

IDIOTYPIC SELF BINDING OF A DOMINANT GERMLINE IDIOTYPE (T15)

Autobody Activity is Affected by Antibody Valency

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Recently we have described (1) an IgM antibody that has the unique property of specific self binding. The 11E7-1 hybridoma was isolated by fusion with a *nu/nu* BALB/c mouse immunized with TEPC 15 myeloma protein (T15). The mAb 11E7-1 binds to PC, to insolubilized T15, and to 11E7-1 itself. The binding is specifically inhibited by free PC and PC analogs. Thus, this antibody has a dual specificity of binding to PC and of expressing an idiotope for PC inhibitable self binding. Because of the simultaneous expression of complementary paratope and idiotope it has been termed "autobody" (2-4).

The specificity of self binding appears to be extremely stringent, since as little as 10^{-7} M PC inhibits 50% self binding in ELISA. Furthermore, the strength of inhibition by PC, glycerophosphorylcholine (GPC),¹ and choline follows the same pattern as the PC fine specificity of T15 binding to PC (5). This seems to suggest that the PC binding site (paratope) of 11E7-1 is similar to the paratope of the T15 prototype, and also that the PC site is directly involved in the self binding. A rational interpretation of these properties of 11E7-1 implies that this antibody expresses a paratope for the antigen and its complementary internal image idiotope (6).

11E7-1 also expresses the T15 idiotope because it reacts with an anti-T15 mAb, F6-3, that reacts with T15 idiotope-positive antibodies of different isotypes (7). This finding led us to speculate that 11E7-1 may be part of a self-regulating idiotypic cellular loop controlling the normal anti-PC response or may cause complex deposition on pathogenic targets. Regardless of the biological meaning of a complementary self-binding antibody, this unique dual expression of paratope and idiotope has important ramifications for the network hypothesis.

In this update on our studies of the 11E7-1 autobody, we addressed the question of the structural origin of the autobody properties. The PC-specific self binding could be caused by a somatic mutation of the T15 prototype structure in which the PC paratope remained intact and an internal image idiotope has

This work was supported in part by U.S. Public Health Service grant AG-04180 from the National Institute on Aging, Department of Health, Education and Welfare.

¹ *Abbreviations used in this paper:* BBS, borate-buffered saline; CDR2, complementarity determining region 2; GPC, glycerophosphorylcholine; NP, 4-hydroxy-3-nitro-phenyl.

been created. Sequence analysis of the variable L and H chain regions of 11E7-1 would indicate the presence of structural changes in the germline T15.

Alternatively, the self-binding property may be inherent in the T15 prototype structure and its expression may be subject to enhancement by Ig subunit-mediated effects. While under normal experimental conditions of working with T15⁺ antibodies in solution the self-binding activity is not well expressed, it may be seen after stabilization of the T15 structure absorbed to surfaces. In this case, the valency of interactions between surface-bound T15 and T15 in solution could be an important factor to promote self binding.

Our data reported here clearly demonstrate that the variable region sequence of L and H chains of 11E7-1 is identical to the germline sequence of T15, and that the self-binding property of 11E7-1 is shared by other T15 prototype antibodies. Therefore, self binding is an inherent characteristic of the T15 prototype. We also show that the self-binding ability is a function of the valency of the T15 structure.

Materials and Methods

Myeloma and Hybridoma Proteins. Myeloma cell line T15 was obtained from Litton Bionetics, Kensington, MD, and the antibody was purified from ascitic fluid by a PC-Sepharose column. The anti-TNP myeloma antibody MOPC 315 (M315) was the kind gift of Mr. G. Radzinski, Roswell Park Memorial Institute, Buffalo, NY. mAb 11E7-1 was prepared in our laboratory and purified from ascites by affinity chromatography on T15-Sepharose column or PC-Sepharose column (1). mAb 4F1 was prepared in our laboratory and purified from ascites by affinity chromatography on M167-Sepharose column (8). HPCG14 hybridoma protein was a gift from Dr. P. Gearhart, Johns Hopkins University, Baltimore, MD (9). The antiphthalate hybridoma antibody 3A4 was a gift from Dr. R. B. Bankert, Roswell Park Memorial Institute. The anti-human T cell leukemia mAb SN2 was a gift from Dr. B. K. Seon, Roswell Park Memorial Institute (10). mAbs 10N 3/2 and LN 12/4 were gifts from Dr. C. Bona, Mount Sinai Medical School, New York. mAb F6-3 was prepared in our laboratory (7) and was purified on a T15-Sepharose column.

