IDIOTYPE EXPRESSION IN THE IMMUNE RESPONSE TO THE HAPTEN TRINITROPHENYL

by

Peter G. Foiles

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

May 1983

APPROVED:

[redacted]

(Professor In Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

TABLE OF CONTENTS

LIST OF TABLES

PAPER I

LIST OF FIGURES

PAPER I

STATEMENT OF QUESTION

Jerne was the first to present a model of how idiotypes could be used in the regulation of the antibody response (1). Since then a large amount of experimental data has been gathered which supports the basic premise that the idiotypes are not expressed at random but are regulated in some fashion. Examples of this are responses to the haptens phospholcholine (PC), arsonate (Ars) and (4 hydroxy-3 nitrophenylacetyl (NP), where a substantial proportion of the response consists of antibody of a single idiotype (2-4). Dominance of an antibody response by a single idiotype is an exception since most antibody responses are heterogeneous with respect to idiotype. The specific examples listed above and similar systems have become models for studying the regulation of idiotype expression since their large representation in the response makes them easy to study, the assumption being that whatever regulatory mechanisms are used in these responses would be representative of those used in all responses. In the work for this thesis I have used the response to the hapten trinitrophenyl (TNP), which does not have a dominant idiotype, to approach the question of idiotype regulation. This approach was made possible by the use of hybridoma technology which allows one to select a panel of individual idiotypes from a diverse response.

This approach allowed us to ask whether the expression of idiotype is regulated at the level of antibody affinity, genetics, or cellular mechanisms.

LITERATURE REVIEW

Idiotypes are antigenic determinants on antibody molecules which are unique to antibodies of a given specificity and are therefore associated with the variable region of the antibody molecule. Jerne has proposed an idiotype network theory (1) which states that idiotypic determinants can be used as recognition points for regulatory control mechanisms during an immune response. In the years since this theory was proposed a growing body of experimental evidence has accumulated in its support.

The clinical relevance of idiotypes is apparent from a number of different studies. A spontaneous auto anti-idiotype recognizing the Coombs Positive autoantibodies of the NZB mouse has been described (5). A naturally occurring autoimmune thyroiditis in rats has been treated with low dose irradiation and anti-idiotype (6) and an experimental autoimmune nephritis can be blocked with anti-idiotype (7). Antibodies to the acetycholine receptor in patients with myasthenia gravis have been found to have a common idiotype (8). Anti-idiotypic antisera have also been used to follow tumor load in lymphoma patients (8) and to enhance immunity to trypanosomes (10). These kinds of studies indicate that an understanding of idiotypic regulations may have important clinical applications in the future.

As mentioned above the expression of idiotype can be regulated at one or more of three levels: antibody affinity, molecular mechanisms and/or cellular mechanisms. I shall review each of these starting with antibody affinity.

Since many idiotypic determinants are in or near the antibody combining site they can serve as genetic markers for those sites. It is possible then that a given idiotype is expressed in a response simply because it is associated with a high affinity binding site. This possibility is easily tested by determining the antibody affinity of a dominant idiotype and comparing it to the affinities of non-dominant idiotypes. Studying the T15 idiotype, which is associated with the PC binding myeloma protein of Tepc-15, Rodwell et al. showed that there is affinity maturation in the anti-PC response with the secondary non T15 IgG antibodies having higher affinity than the primary T15 IgM antibodies (11). They argue for an antigen driven selection mechanism for high affinity antibodies but do not explain the T15 dominance seen in the primary response. Briles et al. compared T15 positive hybridomas to ones expressing M603 or M511 idiotypes in the ability to protect mice from a S. pneumoniae challenge and found the T15 idiotype to be optimal (12). They proposed that this was a force in T15 being selected as the dominant anti-PC idiotype. But Briles and Davie have also shown that commitment

to a specific spectrotype of anti-carbohydrate antibody, as defined by isoelectric focusing, can occur before the antigen is seen (13). These data would argue that antibody affinity alone is not the major regulator of idiotype expression.

The second possible level of idiotype regulation is molecular. At this level the expression could be regulated by the heavy chain allotype, Ir genes, isotype, or the simple presence or absence of the proper variable region gene in the genome. An early explanation of the dominance of the T15 idiotype in the response of the BALB/c mouse to phosphocholine was that in these mice T15 was the only variable region gene available for anti-PC antibody. This was shown not to be the case in two ways. First Gearheart et al. looked at the heterogeneity of anti-PC precursors and found that T15 positive precursors were only a small fraction of the total (14). Second, it is possible to suppress the expression of a given idiotype by administering anti-idiotypic antibody from birth. When Augustin and Cosenza used this method with T15 they were able to get substantial anti-PC responses that were T15 negative (15). Similarly, Berek et al. were able to generate PC specific hybridomas from mice neonatally supressed with anti-T15 idiotype (16). These studies were interpreted to mean that BALB/c mice have genes which code for non T15 PC binding antibodies but for whatever reason they

are not normally used.

This conclusion is strengthened by the observation that many idiotypes appear to be germline genes present throughout a species and even across species lines. Peare and Claflin found T15 positive antibodies in a number of inbred strains of mice and even in wild mice (17). The only difference noted was a somewhat greater heterogeneity of the T15 antibodies in strains other than BALB/c. Crews et al. looked at the amino acid sequence of nineteen monoclonal anti-PC antibodies and found all could have derived from a single gene by somatic mutation (18). In addition Estess et al., using restriction fragment analysis, concluded that the major crossreactive idiotype of the anti-arsonate response was derived from at most two genes (19). Sharing of amino acid sequences in the first hypervariable region between mouse and human anti-PC antibodies has been shown by Riesen et al. (20). Petit et al. have described a major idiotype in the response of rats to GAT (21). The idiotype is found on a number of inbred strains of rat as well as mouse and guinea pig. It appears that dominant idiotypes can be coded for by germline or at least closely related genes which are present throughout the species and even across species but that these are not necessarily the only genes that can be used.

The genetic control of idiotype expression is most often found to be linked to the immunoglobulin genes

themselves, ie., the heavy chain allotype locus (IgH). This is most clearly shown in the anti-NP response of the C57BL/6 mouse where the response is dominated by a single idiotype, NPb, which requires the lambda light chain, the IgH b allotype and is heteroclitic. A second idiotype, Npa, is a minor idiotype of the C57BL/6 response but is a major idiotype in the response of BALB/c mice which express the IgH a allotype (22.) Anti-NP hybridoma antibodies were isolated from C57BL/6 and BALB/c mice (23). Those from C57BL/6 (NPb) could be placed into six groups based on their idiotypes determined serologically. One of the group showed crossreactivity with the NPa positive hybridomas from the BALB/c mouse. In addition the NPa monoclonal antibodies were much more homogeneous consisting of a single serological group. Their findings suggest that the NPa and a portion of the NPb response are derived from a single germline gene. These investigators are studying DNA sequences of the genes to clarify this point.

Similar findings linking allotype and idiotype were made in the azophenylarsonate (Ars) response where the major idiotype (CRIa) in the A/J mouse is linked to the IgH a locus (24). CRIa can be expressed on more than one allotype but restriction to certain allotypes has been shown in allotype congenic mice (25). A second major idiotype defined in the BALB/c mouse (CRIc) is also allotype linked and is similar to a minor idiotype found in the A/J

response (26, 27).

Allotype congenic mice were also used to show that the PC binding idiotype M511 was controlled by the IgH locus but that PC binding idiotypes T15 and M603 are not (28). These and other studies have clearly established that there is often, but not always, a strong association between IgH allotype and idiotype implying that allotype alone is not the only regulator of idiotype expression.

Another point of debate on idiotype expression has been the location of the idiotypic determinant: heavy chain, light chain or both. The method for determining chain association or determinants is to separate the heavy and light chains and test directly which expresses the idiotype. In the first anti-Ars response the CRI was found to be on the heavy chain (29). In keeping with this, it was found that CRI negative antibodies could use the same light chain as CRI positive molecules and still not express the CRI (30). Metzger and Roux, looking at rabbit anti-a1 allotype idiotypes also found that the dominant idiotypic determinant was on the heavy chain (31). The role of the light chain is not unimportant, however. For example, Roland and Cazanave, also looking at rabbit anti-allotype idiotype, found both the heavy and light chain to be required for expression of the idiotype (32).

The NPb idiotype system provides a unique system

to study the role of light chain in the expression of idiotypic determinants. This idiotype is only found on antibodies with lambda light chains (2) so when SJL mice, which cannot make lambda chains, were tested they did not express the NPb idiotype (33). However, the NPb idiotype is still probably a heavy chain determinant since the heteroclitic nature of the NPb idiotype is dependent on the proper heavy chain for expression (35). SJL mice also use a different heavy chain. This may be the result of a requirement for a particular heavy and light chain combination in order to form a functional binding site (34). So, even though most idiotypes are physically located on the heavy chain, certain light chains may be required to give the proper conformation to the molecule. This phenomenon has also been seen in the anti-NP and anti-TNP responses (36, 37).

While it is clear that Ir genes can regulate the response to certain antigens, their role in idiotype expression is not clear. Ju et al. found that in the Ir gene regulated anti-GAT response similar idiotypes were expressed in responder and non-responder (actually low responder) strains (38). However, Beckoff et al. found in the anti-DNP response in guinea pig, which is under Ir gene control, that responder strain T cells were required for expression of the idiotype (39). This discrepancy could be explained by the observation of Asano et al. that Ir genes affect different B cell subpopulations

differently (40). In any event, if Ir genes do play a role in idiotype expression it may be through the action of regulatory T cells which will be discussed next.

T cells are the most likely cellular regulators of idiotype expression. These cells have been divided into helper and suppressor populations. Helper cells can be further divided, as Janeway et al. done, into those cells which interact with B cells through an antigen bridge (Th_1) and those which recognize isotype, allotype or idiotype on B cells (Th_2)(46). The Th_2 cell has been the most extensively studied in terms of idiotype regulation. Bottomly has proposed that the Th_2 cell requires prior exposure to the immunoglobulin determinant in order to function. This was demonstrated in two systems. The first is the CBA/n mouse, which lacks the ability to develop a normal anti-PC response, though it can make IgE anti-PC antibody (41). Bottomly and Mosier showed that this mouse also lacked Th_2 cells specific for the T15 idiotype (42). In addition, mice which have been treated from birth with anti mu chain antibodies do not have Ig positive cells and do not have Th_2 cells (43). MHC reactive T cells were able to help in an anti-PC response but the response was not T15 idiotype positive (44). Th_2 cells have been demonstrated in the anti-DNP, anti-Ars and anti-staphylococcal nuclease responses (45-47).

However, Bottomly and Mosier's contention that

CBA/n mice lack T15 specific Th_2 cells has been seriously questioned by a number of investigators. Quintans et al. have shown in vivo (48) and Quintans et al., Kenny et al. and Teal have shown in vitro (49-51) that adequate T cell help exists in CBA/n mice to give T15 positive responses. These data do not invalidate the existence of Th_2 cells but do question the necessity for prior exposure of these cells to immunoglobulin. The data also strengthen the argument that the lack of an IgM anti-PC response in CBA/n mice is due to a B cell defect (discussed below).

There are two types of suppressor T cells which are involved in the regulation of idiotype: those which bind antigen and share idiotypic determinants with B cells, and those which recognize the idiotype directly. Idiotype positive suppressor cells have been demonstrated in a number of systems (16, 52-55). The administration of anti-idiotype can either enhance or suppress the function of these cells, depending on the conditions of the experiment (56-59).

T supressor cells recognizing idiotype directly have also been well documented (60-63). Some investigators have found these cells to be functionally restricted by MHC and/or allotypic determinants (64, 65), while others have not (61).

It should also be mentioned that T cells involved in strictly cellular responses such as DTH can also be

regulated by idiotypic determinants present on their surface. Thomas et al. and Sy et al. have been able to induce DTH reactions (66, 67), and in addition Sy has induced DTH suppressor cells with anti-idiotype (68).

A second cellular mechanism which may affect the expression of idiotype is the existence of functionally distinct B cell subpopulations. Antigens differ in their requirement for T cell help (69-71). Functionally different B cells respond when antigens differing in their T cell dependency are used (72-74). These cells can be physically separated (75) and differ in their susceptibility to tolerance induction (76, 77) and drug sensitivity (78, 79). They also appear at different times during ontogeny (80, 81) and even differ in the memory respone (82).

The CBA/n mouse provides an interesting model to study the role these B cell subpopulations play in idiotype expression. These mice have an x-linked immune defect which prevents them from responding to certain thymus independent antigens (TI-2)(83, 84), but not to others (TI-1) (85, 86). On the basis of their surface immunoglobulin CBA/n B cells appear to be immature (90-91). In addition, these mice do not respond to a primary exposure to PC antigens (87-89) but can be made to respond at low levels with predominantly T15 negative antibody on repeated stimulation and/or the use of LPS as a carrier (92-94). Defects in isotype distribution have also been found (90-91) with the

lack of IgG3 expression possibly being the result of a missing T cell population (51).

In addition to the different B cells which respond to TI and TD antigens, virgin and memory B cells also differ in expression of idiotype. In the responses to Ars, NP and PC, the idiotype dominance seen in the primary response is lost (95-97), although in the memory response dominance by the primary idiotype may persist among certain isotypes (97).

As is evident by the above discussion, the expression of idiotype is a highly complex process which can be affected at many different levels. To help further our understanding of this process, I have examined the expression of idiotypes found in the anti-TNP response at each of these levels. To accomplish this, I have used hybridoma technology similar to studies in other systems (18, 96, 98, 101). To correlate binding specificity with idiotype expression, the binding characteristics of the hybrids were determined in detail. Genetic influences were tested by examining several mouse strains which differ at both IgH and MHC loci. Cellular influences were studied by comparing idiotype in TI and TD responses, both in CBA/n mice and in mice which had undergone graft-vs-host reaction which has been shown to affect B cell subpopulations differentially (99, 100).

The availability of these hybridomas allowed me

to ask whether hapten inhibition of hemolytic plaques or binding in an ELISA were valid measures of antibody affinity. These methods were developed to overcome certain problems with the classical method of determining antibody affinity, that of equilibrium dialysis (102, 103). Equilibrium dialysis requires a relatively large amount of antibody for the analysis, making it difficult to analyze antibody produced in in vitro assays. The above mentioned methods do not have this disadvantage; however, their validity has been questioned. North and Askonas compared the affinity of antibody produced by cells cloned in splenic fragments in two different systems: by a modified Farr technique and by hapten inhibition of hemolytic plaques (104). They found that the two methods were not in complete agreement. On the other hand, theoretical analysis of the hapten inhibition systems has shown that under the proper conditions these methods should give reliable results (105, 106). And inhibition of plaques, ELISA or radioimmune assays have been used by a number of investigators (107-109).

The availability of cloned cell lines producing defined antibodies made it possible to compare directly, affinity measurements made by the plaque inhibition, ELISA and equilibrium dialysis assays. When this comparison was made it was found that the inhibition methods did not agree with equilibrium dialysis but did reflect, reliably, large

differences in affinity.

In addition to the research on idiotype expression, I have studied the population of B cells which is able to form colonies in soft agar. This population of cells overlaps with the mature B cells which are not present in the CBA/n mouse (110). The defect in the CBA/n mouse has been linked to a single gene on the X chromosome (Xid) so the lack of colony forming cells can be used as a phenotypic marker for this gene. Precisely which cell type gives rise to colony forming cells is not clear. The frequency of colony forming cells varies with the type of mitogen used, and when mixtures of mitogens are used the frequencies of colony forming cells appear additive (111). This suggests that these cells are heterogeneous with respect to mitogen stimulation. In addition, the surface phenotype of colony forming cells varies, depending on their developmental state. Colony forming cells from fetal and newborn tissues are both IgD and Ia negative, while colony forming cells from adult tissues are positive for both of these markers (112-113).

I have used colony forming cells to monitor the Xid gene in two situations. We have shown that the induction of graft-vs-host disease can restore response to TNP-ficoll (TI-2 antigen) in CBA/n F_1 male mice (114). In this study I found that colony forming cells were not restored by this procedure, indicating that the Xid defect

is only partially reversible by this procedure. It was shown previously that increased levels of colony forming cells occur in mice with a spontaneous autoimmune disease (NZB) and that the presence of the Xid gene retards the development of autoimmunity (115-117). In a second collaborative study (118) we showed that the Xid gene was also able to prevent the development of spontaneous autoimmune disease in BXSB mice. These mice were derived from a cross between SB/Le and C57BL mice and are characterized by a lupus-like autoimmune disease which is more severe in male mice. We found that even though these mice did not develop autoimmune disease in the presence of the Xid gene, colony forming cells were not increased, suggesting complex effects of the Xid gene on immune cells involved in autoimmune disease.

REFERENCES

1. Jerne, N. K. 1974. Towards a network theory of the immunce system. Ann. Init. Pasteur. 1250:373.

2. Jack, R. S., T. Imanishi-Kari, and K. Rajewsky. 1977. Idiotypic analysis of the response of C57Bl/6 mice to the (4-hydroxy-3-nitrophenyl) acetyl (NP) group. Eur. J. Immun. 8:559.

3. Claflin, J. L., R. Leiberman, and J. M. Davie. 1974. Clonal nature of the immune response to phosphorylcholine II. Idiotypic specificity and binding characteristics of anti-phosphorylcholine antibodies. J. Immunol. 112:1747.

4. Keuttner, M. G., A. Wang, and A. Nisonoff. 1972. Quantitative investigations of idiotypic antibodies. IV. Idiotypic specificity as a potential genetic marker for the variable region of mouse immunoglobulin polypeptide chains. J. Exp. Med. 135:579.

5. Cohen, P. L., and R. A. Eisenberg. 1983. Anti-idiotypic antibodies to the Coombs antibody in NZB F_1 mice. J. Exp. Med. 156:173.

6. Zanetti, M., and P. E. Bigazzi. 1981. Anti-idiotypic immunity and autoimmunity. I. *In vitro* and *in vivo* effects of anti-idiotypic antibodies to spontaneously occurring autoantibodies to rat thyroglobulin. Eur. J. Immun. 11:187.

7. Neilson, E. G. and G. M. Phillips. 1982. Suppression of interstitial nephritis by auto anti-idiotypic immunity. J. Exp. Med. 155:179.

8. LeFevert, A. K., R. W. James, C. Alliod, and B. W. Falp. 1982. A monoclonal anti-idiotypic antibody against anti-receptor antibodies from myasthenia sera. Eur. J. Immun. 12:790.

9. Hatzubui, A., D. G. Maloney, and A. Levy. 1981. The use of a monoclonal anti-idiotype antibody to study the biology of a human B cell lymphoma. J. Immun. 126:2397.

10. Sacks, D. L., K. M. Esser, and A. Sher. 1982. Immunization of mice against African trypanosomiasis using anti-idiotypic antibodies. J. Exp. Med. 155:1108.

11. Rodwell, J. D., P. J. Gearheart, and F. Karusch. 1983. Restriction in IgM expression. IV. Affinity analysis of monoclonal antiphosphorylcholine antibodies. J. Immun. 130:313.

12. Briles, D. E., C. Forman, S. Hudak, and J. L. Claflin. 1982. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against S. pneumoniae. J. Exp. Med. 156:1177.

13. Briles, D. E., and M. J. Davie. 1980. Clonal nature of the immune response. II. The effect of immunization on clonal commitment. J. Exp. Med. 152.151.

14. Gearheart, P. J., N. H. Sigal, and N. R. Klinman. 19745. Heterogeneity of the BALB/c anti-phosphorylcholine response at the precursor cell level. J. Exp. Med. 141:56.

