

NOVEL IDIOTYPIC AND ANTIGEN-BINDING  
CHARACTERISTICS IN TWO ANTI-DINITROPHENYL  
MONOCLONAL ANTIBODIES\*

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Idiotypic identification of monoclonal antibodies has been used to define and enumerate unique antibody specificities (clonotypes) within the murine repertoire of B cells specific for phosphorylcholine (PC) (1-3). While the response in the BALB/c strain is dominated by a clonotype which is identical to the TEPC 15 (T15) plasmacytoma protein (1, 4, 5), antibodies not identical to T15 comprise 20-30% of the anti-PC in vitro response and appear to be heterogeneous by idiotypic cross-reactivity and hapten inhibition (3). Using a rabbit anti-idiotypic antibody to T15 (R anti-T15), which recognizes a different portion of the variable region than the murine anti-T15 (M anti-T15) (3, 6), a group of clonotypes was identified which reacts with R anti-T15 and anti-Fab on a 1:1 weight basis but has only weak cross-reactivity for the M anti-T15 (3). Clonotypes identified as R anti-T15 negative and M anti-T15 positive were not found in the PC-specific repertoire. However, this latter antibody specificity may not have PC-binding properties, since the antigen-binding portion of the variable region recognized by R anti-T15 serum is not present. It may be predicted, therefore, that a family of clonotypes should exist which share the same variable region "framework" residues with T15 myeloma protein, and thus should be recognized by M anti-T15, but have combining sites sufficiently distinct to bind other antigens.

In order to test this prediction, a large number of homogeneous antibodies generated in the splenic focus system in response to dinitrophenyl (DNP) were screened for their ability to react with M anti-T15 as well as to bind PC. The discovery of a R anti-T15<sup>-</sup>, M anti-T15<sup>+</sup> clonotype within the DNP repertoire confirms the above hypothesis and points to the novel idiotypic relationships which may be found among homogeneous antibodies binding diverse antigens. In addition, the maximum frequency with which this clonotype occurs within the B-cell pool has been estimated.

Materials and Methods

*Antigens and Animals.* The preparation of *Limulus polyphemus* hemocyanin (Hy), DNP-Hy, DNP-bovine serum albumin (BSA), and PC-BSA has been described previously (1, 2, 7). 6- to 8-wk-old BALB/c mice (Institute for Cancer Research, Philadelphia, Pa.) received intraperitoneal

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injections of 100  $\mu$ g Hy in complete Freund's adjuvant. 6–12 wk after carrier priming, these mice were used as recipients in adoptive transfer of adult, nonimmune spleen cells. 4–6 h before cell transfer, the recipients received 1,300 R of total body irradiation from a cesium source.

*Splenic Focus Technique.* Suspensions of adult BALB/c spleen cells were prepared as described previously (7), and  $4 \times 10^6$  viable cells injected intravenously into Hy-primed, irradiated, syngeneic, adult recipients. Fragment cultures were prepared from spleens of recipient mice 16 h after cell transfer (7). The fragments were individually stimulated with DNP-Hy ( $10^{-6}$  M DNP), and the culture fluids changed every 2–3 days. Fluids collected 9–13 days after stimulation were assayed for anti-hapten antibody and idiotype.

*Radioimmunoassays for Antibody and Idiotype.* 20  $\mu$ l of culture fluids were added to PC-BSA or DNP-BSA immobilized on polyvinyl plates (Cooke Laboratory Products Div., Dynatech Laboratories, Inc., Alexandria, Va.) (8). Bound anti-hapten antibody was detected by the subsequent binding of  $^{125}$ I-labeled purified rabbit anti-mouse Fab antibody (7). The amount of antibody was quantitated using T15 protein or purified anti-DNP antibody as standards.

