IMMUNOGLOBULIN WITH COMPLEMENTARY PARATOPE
AND IDIOTOPE

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The surface of the immunoglobulin structure plays a multifunctional role within the immune network. The variable structures of antibodies express a large repertoire of idiotypic specificities (idiotopes) and are also responsible for the vast diversity of different binding sites (paratopes). Crystallographic analyses of antibodies have identified V regions that form a binding site or pocket for binding with antigen (1). The sequence regions that participate in the antigen-binding site have been correlated closely with the most variable sequence regions of the domain, and were described as complementarity-determining regions (CDR) (2). For communication with antibodies and receptors of immunocompetent cells, the Ig surface has developed another variable repertoire that expresses the diversity of idiotopes. Very little is known about the structural correlates of idiotypic determinants except that they must be accessible for antibodies to bind to. This implies that idiotopes are autoantigens, as they are recognized as self antigens by the immune system (3). The size of the idiotope repertoire produces the statistical necessity that the shapes of other structures are mimicked. This stereochemical mimicry generates what immunologists call internal antigens or homobodies (4, 5). Since the expression of idiotopes is, at least in part, made by an independent topographical area of the Ig surface (6), it should be possible for the same Ig structure to possess idiotope and paratope of complementary configuration. Mimicry of Ig determinants by idiotopes has been described recently by Bona and colleagues (7), and by Chen and coworkers (8). The term epibody was developed to signify the presence of an antibody recognizing both an idiotope and an epitope on the original antigen. In their example, the antigenic determinant mimicked by an idiotypic determinant may be the target for an antiidiotypic antibody (epibody). Herein, we describe an antibody that mimicks the antigen for its own paratope site. The specificity of the antibody is for phosphorylcholine (PC), and PC is mimicked and expressed as idiotope by the same antibody. Coexpression by the same molecule of paratopes and idiotopes that possess complementary structures may have profound implications for the understanding of the network concept (8), the role in autoimmunity (9), and the design of idiotype vaccines (10).

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Materials and Methods

Mice. Athymic BALB/c nu/nu mice, 6 wk old, were obtained from our own breeding colony. Originally these mice were obtained from the National Institutes of Health, Bethesda, MD.

Myeloma and Hybridoma Proteins. Myeloma cell lines TEPC15 (T15), MOPC167 (M167), MOPC503 (M603), and MOPC (M511) were obtained from Litton Bionetics, Kensington, MD, and were purified from ascitic fluid by PC-Sepharose column. Hybridoma cell lines HPCG11 and HPCG12 were gifts from Dr. P. Gearhart, Johns Hopkins University, Baltimore, MD, and were purified from ascitic fluid on a PC-Sepharose column. HPCG13 and HPCG14 hybridoma proteins were gifts from Dr. P. Gearhart (11). The anti-T15 hybridoma F6-5 was prepared in our laboratory (12) and was purified on T15-Sepharose column. The anti-M167 hybridoma 4F1 was prepared in our laboratory and was purified on M167-Sepharose column. The anti-TNP myeloma proteins MOPC315 (M315) and MOPC460 (M460) were purified on TNP-Sepharose column. mAb 11E7-1 was purified from ascites by affinity chromatography on T15-Sepharose column or PC-Sepharose column.

Immunization and Fusion. Athymic BALB/c nude mice were primed with 100 μg i.p. of T15 IFA three times at 3-wk intervals. 3 d after the last immunization, splenic lymphocytes were fused with P3-X63Ag8.653 myeloma cells, and hybrids were selected in the HAT medium. Culture supernatants were tested by ELISA. One clone, 11E7, an IgM k-producing hybridoma, was selected, subcloned twice by limiting dilutions, expanded in tissue culture, and then carried as ascitic tumor in BALB/c mice.