15. Augustin, A., and H. Cosenza. 1976. Expression of new idiotype following neonatal idiotypic suppression of a dominant clone. Eur. J. Immunol. 6:477.

16. Berek, C., M. H. Schreier, C. L. Sidman, J. C. Saton, H. P. Hocker, and H. Cosenza. 1980 phosphorylcholine-binding hybridoma protein of normal and idiotypically suppressed BALB/c mice. I. Characterization and idiotypic analysis. Eur. J. Immun. 10:258.

17. Pease, L., and J. L. Claflin. 1981. Clonal regulation in the response to phosphorylcholine. II. Heterogeneity among T15 idiotype positive antibody in inbred and wild mice. Eur. J. Immunol. 11:662.

18. Crews, S. Griffen, J., H. Huang, K. Calame, and L. Hood. 1981. A single V_H gene segment encodes the immune response to phosphorylcholine. Somatic mutation is correlated with the class of the antibody. Cell. 25:59.

19. Estess, P. F. Otani, E. C. B. Miller, J. D. Capra, and P. W. Tucker. 1982. Gene rearrangements in monoclonal A/J anti-arsonate antibodies. J. Immun. 129:2319.

20. Riesen, W. F., D. G. Brown, and J. C. Jaton. 1976. Human and murine phosphorylcholine binding immunoglobulin. Conserved subgroup and first hypervariable region of heavy chains. Proc. Nat. Acad. Sci. 73:2096.

21. Petit, C., M. Silbert, O. Somme, L. Teckerg, J. C. Magic, M. E. Dorf, and J. Theze. 1982. Analysis of major anti-idiotype associations with anti-GAT antibodies. Molec. Immun. 19:1139.

22. Karjalainen, K. 1980. Two major idiotypes in mouse anti-(4-hydroxy-3-nitrophenyl) acetyl (NP) antibodies are controlled by allelic genes. Eur. J. Immun. 10:132.

23. White-Scharf, M. E., and T. Imanishi-Kari. 1982. Crossreactivity of the NP^a and NP^b idiotypic responses of BALB/c and C57Bl/6 mice to (4-hydroxy-3-nitrophenyl) acetyl (NP). Eur. J. Immun. 12:935.

24. Pawlak, L. L., E. B. Mushinski, A. Nisonoff, and M. Potter. 1973. Evidence for linkage of the IgCH locus to a gene controlling the idiotypic specificity of anti-p-azophenylarsonate antibodies in strain A mice. J. Exp. Med. 137:22.

25. Pawlak, L. L., and A. Nisonoff. 1973. Distribution of a crossreactive idiotypic specificity in inbred strains of mice. J. Exp. Med. 137:855.

26. Brown, A. R., and A. Nisonoff. 1981. An intrastrain crossreactive idiotype associated with anti-p-azophenylarsonate antibodies of BALB/c mice. J. Immun. 126:1263.

27. Brown, A. R., E. Lamoyi, and A. Nisonoff. 1981. Relationship of idiotypes of the anti-p-azophenylarsonate antibodies of A/J and BALB/c mice. J. Immun. 126:1268.

28. Ruppert, V. J., K. Williams, and J. L. Claflin. 1980. Specific clonal regulation in the response to phosphocholine. I. Genetic analysis of the response of a distinct idiotype (M511 id). J. Immun. 124:1068.

29. Milner, E. C. B., and J. D. Capra. 1983. Structural analysis of monoclonal antiarsonate antibodies. Idiotypic specificaties are determined by the heavy chain. Molec. Immun. 20:39.

30. Gill-Pagaris, L. A., E. Lamoyi, A. R. Brown, and A. Nisonoff. 1981. Properties of a minor cross-reactive idiotype associated with anti-o-azophenylarsonate antibodies of A/J mice. J. Immun. 126:75.

31. Metzger, D. W., and K. H. Roux. 1982. A predominant idiotype on rabbit anti $V_H$al allotype antibodies; sharing by both major and minor V_H subgroups. J. Immun. 129(3):1138.

32. Roland, J., and P. A. Cazanave. 1981. Rabbits immunized against b6 allotype express similar anti b6 antibodies. Eur. J. Immun. 11:469.

33. Cramer, M., U. Krawinkel, I. Melchers, T. Imanishi-Kari, Y. Ben-Reslah, D. Givol, and K. Rajewsky. 1979. Isolated hapten binding receptors of sensitized lymphocytes. IV. Expression of immunoglobulin variable regions in (4-hydroxy-3-nitrophenyl) acetyl (NP)-specific receptors isolated from murine B and T lymphocytes. Eur. J. Immun. 9:332.

34. Imanishi-Kari, T., E. Rajavolgyi, T. Takemori, R. S. Jack, and K. Rajewsky. 1979. The effect of light chain gene expression on the inheritance of an idiotype associated with primary anti-(4-hydroxy-3-nitrophenyl) acetyl (NP) antibodies. Eur. J. Immun. 9:324.

35. Stashenko, P., and N. R. Klinman. 1980. Analysis of the primary anti-(4-hydroxy-3-nitrophenyl) acetyl (NP) responsive B cells in BALB/c and $B_{10}D_2$mice. J. Immun. 125:531.

36. Barstad, P., J. Hubert, M. Hunkapiller, A. Goetz, J. Schilling, B. Black, B. Eaton, J. Richards, M. Weigert, and L. Hood. 1979. Immunoglobulin with hapten binding activity: structure function correlation and genetic implications. Eur. J. Immun. 8:497.

37. Zeldis, J. B., W. H. Konigsberg, E. F. Richards, and R. W. Rosenstein. 1979. The location and expression of idiotypic determinants in the immunoglobin variable region. II. Chain location of variable region determinants. Molec. Immun. 16:371.

48. Quintans, J., M. R. Loken, Z. S. Quan, R. F. Deck, and B. Regueiro. 1981. Alteration of clonal profile. II. Studies on the capacity of BALB/c splenic B cells to perpetuate responsiveness to phosphorylcholine and T15 idiotype dominance. Eur. J. Immun. 11:236.

49. Quintans, J., Z. S. Quan, and M. A. Arias. 1982. Mice with the Xid defect have helper cells for T15 idiotype dominant anti-phosphorylcholine primary and secondary plaque forming cell responses. J. Exp. Med. 155:1245.

50. Kenny, J. J., L. S. Wicker, G. Guelde, and I. Scher. 1982. Regulation of T15 idiotype dominance. I. Mice expressing the Xid immune defect provide normal help to the T15 positive B cell precursors. J. Immun. 129:1534.

51. Teale, J. M. 1983. Abnormalities in isotype expression in CBA/n mice due to stimulatory environment rather than a B cell defect. J. Immun. 130:72.

52. Cerny, J., C. Heusser, R. Wallich, O. J. Hammerling, and D. D. Eardly. 1982. Immunoglobin idiotypes expressed by T cells. I. Expression of distinct idiotypes detected by monoclonal antibodies on antigen-specific suppressor T cells. J. Exp. Med. 156:719.

53. Cerny, J., M. J. Caulfield, and M. Siat. 1980. Regulation of the T15 idiotype positive, T-independent immune response to Streptococcus pneumoniae R36a (Pn). I. Specific suppressor T cells induced by Pn in vitro are antigen specific and idiotype positive. Eur. J. Immun. 10:661.

54. Fung, J., and H. Kohler. 1980. Mechanism of neonatal idiotype suppression. II. Alterations in the T cell compartment suppress the maturation of B cell precursors. J. Immun. 125:2489.

55. Sherr, D. H., S. Ju, J. Z. Weinberger, B. Benacerraf, and M. E. Dorf. 1981. Hapten specific T cell responses to 4-hydroxy-3-nitrophenyl acetyl. VII. Idiotype specific suppression of plaque forming cell responses. J. Exp. Med. 153.640.

56. Bernable, R. R., A. Coutinho, P-A. Cazenave, and L. Forni. 1981. Suppression of a recurrent idiotype results in profound alterations of the whole B cell compartment. Proc. Nat. Acad. Sci. 78.6416.

57. Kelso, G., M. Reth, and K. Rajewsky. 1981. Control of idiotype expression by monoclonal anti-idiotype and idiotype-bearing antibody. Eur. J. Immun. 11:418.

58. Eichmann, K. 1974. Idiotype suppression. I. Influence of the dose and the effector functions of anti-idiotypic antibody on the production of an idiotype. Eur. J. Immun. 4:296.

59. Eichmann, K. 1975. Idiotypy suppression. II. Amplification of a supressor T cell with anti-idiotypic activity. Eur. J. Immun. 5:511.

60. Bona, C., and W. E. Paul. 1979. Cellular basis of regulation of expression of idiotype. I. T-suppressor cells specific for MOPC-460 idiotype regulate the expression of cells secreting anti-TNP antibodies bearing 460 idiotype. J. Exp. Med. 149:592.

61. Hirai, Y., Y. Dohi, M-S Sy, M. I. Greene, and A. Nisonoff. 1981. Suppressor T cells induced by idiotype coupled cells functions across an allo-type barrier. J. Immun. 126:2064.

62. Milburn, G. L., and R. O. Lynch. 1982. Immuno-regulation of murine myeloma in vitro. II. Suppression of MOPC-315 immunoglobulin secretion and synthesis by idiotype specific suppressor T cells. J. Exp. Med. 155:852.

63. Abbas, A. K., M. Takaoki, and M. F. Greene. 1981. T lymphocyte mediated suppression of myeloma function in vitro. IV. Generation of effector suppressor cells specific for myeloma idiotypes. J. Exp. Med. 155:1216.

64. Juy, D., D. Primi, P. Sanchez, and P-A. Cazenave. 1982. Idiotype regulation. Evidence for the involvement of IgH-C-restricted T cells in the M460 idiotype suppressive pathway. Eur. J. Immun. 12:24.

65. Speck, N. A., and S. K. Pierce. 1982. Antibody specific immunoregulation is restricted by the major histocompatibility gene complex. Eur. J. Immun. 12:972.

66. Thomas, W. R., G. Morahan, I. P. Walker, and J. A. F. P. Miller. 1981. Induction of delayed type hypersensitivity to azobenzenearsonate by a monoclonal anti-idiotype antibody. J. Exp. Med. 153:743.

67. Sy, M. S., A. R. Brown, B. Benacerraf, and M. I. Greene. 1980. (Antigen and receptor driven regulatory mechanism. I. Induction of suppressor T cells with anti-idiotypic antibodies.) J. Exp. Med. 151:896.

68. Sy, M. S., B. A. Bach, Y. Dohi, A. Nisonoff, B. Benacerraf, and M. I. Greene. 1979. (Antigen and receptor driven regulatory mechanisms. III. Induction of delayed type hypersensitivity to azobenzenearsonate with anti crossreactive idiotypic antibodies. J. Exp. Med. 150:1216.

69. Gershon, R. K., and K. Kondo. 1970. Cell interaction in the induction of tolerance; the role of the thymic lymphocyte. Immunol. 18:723.

70. Andersson, G., and H. Blomgren. 1971. Evidence for thymus independent humoral antibody production in mice against polyvinylpyrolidone and E. Coli lipopolysaccharide. Cell. Immunol. 2:411.

71. Playfair, J. H. L., and E. C. Purves. 1971. Separate thymus dependent and thymus independent antibody forming cell precursors. Nat. 231:149.

72. Quintans, J., and H. Cosenza. 1976. Antibody response to phophorylcholine in vitro. II. Analysis of T dependent and T independent responses. Eur. J. Immun. 6:399.

73. Lewis G. K., and J. W. Goodman. 1977. Carrier-directed anti-hapten responses by B cell subsets. J. Exp. Med. 146:1.

74. Jennings, J. J., and M. B. Rittenberg. 1976. Evidence for separate sub-populations of B cells responding to T-independent and T-dependent antigens. J. Immun. 117:1749.

75. Gorczynski, R., and M. Feldmann. 1975. B cell heterogeneity difference in the size of B lymphocytes responding to T-dependent and T-independent antigens. Cell. Immunol. 18:88.

76. Cambier, J. C., E. S. Vitetta, J. W. Uhr, and J. R. Kettman. 1977. B cell tolerance. II. Trinitrophenyl human gamma-globulin induced tolerance in adult and neonatal murine B cells responsive to thymus dependent and independent forms of the same hapten. J. Exp. Med. 145.778.

77. Tite, J. P., S. Marshal-Clarke, and J. H. L. Playfair. 1978. Differential tolerance of thymus independent and thymus dependent antibody responses. Immunol. 34:337.

78. Galanaud, P., M. C. Crevon, and J. Dormont. 1976. Effect of azothioprine on in vitro antibody responses. Differential effect on B cells involved in thymus dependent and thymus independent responses. Clin. Exp. Immunol. 22:139.

79. Kunkl, A., and G. B. Klaus. 1980. Selective effect of cyclosporin A on functional B cell subsets in the mouse. J. Immun. 125:2526.

80. Sherwin, W. K., and D. T. Rowlands, Jr. 1975. Determination of the hierarchy of humoral immune responsiveness during ontogeny. J. Immun. 115:1549.

81. Howard, J. O., and C. Hale. 1976. Lack of neonatal susceptibility to induction of tolerance by polysaccharide antigens. Eur. J. Immunol. 6:486.

82. Tittle, T. V., and M. B. Rittenberg. 1978. Distinct subpopulations of IgG memory B cells respond to different molecular forms of the same hapten. J. Immun. 121:936.

83. Scher, I., A. Ahmed, D. M. Strong, A. D. Steinberg, and W. E. Paul. 1975. X-linked B-lymphocyte immune defect in CBA/n mice. I. Studies of the function and composition of spleen cells. J. Exp. Med. 141:788.

84. Scher, I., A. D. Steinberg, A. K. Berning, and W. E. Paul. 1975. X-linked B lymphocyte immune defect in CBA/n mice. II. Studies of the mechanism underlying the immune defect. J. Exp. Med. 142:637.

85. Mosier, D. E., I. Scher, and W. E. Paul. 1976. In vitro responses of CBA/n mice; spleen cells with an X-linked defect that precludes immune responses to several thymus independent antigens can respond to TNP-lipopolysaccharide. J. Immun. 117:1363.

86. Mond, J. J., I. Scher, D. E. Mosier, M. Blaise, and W. E. Paul. 1978. T-independent responses in B cell defective CBA/n mice to Brucella abortus and to TNP-conugation of Brucella abortus. Eur. J. Immun. 8:459.

87. Mond, J. J., R. Lieberman, J. K. Inman, D. E. Mosier, and W. E. Paul. 1977. Inability of mice with a defect in B-lymphocyte maturation to respond to phosphorylcholine on immunogeneic carriers. J. Exp. Med. 146:1138.

88. Quintans, J. 1977. The "patchy" immunodeficiency of CBA/n mice. Eur. J. Immun. 7:749.

89. Quintans, J., and R. Benca-Kaplan. 1978. Failure of CBA/n mice to respond to thymus dependent and thymus independent phosphorylcholine antigens. Cell. Immunol. 38:294.

90. Finkelman, F. P., A. H. Smith, I. Scher, and W. E. Paul. 1975. Abnormal ratio of membrane immunoglobulin classes in mice with an X-linked B-lymphocyte defect. J. Exp. Med. 142:1316.

91. Scher, I., S. O. Sharrow, and W. E. Paul. 1975. X-linked B-lymphocyte defect in CBA/n mice. III. Abnormal development of B-lymphocyte population defined by their density of surface immunoglobin. J. Exp. Med. 144:507.

92. Kohler, H., S. Smyk, and J. Fung. 1981. Immune response to phosphorylcholine. VIII. The response of CBA/n mice to PC-LPS. J. Immun. 126:1790.

93. Kenny, J. J., G. Guelde, J. L. Claflin, and I. Scher. 1981. Altered idiotype response to phosphocholine in mice bearing an X-linked immune defect. J. Immun. 127:1629.

94. Clough, E. R., D. A. Levy, and J. J. Cebra. 1981. CBA/n X BALB/c F_1 male and female mice can be primed to express quantitatively equivalent seconary anti-phosphocholine responses. J. Immun. 126:387.

95. Henry, C., and A. Lucan. 1982. The relation of idiotype expression to isotype and allotype in the anti-P-azobenzenearsonate response. Eur. J. Immun. 12:175.

96. Reth, M., G. J. Hammerling, and K. Rajewsky. 1978. Analysis of the repertoire of anti-NP antibodies in C57Bl/6 mice by cell fusion. I. Characterization of antibody families in the primary and hyperimmune response. Eur. J. Immun. 8:393.

97. Chang, S. P., and M. B. Rittenberg. 1981. Immunologic memory to phophorylcholine in vitro. I. Asymetric expression of clonal dominance. J. Immun. 126:985.

98. Mariuzza, R., and M. Strand. 1981. Chemical basis for diversity in antibody specificity analyzed by hapten binding to monoclonal anti (4-hydroxy-3-nitrophenyl) acetyl (NP) immunoglobulin. Molec. Immunol. 18:847.

99. Golding, H., and M. B. Rittenberg. 1980. Differential sensitivity of IgG memory subpopulations to allogeneic thymocytes: positive and negative signals. J. Immunol. 124:1284.

100. Kaattari, S. L., and M. B. Rittenberg. 1982. Conconavalin A supernatent recruits antigen insensitive IgG memory B lymphocyte precursors into an antigen sensitive precursor pool. J. Immunol. 128:720.

101. Kearney, J. F., R. Barletta, Z. Quan and J. Quintans. 1981. Monoclonal vs. heterogeneous anti-H-8 antibodies in the analysis of the anti-phosphorylcholine response in BALB/c mice. Eur. J. Immunol. 11:877.

102. Andersson, P. 1970. Studies on the regulation of avidity at the level of the single antibody cell. The effect of antigen dose and time after immunization. J. Exp. Med. 132:77.

103. Davie, J. M., and W. E. Paul. 1972. Receptors on immunocompetent cells. V. Cellular correlates of the "maturation" of the immune response. J. Exp. Med. 135:660.

104. North, J. R. and B. A. Askonas. 1974. Analysis of affinity of monoclonal antibody responses by inhibition of plaque forming cells. Eur. J. Immunol. 4:361.

105. DeLisi, C. 1976. Hemolytic plaque inhibition: the physical chemical limits on its use as an affinity assay. J. Immunol. 117:2249.

106. Jerne, N. K., C. Henry, A. A. Nordin, H. Fuji, A. M. C. Kovos and I. Lefkovitz. 1972. Plaque forming cells, methodology and theory. Transpl. Rev. 18:130.

107. Adorini, C., C. Pini, G. D'Agostano, G. DiFelice, C. Mancini, C. V. Pozzi, S. Vietri and G. Doria. 1981. Induction of agobenzenearsonate (ABA) specific helper and suppressor T cells and in vitro evaluation of their activities in the antibody response to T-dependent ABA-protein conjugates. J. Immunol. 127:1018.

108. Chang, S. P., M. Brown, and M. B. Rittenberg. 1982. Immunologic memory to phosphorylcholine. II. PC-KLH induces two antibody populations that dominate different isotypes. J. Immunol. 128:702.

109. Kresina, R. F., S. M. Rosen, and A. Nisonoff. 1982. Degree of heterogeneity of binding specificity of antibodies to the phenylarsonate group that share a common idiotype. Molec. Immunol. 19:1433.

110. Kincade, P. W. 1977. Defective colony formation by B lymphocytes from CBA/n and C_3H/HeJ mice. J. Exp. Med. 145:249.