M anti-T15, R anti-T15, and rabbit anti-167 were prepared and purified as previously described (1, 3). The rabbit anti-167 was a gift of Dr. Michael Cancro, University of Pennsylvania, Philadelphia, Pa. Idiotypic assays were performed by a modification of a previously described solid-phase inhibition radioimmunoassay (1). Briefly, the appropriate dilution of anti-idiotypic serum was adsorbed to wells of polyvinyl plates. After washing and adding 1% BSA in 0.02 M phosphate-0.15 M NaCl, pH 7.4, to the wells, various concentrations of unlabeled inhibitor proteins or culture fluids were added, followed by 2–4 ng of  $^{125}$ I-labeled T15 or 167 protein. The plates were incubated overnight at 37°C, washed, and counted in a gamma counter. The specificities of these anti-idiotypic sera have been demonstrated (references 1 and 3, and M. Cancro and N. H. Sigal, unpublished results).

## Results and Discussion

Monoclonal anti-DNP antibodies derived from splenic B cells of BALB/c adult mice were studied for the presence or absence of M anti-T15 reactivity and for their ability to bind PC. Two foci of the 680 clones analyzed bound PC, and one of these monoclonal antibodies reacted with M anti-T15, as shown in Table I. Clone P624 reacted with anti-Fab and M anti-T15 on a 1:1 weight basis, implying that all antigenic determinants recognized on the T15 protein by this anti-idiotypic serum were present on this homogeneous anti-DNP antibody as well. Neither of the two foci reacted with R anti-T15 or with anti-167. Thus, clone P624 fulfills the criterion of retaining the T15 variable region determinants recognized by M anti-T15 but not sharing any of the variable region determinants recognized by R anti-T15.

In addition to uncovering novel idiotypic relationships, analysis of cross-reactive monoclonal antibodies in the DNP repertoire can be used to define clonotype subsets and enumerate their frequency in the B-cell pool. Idiotypic cross-reactivity in serum antibody may indicate weak reactivity among a diverse population of antibodies, none of which are identical to the idiotype in question, or alternatively, the relevant idiotype may be present but represent only a small proportion of the serum antibody. The splenic focus system removes this ambiguity, since homogeneous antibodies that show idiotypic cross-reactivity can be operationally defined as a distinct clonotype. While two anti-DNP foci which cross-react with an anti-idiotypic serum or bind to an unrelated hapten cannot be considered identical, they represent subsets of anti-DNP clonotypes, and the repeat frequency of these subsets can be enumerated. Since the generation of 680 anti-DNP clones required an analysis of  $2.5 \times 10^8$  spleen cells and given a 4% cloning efficiency of the splenic focus system (8), a maximum

TABLE I  
*Presence of Hapten Binding and Idiotypic Activity in Two Anti-DNP Monoclonal Antibodies*

Assay*	P624	S40
	<i>ng in 20 <math>\mu</math>l of culture fluid</i>	
DNP binding	1.7	3.8
PC binding	2.0	4.2
Murine T15	2.0	0
Rabbit T15	0	0
Rabbit 167	0	0

\* A sensitive radioimmunoassay was used to quantitate the amount of hapten-specific antibody in 680 anti-DNP monoclonal antibodies, using T15 myeloma protein or purified anti-DNP antibody as standards. Detection of idiotypic determinants was carried out in a competitive solid-phase radioimmunoassay, using T15 or 167 as standards. Of the 680 anti-DNP monoclonal antibodies tested, only clones P624 and S40 demonstrated PC binding and/or idiotypic reactivity.

estimate of the frequency of clones P624 and S40 in the B-cell pool can be calculated to be 1 in  $4 \times 10^6$  splenic B cells. This estimate is, at best, a crude approximation, since neither of these clonotypes repeated in the population analyzed, and, therefore the clonotypes may be much rarer than the calculations imply. Nevertheless, this study, as well as a number of other recent reports (9-12), suggests that the B-cell repertoire is extremely diverse and may consist of greater than  $10^7$  clonotypes (9-13).

