carbohydrates these results also strengthen the hypothesis that the primordial V genes probably evolved to recognize environmental pathogens.

TABLE I

PERCENTAGE OF J606-GAC DETERMINANT BEARING PLASMA CELLS FROM FIVE SPECIES

SPECIES	J606-GAC BEARING PLASMA CELLS OUT OF TOTAL NUMBER OF PLASMA CELLS[a]
Mouse spleen (BALB/c)	179/1695 (10.5%)
Rat spleen	21/167 (12.5%)
Cat Bone Marrow	8/508 (1.5%)
Human PWM stimulated PBL	
1	18.625 (3%)
2	5/816 (0.6%)
3	35/960 (3.6%)
4	7/707 (0.9%)
5	45/844 (5.3%)
Chicken spleen	6/1101 (0.5%)

a) Percentages were determined by cytoplasmic immunofluorescence. Cytocentrifuge cell preparations were stained with 0-1 coupled to rhodamine isothiocyanate and counterstained with an appropriate anti-spleen immunoglobulin specific reagent coupled to fluorescein.

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V. ANALYSIS OF A SUBSET OF J606-GAC RANDOMLY SELECTED HYBRIDOMA ANTIBODIES PRODUCED FROM LPS STIMULATED MOUSE SPLEEN CELLS

The possibility that antibody molecules of different binding abilities could be encoded by a single gene segment appears more likely based on recent molecular analyses of mouse variable region genes. The studies with antibodies to phosphocholine, nitrophenyl, and arsonate (1-3) have shown that a large percentage of the antibody response to individual haptens can be encoded by a single V_H gene segment. Therefore, the question can be raised as to whether antibodies reacting with other specificities could be encoded by these particular gene segments. Utilizing our anti-V_H serum, 0-1, as a probe, we have identified a subset of mouse antibodies which appear to represent the mutated products of one V_H gene segment.

In an effort to obtain monoclonal lines secreting J606-GAC antibodies, we stimulated mouse spleen cells with lipopolysaccharide and after four days in culture we fused the cells to the mouse nonsecreting myeloma cell line P3x63Ag8.653 (4). Growing hybrids were screened for the presence of the J606-GAC determinants with anti-V_H serum, 0-1. Out of 576 growing hybrids, 27 were J606-GAC-positive. Subsequently, amplification of ascitic tumors and subsequent chromatographic purification of the immunoglobulin from six of these tumors were performed.

The preliminary N-terminal amino acid sequence (performed in collaboration with Dr. A. Bhown) of one of these six hybridomas, 4-6,D1-3, in comparison with the known sequences of J606 (5) and GAC-9 (6) is

shown in Table I. These data illustrate that out of eight amino acids which are particularly variant among the first 30 amino acids of mouse heavy chain group III antibodies, only one may potentially be different, whereas for any other group III antibody whose sequence has been determined there are at least three differences. Therefore, these data suggest that the variable regions of these three antibodies are likely to be the products of the same or a very similar gene.

In Table II, the preliminary N-terminal amino acid sequences of the light chains of hybridomas, 4-6,D1-3 and 4-9,B1N-2 (performed in collaboration with Dr. A. Bhown) are compared with the known sequence of J606 (5) and GAC-9 (6). These sequences suggest that these four antibodies represent the products of four different light chains.

Table III contains the antigen and idiotypic characteristics of these six antibodies: 4-6,D1-3, 4-9,B1N-2, 4-1,D4-4, 4-7,B3-2, 4-3,C6-13, and 4-1,D3-6. These data demonstrate that none of the six randomly produced hybridomas has specificity for either GAC or inulin although at this point their specificities remain unknown.

Therefore, these data demonstrate that the J606-GAC antibodies are comprised of antibodies known to bind at least three different antigens even though their variable regions appear similar enough to be encoded by the same gene segments.