111. Kincade, P. W., P. Ralph, and M. A. S. Moore. 1976. Growth of B lymphocyte clones in semisolid cultures is mitogen dependent. J. Exp. Med. 143:1265.

112. Kincade, P. W., C. J. Paige, R. M. E. Parkouse, and O. Lee. 1978. Characterizaton of murine colony forming B cells. I. Distribution, resistance to anti-immunoglobulin antibodies and expression of Ia antigens. J. Immunol. 120:1289.

113. Scott, D. W., J. E. Layton, and G. R. Johnson. 1978. Surface immunoglobulin phenotype of murine spleen cells which form B cell colonies in agar. Eur. J. Immunol. 8:286.

114. Golding, H., P. G. Foiles, and M. B. Rittenberg. 1982. Partial reconstitution of TNP-ficoll responses and IgG3 expression in Xid mice undergoing graft-vs-host reaction. J. Immunol. 129:2641.

115. Taurog, J. E., H. M. Moutsopoulos, Y. J. Rosenberg, T. M. Chused, and A. P. Steinberg. 1979. CBA/n X linked B cell defect prevents NZB B cell hyperactivity in F_1 mice. J. Exp. Med. 150:31.

116. Oshugi, Y., M. E. Gershwin, and A. Ahmed. 1981. Study of congenitally immunologically mutant New Zealand mice. V. B cell function of NZB Xid mice. J. Immunogenet. 8:129.

117. Kemp, J., L. Dohrman, A. Jayawardena, B. Huber, and R. K. Gershon. 1980. B cell subsets in autoimmunity: an autoimmune hemolytic response to mouse erythrocin is moderated by the CBA/n defect and anti-lyb-3 antisera. Cell. Immunol. 53:84.

118. Golding, B., H. Golding, P. Foiles, and J. Morton. 1983. CBA/N X-linked defect delays expression of the Y-linked accelerated autoimmune disease in BXSB mice. J. Immunol. 130:1043.

Monoclonal Antibodies Used to Compare Three Methods of Determining Antibody Affinity

Peter G. Foiles, Ian Todd,
and Marvin B. Rittenberg

Department of Microbiology and Immunology,
Oregon Health Sciences University
Portland, Oregon 97201

ABSTRACT

Four anti-TNP hybridoma cell lines are characterized in terms of antigen binding. Three of the hybrids are derived from secondary responses and are IgG_1 K and the fourth is from a primary response and is a IgM K. By comparing measurements of antibody affinity made by equilibrium dialysis, ELISA, and hemolytic plaque inhibition experiements we were able to test directly if ELISA and hemolytic plaque inhibition are valid measures of antibody affinity. By equilibrium dialysis the K_o ranged from 8.5×10^{-8} to 2.0×10^{-8} for the IgG monoclonals and was 6.2×10^{7} for the monoclonal IgM. The values for K_o estimated from plaque inhibition or ELISA data were appreciably lower, giving values for the binding constant that were one to two orders of magnitude less than had been determined by equilibrium dialysis. Depite these quantitative differences the relative order of binding was essentially similar by all three methods with the IgM monoclonal having the lowest avidity for antigen.

INTRODUCTION

Inhibition of hemolytic plaques by monovalent antigen was developed as a method of determining the avidity of antibody produced by a population of antibody-producing cells (Andersson, 1970; Davie et al., 1972; Jerne, et al., 1972). The validity of this method has been in dispute for several years. North and Askonas using antibody from cells cloned in splenic fragments found poor correlation between avidity measurements made by inhibiting hemolytic plaques and those made on the secreted antibodies by a modified Farr technique (North et al. 1974). DeLisi, on the other hand, in a careful theoretical analysis argued that under the proper experimental conditions of inhibitor and antibody valence, differences in the 50% inhibition points should reflect differences in affinity (DeLisi, 1974, 1975, 1976), and this method has been used in a number of investigations to determine affinity differences (Adorini et al., 1981; Goidl et al., 1975; Miller et al., 1972; Wu et al., 1972; Moller et al., 1973). Avidity measurements have also been made by hapten inhibition of ELISAs and radioimmune assays (Chang et al., 1982; Kresina et al., 1982). In these assays, as in the plaque assay, the antigen exists in a multideterminant solid phase which could influence the results (Jerne et al., 1972; DeLisi, 1976).

In this study we used four anti-trinitrophenyl (TNP) hybridoma cells as plaque-forming cells and their purified monoclonal products to assess antigen binding characteristics by various techniques. The comparison of affinity measurements made on these antibodies by equilibrium dialysis to those made by inhibition of hemolytic plaques or by inhibition of binding in an ELISA allowed a direct test of the validity of the latter two assays. This direct comparison showed that values of K_o estimated by hapten inhibition of hemolytic plaques or ELISA were one to two orders of magnitude lower than those obtained thermodynamically through equilibrium dialysis. This was true of both IgM and IgG monoclonal antibodies. For a given method of analysis, however, the IgM preparation was consistently found to be of lowest avidity.

MATERIALS AND METHODS

Animals. BALB/c mice were purchased from the Fred Hutchison Cancer Research Center, Seattle, WA and housed in our animal care quarters. Mice were immunized with TNP-KLH as described previously for memory responses (Tittle and Rittenberg, 1978) or for primary responses (Golding et al., 1982).

Hybridomas. The NS-1 and SP2/0 myeloma fusion

partners were obtained from Dr. Ed Clark (Department of Genetics, University of Washington, Seattle) and the Salk Cell Distribution Center, respectively. Hybridomas were produced by a modification of the method of Oi and Herzenberg (Oi et al., 1980). Briefly, 10^8 spleen cells and 10^8 fusion partners were washed in serum-free RPMI-1640 medium and pelleted at 400 x G for 5 min. in a 50 ml conical centrifuge tube. One ml of a 50% PEG 1500 solution (BDH Chemicals, Ltd., Poole, England) was then added followed by slow dilution with 8 ml of serum-free medium. The cells were then pelleted as before and re-suspended at 5 x 10^6/ml in HAT medium including 10^8 thymocytes as fillers. 0.2 ml of this cell suspension was then plated in wells of 96-well culture plates (Falcon #3072, Oxnard, CA). Cultures were fed every 5 days with 0.1 ml of HT medium. Generally, approximately 50% of the wells showed growth of clones by two weeks at which time they were screened for antibody production. Positive clones were selected in an ELISA using TNP-BSA as antigen bound to the plates (Golding et al., 1982). The clones were expanded and cloned by repeated limiting dilution in thymocyte fillers (Oi et al., 1980).

Antibody Assays. Anti-TNP plaque-forming cells were determined by the slide method of Cunningham and Szenberg (Cunningham et al., 1968) using TNP coated sheep

red cells (Rittenberg and Pratt, 1969). For hapten inhibition studies free hapten was added directly to the plaquing mixture. An ELISA was also used as previously described (Golding et al., 1982) for quantification of idiotype content and fine specificity analysis. For these inhibition studies free hapten or anti-idiotype was added with the antibody to the wells and incubated as usual to allow competition with the hapten-BSA bound to the plate.

Equilibrium Dialysis. Equilibrium dialysis was done using ^{3}H-DNP-lysine (New England Nuclear, Boston, MA). This material was purified before use by thin layer chromatography on silica gel G (Eastman Kodak, Rochester, NY) with 25 parts butanol, 4 parts acetic acid and 10 parts water as the solvent system. Enough ^{3}H-DNP-lysine was used to provide approximately 100,000 counts per minute with the balance of the hapten being unlabeled DNP-lysine (Sigma Chemical Co., St. Louis, MO). An apparatus conisting of 12 pairs of chambers was used. The members of a pair were separated by a boiled dialysis membrane. Opposing chambers were filled with 0.7 ml of antibody or hapten and allowed to equilibrate at 4°C for 48 hrs. The antibody concentration was 10^{-7} molar and the hapten concentrations ranged from 4×10^{-7}M to 7×10^{-9}M for the IgG antibodies and from 10^{-5}M to 10^{-8}M for the IgM antibody. Samples were counted in a Bechman liquid scintillation counter. The amount of antibody bound was determined by the difference in hapten

concentration in the two chambers. Control experiments showed that nonspecific binding to the dialysis membrane by irrelevant mouse IgG was less than 3%. Data were plotted as 1/b vs. 1/c where b equals the concentration of bound hapten at free hapten concentration c (Liu et al., 1980).

Purification of Monoclonal Anti-TNP Antibodies by Affinity Chromatography. The hybridoma proteins were purified from ascitic fluid by affinity chromatography using protein A or DNP-lysine bound to CNBr activated Sepharose 4B (Pharmacia, Uppsala, Sweden). Ascitic fluid containing IgG1 hybridoma antibodies (TK-1, TK-3, TK-4) was passed over the protein A column and eluted with pH 5.4 M citrate buffer (Ey et al., 1978). This material was then passed over the DNP column and the specific antibody eluted with DNP-Gly (0.1 M, pH 8.4) followed by extensive dialysis against pH 7.2 phosphate buffer (Goetzl et al., 1970). TK-5, MOPC-315 and MOPC-460 proteins were purified using only the DNP column.

Gels. SDS-PAGE analysis of the whole Ig molecule was done in 6%-18% continuous gradient gels (Laemmli, 1970). Five to 10 mg of protein was run and stained with coomassie blue.

Isoelectric focusing analysis of whole Ig molecules was done on LKB ampholine PAG plates, Ph 3.5-9.5 (LKB Instruments, Inc., Rockville, MD). Approximately 10

mg of protein was applied at the anodic end. Focusing was carried out for 2 hours at 15 W. Gels were stained with coomassie blue. Isoelectric focusing of isolated light chains was performed using a modification of the method of Perlmutter (Perlmutter et al., 1977). The SDS-PAGE gels were run on a flat bed apparatus (Pharmacia, Uppsala, Sweden). The appropriate gel slice containing the isolated L chains was re-equilibrated in 8M urea, 1% NP-40 prior to loading on an IEF slab gel containing 6M urea, 2% ampholytes (pH 5-9, Bio-Rad, Richmond, CA) and 0.5% NP-40. Following fixation of the gel, the ampholytes were extensively eluted with a solution of copper sulfate in ethanol-acetic acid. Copper ions were removed by soaking in EDTA solution and the protein bands silver stained as described by Morrissey (1981).

Haptens. DNP-lysine (DNP-Lys) was purchased from Sigma Chemical Co., St. Louis, MO). TNP-epsilon amino-caproic acid (TNP-EACA) was prepared by the method of Benacerraf and Levine (1962).

RESULTS

Hybridomas TK-1, TK-3 and TK-4 were derived by a protocol designed to obtain hybrids which were representative of the in vitro IgG secondary response to TNP-keyhole limpet hymocyanin (TNP-KLH). In these experiments spleen

cells from day nine of a secondary *in vitro* culture with antigen (Tittle and Rittenberg, 1978) were used as fusion partners for NS-1 cells. Hybridoma TK-5 was derived from a fusion where spleen cells from a primary *in vivo* response to TNP-KLH were fused to NS-1 to provide a representative clone from a primary response. Immunodiffusion analysis with commercial antisera specific for mouse heavy and light chains showed all three of the hybrids from the memory pool -- TK-1, TK-3 and TK-4 to have IgG_1 heavy chains and K light chains while TK-5 from the primary pool was an IgM protein also bearing the K light chain (not shown).

The SDS-PAGE analysis in Figure 1 shows the three hybrids from the secondary response (TK-1, TK-3, TK-4) typical of IgG antibodies, to have heavy chain molecular weights of approximately 50 K. The double light chain bands seen with TK-4 are most likely due to the incorporation of an NS-1 light chain into a secreted molecule. Similar findings have been reported recently by Giles *et al*. (1982). The molecular weight of the TK-5 heavy chain is typical of an IgM antibody. We attribute the extra heavy chain band to differences in glycosylation although this has not been established. The nonreducing isolectric focusing gels (Fig. 2) show the restricted heterogeneity expected of hybridoma proteins. TK-5 was not focused because IgM molecules are too large to enter the gel.

The light chains of these antibodies were compared by two dimensional gel electrophoresis. In addition to the light chains from the four hybridomas the light chain of MOPC 21 (the parental line of the fusion partner NS-1 (Cowan et al. 1974)) was run since some hybridoma molecules may incorporate the NS-1 light chain (Giles et al., 1982). Based on spectrotype Fig. 3 shows a light chain pattern for the TK-3 and TK-4 hybridomas partially comparable to the MOPC 21 light chain pattern. However, both TK-3 and TK-4 also have bands that differ distinctly from the MOPC-21 pattern. In contrast TK-1 and TK-5 have spectrotypes which suggest they lack the MOPC-21 light chain.

Representative plots from three to five equilibrium dialysis experiments for each antibody are shown in Figure 4. The double reciprocal plots of bound vs. free hapten show a near perfect fit to a straight line (correlation coefficient = 0.99) indicating a high degree of binding site homogeneity as expected of monoclonal antibodies. The association constants (Ko) calculated from the equilibrium dialysis data are summarized in Table I. As expected the three IgG antibodies (TK-1, TK-3, TK-4) were of higher affinity than the IgM antibody (TK-5) which was obtained from a hybridoma made from the primary B cell pool.

The intrinsic association constant determined from equilibrium measurements provides the standard for which other measurements of antibody binding are estimates. The availability of both cloned hybridoma cells and their products made it possible to compare hapten inhibition of hemolytic plaques and hapten inhibition of binding in an ELISA with equilibrium dialysis. The results of the three methods are compared in Table I. The data can be analyzed in two ways: direct comparison of the different methods with a single antibody or comparison of the affinities of the antibodies relative to each other in the three different systems. In the former analysis one would not expect the values to be the same since there are a number of differences between the systems, among them the temperature which varies from 4°C to 37°C in the three systems and the multideterminant nature of the antigen used in the plaque and ELISA methods. Thus, not surprisingly, the absolute values for Ko in the latter methods differed measurably from the equilibrium dialysis values with a minimum of 10-fold more hapten required to cause 50% inhibition of the solid phase reactions compared to that required to saturate 50% of the antibody sites at equilibrium.

An additional difference between solid phase assays and equilibrium experiments is that solid phase assays are based on two forms of hapten (one in solid

phase, one in solution) competing for the same combining site while in equilibrium studies the proportion of combining sites filled by a single form of hapten (in solution) is measured. Consequently affinity measurements made in solid phase assays are actually the relative affinity of the antibody for the solid phase antigen versus the fluid phase hapten. This could be misleading when comparing antibodies with different fine specificity patterns for the antigen/hapten pairs used. As presented in Table II, TK-3 has a 100-fold greater affinity for TNP than for DNP while the other antibodies bind the two haptens similarly. While the affinity of TK-3 and TK-4 for DNP-lys are identical by equilibrium dialysis (Table I), TK-3 has lower affinity for DNP in the solid phase assays especially plaque inhibition (Table II) where TNP is in the solid phase and DNP in solution. We believe this to be due to a combination of both differences in fine specificity and to differences between the ELISA and plaque inhibition assay systems. In the ELISA, determination of bound antibody is made after equilibrium has been reached and the excess inhibiting antigen has been removed while in plaque inhibition, determination of bound antibody (red cell lysis) occurs simultaneously with the establishment of equilibrium with the inhibitor and may even result from a transient association of antibody with the red cell surface. Consequently it might be harder to prevent

antibody-mediated red cell lysis than to inhibit antibody binding in an ELISA which would predict a lower affinity for TK-3 when estimated with DNP in plaque inhibition as shown in Table I; it should be noted, however, that K_o values by plaque inhibition were not uniformly greater than those obtained by ELISA for IgG hybridomas. Despite these difficulties the solid phase methods were generally able to determine the relative affinity between IgG and IgM antibodies, thus TK-5 was consistently shown to have the lowest affinity.

DISCUSSION

These hybridoma lines were developed to identify dominant clones in the IgG anti-TNP memory pool. The monoclonal nature of these cell lines was confirmed by the homogeneity of the IEF patterns and linearity of the antigen binding data from the equilibrium dialysis experiments. To determine if NS-1 light chains were present in any of the hybridoma molecules the light chains were separated by SDS-PAGE and then run on isoelectric focusing gels. There are NS-1-like light chains present in TK-3 and TK-4 which is consistent with other reports on hybridomas made with NS-1 (Giles et al., 1982). In terms of heterogeneity TK-1 and TK-3 have unique spectrotypes while TK-4 and TK-5 have similar spectrotypes. The availability

of these monoclonal lines made it possible to compare methods of assessing the affinity of anti-TNP combining sites.

DeLisi has shown theoretically that under conditions such as those used here (inhibition of antibody binding to a multivalent antigen with a univalent inhibitor) that the 50% inhibition point should reflect antibody affinity; however, no experimental evidence was given (DeLisi et al., 1976). The use of hybridoma antibodies allowed utilization of a homogeneous antibody population in order to determine its affinity by equilibrium dialysis and then to test directly the correspondence of affinity measurements made by inhibition of hemolytic plaques or ELISA.

As could be expected from the differences in methods, the K_o values obtained by hapten inhibition of plaque formation and ELISA were not the same as the values obtained by equilibrium dialysis. Both non-thermodynamic methods underestimated the actual affinities of the four anti-TNP hybridomas. These differences reflect the fact that markedly different phenomena are measured in equilibrium dialysis as compared to hapten inhibition of plaque formation or ELISA. While equilibrium dialysis involves the direct binding of antibody to soluble monovalent hapten, both plaque inhibition and ELISA inhibition measure the ability of soluble monovalent hapten to inhibit the

binding of antibody to a multideterminant solid phase antigen. However, the ELISA hapten inhibition measurements produced the same relative order of affinities as equilibrium dialysis (K_o of TK-3> TK-4> TK-1> TK-5). A somewhat different order of affinity was indicated by plaque inhibition among TK-1, TK-3 and TK-4 although all three methods agreed that TK-5 possessed the lowest affinity. It is apparent that while plaque inhibition or ELISA inhibition can be used to determine the relative affinities of antibodies which differ markedly in affinity (TK-4 and TK-5), these methods are less reliable in differentiating among antibodies of similar affinity (TK-1, TK-3 and TK-4).

These results are in agreement with those of North and Askonas (North et al., 1972), who also found inconsistencies with plaque inhibition as a measure of antibody affinity. The conditions used in this study are not precisely the same as those recommended by DeLisi (1976) since we used directly haptenated red cells. This would result in a lower density of hapten and shold affect the binding of pentameric IgM molecules to a greater extent than IgG molecules. Despite this, we found that the IgM hybridoma TK-5 was the antibody whose affinity relative to the other antibodies was the most reliably predicted. Since haptenated protein was used in the ELISA assay, this should have met the requirements recommended by DeLisi. The ELISA

assay did, in fact, come the closest to predicting accurately the relative affinities of the four antibodies.

These results do not invalidate the use of either of these two methods for comparing the affinity of a single antibody for hapten analogs since the factors causing the aberration would remain constant for a single method. An example of this is seen in Table II where TK-3 is shown to have at least 100-fold greater avidity for TNP than for DNP in both plaque inhibition and ELISA. Since all conditions were constant in each test except for the hapten used, the most reasonable explanation for the observed differences is affinity for the hapten in question. This marked preference for TNP over DNP was not seen for the other hybridoma antibodies and could not be revealed by equilibrium dialysis with DNP-LYS. Thus, it is likely that the value of K_o calculated from equilibrium dialysis does not reveal the true specificity of TK-3; the relative values obtained by the solid phase assays suggest that equilibrium measurements using TNP-LYS would provide a K_o of approximately 10^{-10}.