The R anti-T15<sup>-</sup>, M anti-T15<sup>+</sup> clonotype was not detected in an analysis of 404 monoclonal anti-PC antibodies which were assayed for the presence of both idiotypes (3). There are two possible explanations for this puzzling result. First, due to differences in antigen-binding site amino acid residues, this clonotype's affinity for PC may be sufficiently low such that precursor cells producing this specificity are not triggerable by this hapten. While the radioimmunoassay used in this study is relatively affinity independent (14), primary B cells appear to require an affinity threshold for stimulation (7, 13). Second, it is possible that the R anti-T15<sup>-</sup>, M anti-T15<sup>+</sup> clonotype is so rare that an insufficient number of anti-PC monoclonal antibodies were examined to identify this specificity. Although enough anti-PC foci were analyzed to detect a specificity that occurred at a frequency of 1 in  $2.5 \times 10^7$  B cells, its absence in this population may reflect the enormous diversity of the B-cell repertoire. Since a low frequency of PC-specific monoclonal antibodies binds DNP (unpublished results), clone S40 may belong to the R anti-T15<sup>-</sup>, M anti-T15<sup>-</sup> subset that constitutes approximately 20% of the PC-specific repertoire (3) and represent a rare clonotype that is stimulated by both PC and DNP.

The discovery of an R anti-T15<sup>-</sup>, M anti-T15<sup>+</sup> clonotype within the DNP-specific repertoire bears a distant relationship to the finding of Oudin and Cazenave (15), who demonstrated similar idiotypic specificities in rabbit antibodies with a variety of antigen-binding properties. In contrast to this early report, the present study was performed in a well-defined idiotypic system, using anti-idiotypic sera against purified T15 myeloma protein. Analysis of monoclonal antibodies also has eliminated the question of whether the common idiotypic reactivities identified in serum antibody represented a diverse array of cross-reactive specificities which shared only partial identity with the original

idiotype. While the difficulties of this earlier study must be kept in mind, the results of the present investigation, in general, support the conclusions of Oudin and Cazenave (15).

Elucidation of idiotypic relationships among clonotypes occupies a central position in the delineation of the mechanism responsible for the generation of antibody diversity. While it is possible that the reactivity of P624 for PC and its recognition by M anti-T15 represent the fortuitous occurrence of cross-reactive determinants, the ability of this clonotype to react with both hapten and anti-idiotypic on a 1:1 weight basis suggests that it is a variant within the T15 family of clonotypes. Theorists who postulate that somatic events generate the repertoire might predict that a specificity such as P624 arose through a series of mutations in the T15 germ-line sequence. More interesting, however, is the possibility that the R anti-T15<sup>-</sup>, M anti-T15<sup>+</sup> clonotype may represent a distinct set of hypervariable region sequences inserted into the T15 framework, thus bearing an inverse relationship to the previously identified R anti-T15<sup>+</sup>, M anti-T15<sup>-</sup> clonotype. Amino acid sequencing of such homogeneous monoclonal antibodies may provide significant insight in the discrimination between these alternatives. In addition, this study points out the utility of the splenic focus technique in generating vast arrays of homogeneous antibodies which can be used to investigate idiotypic cross-reactions in the homologous antigen system or to search for cross-reactive clonotypes within antibody populations binding other antigens.

### Summary

Monoclonal anti-dinitrophenyl antibodies generated in the splenic focus system from B cells of adult BALB/c mice were studied for the presence or absence of murine anti-T15 (M anti-T15) reactivity and for their ability to bind phosphorylcholine (PC). Two foci of the 680 clones analyzed bound PC, and one of these antibodies reacted with M anti-T15 and anti-Fab on a 1:1 weight basis. The discovery of a clonotype reactive with M anti-T15 but not with rabbit anti-T15 (R anti-T15) serum, the converse of the R anti-T15<sup>+</sup>, M anti-T15<sup>-</sup> clonotype identified in the PC-specific repertoire, points to the novel idiotypic relationships which may be found among homogeneous antibodies binding diverse antigens. The R anti-T15<sup>-</sup>, M anti-T15<sup>+</sup> clonotype may represent a distinct set of hypervariable region sequences inserted into the T15 framework or may be a somatic variant of the T15 germ-line sequence. In addition, the maximum frequency with which this clonotype occurs within the B-cell pool is estimated.