ELISA. For hybridoma screening, 96-well microtiter plates (Becton Dickinson and Co., Oxnard, CA) were coated for 18 h with 0.1 ml of purified T15 (2 μg/ml) in PBS, washed three times, and incubated for 1 h with 1% BSA in PBS. After washing, 20 μl of culture supernatant was added to each well, with 80 μl of 1% BSA in borate-buffered saline. After washing, alkaline phosphatase-coupled goat anti-mouse IgG and IgM (Southern Biotechnology Associates, Birmingham, AL) were used to detect antibodies binding to T15-coated plates. Substrate was added, and the OD at 405 nm was measured. For further details on other assays, see legends for figures.

RIA. Microtiter plates were coated for 18 h with 0.1 ml of purified antibodies (10 μg/ml) or PC-BSA (2 μg/ml) in PBS, washed three times, and incubated for 2 h with 1% BSA in PBS. After washing, 125I-labelled 11E7-1 was added and incubated for 18 h. After incubation, the wells were extensively washed and the bound radioactivity of individual wells was determined in a gamma counter (Packard Instrument Co., Downers Grove, IL). Tests were done in quadruplicate.

Immunoblotting. The binding of the 125I-labelled 11E7-1 to different antibodies and PC-BSA was tested by immunoblot. Nitrocellulose papers (Bio-Rad Laboratories, Richmond, CA) were loaded with 5 μl of various antibodies (1 mg/ml) or PC-BSA (0.2 mg/ml), dried, and blocked for 1 h with 5% BSA in 50 mM Tris. The nitrocellulose sheets were then incubated for 3 h at room temperature with 5% BSA/50 mM Tris containing 125I-labelled 11E7-1 (10⁶ cpm/ml) in the absence or presence of PC (10⁻⁴ M). After incubation, the nitrocellulose sheets were extensively washed, dried, and exposed to Kodak XAR-5 film at -70°C for 3 h.

Results

Dual Binding to PC and T15. A hybridoma antibody was isolated from a fusion of BALB/c nude mice that were immunized with the T15 myeloma protein. T15 is a PC-binding myeloma and expresses the dominant idiotype in the response of BALB/c mice to immunization with PC antigens (13). The 11E7 hybridoma was selected for binding to insolubilized T15 using an ELISA. Surprisingly, supernatant of 11E7 also bound to insolubilized PC-BSA. 11E7 was recloned twice, and the subclone 11E7-1 was used throughout this study. In Fig. 1, the dual binding to PC and T15-coated plates in ELISA are shown using the
culture supernatant of 11E7-1. As serial dilutions of supernatant are added, the binding to PC-BSA decreases while the binding to T15-coated plates increases. As control, no binding to M167 plates is observed with any dilution. These results indicate that the 11E7-1 hybridoma has a dual specificity for PC and T15. Consequently, it should be possible to purify the antibody by affinity chromatography over either PC immunoabsorbent or T15 immunoabsorbent. 11E7-1 ascites was raised in BALB/c, passed over PC-Sepharose, and eluted with $10^{-3}$ M PC (14). 11E7-1 was also adsorbed to T15-Sepharose and eluted with 1 M acetic acid. The eluants from both immunoabsorbents were tested in ELISA for binding to PC-BSA and to T15. Similar binding to T15 and PC-BSA of affinity-purified 11E7-1 hybridoma as well as the 11E7-1 culture supernatant (Fig. 1) was observed (data not shown).

Specificity of 11E7-1. 11E7-1 supernatant was added to plates coated with different PC-binding antibodies and non-PC-binding control antibodies. As seen in Fig. 2, three patterns are observed. 11E7-1 binds best to a group of three antibodies, T15, HPCG14, and MOPC 603; the binding to HPCG12 and HPCG11, both T15 + PC hybridomas, is lower, while no binding to M167, MOPC511, HPCG13 (M167 idiotype), and MOPC460 (DNP-binding) is obtained.