Acknowledgments: The authors wish to thank McKay Brown and Dr. Miriam Bertram for performing the IEF and SDS-PAGE analyses on the whole molecules. In addition we are grateful to Dr. Sandra P. Chang for a critical and helpful review of the manuscript and to Dr. E. Clark for providing NS-1 cells.

REFERENCES

Adorini C., Pini C., D'Agostaro G., DiFelice G., Mancini C., Pozzi C.V., Vietri S., & Doria G. (1981) Induction of azobenzenearsonate (ABA) specific helper and suppressor T cells and in vitro evaluation of their activities in the antibody response to T-dependent ABA-protein conjugates. J. Immunol. 127, 1018-1023.

Andersson P. (1970) Studies on the regulation of avidity at the level of the single antibody cell. The effect of antigen dose and time after immunization. J. Exp. Med. 132, 77-88.

Benacerraf B. & Levine B.B. (1962) Immunological specificity of delayed and immediate hypersensitivity reactions. J. Exp. Med. 115, 1023-1035.

Chang S.P., Brown M., & Rittenberg M.B. (1982) Immunologic memory to phosphorylcholine. II. PC-KLH induces two antibody populations that dominate different isotypes. J. Immunol. 128, 702-706.

Cowan N.J., Secher D.S., & Milstein C. (1974) Intracellular immunoglobulin chain synthesis in non-secreting variants of a mouse myeloma: Detection of inactive light chain messenger RNA. J. Mol. Biol. 90, 691-701.

Cunningham A. & Szenberg A. (1968) Further improvement in the plaque technique for detecting single antibody forming cells. Immunol. 14, 599-600.

Davie J.M., & Paul W.E. (1972) Receptors on immunocompetent cells. V. Cellular correlates of the "maturation" of the immune response. J. Exp. Med. 135, 660-674.

DeLisi C., & Goldstein B. (1974) On the mechanism of hemolytic plaque inhibition. Immunochem. 11, 661-665.

DeLisi C., & Goldstein B. (1974) The kinetics of hemolytic plaque formation. II. Inhibition of plaques by hapten. J. Theoret. Biol. 51, 313-335.

DeLisi C. (1975) The kinetics of hemolytic plaque formation. IV. IgM plaque inhibition. J. Theoret. Biol. 52, 419-440.

DeLisi C (1976) Hemolytic plaque inhibition: the physical chemical limits on its use as an affinity assay. J. Immunol. 117, 2249-2257.

Estess P., Lamoyi E., Nissonoff A. & Capra J.D. (1980) Structural studies on induced antibodies with defined idiotypic specificities. IX. Framework differences in the heavy- and light-chain-variable regions of monoclonal anti-p-azophenylarsonate antibodies from A/J mice differing with respect to a cross-reactive idiotype. J. Exp. Med. 151, 863-875.

Ey P.L., Prowse S.J., & Jenkin C.R. (1978) Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. Immunochem. 15, 429-436.

Giles R., Klapper D. & Clem L.W. (1982) Intrasubunit homogeneity in heterogeneous IgM antibodies to the DNP moiety derived from a murine hybridoma cell line. Molec. Immunol. 19, 747-754.

Goidl E.A., Birnbaum O., & Siskind G.W. (1975) Determination of antibody avidity at the cellular level by the plaque inhibition technique: Effect of valence of the inhibitor. J. Immunol. Meth. 8, 47-52.

Golding H., Foiles P.G. & Rittenberg M.B. (1982) Partial reconstitution of TNP-ficoll responses and IgG3 expression in Xid mice undergoing graft-vs.-host reaction. J. Immunol. 129, 2641-2646.

Jerne N.K., Henry C., Nordin A.A., Fuji H. Koros, A.M.C., & Lefkovits I. (1972) Plaque forming cells, methodology and theory. Transpl. Rev. 18, 130-191.

Juy D., Primi D., Sanchez P. & Cazenave P. (1982) Idiotype regulation: evidence for the involvement of Igh-C-restricted T cells in the M-460 idiotype suppressive pathway. Eur. J. Immunol. 12, 24-30.

Kresina R.F., Rosen S.M., & Nisonoff A. (1982) Degree of heterogeneity of binding specificities of antibodies to the phenylarsonate group that share a common idiotype. Molec. Immunol. 19, 1433-1439.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Liu F., Bohn J.W., Ferry E.L., Yamamoto H., Molinaro C.A., Sherman L.A., Klinman N.R. & Katz D.H. (1980) Monoclonal dinitrophenyl-specific murine IgE antibody: Preparation, isolation and characterization. J. Immunol. 124, 2728-2737.

Miller G.W., & Segre D. (1972) Determination of relative affinity and heterogeneity of mouse anti-DNP antibodies by a plaque inhibition technique. J. Immunol. 109, 74-83.

Moller E., Bullock W.W., Makela O. (1973) Affinity of T and B lymphocyte receptors for hapten determinants. Eur. J. Immunol. 3, 172-179.

Morrissey J.H. (1981) Silver stains for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Analytical Biochem. 117, 307-310.

Nadler P.I., Miller G., Sachs D.H. & Hodes R.J. (1982) The expression and functional involvement of nuclease-specific idiotype on nuclease-primed helper T cells. Eur. J. Immunol. 12, 113-120.

North J.R., & Askonas B.A. (1974) Analysis of affinity of monoclonal antibody responses by inhibition of plaque forming cells. Eur. J. Immunol. 4, 361-366.

Oi V.T. & Herzenberg L.A. (1980) Selected methods in cellular immunology. Edited by B.B. Mishell and S.M. Shiigi pp. 351-372, W.H. Freeman and Co., New York.

Perlmutter R.M., Briles D.E. & Davie J.M. (1977) Complete sharing of light chain spectrotypes by murine IgM and IgG anti-streptococcal antibodies. J. Immunol. 118, 2161-2166.

Reed L.J., & Muench H (1938) A simple method of estimating fifty percent endpoint. Am J. Hyg. 27, 493-497.

Rittenberg M.B. & Pratt K. (1969) Anti-trinitrophenyl (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. Proc. Soc. Exp. Biol. Med. 132, 575-581.

Tittle TV., & Rittenberg M.B. (1978) Expression of IgG memory response <u>in vitro</u> to thymus-dependent and thymus-independent antigens. Cell. Immunol. 35, 180-190.

Wu C-Y, & Cinader B. (1972) Dose and time dependent changes in the binding capacity of IgM antibody. Eur. J. Immunol 2,398-405.

TABLE I

AFFINITY MEASUREMENTS BY THREE DIFFERENT METHODS

	Equilibrium Dialysis Ko x 10^{-7}	Plaque Inhibition Ko x 10^{-7}	ELISA Ko x 10^{-7}
TK-1	0.85±0.29	25±9	28±13
TK-3	0.2±0.02	91±36	17±6
TK-4	0.25±0.05	2.9±0.2	8.1±2.5
TK-5	6.2±2.9	540±60	128±38

Ko — The molar concentration of DNP-Lys needed for 50% saturation of binding in equilibrium dialysis determined by double reciprocal plots (Liu et al. 1980) or 50% inhibition of binding in plaque inhibition or ELISA. The 50% inhibition end points in the solid phase reactions were determined by method of Reed and Meunch (1938).

TABLE II

SPECIFICITY OF HYBRIDOMAS FOR TNP AND DNP HAPTENS

Hybridoma	Inhibitor	Plaque Inhibition	ELISA
TK-1	DNP-Lys	28±9[a]	28±13
	TNP-EACA	2.1±2	15.5±7
TK-3	DNP-Lys	91±36	17±6
	TNP-EACA	0.85±0.26	0.02±.006
TK-4	DNP-Lys	2.9±0.2	8.1±2.5
	TNP-EACA	11.7±0.6	5.8±2.6
TK-5	DNP-Lys	540±60	128±38
	TNP-EACA	384±45	250±77

a) Values are concentration of inhibitor needed for 50% inhibition x 10^{-7}.

FIGURE 1

Analysis of anti-TNP hybridomas by SDS-PAGE in a 6%-18% continuous gradient. Protein stained with coomassie blue. A) TK-5; B) TK-4; C) TK-3; D) TK-1; E) MW standards. 7.5 mg of each protein were used.

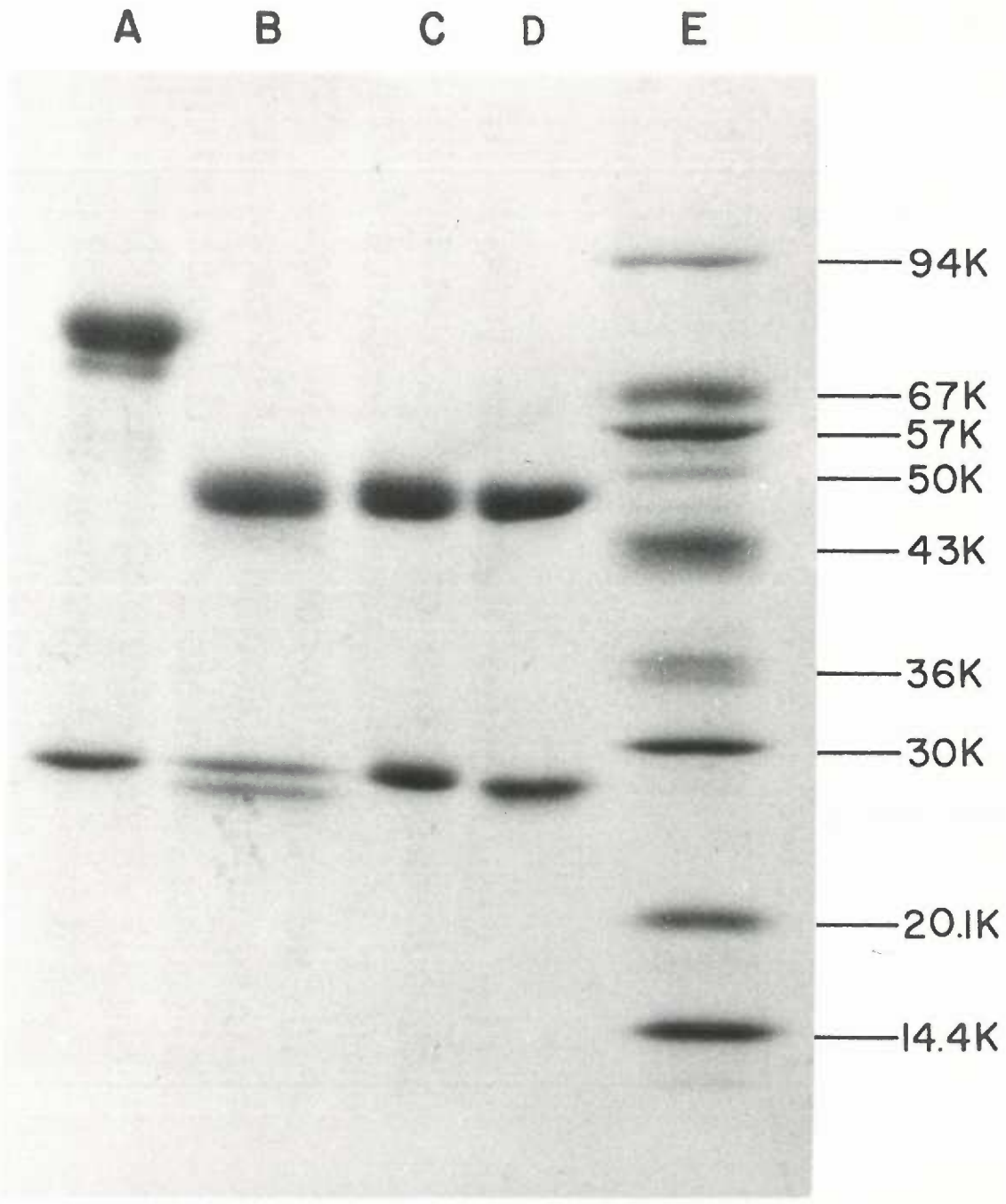

A
B
C
D
E
94K
67K
57K
50K
43K
36K
30K
20.1K
14.4K

FIGURE 2

IEF gels. 10 mg of each hybridoma were applied to the anodic end of a polyacrylamide gel with a pH gradient of 3.5 to 9.5. After focusing the protein was stained with coomassie blue. A) myoglobin standard; B & C) TK-4; D) TK-3; E) TK-1.

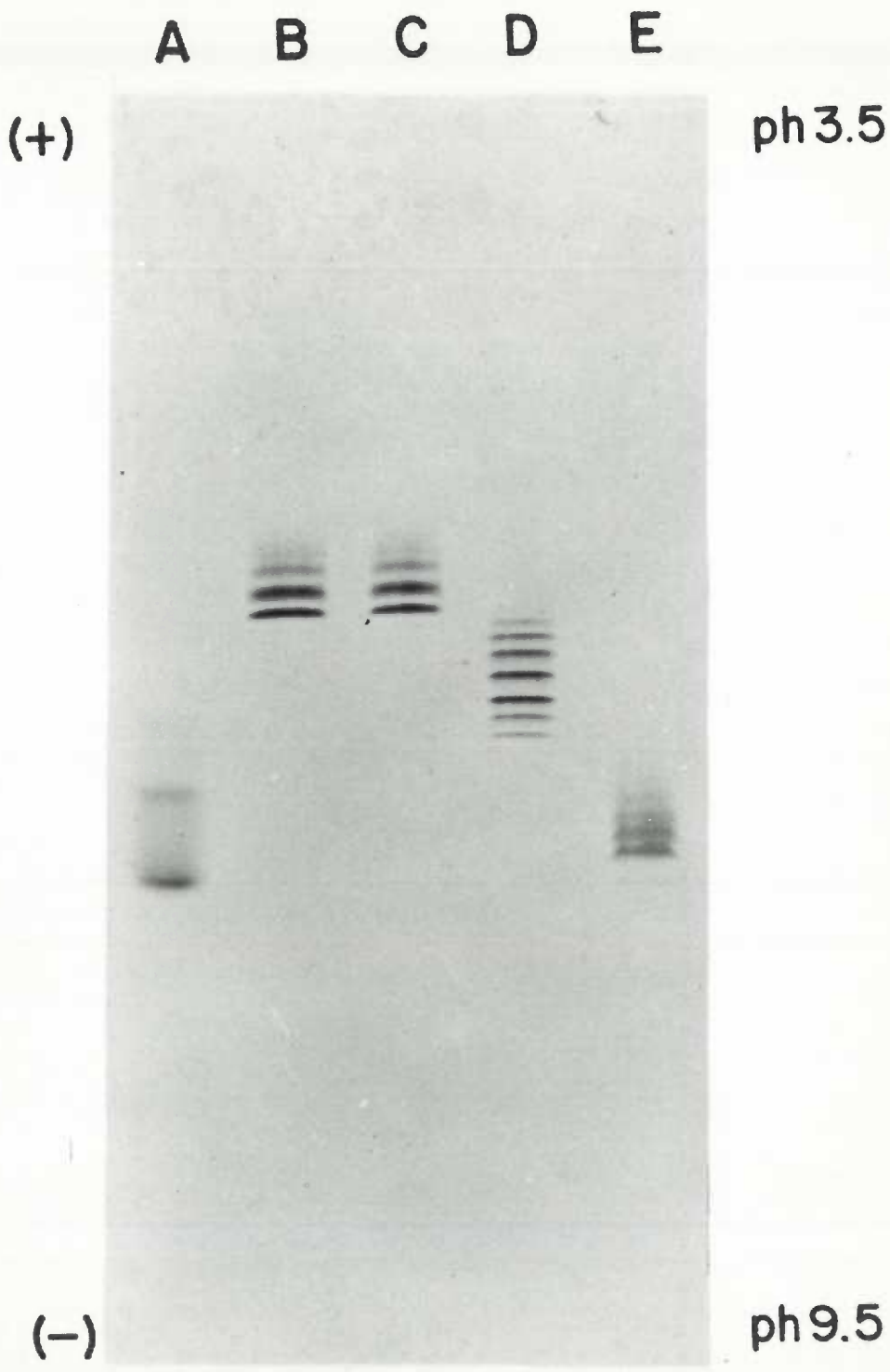

A
B
C
D
E
(+)
ph 3.5
(−)
ph 9.5

FIGURE 3

Two dimensional SDS-PAGE, IEF gel. Light chain bands from SDS-PAGE to polyacrylamide gel with a pH gradient of 5.0 to 9.0. After focusing the protein was silver stained. A) TK-1; B) TK-3; C) MOPC-21; D) TK-4; E) TK-5.