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### References

1. Gearhart, P. J., N. H. Sigal, and N. R. Klinman. 1975. Heterogeneity of the BALB/c anti-phosphorylcholine antibody response at the precursor cell level. *J. Exp. Med.* 141:56.

2. Sigal, N. H., P. J. Gearhart, and N. R. Klinman. 1975. The frequency of phosphorylcholine-specific B cells in conventional and germfree BALB/c mice. *J. Immunol.* 68:1354.
3. Gearhart, P. J., N. H. Sigal, and N. R. Klinman. 1977. The monoclonal anti-phosphorylcholine antibody response in several murine strains: genetic implications of a diverse repertoire. *J. Exp. Med.* 145:876.
4. Cosenza, H., and H. Köhler. 1972. Specific inhibition of plaque formation to phosphorylcholine by antibody against antibody. *Science (Wash. D. C.)*. 176:1027.
5. Claflin, J. L., R. Lieberman, and J. M. Davie. 1974. Clonal nature of the immune response to phosphorylcholine. I. Specificity, class, and idiotype of phosphorylcholine-binding receptors on lymphoid cells. *J. Exp. Med.* 139:58.
6. Claflin, J. L., and J. M. Davie. 1974. Clonal nature of the immune response to phosphorylcholine. IV. Idiotypic uniformity of binding site-associated antigenic determinants among mouse antiphosphorylcholine antibodies. *J. Exp. Med.* 140:673.
7. Klinman, N. R. 1972. The mechanism of antigenic stimulation of primary and secondary clonal precursor cells. *J. Exp. Med.* 136:241.
8. Klinman, N. R., A. R. Pickard, N. H. Sigal, P. J. Gearhart, E. S. Metcalf, and S. K. Pierce. 1976. Assessing B-cell diversification by antigen receptor and precursor cell analysis. *Ann. Immunol. (Paris)* 127C:489.
9. Kreth, H. W., and A. R. Williamson. 1973. The extent of diversity of anti-hapten antibodies in inbred mice: anti-NIP (4-hydroxy-5-iodo-3-nitro-phenacetyl) antibodies in CBA/H mice. *Eur. J. Immunol.* 3:141.
10. Pink, J. B. L., and B. Askonas. 1974. Diversity of antibodies to crossreacting nitrophenyl haptens in inbred mice. *Eur. J. Immunol.* 4:426.
11. Ju, S-T., A. Gray, and A. Nisonoff. 1977. Frequency of occurrence of idiotypes associated with anti-*p*-azophenylarsonate antibodies arising in mice immunologically suppressed with respect to a cross-reactive idiotype. *J. Exp. Med.* 145:540.
12. Köhler, G. 1976. Frequency of precursor cells against the enzyme  $\beta$ -galactosidase. An estimate of the BALB/c strain antibody repertoire. *Eur. J. Immunol.* 6:340.
13. Klinman, N. R., and J. L. Press. 1975. The B-cell specificity repertoire: its relationship to definable subpopulations. *Transplant. Rev.* 24:41.
14. Klinman, N. R., and R. B. Taylor. 1969. General methods for the study of cells and serum during the immune response: the response to dinitrophenyl in mice. *Clin. Exp. Immunol.* 4:473.
15. Oudin, J., and P. A. Cazenave. 1971. Similar idiotypic specificities in immunoglobulin fractions with different antibody functions or even without detectable antibody function. *Proc. Natl. Acad. Sci. U. S. A.* 68:2616.