To further analyze the fine specificity of the 11E7-1 binding to PC antibodies, inhibition with PC and analogs was performed in a direct-binding ELISA using enzyme-labeled 11E7-1. In Table I, the concentrations of inhibitors giving 50% inhibition ($ID_{50}$) of 11E7-1 binding are shown. PC and glycerophosphorylcholine are by far the best inhibitors, followed by choline, which is $\sim$1,000 times less effective. This inhibition pattern is reminiscent of the hapten specificity of T15 and HOPC8 described earlier (14). In Table I, the 11E7-1 binding to insolubilized 11E7-1 is shown together with the $ID_{50}$, using PC and analogs. The observed 11E7-1 self-binding in Table I will be further examined in the following section.
FIGURE 2. Antiidiotype activity of 11E7-1. The assays were performed in a similar manner as in Fig. 1, except the plates were coated with T15, HPCG14, M603, M511, M167, M460, HPCG12, HPCG13, and HPCG11.

TABLE I

| Plates coated with:* | ID₅₀ (mM) of 11E7-1 binding by: |
|----------------------|---------------------|
|                       | Choline | GPC | PC |
| T15                  | 1.5     | 0.07 | 0.007 |
| HPCG14               | 1.3     | 0.03 | 0.002 |
| M603                 | 1.9     | 0.02 | 0.006 |
| 11E7-1               | 1.1     | 0.005 | 0.001 |

* Microtiter plates were coated for 18 h at 4°C with 2 μg/ml of different antibodies, and then incubated with 25 ng of enzyme-coupled 11E7-1 for 18 h at 4°C in the presence of different concentrations of PC analogs.

**Self-binding of 11E7-1.** The results presented so far suggest that 11E7-1 expresses both a paratope site for PC and an internal image idiotope of the PC hapten. If so, we should be able to detect self-binding of 11E7-1 or self-aggregation. To test for self-binding, affinity-purified, enzyme-labeled 11E7-1 was added to plates coated with 11E7-1, T15, or M167. The data shown in Fig. 3 demonstrates that 11E7-1 binds to T15 and to itself, but not to M167. Furthermore, self-binding to 11E7-1 was completely inhibited by PC and analogs, as shown in Table I.

The binding specificity of 11E7-1 was further examined in RIA and immunoblotting. 11E7-1 was iodinated and allowed to bind to insolubilized 11E7-1, a
FIGURE 3. 11E7-1 is self-binding. Microtiter plates were coated for 18 h with 100 µl/well of 11E7-1 or T15 (2 µg/ml) in PBS, washed three times, and incubated for 1 h with 1% BSA in PBS. After washing three times, enzyme-coupled 11E7-1 in 1% BSA/0.05% Tween 20/borate-buffered saline solution was added. After washing three times, color was developed with substrate, and the OD at 405 nm was measured.

TABLE II

| Plates coated with:* | Isotypes | Binding* of 125I-11E7-1 (cpm) |
|----------------------|----------|--------------------------------|
|                      |          | Without PC | With PC (10^-3 M) |
| 11E7-1               | με       | 10,080 ± 315 | 136 ± 28         |
| 4F1                  | με       | 125 ± 16     | 100 ± 8          |
| T15                  | αε       | 11,157 ± 862 | 111 ± 54         |
| M315                 | αδ       | 289 ± 18     | 108 ± 14         |
| F6-3                 | γ1ε      | 13,069 ± 735 | 12,972 ± 466     |
| PC-BSA               | --       | 13,769 ± 276 | 119 ± 10         |

* Microtiter plates were coated for 18 h at 4°C with 10 µg/ml of 11E7-1, 4F1, T15, M315, F6-3, or 2 µg/ml of PC-BSA, then incubated with 10 ng of 125I-11E7-1 (20,000 cpm) for 18 h at 4°C in the absence of PC or in the presence of PC (10^-3 M).

* Binding is expressed as mean ± SD of cpm (n = 4).

panel of other antibodies, and to PC-BSA. The results in Table II confirm data obtained with ELISA. 4F1 is an unrelated hybridoma that has specificity for M167 (15).