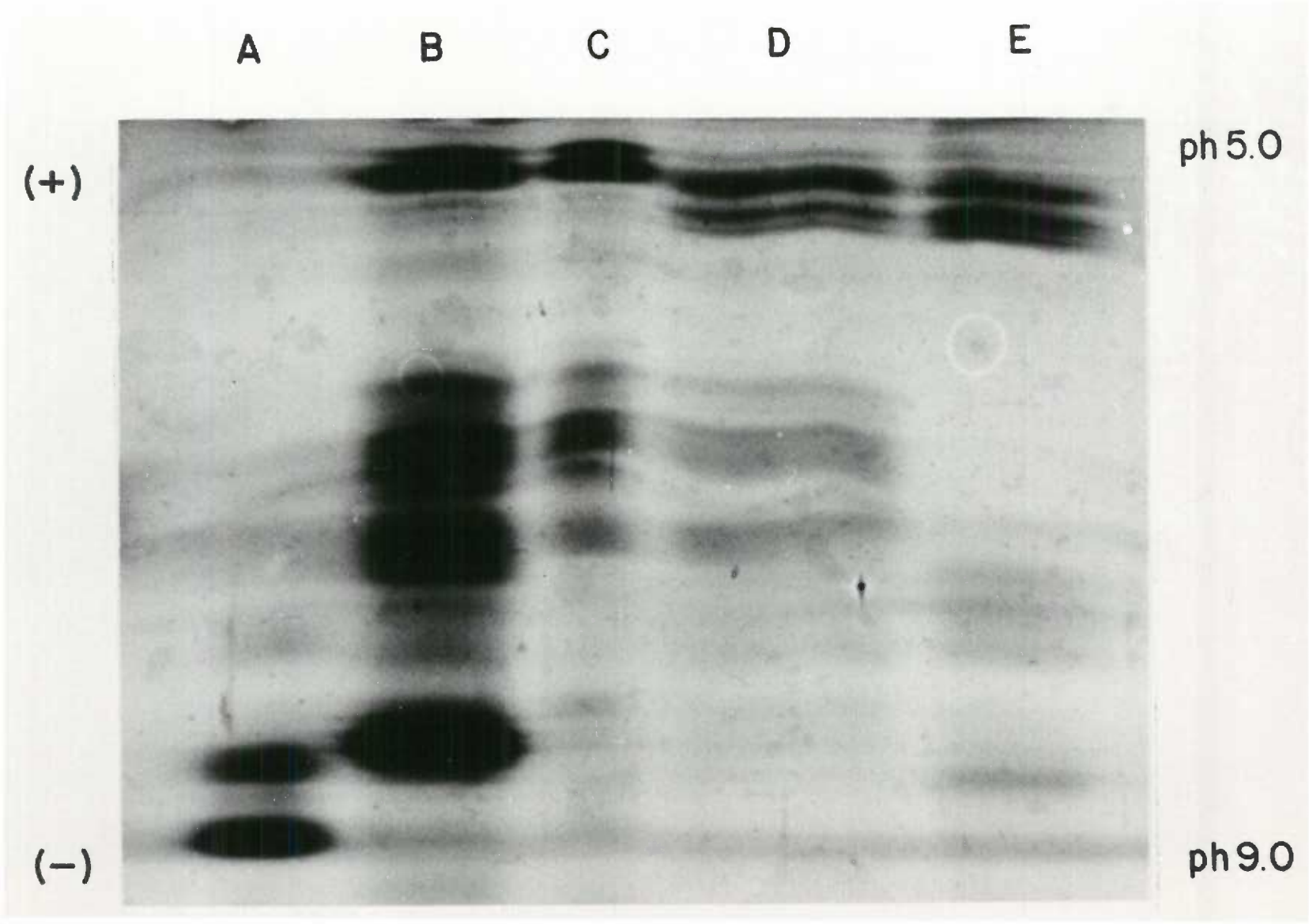
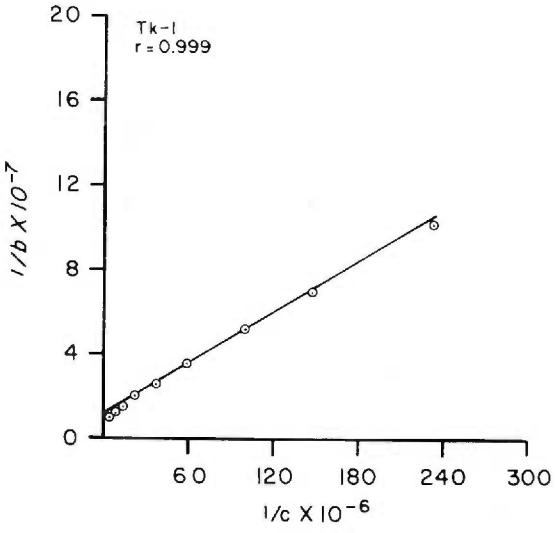
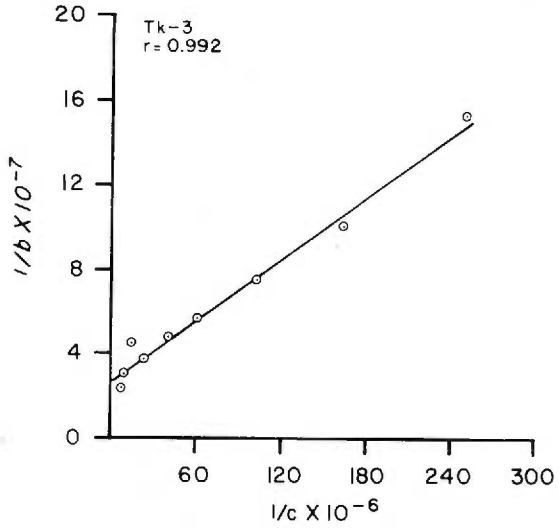
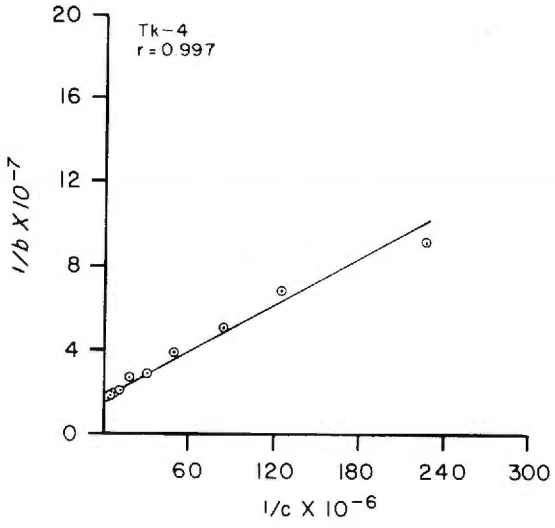

A
B
C
D
E
(+)
(−)
ph 5.0
ph 9.0

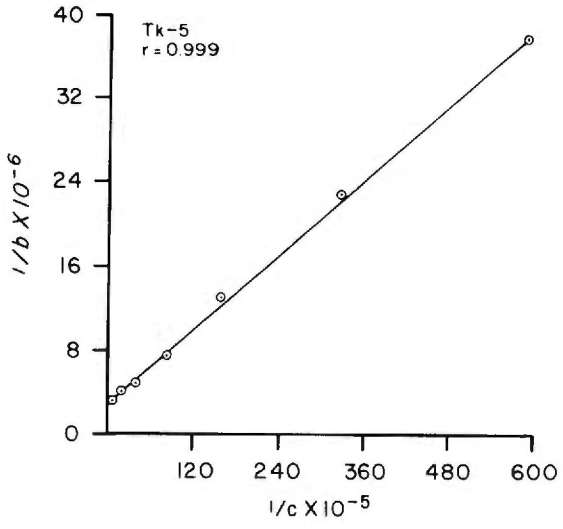
FIGURE 4

Equilibrium dialysis binding data. b = concentration of bound hapten. c = concentration of free hapten. r = correlation coefficient by linear regression analysis. See Materials and Methods for Details.


Tk-1
r = 0.999
1/b X 10⁻⁷
1/c X 10⁻⁶
0
4
8
12
16
20
60
120
180
240
300


Tk-3
r = 0.992
1/b X 10⁻⁷
1/c X 10⁻⁶
0
4
8
12
16
20
60
120
180
240
300


Tk-4
r = 0.997
1/b X 10⁻⁷
1/c X 10⁻⁶
0
4
8
12
16
20
60
120
180
240
300


Tk-5
r = 0.999
1/b X 10⁻⁶
1/c X 10⁻⁵
0
8
16
24
32
40
120
240
360
480
600

The results of the first manuscript show that I have produced four anti-TNP hybridoma cell lines. These antibodies represent at least three different binding sites for TNP based on their affinity for this hapten. In addition I have shown that hapten inhibition of binding in ELISA or hemolytic plaque assays can be used to estimate antibody affinity. In the work that follows I will further characterize these antibodies in terms of fine specificity for antigen and idiotype. The final section will examine how these idiotypes are expressed in different antibody responses.

MATERIALS AND METHODS

Mice -- A/J, AKR, C57Bl/6, CBA/ca, CBA/l, BXSB, SJL, and B_6D_2 mice were obtained from the Jackson Laboratory, Bar Harbor, ME. BALB/c, CBA/n and all F_1 crosses were bred in our facilities.

Antigens -- TNP-KLH was produced by trinitrophenylation of keyhole limpet hemocyanin according to Rittenberg and Amkraut (1) and had a molar ratio of TNP_{1356}-KLH based on a MW of $8x10^6$ for KLH. TNP-AECM-ficoll was produced according to Inman (2) and had a molar ratio of TNP_{75}-ficoll based on a MW of 400,000 for ficoll.

Immunization Protocols -- Most primary responses were induced in mice which had been carrier primed with three weekly injections of 100ug KLH-bentonite based on the method of Gallily and Garvey (3). 20ug of TNP-ficoll or 100ug TNP-KLH bentonite was then given Ip. Mice involved in GVH studies were not carrier primed and received 10ug TNP-ficoll Ip or 100 ug TNP-KLH mixed with an equal volume of maalox and 10^9 Bordetella pertussis organisms (Eli Lilly Co., Indianapolis, IN). Secondary responses were generated in mice which were primed with 100 ug TNP-KLH bentonite every other week for three Ip injections (4). Mice were rested for at least two months before secondary challenge. Pools of four to six mice were bled

at the indicated times and the serum separated.

Graft-vs-Host Reaction (GVH) -- GVH was induced by iv injection of parental lymphocytes into F_1 recipients shortly before antigen administration. Donor lymphocytes were obtained from two to three month old mice.

Class and Sub-Class Specific Antibodies -- Anti-u antibody was obtained from KPL Labs (Gaithersburg, MD) and was passed over a TK-3 (IgG1K) column. Rabbit anti-Gi, anti G2a and anti-G2b antibodies were purchased from Bionetics Research Products (Kensington, MD). These reagents were purified by elution from TK-1 (IgG1) RPC-5 (IgG2a) or MOPC 195 (IgG2b) immunoadsorbent columns. These reagents were coupled to alkaline phosphatase (Sigma Chem. Co., St. Louis, MO) by the method of Kerney et al. (5).

ELISA Assays -- The amount of each isotype was determined by comparison to a standard hyperimmune anti-phosphocholine antiserum containing known amounts of IgM, IgG1, IgG2a, IgG2b and IgG3 antibodies. Dilutions of this antibody were bound to PC-histone coated polystyrene microtiter plates (Costar, Cambridge, MA) to form a standard curve in which the amount of isotype bound is related to the OD_{405} generated with alkaline phosphatase conjugated class or sub-class specific antibodies. Appropriate dilutions of experimental anti-TNP antiserum were bound to TNP-BSA coated plates for 90 minutes followed by three

washes with PBS-Tween 20 (0.05%) and three with plain PBS. The optimal concentration of AP-conjugated class or sub-class specific antibodies was added to both the TNP and PC coated wells for 90 minutes followed by six washes as above. The enzyme substrate P-nitrophenylphosphate (1 ug/ml; Sigma) in 0.9M diethanolamine ph 9.8 containing mM MgCl was added to the plates and the reaction stopped by addition of ethylene diamine tetraacetate to a final concentration of 20 mM.

The amount of idiotype present in a sample was determined by a modification of the ELISA just described. A dilution series of the test serum was made in the absence of the anti-idiotype antibody and used to measure the inhibition of binding of the anti-TNP antibody to the plate in the presence of anti-idiotype.

B-Colonies in Methylcellulose -- The ability of spleen cells to form B-colonies was determined using a modification of the method of Kurland et al. (6). $3x10^5$ spleen cells in complete medium plus 50 ug/ml lipopolysaccharide (LPS) from E. coli 055:B5 (Difco Labs, Detroit, MI) and 1.5% carboxymethyl cellulose (Sigma, medium viscosity) were layered on top of macrophage feeder layers consisting of 10^5 adherent peritoneal exudate cells covered with 0.5% agar plus $1.4x10^{-7}$M Indomethacin. Dishes were incubated at 37°C and the number of colonies formed was determined on day six using a Bausch and Lomb

dissecting microscope (only colonies containing more than 50 cells were scored).

Organ Weights -- Brachial lymph nodes and spleens were immersed in normal saline and weighed shortly after removal using a Mettler chemical balance.

Anti-Idiotype -- Rabbit anti-idiotype antibodies were prepared by immunizing rabbits subcutaneously with 100 ug of antibody protein in Complete Freund's Adjuvant followed by two monthly injections (100 ug) in Incomplete Freund's Adjuvant. The rabbits were bled one week after the third injection and the serum collected. It was heat inactivated and then absorbed on a normal mouse serum (BALB/c) column. Antibody to TK-5 was absorbed with MOPC-21 (IgG1), RPC-5 (IgG2a), M195 (IgG2b), Y5606 (IgG3), MOPC-104E (IgM) and MPC-603 (IgA). Antibodies to TK-1 and TK-4 were absorbed with other anti-TNP proteins, i.e., anti-TK-1 was absorbed with TK-3, TK-4 and TK-5. The anti-idiotype was then bound to an idiotype column and eluted with DNP-glycine to isolate binding site-associated anti-idiotype. A murine hybridoma antibody AI-31 was made by immunizing a BALB/c mouse with TK-3 protein coupled to KLH with glutaraldehyde (7). These spleen cells were then fused with SP2/0 cells as described above and screened for antibodies that inhibited binding of TK-3 to TNP-bovine serum albumin. In addition a rabbit anti-MOPC 315/460 idiotype was kindly provided by Dr. Robert Rosenstein. (8)

Haptens -- TNP-lysine (TNP-lys), TNP-phenylalanine (TNP-Phe), and DNP-epsilon aminocaproic acid (DNP-EACA) were purchased from ICN Nutritional Biochemicals, Cleveland, OH. DNP-lysine (DNP-Lys) and DNP-glycine (DNP-Gly) was purchased from Sigma Chemical Co., St. Louis, MO. 2-4-dinitronaline (2,4 DNA) and 4-nitrophenylbutyric acid (4NPBA) were purchased from Aldrich Chemical Co., Milwaukee, WI. TNP-epsilon aminocaproic acid (TNP-EACA) was prepared by the method of Benacerraf and Levine (9).

DETERMINATION OF ANTIBODY VALENCE

In addition to the thermodynamic information on binding which can be derived from equilibrium dialysis data, the number of binding sites per molecule (valence) can be determined. The usual method is that of Scatchard plots (10) which are shown in Figure 1. Table I shows the combined results of from three to five replicate experiments for each antibody. The valence of IgG and IgM molecules is known to be 2 and 10, respectively. The values obtained are two to threefold lower than expected.

Difficulty in determining the correct valence of antibody molecules, particularly of IgM, has been reported previously. The valence of pentameric IgM was first reported to be from 4 to 6 based on equilibrium dialysis experiments (11, 12). The reason for the low valence was

due to some of the combining sites of the molecule having very low affinity for antigen and therefore going undetected (13). The valence of the IgA myeloma protein M-315 was originally found to be 1, not the expected 2 (14). The difficulty in this case was that at the low antibody concentrations used in the equilibrium dialysis experiments there was some denaturation of the antibody leading to loss of binding sites (15). With the addition of carrier protein such as gelatin into the system the expected valence of 2 for IgA was obtained. Recently Liu, et al. reported on an IgE monoclonal anti-DNP antibody made with the nonsecreting fusion partner SP2/0 (16). While they reported the affinity of the antibody for DNP from equilibrium dialysis data, the valence was not derived. When this value is calculated from their data it is found to be 1, not the expected 2. Since they did not report the valence, they give no explanation of the low values.

In the data presented in Fig. 1 and Table I, the low values of hybridomas TK-3 and TK-4 can be explained, at least in part, to the presence of NS-1 light chains resulting in the formation of binding sites which do not bind TNP. The presence of these chains was confirmed biochemically as shown in the first manuscript. This could not be the explanation for the IgE observation mentioned above, since the SP2/0 fusion partners used in those experiments cannot contribute light chains. There

are no NS-1 light chains in the TK-1 or TK-5 hybridomas and their valence is still half that expected. Three possible explanations are: 1) that some of the binding sites were blocked by hapten during purification of the protein; 2) that the determination of the protein concentration was in error; or 3) that binding sites were lost due to the low protein concentration as mentioned above. The observation that TK-1 protein isolated only by absorption onto protein A sepharose also had a valence of 1 argues against hapten blockade of binding sites. I have determined the protein concentration by four separate methods, OD_{280}, Lowry, Bio-rad and Nessler nitrogen determination, and found close agreement by all four methods. The addition of gelatin in equilibrium dialysis experiments with the hybridoma protein TK-1 did not modify the valence obtained from the already reported value of 1. The low valence of TK-5 may be due to the phenomenon of low affinity binding sites as discussed above. The low valence of the IgG monoclonals is more difficult to explain. Even with the interference of NS-1 light chains, there would have to be at least one active binding site in order for the antibody to be recovered in the purification protocol. Since even purification by protein A binding and subsequent elution result in low valence it seems likely that the low pH used for elution or low protein concentrations result in the loss

of functional binding sites.

I believe the equilibrium dialysis methodology I used to be valid in general for the following reasons. The binding data plot in a straight line as expected, the value for the binding constant determined by Dr. Feldbush of 1.1×10^7 agrees well with my value of 1.2×10^7 for TK-1, and lastly, the presence of NS-1 light chains in TK-3 and TK-4 is reflected in the valences I obtained for those antibodies.

CHARACTERIZATION OF HYBRIDOMA ANTIBODIES IN TERMS OF IDIOTYPE AND FINE SPECIFICITY

The fine specificity of the four hybridomas and the myeloma protein M460 was investigated by comparing the binding of eight TNP analogs utilizing the ELISA. Binding to the analogs was compared using TNP-EACA as the reference hapten (Table II) A value greater than 1 indicates greater affinity for that hapten than for TNP-EACA and a value less than 1 indicates an affinity for that hapten lower than that for TNP-EACA. As can be seen in Table II, there are several features that distinguish the binding sites of the five antibodies from one another. For example, TK-1, TK-4, TK-5 and M460 do not distinguish appreciably between the DNP and TNP determinants as indicated by the inhibition ratios in Table II. Although the TK-5 and M460 sites appear distinct because they bind these

haptens, compared to TK-3, only ten percent or one percent as well, respec- tively. A distinct combining site which has a marked preference for TNP analogs over DNP analogs is represented by TK-3, the data make it clear that the preference is for TNP itself and not for the amino acid carrier as seen by comparing the binding to TNP-lys and DNP-lys. Secondly, it can be seen that while TK-1 and TK-4 have similar fine specificity patterns with most hapten analogs tested, they are not identical since they differ appreciably in binding to TNP-Phe and 4-NPBA. Binding to the latter which has a a single nitro group on the phenyl ring was markedly low for all of the antibodies tested. The M460 antibody was unique in that it had somewhat greater affinity for the hapten analogs TNP-Lys and DNP-Gly than for TNP-EACA.

Rabbit antisera specific for idiotypic dermini-nants of TK-1, TK-4 and TK-5 and a murine hybridoma anti-body specific for an idiotypic determinant on TK-3 were prepared as described. In addition, we used a rabbit antiserum (anti-315/460) specific for an idiotype expressed by both M-315 and M460 (8). The specificity patterns of the various anti-idiotypic antibodies are shown in Figure 2. As seen in Figure 2A, the rabbit anti-TK-1 idiotype blocked binding by TK-1 and M460 in a similar fashion. Thus, it appears that anti-TK-1 idiotype recognizes a determinant common to both TK-1 and M460 and, accordingly,

must recognize a public idiotype. However, since this antiserum did not block M-315, this putative public idiotype must be distinct from the 315/460 public idiotype described by Rosenstein, et al. The mouse monoclonal AI-31 (anti-TK-3) and the rabbit anti-TK-4 and TK-5 antibodies (Figures B, C, E, respectively) behave monospecifically, each recognizing a determinant unique to their given anti-TNP antibody; consequently, these must be considered private determinants. The anti-M-315/M460 public idiotype of Rosenstein, et al., does not recognize any of the four anti-TNP hybridomas including TK-1 (Figure 4D) confirming the conclusion derived fron analysis with anti-TK-1 idiotype. These data demonstrate that each of the anti-TNP antibodies reacts uniquely with the panel of idiotype specific antisera. Table III summarizes the characteristics of these anti-idiotypic antisera.

IDIOTYPE EXPRESSION

There are three questions I wanted to ask initially concerning the expression of the idiotypes I had defined. One, is there genetic control of expression; two, does the nature of the carrier affect expression; and three, are the idiotypes expressed equally in primary and secondary responses. To answer these questions, six strains of mice differing at both the MHC and Igh loci

were challenged with TNP coupled with either keyhole limpet hemocyanin (KLH) or ficoll and their serum collected. For secondary responses the animals were first primed with TNP-KLH and then rested for three months before secondary challenge. For the primary response, the mice were bled on days five, seven and nine, and the secondary on days three, five and seven. In both cases the antibody titer in ELISA peaked for the last two bleedings. The last bleeding was used in all experiments. One-way analysis of variance was used to analyze the data on genetic influences on idiotype expression. The null hypothesis for this analysis is that the levels of idiotype expressed for all six strains of mice are equal. This hypothesis can safely be rejected (indicating genetic influence on idiotype expression) when P is less than 0.05. Ninety-five percent confidence intervals calculated from the standard error are given for comparison of the combined idiotype levels.

Table IV gives the genetic makeup of the mouse strains used. Tables V and VI give the percent of the primary IgM response which is inhibitable by a given anti-idiotypic antiserum. Tables VII and VIII give the percent of the secondary IgG response inhibitable with these anti-idiotype antisera.