F(ab')₂ Fragmentation and H and L Chain Separation of T15. The F(ab')₂ fragment of the T15 antibody was prepared by pepsin digestion according to Lamoyi and Nisonoff (11). After digestion, various digestion fractions were separated by passing through a Sephacryl S-200 column; the peak fractions following the undigested Ig peak were pooled and found to be F(ab')₂ fragments. Isolated T15 H and L chains were prepared by the method of Berek et al. (12). 10 mg of monomeric T15 was dialyzed overnight against 6 M urea in the presence of 1 N acetic acid. Chains were separated on a Sephacryl S-200 column equilibrated in dialysis buffer.

Preparation of Monomeric, Dimeric T15, and Pentameric 11E7-1. Dimeric T15 and a pentameric 11E7-1 were purified directly from ascites by a PC-Sepharose column. To obtain a monomeric T15, ascites was reduced and alkylated before purification using 5 mM DTT and 0.01 M iodoacetamide at the final concentration. The purities and molecular weights of the antibodies were confirmed by HPLC analysis. 10 μ l of each antibody was run through a Superose 6HR 10/30 column. A buffer containing 0.05 M phosphate and 0.15 M NaCl at pH 7.0 was used as the eluent at a flow rate of 0.6 ml/min. The column effluent was monitored at 254 nm.

ELISA. A microtiter plate was coated for 18 h with 0.1 ml/well of T15 (2 μ g/ml) in PBS, washed three times, and incubated for 1 h with 1% BSA in PBS. After washing three times, 25 ng of enzyme-coupled 11E7-1 in 1% BSA and 0.05% Tween 20/borate-buffered saline (BBS) was added in the absence or presence of inhibitors. Enzyme-coupled goat anti-mouse IgM was used for detecting antibody binding to the microtiter plate.

After washing three times, color was developed with substrate and the OD₄₀₅ was measured.

Radioimmunoassay. Microtiter plates were coated for 18 h with 0.1 ml of purified antibodies (5 µg/ml) or PC₇-BSA (2 µg/ml) in PBS, washed three times, and incubated for 1 h with 1% BSA in PBS. After washing, ¹²⁵I-labeled antibodies were added in the absence or presence of inhibitors. After 18 h of incubation, the wells were extensively washed and the bound radioactivity of individual wells was determined in a gamma counter (Packard Instrument Co., Inc., Downers Grove, IL). For further details, see legends for figures and tables.

RNA Preparation. Total cellular RNA was prepared by modification of the guanidinium monothiocyanate method (13). Poly(A)⁺ RNA was isolated on oligo(dT)-cellulose columns.

Synthesis and Labeling of Oligonucleotide Primer. The oligonucleotide primer from the 5' end of the mouse κ chain constant region was obtained as a gift from Dr. P. W. Tucker, University of Texas Health Science Center, Dallas, TX. Other oligonucleotide primers were synthesized with a DNA synthesizer (No. 380A; Applied Biosystems, Inc., Foster City, CA) in our laboratory. The primers were labeled at the 5' end with γ-[³²P]ATP and T4 polynucleotide kinase (International Biotechnologies, Inc., New Haven, CT) (14).

Synthesis and Sequencing of cDNA. cDNA was synthesized with Avian myeloblastosis virus reverse transcriptase (Life Science Inc., St. Petersburg, FL) as described by Sood et al. (15). The cDNA was purified by electrophoresis through 40 cm of 6% polyacrylamide/7 M urea preparative gels. Sequencing of cDNA was performed by the chemical degradation method (14).

Protein Sequence Analysis. The L chain NH₂-terminal sequence was determined on a protein sequencer (No. 470 A; Applied Biosystems, Inc.) in conjunction with an on line No. 120 PTH analyzer (Applied Biosystems, Inc.).