Iodinated 11E7-1 binds strongly to plates coated with 11E7-1, T15, F6-3 (an anti-T15 hybridoma) (16), and PC-BSA at >50% of added total counts. The binding to 11E7-1, T15, and PC-BSA is completely inhibited by free PC, while the binding to F6-3 is not. To confirm these results from the RIA and ELISA, iodinated 11E7-1 was used in immunoblotting (see Fig. 4). Labelled 11E7-1 reacts with T15, 11E7-1, F6-3, and PC-BSA, but not with MOPC315 and 4F1. Blotting in the presence of 10^-5 M PC is inhibited for 11E7-1, T15, and PC-BSA but not for F6-3. No binding is seen to 4F1 and MOPC315. These blotting patterns are in complete agreement with the data obtained in ELISA and RIA.
Expression of T15 Idiotype by 11E7-1. To evaluate the idiotype family relationship of the PC-binding 11E7-1, the 11E7-1 binding to the anti-T15 mAb F6-3 (16) was determined. As already discussed, 11E7-1 binds to insolubilized F6-3 in RIA and immunoblotting (see Table II and Fig. 4). F6-3 is a T15-specific antiidiotypic antibody that does not recognize the idiotope related to the PC paratope. The binding of F6-3 to 11E7-1 has the same characteristics as its binding to T15. It is not inhibited by free PC (see Table II and Fig. 4). These results indicate that 11E7-1 expresses a classical T15 idiotope, and suggests that 11E7-1 belongs to the T15 idiotype family. We are presently investigating to which VH and VL gene families 11E7-1 belongs.

Discussion

The first critical question regarding the dual specificity of 11E7-1 concerns the monoclonality of the hybridoma. 11E7-1 was recloned twice by limiting dilution. The cell number was plated each time at 0.3 cell/well, which excludes multiclonality at 0.9984 probability. Additional evidence for monoclonality is the stability of the dual binding properties over >7 mo of passage.

The question of whether 11E7-1 consists of a mixed molecule cannot be answered by serological and immunological techniques, but will have to be addressed by isolating and/or sequencing the V\textsubscript{H} and V\textsubscript{L} genes. These studies are underway. Regardless of the answer to the question of mixed Ig molecules...
in 11E7-1, the results deserve to be discussed with regard to the idiotype network (16), to structural consideration on the Ig molecules (17, 18), to a possible role in autoimmunity (19), and to the design of idiotype vaccines (20).

The coexpression of complementary paratope and idiotope sites on the same molecule has some important ramifications for the topography of idiotopes and paratopes. According to the classical view, the binding site of an antibody resides in distinct domain of the molecule, which is characterized by a pocket, groove, or cleft. This model is supported by x-ray crystallographic data of several Fab fragments, which all have a concave surface area for harboring the hapten or antigen. This view might be adequate for antihapten antibodies, but has to be modified to account for antibodies binding to protein antigens. Recently, models of antibody-antigen complexes (21) were discussed that involve rather large and flat areas of the antibody, making multiple contacts with the antigen surface. Thus, not all paratopes must be pocket-like structures.

In support of a nonpocket domain, a recent report by Schiffer and colleagues (16) shows a different configuration of the antibody structure. The Loc \( \lambda \) chain dimer of Schiffer has, in place of the typical pocket, a protrusion that closes the antigen-binding cleft. By virtue of having a protrusion instead of a pocket, Loc becomes a model for an idiotope-presenting structure. One could speculate that the 11E7-1 structure is a hybrid between a pocket and a protrusion model. On the other hand, the simultaneous expression of complementary paratope and idiotope makes a strong argument for the segregated topographical location of paratopes and idiotopes. This supports the notion of distinct paratopic and idiotopic minidomains (6). However, in a functional sense, the distinction between paratope and idiotope becomes blurred. There is, a priori, no functional superiority of the PC binding site over the PC internal image, as both participate equally in the regulation of the immune network (22).

The properties of 11E7-1 are similar but distinct from the kind of molecule described as epibody by Bona et al. (7) and by Chen et al. (8). These authors have characterized an antidiotypic antibody as an epibody, which reacts not only with an idiotope on a monoclonal human anti-IgG autoantibody (rheumatoid factor, RF), but also with the Fc fragment of human IgG. These results also strongly suggest that an epibody can recognize shared determinants on the L chain of human RF and human IgG resulting from the Ser-Ser-Ser sequence. In contrast to the epibody described by Bona et al. (7), the 11E7-1 molecule expresses the idiotypic determinant for its own paratope. Thus, 11E7-1 exhibits self-binding activity. Accordingly, we propose the term autobody to specify the potential for self-binding.