We will start by examining the primary response

to TNP-KLH and to TNP-ficoll in Tables V and VI respectively. TK-3 idiotype is not present to a significant degree in any of the strains in either the TD or TI responses with the exception of the SJL mouse in the primary TNP-KLH response. The same is true of the secondary response. The TK-3 anti-idiotype is the only monoclonal anti-idiotype used and so recognizes a single idiotope. In contrast, the other idiotypes are from heteroantisera and may recognize multiple idiotopes. This could account for the low to nonexistent suppression of binding seen with this anti-idiotype.

That the strains differ in their level of expression of the idiotype panel is clearly shown in both the TI and TD responses. In the TNP-KLH response TK-1, TK-4 and the combined idiotype all differed significantly, while in the TI response TK-1 and combined idiotype differed significantly. Since these mice have different genetic backgrounds including the H-2 and Igh loci (Table IV), it is likely that these genetic differences are responsible for the differing degree of expression of the idiotype panel in these mice. The expression of the idiotype panel also seems to be affected by the carrier used in at least one strain. This is most clearly shown in the A/J mouse where there was a sixfold drop in the combined idiotype level. Because of variability the threefold drop seen in BALB/c was not significant. AKR, C57Bl/6 and DBA/1 all

maintained their level of expression. SJL mice seem to make relatively higher levels of idiotype regardless of the carrier used.

In comparing the primary response to the secondary response (Tables V and VI to VII and VIII) there is a marked increase in the expression of the idiotypes. In the TNP-KLH response the C57Bl/6 and DBA/1 strains which lack idiotype expression entirely in the primary now express idiotype at about the fifteen percent level. AKR and BALB/c show lower increases, while A/J remain the same and SJL shows a significant drop. The difference in the TI response is even more striking. With the exception of BALB/c and SJL, which stay about the same, all the strains show significant increases in idiotype expression.

The expression of idiotype in the TNP-KLH secondary response is more uniform. There is no significant difference in the level of combined idiotype or of any of the individual idiotypes except for TK-1 where AKR, BALB/c and DBA/1 have approximately twice the amount of idiotype. The most surprising result in the secondary response is the high level of idiotype expression in the TNP-ficoll response. All of the hybridomas expressing these idiotypes were derived from thymus-dependent responses. I would have predicted that if there were differences between thymus-independent and thymus-dependent responses that there would be more idiotype in the thymus-dependent than

the thymus-independent response, but this clearly was not the case. However, the hybridomas were derived from BALB/c mice and these mice were the only strain to show reduced levels of idiotype in the TNP-ficoll response. This may be the best evidence for genetic control of idiotype expression, since the difference between the strains is not just in the level of idiotype expressed but in how the levels are modified in response to different immunization protocols.

The final two questions I will address are: What is the relationship, if any, between isotype and idiotype, and what effect to T cell signals in the form of GVH have on idiotype expression? The experiments to answer the first question overlap with those addressed to the second. In the primary response of BALB/c mice to TNP-KLH and TNP-ficoll individual isotype responses were analyzed for idiotype content. Secondary responses of a number of mouse strains were analyzed for their expression of individual IgG subclasses in the distribution of idiotype among these subclasses. For the second question, normal or defective Xid mice which had or had not undergone GVH were tested for idiotype expression and individual isotypes.

Table IX shows the result of the BALB/c studies. It appears that TK-4 idiotype in the TNP-KLH response is associated with the IgG3 isotype. However, this is not a

consistent finding since the TNP-ficoll response where the IgG3 subclass is in much higher proportion to the TK-4 idiotype is essentially missing. The other idiotypes are present at low levels and in no particular association with any isotype. The level of M315/460 idiotype was lower than expected from the results of Rosenstein (17). However, they assayed for their public idiotype at forty days after priming, whereas I am looking on day nine. The level of idiotype expression could easily vary with time after immunization.

Table X shows the subclass distribution for the secondary IgG response of five different strains. The most striking observations are: 1) the almost complete dominance of the response by the IgG1 and IgG2b subclasses, and 2) the similarity in subclass distribution between the TNP-KLH and TNP-ficoll responses. These results are compatible with a previous report which showed that the subclass distribution of a secondary response, in mice primed with a thymus-dependent antigen, has the characteristics of a TD response even when a TI antigen is used (18). The one possible exception to this observation is the response of the C57bl/6 mouse where the TNP-ficoll response does induce increased expression of IgG3 and reduced expression of IgG1 typical of a TI-2 antigen response.

To assess the association, if any, between subclass and idiotype responses in which one or more idiotypes comprised at least ten percent of the response were tested for the distribution of those idiotypes in the different subclasses. These data are shown in Table XI. Differences in idiotype expression do not appear to correlate with differences in isotype expression. For example, in the response of the AKR mouse, there was a dramatic increased expression of the idiotype panel when the TI-2 antigen was used in comparison to the TD form, yet the distribution of the subclasses for these two responses was identical. In the case of the TK-1 idiotype and the AKR mouse response, the shift to IgG1 predominance in the TI-2 response is not matched by a change in the overall subclass distribution or by a shift in the expression of idiotype. In the DBA/1 mouse, however, the shift in TK-1 idiotype subclass distribution is mirrored by a similar shift in the overall subclass distribution. Taken together, these observations on primary and secondary responses do not show any consistent relationship between a given idiotype and a given isotype.

Table XII shows the TNP-KLH response of B_6 D_2 mice with and without GVH. The only apparent association of isotype and idiotype is that the vast majority of idiotype is associated with the dominant isotypes IgG1 and IgG2b. GVH had the effect of reducing the response and

reducing the proportion of the response which was idiotype positive. The working hypothesis, as stated earlier, is that GVH stimulates a shift of B_2 cells to B_1 cells. From this one would predict that the TNP-ficoll response should have had an increased proportion of idiotype in the presence of GVH. As seen in Table XIII, this prediction is incorrect. While the IgM response is increased with GVH as expected, the high proportion of TK-5 idiotype drops to zero. What little idiotype is present in the IgG response is also reduced in the presence of GVH.

Tables IX and X show the effect of GVH in F_1 mice expressing the defective Xid gene. The results of the TNP-KLH response mimic exactly those in B_6 D_2 mice (compare Tables XII and XIV). The TNP-ficoll results are shown in Table XVIII. In this situation the response to TNP-ficoll in the absence of GVH is zero due to the Xid gene. As shown earlier, however, GVH can partially restore this response. The level of idiotype in this response would be similar to the TNP-KLH response with GVH except for the high level of TK-5 idiotype seen in the IgG1 subclass. The effect of GVH on the expression of these idiotypes, then, is to consistently give lower levels of expression than if GVH were not involved. There was no evidence for a consistent association of any of the idiotypes with any isotype.

B_6D_2 mice are F_1 offspring of a cross between

C57Bl/6 and DBA/2 mice. DBA/2 mice differ from DBA/1 mice only in the H-2 region. The expression of idiotype by the F_1 cross appears to be higher than in either of the parental strains tested (Tables V through IX compared to Table XIII). This is also true of the CBA/n by BALB/c F_1 mice, although I have no information on idiotype expression in the CBA background. That F_1 mice can express idiotype at a higher level than either of the parental strains is an indication of the complex genetics involved in regulation of idiotype expression.

STUDIES ON COLONY FORMING B CELLS

As discussed in the introduction, colony-forming B cells can be used as a phenotypic marker for the Xid gene of the CBA/n mouse. The best defined defect of this gene is the apparent lack of a relatively mature population of B cells (19). One of the important questions in the study of autoimmune disease is which cell populations(s) is/are involved in the disease process. Since the presence of the Xid gene appears to result in the loss of a particular subset of B cells the question can be asked are these cells involved in autoimmune disease. The Xid gene has been shown to modify the expression of the spontaneous autoimmune disease of NZB mice (20, 21). These mice also have elevated numbers of colony forming B cells (22).

BXSB mice also have a spontaneous autoimmune disease which is unique since males are affected to a much greater extent than are females. This male preponderance is not due to hormonal influences, but has been linked to the Y chromosome (23, 24). The question I wished to address was whether BXSB mice were similar to the NZB in having increased numbers of colony forming B cells and is their disease reduced by the presence of the Xid gene? The results of this study were published in a collaborative report with Drs. B. Golding, H. Golding and J. Morton (25).

To determine the effect of the Xid gene in BXSB F_1 crosses between BXSB and normal or Xid defective CBA/n mice were made. The presence of autoimmune disease was shown by increases in spleen and lymph node weight. Table XVI shows development of an autoimmune disease in both the parental BXSB strain and the F_1 offspring in which spleen and lymph node enlargement developed progressively between five and eight months. The F_1 females do not show signs of disease, which is consistent with the course of the disease seen in BXSB females who develop autoimmunity that is less severe and occurs later in life than in the male. While the presence of the CBA/ca mouse genes delays development of disease in the male, the disease does clearly develop. Table XVI shows the data for the crosses involving the Xid gene. Three points are

apparent: first, the presence of the Xid gene prevents the development of autoimmune disease even in 10-month-old mice. Second, in the reverse cross BXSB X CBA/n the presence of BXSB autosomal and normal X chromosome genes results in the appearance of some disease but at reduced levels compared to when the defective X chromosome is present. And lastly, colony forming cells do not appear to be enhanced in BXSB mice with the disease.

Thus, the answers to the two questions posed above are, no, colony forming cells are not increased in BXSB mice, but yes, the Xid gene is able to protect against development of the disease. One might have predicted that if the cells controlled by the Xid gene were necessary, as shown, that in the presence of disease Xid-controlled cells (colony forming cells) would be elevated as is the case in the NZB. This was not seen and may reflect the pleiotropic nature of the Xid defect. While the hallmark of the Xid gene is the inability to respond to TI-2 antigens, there are also abnormalities in the ability to respond to thymus replacing factor and in the isotype profile and level of response to some TD antigens (26-28). Colony forming B cells are not necessarily the cells involved in autoimmune disease development in NZB or BXSB mice, but may be one of many functionally different subsets controlled by the Xid gene.

As discussed in the introduction, B cells can be

divided into two populations, B_2 cells which respond to TD antigens and B_1 cells which respond to TI-2 antigens and may be missing from the CBA/n mouse. Previous work has suggested that allogeneic T cell signals may be able to trigger differentiation of B_2 cells to B_1 cells (29, 30). Since B_2 cell development appears to be under the control of the Xid gene, it was thought that allogeneic effects in the form of graft-vs-host disease might be able to correct the Xid defect. Antigen responses to TNP-ficoll and isotype profile in colony forming cells were used as markers for the appearance of B cells in CBA/n mice which had undergone graft-vs-host disease (GVH). As shown in Table XVII, CBA/n mice previously unresponsive to TNP-ficoll were able to give low responses to this antigen if they had undergone GVH. The isotype profiles show increased levels of IgG1 antibody as would be expected of a B_2 response. Colony forming cells, however, were not restored by this regimen.

It appears then that graft-vs-host disease can stimulate the development of some B_1 cells in CBA/n mice, but this development is not complete. Whether this is in some way associated with the failure of B colony forming cell restoration is not clear, but since colony forming ability is not regained to any degree it seems unlikely to be linked to B_1 cell development. This observation is compatible with those made in the

previous section on autoimmune mice. In that situation B cells appeared to be necessary for disease development but colony forming cells were not elevated in the presence of disease, suggesting that they were not representative of all B_1 cells. Those observations, coupled with the graft-vs-host studies, are consistent with the model where colony forming cells are a subset of cells within the B_1 population. Additional studies, perhaps looking for surface markers, are clearly needed to clarify this point.

DISCUSSION

To better understand the binding site associated with the idiotypes I was studying, their fine specificity for antigen was tested. By testing the ability of anti-hapten antibodies to bind hapten analogs, a measure of the similarity of the binding sites can be made which in some instances was more informative than equilibrium dialysis using a single hapten. The panel of eight hapten analogs defines four different binding sites. The first is that of TK-5, whose distinguishing characteristic is its low affinity for TNP and DNP relative to that of the other antibodies. TK-1 and TK-4 contain the second type of binding site, although the sites on these two molecules are not identical since they clearly differ in their ability to bind TNP-Phe. They are distinct from TK-5 in

having a tenfold higher affinity for TNP which was also apparent from equilibrium dialysis data. The third site is that of TK-3 which has a very high affinity for TNP and a marked preference for TNP over DNP not seen for the other antibodies. The fourth binding site is represented by myeloma protein M460 which displayed the lowest affinity of any of the antibodies tested. M460 also differed from the other sites by its relatively high binding values for TNP-LYS and DNP-GLY.

Examination of the idiotypes of the hybridomas indicated each was unique and that only one (TK-1) shared a determinant with another anti-TNP antibody (M460). This cross-reactivity is surprising in view of the clear differences seen between TK-1 and M460 in both affinity and fine specificity as shown in Table V. Table V shows TK-1 and TK-4 to have very similar fine specificity patterns and since the anti-idiotypes were purified by hapten elution and were, therefore, presumably, binding site specific, we expected the idiotypes of these antibodies to be similar also. This was not the case. However, since antibodies against each of these proteins did not recognize the other, the idiotype differences between TK-1 and TK-4 may reflect the differences in affinity for TNP-Phe. It should be noted that these proteins could also have shared idiotopes but antibodies against these determinants would

have been removed in the purification of these hetero-antisera (see Materials and Methods).

Although the IEF analysis of the light chains shown in the first manuscript showed heterogeneity, this is not clear evidence placing the idiotype on the light or heavy chain. In most idiotype systems the expression of the idiotype is linked to the Igh allotype locus (31-32), but restriction of light chains in relation to idiotypes has also been reported (33-35). The reasons advanced for these restricted associations of heavy and light chains are either that the idiotype is physically present on both chains or, as is usually the case, that the heavy chain requires a particular light chain in order to produce a functional binding site (35).

These results are in agreement with previous work. When fine specificity analysis and idiotype have been compared in other systems, antibodies sharing idiotype usually, but not always, shared fine specificity patterns (36). There have also been antibodies described which express idiotype but do not bind the relevant antigen (37). In this case, TK-3 and TK-5 clearly differ in both fine specificity and idiotype, but TK-1 and TK-4 exhibit only minor differences in fine specificity (Table V TNP-Phe and 4-NPBA) and yet have distinct idiotypes. These analyses are also consistent with the results of other studies describing the complexity of the anti-TNP/DNP antibody

response (38-39). The expression of TK-1 among memory anti-TNP hybridomas suggests that variable regions related to that defined by M460 are represented in long-term memory; however, our results also suggest that the anti-TNP memory pool contains a number of other possibly unrelated variable regions at least partially defined by TK-3 and TK-4.

The main thrust of these studies was to examine the expression of the idiotypes which I had defined in a number of different situations to see if their expression was regulated in any way. The first observation was that the genetic makeup of the animal does affect the level of idiotype expression. The nature of this apparent genetic control cannot be clarified without the use of congenic strains of mice, but previous work would suggest that Igh linked genes are the most likely (31, 40). One genetic difference seen, which is probably not attributable to the Igh locus, is that BALB/c mice did not have increased levels of idiotype expression in the TNP-ficoll versus the TNP-KLH secondary response. This observation also applies to the question, do B_1 and B_2 cells differ in idiotype expression. The answer is yes, especially in the secondary response, but the difference is not consistent. Five of the strains show a marginal to marked increase in idiotype when challenged with TNP-ficoll compared to challenge with TNP-KLH, but one, BALB/c, was reduced. This argues against

the hypothesis that idiotype preference is an intrinsic property of the B cells themselves and for the hypothesis that idiotypes are selected for by regulatory cells which are themselves under some form of genetic control.

Virgin and memory cells also appear to differ in their expression of this idiotype panel, which is in agreement with findings in other systems (36, 41-42). Since three of the idiotypes tested were from a secondary response, it was not surprising that these idiotypes were more prevalent in those responses. TK-5 idiotype, however, is derived from a primary response and was also expressed at a higher level in secondary, not primary, responses. The assumption that hybridomas derived from a response accurately reflect the idiotypic profile of that response has conflicting experimental data to support it. Hybridomas derived from the anti-NP and anti-DNP systems have been found to have idiotypes prevalent in the response from which they came (42, 43), but in responses to two other haptens, phthalate and oxazolone, the frequency of serum and hybridoma idiotypes did not agree (44, 45).

In a study similar to those reported here, Scott and Fleischman describe idiotypes which were expressed in primary anti-DNP responses and were only found in association with certain isotypes (43). In contrast, none of the idiotypes which I studied showed any consistent association with a particular immunoglobulin isotype. Rather, they

were found in whichever isotype or isotypes were predominant in a given response. These two observations are not necessarily in conflict since association with an isotype may be a property of only certain idiotypes.

The effect of GVH on idiotype expression was in all cases to reduce the expression of the idiotype. This could have occurred by one of two mechanisms. First, the B_2 to B_1 shift, which GVH apparently causes (20-21), could alter idiotype expression. However, the change seen in idiotype expression is a reduction, the proposed shift in responding cell populations should have resulted in an increased expression of idiotype. Alternatively, helper and/or suppressor T cells, activated by the GVH, could alter the idiotype expression.

Taken together, all of these observations suggest a model for the expression of this panel of idiotypes. A summary of my findings is that this panel of idiotypes is primarily associated with a secondary TNP-ficoll response and to a lesser extent to the secondary TNP-KLH response. Inbred strains of mice differed in the amount of idiotype expressed and in one case in the distribution of the idiotypes between TNP-KLH and TNP-ficoll responses. None of the idiotypes were consistently restricted to expression with a particular isotype and the presence of GVH resulted in reduced levels of idiotype expression. A model which incorporates these observations is that these idiotypes

are present at low levels during the primary response but upon secondary stimulation, especially by TI-2 antigen, they are selectively expanded. This selective expression could be the result of either positive or negative regulatory events, most probably mediated by T cells. If GVH is present, however, then different and/or additional idiotypes are selected, resulting in a lower proportional expression of the idiotype panel. These new idiotypes could be the result of somatic mutation or expression of idiotypes which require the extra T cell help provided by GVH.

The differences in idiotype expression between mouse strains differing at the H-2 and Igh loci are consistent with the association of idiotype with certain Igh haplotypes seen in other studies (31, 40). The influence of T cells on idiotype expression has been well established (46-48) and my data concerning GVH would suggest that regulatory T cells are functioning in this system, although this was not pursued in detail.

The differences I observed between primary and secondary responses in the level of idiotype expressed is somewhat different from that seen by others (36, 43, 44). In most idiotype systems the idiotype studied was derived from a primary response and is expressed to the greatest extent in that response. When secondary responses are examined, the expression of the idiotype is either reduced

or lost. The question has been: What is the origin of the "new" idiotypes expressed in the secondary response? In my studies, most of the idiotypes were derived from a secondary response and are expressed to the greatest extent there, but are also found at low levels in the primary response. This suggests that the origins of the "new" idiotypes in the secondary response are minor clones present in the primary response. Further, if somatic mutation is the source of these minor clones then it must be an early event in relation to the development of memory cells.

There are at least three additional areas in which this research could continue. The first would be the kinetics of idiotype expression. I only looked at idiotype expression on one day, it is quite possible that as in the case of M460 idiotype (48), idiotype expression could vary with time after immunization. Secondly, the use of congenic strains of mice and/or F_1 crosses would allow a more precise determination of genetic controls of idiotype expression. Lastly, it should be determined if there are T cells which recognize the idiotypes studied here or express these idiotypic determinants on their surface. The role of these T cells in regulation of idiotype expression could then be determined.