Results

The Variable Region is Involved in Self Binding of 11E7-1. A previous report (1, 2) suggested that the 11E7-1 antibody (IgMκ) has two functional sites; one is a paratope for binding to the PC antigen and the other is an idiotope that is complementary to its own paratope. Through this paratope and idiotope, 11E7-1 exhibit the anti-idiotypic and self-binding activities. To investigate whether the variable region is involved in the self binding of 11E7-1, the F(ab')₂ fragment of T15 was used as an inhibitor in the binding of 11E7-1 to T15. Since we will demonstrate later that T15 has the same variable region sequence and self-binding activity as 11E7-1, the F(ab')₂ fragment of T15 represents the variable region of 11E7-1. Plates were coated with monomeric T15 and incubated with enzyme-labeled 11E7-1 in the presence of inhibitors. As seen in Fig. 1, intact 7s T15 was the best inhibitor; the F(ab')₂ of T15 was also an effective inhibitor, while the free L chain or H chain could not inhibit the binding. These results indicate that the variable region of T15 is the target for binding by 11E7-1.

Next, the hapten specificity of the self binding was analyzed. Plates were coated with 11E7-1 and incubated with ¹²⁵I-labeled 11E7-1 in the presence of various haptens. As seen in Table I, only PC was able to inhibit the self binding. This confirms our previous findings (1). Since 4-hydroxy-3-nitro-phenyl (NP), DNP, and α1-6 dextran do not inhibit the self binding, self binding is highly specific, involving the binding site (paratope) for PC. Although these data do not exclude the possibility that 11E7-1 binds to T15 via the Fc region, we can eliminate this possibility because of the following observations (see also Table II): (a) 11E7-1 or T15 does not bind to other IgM antibodies, and (b) antibodies of different

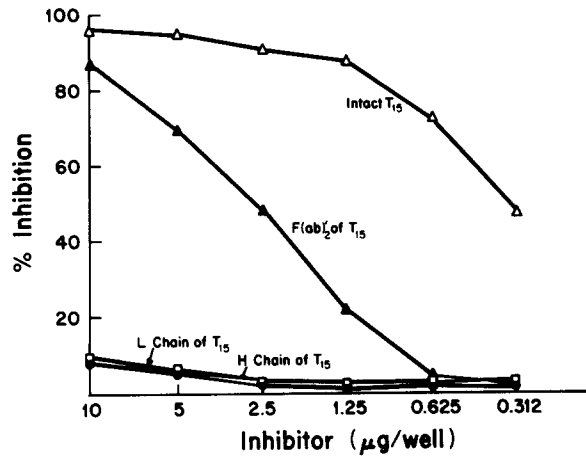


FIGURE 1. Inhibition of 11E7-1 binding to T15 by F(ab')₂ of T15. The microtiter plate was coated for 18 h at 4°C with 2 µg/ml of T15, then washed and blocked by 1% BSA in PBS. After washing, 25 ng of enzyme-labeled 11E7-1 was added in the presence or absence of inhibitors. The percent inhibition were calculated.

TABLE I
Inhibition of Self Binding of 11E7-1 by Different Haptens

Inhibitors (10 ⁻⁵ M)	Binding of ¹²⁵ I-11E7-1	Inhibition
	cpm*	%
Nil	20,936 ± 280	0
PC	135 ± 6	99.8
NP‡	21,015 ± 493	-0.4
DNP§	20,801 ± 378	0.6
Dextran [¶]	20,932 ± 382	0

Microtiter plates were coated for 18 h at 4°C with 100 µl/well of 11E7-1 (5 µg/ml) in PBS, washed three times, and incubated for 1 h with 1% BSA in PBS. After washing, ¹²⁵I-11E7-1 (45,000 cpm) was incubated in the absence of inhibitor or in the presence of inhibitors (10⁻⁵ M).

* Binding is expressed as mean ± SD of cpm (n = 3).

‡ [(4-Hydroxy-3-nitro-phenyl)acetyl]caproate was used as the NP hapten.

§ Dinitrophenyl glycine was used as the DNP hapten.

¶ Nigerose was used as the dextran hapten.

isotype but of identical variable region, such as 11E7-1, have the self-binding and antiidiotype activities. From these observations, we conclude that the binding target and binding site on 11E7-1 are both in the variable region. In other words, the self binding involves a complementary idiotope and paratope in the variable region. The failure of H or L chain to inhibit the self binding indicates that the complementary self-binding idiotope is of combinatorial nature requiring interaction of both chains.