The evaluation of the 11E7-1 properties was greatly facilitated by using the small hapten PC as inhibitor for the binding activities of 11E7-1. Free PC inhibited both binding functions of 11E7-1, the binding as anti-PC antibody and the expression of the internal PC antigen image. These inhibition data produce a clear pattern demonstrating the dual functionality of 11E7-1. If PC is used as competitive inhibitor of 11E7-1 self-binding, we cannot identify the site of action of PC. In these inhibition experiments, insolubilized 11E7-1 was coincubated with PC and labeled 11E7-1. In another experiment, insolubilized 11E7-1 and insolubilized T15 were first incubated with PC for 18 h. The plates were then
washed to remove unbound PC. Radiolabeled 11E7-1 was added for 4 h, and the bound radioactivity was measured. The binding of labeled 11E7-1 to T15, prereacted with PC, was >80% inhibited. This shows that the PC paratope of T15 is the target for binding by 11E7-1. Binding of labeled 11E7-1 to PC-preincubated, insolubilized 11E7-1 was only inhibited by 33% (data not shown). This indicates that, after the paratope of 11E7-1 has been occupied by PC, another site on the insolubilized 11E7-1 is still available for 11E7-1 binding. This site is operationally defined as the internal image of PC. These results support the model in which 11E7-1 self-binding involves two sites of interaction, which can be experimentally distinguished.

The meaning of a dual-function antibody within the immune network can at this time only be the subject of speculation. At face value, the expression of internal antigen and complementary paratope constitutes a mininetwork or closed feedback loop. Biologically, such an antibody may be the product of an aberrant clone, and we expect that such a clone would be eliminated by the surveillance of the immune network. In this view, 11E7-1 represents a shortened regulatory circuit without any consequences for the stability of the immune network balance.

An important issue in the discussion of the biological implication of antibodies with complementary relations of idiotopes and paratopes is the potential for self-binding. We attempted to find soluble complexes of purified 11E7-1, but failed. Evidently, 11E7-1 does not form self-aggregates in solution, but shows its self-affinity only after adsorption to surfaces. This property of self-binding revealed only after adsorption could be activated if 11E7-1 has attached to a PC-presenting infectious organism, such as after infection with Streptococcus pneumoniae (23). Having bound a PC antigen, 11E7-1 now can bind other 11E7-1 molecules, thus enforcing the biological defense mechanism and at the same time acting as buffer for antidiotype suppressive mechanisms.

Regardless of the role of a dual function antibody in the immune network, an antibody with such unique properties might be involved in mechanisms underlying autoimmune reactions (9). Excessive biosynthesis of a self-aggregating antibody with specificity for self antigens could lead to severe immune complex deposits in critical tissues. Characteristic for such kinds of autoimmune deposits would be the lack of nominal antigen material in the deposits.

Finally, the properties of 11E7-1 might help to advance the rational design of idiotype vaccines (20). Idiotype vaccines modeled after the autobody model (9) would transfer immediate passive immunity via its antibody quality, and long-term active immunity by virtue of being an antigen image. Further biological and structural studies on 11E7-1 are needed to address more decisively its possible use as a supervaccine.

Summary

A hybridoma antibody (11E7-1) was isolated from a myeloma fusion with nu/nu BALB/c immunized against the T15 idiotype. This IgM antibody exhibited a dual specificity, binding both to PC and to anti-PC antibodies from two idiotype families. Binding to PC and anti-PC antibodies are completely inhibited by PC analogs. Furthermore, the hybridoma antibody binds to itself. Self-binding is
also inhibited by PC analogs. From these data, we suggest that 11E7-1 hybridoma antibody has a PC-specific paratope site, and at same time expresses the internal PC antigen idiotope. The term autobody is proposed to signify its self-binding and potential role in autoimmunity. Autobodies may have a unique role in the network of immune system. Furthermore, it may be a model for designing idiotype vaccines.

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