TABLE I

N-TERMINAL AMINO ACID SEQUENCES OF J606-GAC DETERMINANT BEARING HEAVY CHAINS

Hybridoma +/or Myeloma Antibody	1 10 20 30
J606	EVKLEESGGGLVQPGGSMKLSCVASGFTFS[a]
GAC-9	_____[b]
4-6,D1-3	_____A_____
4-9,BIN-2	?[c]

a) N. Johnson, J. Slankard, L. Pauland, L. Hood. 1982. J. Immunol. 128:302.

b) R. Perlmutter, personal communication.

c) Preliminary evidence indicates this chain may be blocked.

TABLE II

N-TERMINAL AMINO ACID SEQUENCES OF LIGHT CHAINS

FROM J606-GAC DETERMINANT BEARING HYBRIDOMA ANTIBODIES

Hybridoma +/or myeloma Antibody	1 10 20
J606	DVQMIQSPSSLSASLGDTVTMTCQA[a]
GAC-9	-IV T AAP VPVTP ES STS AS[b]
4-6,D1-3	-I—T-TT— ?———?—T???-
4-9,BIN-2	-I—T—?—?—?——????-ER-?

a) N. Johnson, J. Slankard, L. Paul and L. Hood. 1982. J. Immunol. 128:302.

b) R. Perlmutter, personal communication.

TABLE III

SUMMARY OF ANTIGEN BINDING AND IDIOTYPIC ANALYSIS OF SIX J606-GAC DETERMINANT-BEARING HYBRIDOMA PROTEINS[a]

	Antigen Binding		Idiotype
Antibody	GAC[b]	Inulin	V_H-J606-GAC
J606	-	+	+
GAC-9	+	-	+
4-6,D1-3	-	-	+
4-9,BIN-2	-	-	+
4-1,D4-4	+/-[c]	-	+
4-7,B3-2	-	-	+
4-3,C6-13	-	-	+
4-1,D3-6	-	-	+

a) Hybridoma antibodies prepared by mitogenically stimulating mouse spleen cells with 50 μg/ml LPS and then fusing with the nonsecreting myeloma P3x63Ag8.653 screening by a radioimmune direct binding assay and by cytoplasmic immunofluorescence with purified anti-V_H serum, 0-1.

b) Streptococcal Group-A-carbohydrate.

c) Binds only 1/10 as well as any anti-GAC hybridoma antibody previously analyzed.

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VI. SUMMARY AND CONCLUSIONS

In the five preceding chapters the preparation and characterization of the anti-V_H serum, 0-1, has been described and was shown to identify a distinct subset, J606-GAC, of mouse heavy chain variable region group III antibodies, including all of the anti-inulin myelomas and the majority of the anti-group A carbohydrate hybridomas tested. In addition, this antiserum was shown to recognize the immunoglobulin determinants on 5-15% of mouse splenic B cells. This frequency of occurrence, of J606-GAC bearing B cells, did not change whether the spleens of neonatal, adult or germfree mice were being examined. The distribution of the J606-GAC marker on different heavy chain isotypes was also analyzed, and there appeared to be no preferential associations. In addition, J606-GAC determinant-bearing B cells were found in vertebrates other than mice. These observations provided a basis for discussion of antibody diversity in regard to 1) the potentiality of preferential V_H-C_H pairing, 2) the relative contribution somatic mutations make to the expressed repertoire and 3) the specificity of antibodies directly encoded by germline variable region genes.

The restriction of certain idiotypes and antigen reactivities to IgG antibodies of certain subclasses has suggested to others that there may be preferential pairing of V_H gene segments to certain C_H gene segments. The experiments in Chapter III demonstrated that in the case of the J606-GAC marker there was no preferential association with any particular heavy chain isotype. These data, together with the observa-

tions (Chapters II and V) that the antibodies bearing the J606-GAC marker are probably coded for by one or or a small number of V_H gene segments, indicate that preferential V_H-C_H pairing is unlikely. Therefore, restricted isotype expression of idiotypes and specificities must be due to regulatory mechanisms that come into play after V-D-J-C translocation. Likely control mechanisms leading to isotype restriction might include 1) isotype restricted help and/or suppression; 2) antigen properties such as the ability to activate the alternative complement pathway, which may preferentially expand clones of particular isotypes; and 3) variable stimulation requirements of different B cell subsets.