The Variable Regions of 11E7-1 and S107 are Identical. The results regarding the self-binding properties of 11E7-1 raise the interesting question of the molecular basis of self binding. Since 11E7-1 expresses the T15 idiotype as shown previously (1), the question must be addressed whether 11E7-1 has the sequence of the T15 prototype or a variant of T15.

The H and L chain nucleotide sequences of 11E7-1 were determined by primer extension sequencing. The complete nucleotide sequence of the variable region of the 11E7-1 H chain and a comparison with the H chain of the myeloma

TABLE II
Specificity of Self Binding of Different Polymeric Forms of Antibodies

Plates coated with:	Specificity	Isotype	Binding of ¹²⁵ I-antibody*					
			T15 monomer		T15 dimer		11E7-1 (pentamer)	
			-PC	+PC	-PC	+PC	-PC	+PC
T15 monomer	PC	α, κ	567 ± 7	129 ± 16	9,565 ± 248	117 ± 18	25,151 ± 943	124 ± 21
T15 dimer	PC	α, κ	536 ± 17	131 ± 5	9,367 ± 266	138 ± 7	24,440 ± 481	112 ± 10
M315	TNP	α, λ	143 ± 5	148 ± 14	171 ± 15	138 ± 23	159 ± 14	128 ± 27
11E7-1 (pentamer)	PC	μ, κ	815 ± 52	152 ± 14	12,679 ± 567	143 ± 12	22,486 ± 1,025	156 ± 13
4F11	M167	μ, κ	134 ± 6	138 ± 16	143 ± 25	115 ± 5	188 ± 16	146 ± 14
3A4	Phthalate	μ, κ	142 ± 8	143 ± 21	156 ± 3	134 ± 14	171 ± 10	166 ± 6
10N3/2	Unknown	μ, κ	116 ± 15	119 ± 8	133 ± 14	151 ± 13	172 ± 4	155 ± 9
NL12/4	Unknown	μ, κ	122 ± 9	107 ± 16	127 ± 6	128 ± 26	151 ± 24	143 ± 9
HPCG14	PC	γ_1, κ	426 ± 22	143 ± 8	6,567 ± 191	153 ± 20	6,815 ± 291	137 ± 9
SN-2	Human T leukemia	γ_1, κ	152 ± 12	142 ± 14	172 ± 5	134 ± 15	178 ± 11	160 ± 24
F6.3	T15	γ_1, κ	20,753 ± 642	20,568 ± 781	31,541 ± 568	33,877 ± 208	40,900 ± 196	41,648 ± 253

Microtiter plates were coated for 18 h at 4°C with 5 µg/ml of different antibodies, washed, and blocked by 1% BSA solution. After washing three times, 45,000 cpm of each ¹²⁵I-antibody in 1% BSA, and 0.05% Tween 20/BBS was added in the absence of PC or in the presence of PC (10⁻⁵ M).

* Binding is expressed as mean ± SD of cpm (n = 3).

S107 (16, 17) are shown in Fig. 2. The nucleotide sequence of the 11E7-1 H chain is identical to the sequence of S107. A similar comparison of the 11E7-1 L chain with S107 (18) is shown in Fig. 3. Because the DNA sequence for the leader segment on the L chain differed from the known S107 sequence (data not shown), we confirmed the translated protein sequence for the secreted chain by NH₂-terminal sequence determination. The determined protein sequence agreed with the sequence predicted from the nucleotide sequence (see Fig. 3). Although three nucleotide substitutions were observed in complementarity determining region 2 (CDR2), the translated sequence is identical to the S107 sequence.

Self Binding Is Modulated by Ig Valency. Because of the apparent identity of the variable region sequence of T15 and 11E7-1, the possibility must be considered that T15 also exhibits self-binding activity. To test for T15 self binding, ¹²⁵I-labeled T15 monomer was added to plates coated with a panel of antibodies. The binding of monomeric T15 to T15 prototypes and a variant (HPCG14) is very low but specific, since it can be inhibited by PC (see Table II). This observation prompted us to compare self-binding activities among various polymeric forms of T15 prototype.