SUMMARY

The basic aim of this research was not to dissect a particular control mechanism involved in the regulation of idiotype expression, but to find evidence, in a response which is not dominated by a given idiotype, for or against the existence of specific mechanisms of idiotype regulation which have been described in systems which do have a dominant idiotype. To accomplish this, anti-TNP hybridomas were made and characterized biochemically, in relation to antigen binding, fine specificity and idiotype. Using this panel of idiotypes anti-TNP responses were assayed for idiotype content. These responses differed in terms of genetic background, state of priming, form of hapten carrier used, and the presence or absence of T cell influences in the form of GVH. The results of these studies show the regulation of idiotype expression to be complex. While evidence for restriction of any of these idiotypes with a given isotype is lacking, other genetic and cellular factors which have been described in systems dominated by a single idiotype are clearly at work in the idiotypically heterogeneous anti-TNP response studied here. It appears that the dominance of a response by a single idiotype is due to normal regulatory mechanisms that result in the restrictive expression of idiotype.

REFERENCES

1. Rittenberg, M. B. and A. A. Amkraut. 1966. Immunogeneity of trinitrophenylated hemocyanin: Production of primary and secondary anti-hapten precipitens. J.Immun. 47:421.

2. Inman, J. K. 1975. Thymus independent antigens: the preparation of covalent hapten-ficoll conjugates. J. Immunol. 114:704.

3. Gallily, R. and J. S. Garvey. 1968. Primary stimulation of rats and mice with hemocyanin in solution and adsorbed onto bentonite. J. Immunol. 101:924.

4. Tittle, T. V. and M. B. Rittenberg. 1978. Expression of IgG memory response in vitro to thymus-dependent and thymus-independent antigens. Cell. Immunol. 35:180.

5. Kearney, J. F., P. Barletta, Z. Quan and J. Quintans. 1981. Monoclonal v. heterogeneous anti-H-8 antibodies on the analysis of the anti-phosphorylcholine response in BALB/c mice. Eur. J. Immunol. 11:877.

6. Kurland, J. I., P. W. Kincade and M. A. S. Moore. 1977. Regulation of B. lymphocyte clonal proliferation by stiumlatory and inhibitory macrophage-derived factor. J. Exp. Med. 146:1420.

7. Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochem. 6:43.

8. Rosenstein, R. W., Zeldis, J. B., Konigsberg, W. H. and F. F. Richards. 1979. The location and expression of idiotype determinants in the immunoglobulin variable region. I. Characterization of antibodies directed against the variable region of mouse myeloma immunoglobulins 315 and 460. Molec. Immunol. 16:361.

9. Benacerraf, B. and B. B. Levine. 1962. Immunological specificity of delayed and immediate hypersensitivity reactions. J. Exp. Med. 115:1023.

10. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. Ann. NY Acad. Sci. 51:660.

11. Onoue, K., Y. Yagi, A. L. Grossberg, D. Pressman. 1965. Number of binding sites of rabbit macroglobulin antibody and its subunits. Immunochem. 2:401.

12. Voss, E. W., Eisen, H. N. 1968. Valence and affinity of IgM antibodies to the 2-4 dinitrophenyl (DNP) group. Fred. Proc. 27:684.

13. Onoue, K., A. L. Grossberg, Y. Yagi, D. Pressman. 1968. Immunoglobulin M antibodies with ten combining sites. Science. 162:574.

14. Eisen, H. M., E. S. Simms, M. Potter. 1968. Mouse mylonoma proteins with antihapten antibody activity. The protein produced by plasma cell tumor MOPC-315. Biochem. 7:4126.

15. Underdown, B. T., E. S. Simms, H. N. Eisen. 1971. Subunit structure and number of combining sites of the immunoglobin A myeloma protein produced by mouse plasmacytoma. MOPC-315. Biochem. 10:4359.

16. Lium F-T, J. W. Bohn, E. L. Ferry, H. Yamamoto, C. A. Molinaro, L. A. Sherman, N. R. Klinman and D. H. Katz. 1980. Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation and characterization. J. Immunol. 124:2728.

17. Zeldis, J. B., R. Riblet, W. H. Konigsberg, F. F. Richards and R. W. Rosenstein. 1979. The location and expression of idiotypic determinants in the immunoglobulin variable region. III. Expression of the protein 315 and 460 idiotype determinants in mouse anti-DNP antibodies. Molec. Immunol. 16:657.

18. Golding, H. and M. B. Rittenberg. 1982. In vivo allogeneic effects: shift in the isotype profile of primary TI-2 responses in mice undergoing graft-vs-host reaction. J. Immunol. 129:1878.

19. Scher, I., A. D. Steinberg, A. K. Benniz and W. E. Paul. 1975. X-linked B lymphocyte immune defect in CBA/n mice. II. Studies of the mechanism underlying the immune defect. J. Exp. Med. 142.637.

20. Taurog, J. E., H. M. Moutsopoulos, Y. J. Rosenberg, T. M. Chused and A. D. Steinberg. 1979. CBA/n X-linked B cell defect prevents NZB B cell hyperactivity in F_1 mice. J. Exp. Med. 150:31.

21. Ashugi, Y., M. E. Gershwin and A. Ahmed. 1981. Study of congenitally immunologically mutant New Zealand mice. V. B cell function of NZB Xid mice. J. Immunogenet. 8:129.

22. Kincade, P. W., O. Lee, G. Fernandes, M. A. S. Moore, N. Williams and R. A. Good. 1979. Abnormalities in clonable B lymphocytes and myeloid progenitors in autoimmune NZB mice. Proc. Nat. Acad. Sci. 76:3464.

23. Murphy, E. D. and J. G. Roths. 1979. A Y-chromosome associated factor in strain BXSB producing accelerated autoimmunity and lymphoproliferation. Arthritis and Rheumatism. 22:1188.

24. Eisenberg, R. A. and F. J. Dixon. 1980. Effect of castration on male-determined acceleration of autoimmune disease in BXSB mice. J. Immunol. 125:1959.

25. Golding, G., H. Golding, P. G. Foiles and J. I. Morton. 1983. CBA/n X-linked defect delays expression of the Y-linked accelerated autoimmune disease in BXSB mice. J. Immunol. 130:1043.

26. Press, J. L. 1981. The CBA/n defect defines two classes of T dependent antigens. J. Immunol. 126:1234.

27. Scher, I., A. K. Berning and R. Asofsky. 1979. X-linked B lymphocyte defect in CBA/n mice. IV. Cellular and environmental influences on the thymus dependent IgG anti-sheep red blood cell response. J. Immunol. 123:477.

28. Perlmutter, R. M., M. Nahm, K. E. Stein, J. Slack, I. Zitron, W. E. Paul and J. M. Davie. 1979. Immunoglobin subclass-specific immunodeficiency in mice with an X-linked B-lymphocyte defect. J. Exp. Med. 144:993.

29. Golding, H. and M. B. Rittenberg. 1982. In vitro and in vivo allogeneic effects; differential modulation of B cell subpopulations. J. Immun. 128:1625.

30. Kaattari, S. L. and M. B. Rittenberg. 1982. Concanavalin A supernatent recruits antigen insensitive IgG memory B-lymphocyte precursors into an antigen sensitive precursor pool. J. Immunol. 128:720.

31. Zach, R. S., T. Imanashi-Kari and K. Rajewsky. 1977. Idiotypic analysis of the response of C57Bl/6 mice to the (4-hydroxy-3-nitrophenyl) acetyl group. Eur. J. Immunol. 8:559.

32. Zeldis, J. B., W. H. Konigsberg, F. F. Richards and R. W. Rosenstein. 1979. The location and expression of idiotypic determinants in the immunoglobulin variable region. II. Chain location of variable region determinants. Molec. Immunol. 16:371.

33. Dzierzak, E. A., C A. Janeway, R. W. Rosenstein and P. D. Gottlieb. 1980. Expression of an idiotype (Id-460) during in vivo anti-dinitrophenyl antibody responses. I. Mapping of genes for Id-460 expression to the variable region of immunoglobulin heavy chain locus and to the variable region of immunoglobulin K-light-chain locus. J. Exp. Med. 152:720.

34. Cramer, M., U Krawinkle, I. Melchers, T. Imanashi-Kari, Y. Ben-Neriah, P. Givol and K. Rajewsky. 1979. Expression of immunoglobulin variable regions in (4-hydroxy-3-nitrophenyl) acetyl (NP)-specific receptors isolated from murine B and T lymphocytes. Eur. J. Immunol. 9:332.

35. Barstad, P., J. Hubert, M. Hunkapillar, A. Goetze, J. Schilling, B. Black, B. Easton, J. Richards, M. Weigert, and L. Hood. 1978. Immunoglobulins with hapten binding activity: structure function correlation and genetic implications. Eur. J. Immunol. 8:497.

36. Chang, S. and M. B. Rittenberg. 1981. Immunologic memory to phosphorylcholine in vitro. I. Asymmetric expression of clonal dominance. J. Immunol. 126:925.

37. Wysocki, L. J. and V. C. Sato. 1981. The strain A anti-P-azophenylarsonate major cross-reactive idiotypic family includes members with no reactivity toward P-azophenylarsonate. Eur. J. Immunol. 11:832.

38. Pink, J. R. L. and B. A. Askonas. 1974. Diversity of antibodies to crossreacting nitrophenyl haptens in inbred mice. Eur. J. Immunol. 4:426.

39. Williamson, A. R. 1971. Antibody isoelectric spectra. I. Analysis of the heterogeneity of antibody molecules in serum by isoelectric focusing in gel and specific detection with hapten. Eur. J. Immunol. 1:390.

40. Pawlak, L. L., E. B. Mushinski, A. Nisonoff and M. Potter. 1973. Evidence for linkage of the IgCH locus to a gene controlling the idiotype specificity of anti-P-azophenylarsonate antibodies in strain A mice. J. Exp. Med. 137:22.

41. Henry, C. and A. Lucas. 1982. The relation of idiotype expression to isotype and allotype in the anti-P-azobenzenearsonate response. Eur. J. Immunol. 12:175.

42. Reth, M. G. J. Hammerling and K. Rajewsky. 1979. Analysis of the repertoire of anti-NP antibodies in C57Bl/6 mice by cell fusion. I. Characterization of antibody families in the primary and hyperimmune response. Eur. J. Immunol. 8:393.

43. Scott, M. G. and J. B. Fleischman. 1982. Preferential idiotype-isotype association in antibodies to dinitrophenyl antigens. J. Immunol. 128:2622.

44. Bloor, A. G., Y. Jou, C. Hoeplinger, J. E. Gartner, G. L. Mayers and R. B. Bankert. 1982. Hapten specific B cell repertoire probed by hybridoma technology. Selection and characterization of representative clonotypes from the antibody-forming cell pool. J. Immunol. 128:1443.

45. Kaartinen, M., G. M. Griffiths, P. H. Hamlyn, A. F. Markham, K. Karajalainen, J. L. I. Pelkonen, O. Makela, C. Milstein. 1982. Anti-oxazolone hybridoma and the structure of the oxazolone idiotype. J. Immunol. 130:937.

46. Berek, C., M. H. Schreier, C. L. Sidman, J. C. Jaton, H. P. Hocher and H. Cosenza. 1980. Phosphorylcholine-binding hybridoma proteins of normal and idiotypically suppressed BALB/c mice. I. Characterization and idiotypic analysis. Eur. J. Immunol. 10:258.

47. Janeway, C. A., R. A. Murgita, F. I. Weinbaum, R. Asofsky and H. Wigzell. 1977. Evidence for an immunoglobulin-dependent antigen specific helper T cell. Proc. Nat. Acad. Sci. 74:4582.

48. Bona, C. and W. E. Paul. 1979. Cellular basis of regulation of expression of idiotype. I. T suppressor cells specific for MOPC-460 idiotype regulate the expression of cells secreting anti-TNP antibodies bearing 460 idiotype. J. Exp. Med. 149:592.

TABLE I

Valence of anti-TNP by hybridomas.

Hybridoma	Valence
TK-1	1.0±0.2[a)]
TK-3	0.6±0.1
TK-4	0.6±0.02
TK-5	3.7±1.1

Antibody valence was determined from a Scatchard plot of equilibrium dialysis binding data using ^{3}H-DNP-lysine.

a) Valence ± S.E.

TABLE II

Fine specificity analysis by ELISA -- ratio of inhibition by various analogs of DNP and TNP compared to TNP-epsilon amino caproic acid.

	TK-3	TNP-EACA	TNP-Lys	TNP-Phe	DNP-EACA	DNP-Lys	DNP-Gly	2,4 DNA	4-NPBA
TK-1	0.0013[a]	1.0	0.78[b]	2.4	3.3	1.1	0.59	0.79	0.065
TK-3	1.0	1.0	0.15	0.36	0.001	0.001	0.000053	0.00047	0.000011
TK-4	0.0036	1.0	0.20	0.12	2.0	0.22	0.12	0.43	0.0021
TK-5	0.00016	1.0	0.36	0.15	1.4	1.7	0.34	0.24	0.048
M460	0.000052	1.0	2.98	0.37	0.73	1.73	1.69	0.25	0.16

a $\frac{\text{[TNP-EACA] for 50\% inhibition of TK-3}}{\text{[TNP-EACA] for 50\% inhibition of heterologous antibody]}}$

b $\frac{\text{[TNP-EACA]}}{\text{[Heterologous hapten]}}$

Calculated from concentrations needed to inhibit 50% of binding in ELISA.

TABLE III

Characteristics of anti-idiotype antibodies.

Anti-Idiotype	Source	Specificity
TK-1	Rabbit	TK-1, M460
AI-31	Mouse Monoclonal	TK-3
TK-4	Rabbit	TK-4
TK-5	Rabbit	TK-5
M315/460	Rabbit	M315, M460

TABLE IV

Genotype of inbred mouse strains used.

Strain	H-2	IgH
A/J	a	e
AKR	k	d
BALB/c	d	a
C57Bl/6	b	b
DBA/1	q	c
SJL	s	b
B_6D_2	b/d	b/c
(CBA/n x BALB/c)	k/d	j/a

TABLE V

Idiotype content of primary IgM anti-TNP-KLH response.

Idiotype	A/J	AKR	BALB/c	C57Bl/6	DBA/1	SJL	
TK-1	10.7±2.6[a]	2.6±2.6	3.5±1.8	0	0.3±0.3	13.2±2.5	P<0.005[d]
TK-3	2.6±2.6	2.7±2.7	ND[e]	0	0	11.2±6.9	
TK-4	0.4±0.4	0	7.3±3.7	0	0	0.4±0.4	P=0.037
TK-5	7.6±2.8	0	1.7±1.7	0	0	4.2±2.3	P=0.072
M315/460	3.9±3.4	0	4.6±2.6	0	0.7±0.7	10.7±4.1	P=0.196
Combined[c]	25.2 (16.9-33.4)[b]	5.3 (0-15.7)	17.1 (3.6-30.6)	0	0.9 (0-2.8)	39.8 (12.3-67.2)	

a) Data are arithmetic means of percent inhibition of ELISA by anti-idiotype ± S.E. serum sample pooled from 5-6 mice.

b) 95% comfidence interval calculated from the S.E.

c) Combined idiotype level is determined by addition of the individual idiotype data.

d) P value from one way analysis of variance comparing the idiotype level of the different strains.

e) Primary IgM response not tested. TK-3 idiotype is not present in primary IgG response.

TABLE VI

Idiotype content of primary IgM anti TNP-ficoll response.

Idiotype	A/J	AKR	BALB/c	C57Bl/6	DBA/1	SJL	
TK-1	2.7±2.7	0	0.2±0.2	0	0	9.8+2.2	P=0.038
TK-3	0	0	ND[a)]	0	0	5.1±3.6	
TK-4	1.1±1.1	0	1.5±1.5	0	0	0	P>0.250
TK-5	0	0	3.2±3.2	0	0	5.9±3.3	P>0.250
M315/460	0	0	0	0	0	5.1±2.6	P=0.058
Combined	3.8 (0-11.2)	0	4.9 (0-14.4)	0	0	25.9 (9.9-41.9)	P=0.032

See Table V.

a) IgM response not tested. No TK-3 idiotype in primary IgG response.

TABLE VII

Idiotype content of secondary IgG anti-TNP-KLH response.

Idiotype	A/J	AKR	BALB/c	C57Bl/6	DBA/1	SJL	
TK-1	8.3±1.1	12.5±2.2	17.5±4.2	4.0±0.5	18.1±5.6	0.8±0.3	P=0.020
TK-3	2.3±1.3	1.4±1.4	0.7±0.7	0	0	2.7±2.7	
TK-4	2.9±2.7	0.2±0.2	3.4±2.0	0	0	0	P>0.250
TK-5	3.4±1.9	1.3±1.3	5.8±2.9	2.7±0.6	0	2.3±2.3	P>0.250
M315/460	2.9±1.9	0	0.9±0.9	7.3±0.7	0	1.7±1.7	P=0.102
Combined	19.8 (5.9-33.6)	15.3 (14.5-16.1)	28.3 (24.3-32.8)	13.9 (12.9-14.9)	18.1 (7.2-28.9)	7.4 (3.9-10.9)	P 0.250

See Table V.

TABLE VIII

Idiotype content of secondary IgG response to TNP-ficoll.

Idiotype	A/J	AKR	BALB/c	C57Bl/6	DBA/1	SJL	
TK-1	14.4±3.5	16.7±2.8	0	5.8±0.6	1.8±8.0	13.1±1.2	P=0.077
TK-3	0.6±0.6	1.2±1.0	0	0	0	0	
TK-4	6.1±2.1	11.4±0.2	0	1.2±1.2	0	0	P<0.005
TK-5	5.8±4.3	20.2±2.2	5.1±3.6	5.1±1.4	4.3±2.4	7.3±4.5	P=0.178
M315/460	3.3±2.6	14.3±3.4	0	2.9±1.2	3.1±2.4	2.3±2.1	P=0.045
Combined	30.1 (10.8-49.4)	63.8 (48.9-78.6)	5.1 (0-12)	14.9 (11.0-18.7)	25.4 (15.1-35.7)	22.6 (15.8-29.5)	P 0.005

See Table V.

TABLE IX

Idiotype content of primary BALB/c response to TNP antigens by antibody subclass.

TNP-KLH

ug/ml[a]	IgM 3.6±1.1	IgG3 0.8±0	IgG1 1.1±0.1	IgG2b 0.18±.02	IgG2a 0.01	Total IgG 2.1
TK-1	3.5±1.8[c]	5.3±5.3	0	0.9±0.5	0.1±0.1	6.3±5.6
TK-4	7.3±3.7	15.6±0.2	0	1.2±1.2	0	16.8±0.8
TK-5	1.7±1.7	0	0	1.8±1.3	0	1.8±0.7
M315/460	4.6±2.6	4.4±4.4	0	0	0	4.4±4.4
	17.1 (3.6-30.6)[d]					29.2 (18.1-40.3)

TNP-ficoll

ug/ml	IgM 2.2±.6	IgG3 1.8±0.5	IgG1 0.7±.4	IgG2b .27±0	IgG2a 0.02±.02	Total IgG 2.8
TK-1	0.2±0.2	4.2±4.2	1.1±1.1	2.3±0.2	0	7.6±4.9
TK-4	1.5±1.5	0.9±0.9	0	0.3±0.3	0	1.2±0.8
TK-5	3.2±3.2	2.9±2.7	3.6±1.8	3.2±0.3	0	9.4±4.0
M315/460	0	0	0	0	0	0
	4.9 (0-14.4)					18.2 (0-36.8)

a) ug/ml of antibody ± S.E.

b) Total IgG determined by totaling the subclasses.

c) The percent of the IgM or total IgG response inhibited by anti-idiotype.

d) 95% confidence interval.