Another point of discussion which these analyses have raised is that of the relative contribution somatic mutations make to antibody diversity. These studies in a very different way suggest that somatic mutation of the variable region gene segments during B cell differentiation is a major generator of antibody diversity. The recent molecular studies up to this time have shown that somatic mutation of a single V_H gene segment causes changes in individual idiotypes on antibodies of a single binding specificity (1-3). Our data suggest that somatic mutation probably also accounts for changes in the binding specificities. This implication was based on the high frequency of J606-GAC B cells and antibodies (Chapters II and III) and my finding that antibodies of at least three different binding abilities have sequences similar enough to suggest that they may be the products of one V_H gene segment (Chapter V). Precedence for somatic mutations causing changes in binding specificities has been provided by the <u>in vitro</u> experiments of Cook et al. (4).

There are two alternate interpretations for our data. 1) We may not be looking at the products of a single gene but rather a family of similar genes and 2) changes in the antigen binding specificities may solely be due to different D_H or J_H gene segments, or different light chains. Both of these alternative explanations are very likely and it is impossible to conclusively distinquish between these three possibilities at present. However, answers to some very important questions can be addressed regardless of which possibility turns out to be correct.

The first possibility, that all J606-GAC-bearing antibodies are encoded by one V_H gene segment, brings up the question of the extent to which somatic mutations contribute to antibody diversity. However, if the second possibility, that the J606-GAC bearing antibodies are encoded by a family of similar genes turns out to be correct, then the 0-1 antisera will provide a unique system to examine the majority of the products of a gene family as a whole. It would then be possible to determine 1) the amount of gene recombination that occurs within a V gene family and 2) the relative contribution D and J gene segments make to the binding site. If the third possibility, that different D_H, J_H, or light chains are the cause of the specificity differences, is correct, we would then have a good model for studying V, D, J preferential pairing and the possibility of potential heavy and light chain preferences. Therefore, regardless of which hypothesis is correct, the J606-GAC subset of antibodies provides an interesting model system to study.

Finally, the possibility that germline genes code for antibodies which are important for the organism's protection against possible

pathogens was suggested in chapters I and IV. This hypothesis has the advantage of clarifying the evolutionary significance of J606-GAC antibodies. As my final point, I would like to discuss the possibility that germline genes initially evolved as a protection against pathogens and that they have been maintained, and gene families established, because of the need for regulatory mechanisms. Therefore, in light of the identification of crossreactive V_H or V_L idiotypes such as the ones described by Bosma et al. (5) and Nahm et al. (6) and our own data on the V_H-J606-GAC marker, it would be interesting to reconsider the nature of idiotypic regulatory processes. The questions which could be asked are, what role, if any, in idiotype-anti-idiotype networks do these crossreactive variable region idiotypes play and what effect would these interactions have on an immune response. It is immediately apparent that a network based on a broad idiotype-anti-idiotype network would be fundamentally different from one based on very specific idiotope-anti-idiotope reactions. The primary difference would be that in the former case a greater portion of the repertoire would have the potential to be involved in each network pertubation. Secondly, assuming that the corresponding anti-idiotype is present in as high a frequency as the complementary crossreactive idiotype, it is then likely that regulation could exist prior to and/or without the addition of antigen. In the case of specific idiotope-anti-idiotope interaction, it is difficult to envision how regulation could occur without 1) addition of antigen to expand the idiotope specific clones or 2) exogenous addition of idiotope to expand the anti-idiotope specific clones. Therefore, it would seem likely that both types of regulatory mechanisms should be

should be present. The specific network could fine-tune antigen specific responses and the broadly cross-reactive network could through continuous clonal stimulations provide a system through which diversity could constantly be generated. The necessity of maintaining variable region genes which encoded specificities used in protection against a common pathogen may have been the impetus for the creation of genes encoding anti-idiotypic specificities. The internal stimulation of these complementary structures would then be one mechanism through which an organism could provide enough constant proliferation to allow modifications to occur, which would in turn be maintained through the cross-reactive network.

Therefore, rather than consider these crossreactive determinants as being mitogenic, as has Coutinho (7), it may be more reasonable to consider them as regulatory, as have Paul and Bona (8), and as diversifying.

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