Before using various polymeric and monomeric forms of the T15 prototype antibody, the purity of the preparations must be established. Monomeric T15, dimeric T15, and intact pentameric 11E7-1 were tested on HPLC using a Superose 6HR column. Each antibody preparation yields a single symmetrical peak eluting at the volumes corresponding to monomeric, dimeric, and pentameric Igs (data not shown). These various polymeric forms were tested for their ability to bind to PC. It can be expected that the polymeric forms may be more efficient in binding to insolubilized PC-BSA than the monomeric antibody. As seen in Table III, the pentamer 11E7-1 is about fivefold and the dimer about fourfold more efficient in PC binding than the monomer T15. This difference is not due to the affinity, because the variable regions of T15 and 11E7-1 are

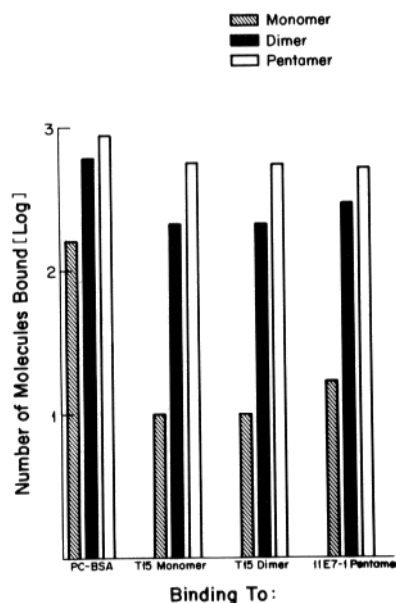


FIGURE 4. Efficiency of self binding by polymeric and monomeric antibodies. The assays were the same as in Tables II and III. The counts per minute of added and bound antibody were converted to number of molecules. The number of added antibodies was standardized to 1,000 molecules.

From these results (Table II and Fig. 4), one can see that the differences in antigen binding among monomer T15, dimer T15, and pentamer 11E7-1 are less pronounced than the differences in self binding. 16% of the monomer T15 binds to PC, while only 1–2% self binding is observed with the monomer. The PC binding of the pentameric 11E7-1 reaches 86% while the self binding is ~50%. It is interesting to note that the increase in the number of self-binding antibody molecules with an increase in the polymeric state is more dramatic than in the number of antigen-binding antibody molecules. These differences need to be considered in an attempt to describe the structural basis and biological significance of self-binding antibodies.

Discussion

The data on the specificity and structure of antibody antibodies demonstrate three major points: (a) the T15 IgA antibody that has the germline sequence of the T15 V_H and V_L prototype, has self-binding activity similar to the IgM 11E7-1 described previously (1); (b) the self binding is greatly influenced by the polymeric state of the T15 prototype structure and is therefore dependent on the valency of the antibody; and (c) the translated protein structure of the IgM 11E7-1 and the IgA S107 are identical to Ig variable regions.

The strong valency dependence of the self-binding property is intriguing for the concept of network regulation because self binding is most pronounced with the IgM isotype that represents the primary response class and also represents the membrane-bound receptor on B cells. The differences in self binding are much greater between different isotypes than the avidity differences in antigen binding. At the stoichiometric level, 50 times more molecules of IgM are self binding than of IgA monomer antibodies. The possibility that these avidity differences are directly caused by the constant region of the IgM are excluded

by showing similar differences with monomeric and dimeric IgA T15 in self binding. Because attempts to produce pure soluble monomeric IgM 11E7-1 were unsuccessful, the self binding of 11E7-1 monomer and pentamer could not be compared. Modulation of the expression of idiotopes by constant region domains have been observed (19–21); however, these differences are not related to the polymeric state of the molecule. Thus, we are inclined to assign the self-binding site to the paratope site for PC or close to this site, mainly because PC inhibits self binding and the binding is affected by valency.

The finding that the protein sequences of 11E7-1 and T15 are identical was at first surprising. It forced us to reexamine whether the T15 IgA antibody also has self-binding properties. It also directed our attention to the question of valency in self binding. Monomeric T15 has very low self-binding activity, as only 1–2% of T15 antibodies may bind to insolubilized T15 or 11E7-1 under the conditions of our assay. This low self binding, although very specific, may have been taken for background binding and therefore neglected in previous work with T15 antibodies, because typically these antibodies are purified on affinity chromatography after mild reduction and alkylation (5). The sequence data demonstrate that the self-binding site is an inherent property of the T15 germline antibody structure.