TABLE X

IgG subclass distribution of secondary anti-TNP responses

Strain	Antigen	Total IgG	IgG3	IgG1	IgG2b	IgG2a
A/J	TNP-K1H	4155±54[a]	1[b]	85	13	1
	TNP-ficoll	403±51	1	92	5	2
AKR	TNP-K1H	1592±364	0	53	41	6
	TNP-ficoll	53±5	4	43	49	4
C57Bl/6	TNP-K1H	157±38	0	92	8	0
	TNP-ficoll	57±18	14	57	29	0
DBA/1	TNP-K1H	734±31	0	89	10	0
	TNP-ficoll	86±20	1	65	33	1
SJL	TNP-K1H	2411±258	0	59	41	0
	TNP-ficoll	105±29	0	62	38	0

a) ug/ml of antibody ± S.E. of anti-TNP antibody from a serum pool of from 5-6 mice.

b) Percentage of the total anti-TNP IgG which is of the indicated subclass.

TABLE XI

Subclass distribution of idiotype in secondary IgG anti-TNP-KlH response.

Strain	Antigen	Idiotype	IgG1	IgG2b
AKR	TNP-KlH	TK-1	43.4[a)]	50.3
	TNP-ficoll	TK-1	80.9	14.3
	TNP-ficoll	TK-5	25.6	72.2
	TNP-ficoll	M315/460	36.0	61.4
DBA/1	TNP-KlH	TK-1	85.6	14.4
	TNP-ficoll	TK-1	62.3	37.7
A/J	TNP-ficoll	TK-1	64.9	35.1
SJL	TNP-ficoll	TK-1	34.8	65.2

a) Percentage of total idiotype positive antibody which is expressed in the indicated subclass.

TABLE XII

Idiotype content of primary B_6D_2 response to TNP antigen by antibody subclass.

TNP-KlH

	IgM	IgG3	IgGl	IgG2b	IgG2a	Total IgG
ug/ml	2.7±0.2	34.4±3.1	261.1+62	117.0±9.4	1.4±0.2	413.9
TK-1	4.1±23	1.9±0.03	24.3±4.2	6.9±15	0.1±.03	33.1±1.0
TK-4	0	1.5±.03	7.4±6.1	3.8±2.3	0	12.7±6.1
TK-5	1.3±1.3	0.8±0.4	16.5±1.4	4.8±0.7	0.1±0.03	22.2±1.9
M315/460	7.2±7.2	0.3±0.3	0	2.4±1.5	0	2.7±1.5
	12.6 (0-15.4)					70.6 (57.4-81.8)

TNP-KlH + GVH

	IgM	IgG3	IgGl	IgG2b	IgG2a	Total IgG
ug/ml	1.8±0.2	17.8±.4	94.2+20	44.5±2.4	1.3±.2	157.8
TK-1	8.6±4.3	2.9±0.4	9.5±6.3	8.5±0.8	0.1±0.1	21.0±3.4
TK-4	0	0.6±0.6	0	3.2±1.8	0	3.8±2.3
TK-5	2.8±1.6	1.1±1.1	0.7±0.5	3.5±1.8	0.1±0.1	5.4±27
M315/460	1.1±1.1	0.1±0.1	0	0	0	0.1±0.1
	12.5 (0-24.8)					30.3 (17.9-42.7)

See Table IX.

TABLE XIII

Idiotype content of primary B_6D_2 anti-TNP-ficoll response by subclass.

TNP-Ficoll

	IgM	IgG3	IgG1	IgG2b	IgG2a	Total IgG
ug/ml	.17±.1	0.6±.1	0.7±.4	0.2±0	.02±.01	1.5
TK-1	ND	5.6±1.2	0	1.0±1.0	0	6.5±2.1
TK-4	ND	0	0	0.6±0.6	0	0.6±0.6
TK-5	43.5±2.5	3.1±3.1	0	1.2±1.2	0	4.3±4.3
M315/460	4.9±0.1	0	0	0	0	0
						11.4 (0-25.1)

TNP-Ficoll + GVH

	IgM	IgG3	IgG1	IgG2b	IgG2a	Total IgG
ug/ml	0.5±.1	0.7±.1	0.5±.5	0.4±.1	.02±.01	1.6
TK-1	6.9±6.9	0	0	0	0	0
TK-4	0	0	0	1.8±1.8	0	1.9±1.9
TK-5	0	0	0	1.1±0.1	0	1.1±0.1
M315/460	0	0	0	0	0.1±0.1	0.1±0.1
	6.9 (0-20.3)					3.1 (0-6.7)

See Table IX.

TABLE XIV

Idiotype content of primary (CBA/n x BALB/c) F_1 male to TNP-K1H

	IgM	IgG3	IgG1	IgG2b	IgG2a	Total IgG
ug/ml	3.0±2.0	1.1±0.6	56.8±3.9	22.1±2.5	1.2±0	81.2
TK-1	30.4±1.8	0.4±0.2	14.2±1.0	9.3±1.1	0	23.9±2.3
TK-4	0	0.5±0.1	4.2±0.9	5.8±1.6	0.3±0	10.8±2.6
TK-5	13.3±1.8	0.2±0.2	10.8±2.8	8.9±2.2	0.2±0.1	20.0±0.6
M315/460	1.9±1.9	0.6±0.6	0	0.6±0.2	0	1.2±0.3
	45.5 (23.2-67.8)					55.8 (47.1-64.5)

TNP-K1H + GVH

	IgM	IgG3	IgG1	IgG2b	IgG2a	Total IgG
ug/ml	0.7±.4	1.2±1.2	25.3±3.8	8.3±.1	0.6±.1	35.4
TK-1	29.3±6.8	0.7±.1	9.7±18	5.5±0.3	0.7±0.1	16.1±2.3
TK-4	6.7±2.9	0.3±0.2	7.1±0.5	2.0±1.4	0.3±0.2	9.4±1.1
TK-5	13.2±3.3	0.5±0.1	6.5±0.1	5.3±1.5	0.5±0.1	12.3±1.4
M315/460	7.3±3.4	0.2±0.1	0	0.5±0.5	0.2±0.1	0.7±0.6
	56.6 (27.5-85.6)					38.3 (30.3-46.3)

See table IX.

TABLE XV

Idiotype content of primary response of
(CBA/n x BALB/c) F_1 males to
TNP-ficoll + GVH

	IgM	IgG3	IgG1	IgG2b	IgG2a	Total IgG
ug/ml	0.4±0.04	0.3±0.2	0.9±.1	.16±0	0.04±0	1.4
TK-1	21.7±1.7	0	0	3.3±0.7	0.6±0.3	3.9±0.5
TK-4	3.3±0.9	0	10.3±3.3	1.4±0.1	0.5±0.2	12.1±3.2
TK-5	39.6±5.8	0	21.2±1.8	3.7±0.4	0.2±2.2	25.1±2.4
M315/460	11.7±1.5	0	3.0±3.0	0.6±0.6	0	3.6±2.4
	76.2 (62.9-89.5)					44.6 (37.4-51.8)

See Table IX.

TABLE XVI

Expression of autoimmune disease in (CBA/ca x BXSB) F_1 mice.

Mouse Strain	Sex	Age (Months)	Spleen Wt. (mg)	Lymph Node Wt. (mg)
CBA/ca	F	7	53±4[a)]	2±0.2
BXSB	M	5	601±49	60±11
	F	21	280±86	9±2
CBA/ca x BXSB	M	5	87±0.4	5±1
		6	162±13	17±9
		7	578±12	23±5
		8	649±35	22±2
	F	6-8	88±3	4±1

a) Data are presented as arithmetic mean ± S.E. of 3-5 mice per group assayed individually.

TABLE XVII

Effect of Xid gene on expression of lymphoproliferative disorder in (CBA/n x BXSB) F_1 males.

Mouse Strain	Sex	Age (Months)	Spleen Wt. (mg)	Lymph Node Wt. (mg)	B Colonies Per 10^5 Spleen Cells
CBA/n x BXSB	M	5	68±17[a)]	5±2	0.5±0.1
		8	72±4	8±2	0.5±0.1
		10	59±7	5±3	0
	F	11	74±9	4±2	118±6
BXSB x CBA/n	M	8	105±13	16±2	48±8
BXSB	M	8	550±30	87±11	48±10

a) Data are presented as mean ± standard error of 5 animals assayed individually for each group.

TABLE XVIII

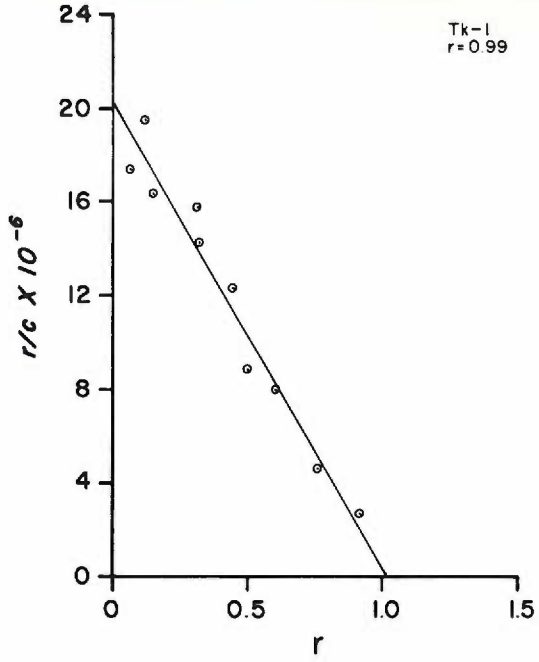
Primary anti-TNP serum antibody response to TNP-ficoll and B colony forming cells in (CBA/n x BALB/c) F_1 males undergoing GVH.

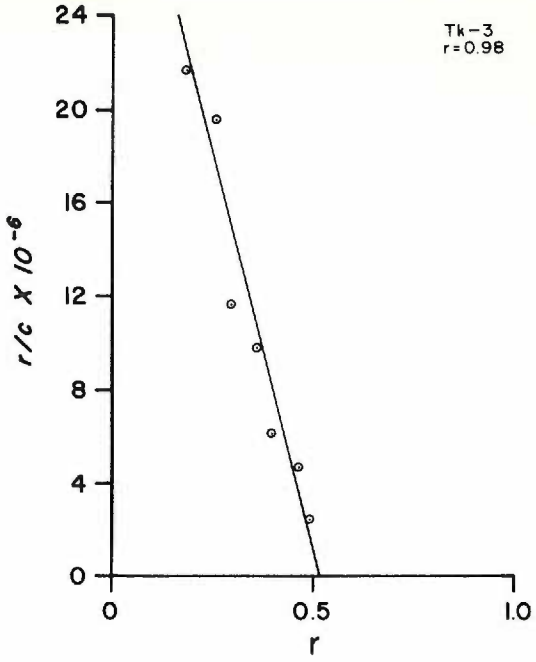
TNP-Ficoll	GVH	IgM	IgG	B Colonies Per 10^5 Spleen Cells
+	-	11.02	15.29	82 ± 6 a)
-	BALB/c	0.03	0.03	0.3 ± 0.09
+	BALB/c	0.12	1.72	0.4 ± 0.09
+	CBA/n	0.18	9.22	0.23 ± 0.08

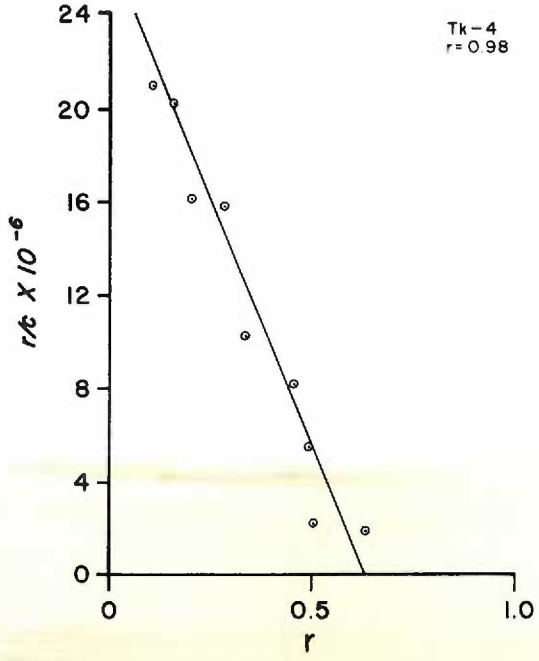
Pooled sera from three to four mice per group were assayed in an ELISA using TNP-BSA coated plates. Values are arithmetic means S.E. were all less than 15%.

a) Arithmetic mean $\pm$ S.E.

Figure 1. Equilibrium dialysis binding data plotted according to method of Scatchard. r = moles of bound hapten per mole of antibody. c = concentration of free hapten.


Tk-1
r=0.99
r/c X 10⁻⁶
r
0
0.5
1.0
1.5
4
8
12
16
20
24


Tk-3
r=0.98
r/c X 10⁻⁶
r
0
0.5
1.0
4
8
12
16
20
24


Tk-4
r=0.98
r/c X 10⁻⁶
r
0
0.5
1.0
4
8
12
16
20
24

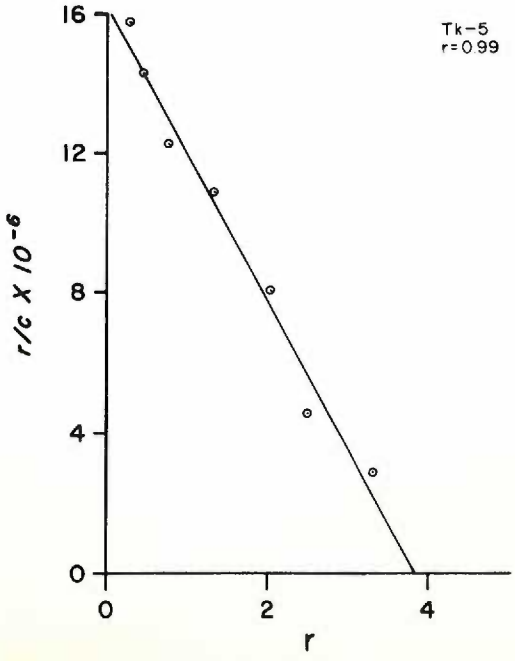
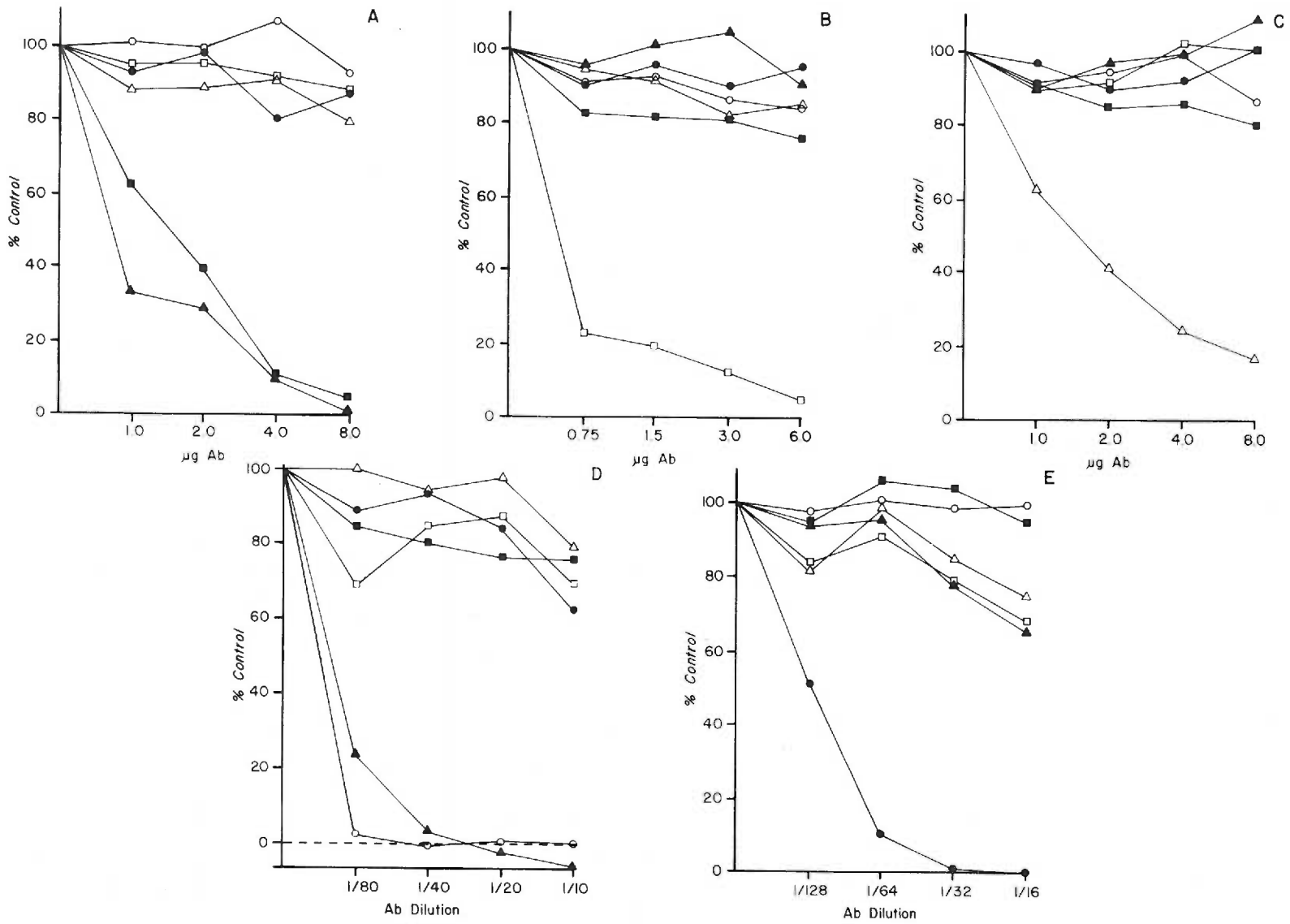

Tk-5
r=0.99
r/c X 10⁻⁶
r
0
2
4
8
12
16

Figure 2. Specificity of anti-idiotype antisera. The binding of various hybridoma or myeloma anti-TNP antibodies to TNP-BSA was assessed in an ELISA in the presence or absence of various anti-idiotype antibodies as inhibitors. 25 mg of anti-TNP antibody was mixed with the indicated amount of anti-idiotype in each of four replicate wells for each experimental point. A) rabbit anti-TK-1 id; B) mouse hybridoma AI-31 (anti-TK-3 id); C) rabbit anti-TK-4 id; D) rabbit anti-MOPC 315/460 shared id; E) rabbit anti-TK-5 id ■ TK-1, □ TK-3, △ TK-4, ● TK-5, ○ M315, ▲ M460.


A
B
C
D
E
% Control
100
80
60
40
20
0
1.0
2.0
4.0
8.0
μg Ab
0.75
1.5
3.0
6.0
1/80
1/40
1/20
1/10
Ab Dilution
1/128
1/64
1/32
1/16

ACKNOWLEDGMENTS

First I would like to thank Dr. Marvin Rittenberg for his guidance in my introduction to the world of science. My wife, Kay, my son, Michael and my parents deserve credit for the understanding, patience and support during these difficult years. And, finally, to my companions in the lab over the years my appreciation for their good humor and support in getting done.