In terms of a structural interpretation of the T15 antibody as an antibody (1), a simultaneous expression of a paratope and an internal PC image in the germline antibody must be assumed. Accepting that the self-binding structure is in part generated by an internal PC image idiotope, it is interesting to compare this idiotope with another T15-associated idiotope detected by the anti-T15 mAb F6-3 (7). The expression of the F6-3 idiotope is only marginally affected by the valency of the antibody. Thus, the self-binding idiotope may have a unique structure and/or function within putative network regulation.

The biological significance of a self-binding antibody expressing complementary paratopes and idiotopes is an interesting subject for speculation. As pointed out previously (1), the 11E7-1 may represent a closed circuit feedback loop in the network. The additional evidence on the importance of the valency for the self-binding property, provided in this report, adds some more specific points on this scenario. For example, it could be that in early ontogeny, emerging B cells committed to the T15 idiotope expression and stimulated by environmental PC antigens bind secreted IgM free of PC antigen. This surface-bound IgM, in turn, would be internalized and could provide a stimulatory signal, thereby initiating clonal proliferation. The same mechanism also could augment presentation of internalized antigen to Th cells. Together, both or either process could produce a clonally dominant expression of the T15 clone seen in some strains of mice (22, 23). Experiments to evaluate this hypothesis are in progress.

With respect to the structural basis of the self-binding site, it is important to remember that the self-binding site is poorly evident in monomeric T15⁺ anti-PC antibodies, but readily detected with increase of Ig polymerization. Because of this dependence on avidity of the overall Ig complex it resembles similar but less pronounced effects on the strength of the classical binding site. It is therefore tempting to assign the self-binding site to the antigen-binding site. An argument in favor of this is that both sites need the interaction of H and L chain variable

domains. However, the increased self binding in polymeric forms is not simply additive but highly synergistic. This effect is reminiscent of classical allosteric mechanisms in biologically active molecules that operate through subunit interactions, like hemoglobin or allosteric enzymes. The inhibition of the self-binding effect by free PC may thus be interpreted as an allosteric effect on the self-binding site. Thus, an alternative model emerges in which two different sites or subsites within the same region have a reciprocal allosteric effector connection, one PC-binding site and an independent self-binding site.

The discovery of an inherent self-binding potential in the germline structure of an idiotype dominant antibody that reacts with environmental and pathogenic organisms raises several interesting questions. First, how can the dual properties of the T15 prototype as antigen binding and antigen-sensitive self binding be interpreted in terms of a three-dimensional model. Since a related three-dimensional model for T15 Fab exists from the crystallographic data on McPC 603 (24), it should be helpful to construct, by computer modeling, a predicted structure for the Fab fragment of T15. Such a model may give clues as to how self binding by T15 may be achieved and which parts of the variable domains are interacting.

Alternatively, the residues involved in self binding could be deduced by testing T15 variants for loss of self binding. If the primary structure of such variants is known, comparison with other variants exhibiting self binding should identify the critical residue(s) and sequence positions. These studies could then be extended by inducing specific mutations into the L and H chain genes and determining the binding and idiotypic properties of their expressed antibodies.

The other interesting aspect of the self-binding T15 prototype is its possible biological function in the idiotype network. T15 is the dominant idiotype in the anti-PC response in BALB/c and appears to be under idiotypic regulation (25–27). The self-binding idiotype could be regulated by itself in a feedback-type loop in which antigen would have a decisive control function, since it inhibits self binding. In another interesting model, self binding of secreted polymeric T15⁺ IgM to the cellular T15⁺ Ig receptor for PC could mimic antigen binding and induce Ig internalization and presentation of a PC-mimicking idiotype to Th cells that recognize T15 idiotypes (28, 29). By this mechanism, the T15 clone could be expanded to a dominant level in the absence of antigenic stimulation. An important question is whether self-binding antibodies occur in other systems that are characterized by clonal dominance. In any event, the description of germline sequences encoding antibody reactivity provides an additional facet of the network system, and experiments designed to more fully delineate the role of such determinants are currently in progress.

Summary

We have previously described (1–3) an IgM antibody that binds to PC, expresses the T15 idiotype, and binds also to itself or T15 if insolubilized. Because of the simultaneous presence of complementary idiotopes and paratopes this type of antibody has been termed antibody. The self binding involves the antigen-binding site because the F(ab')₂ fragment of T15, PC, and no other haptens inhibit the self binding. DNA sequence analysis of 11E7-1 using primer

extension cDNA sequencing showed that the variable sequences of H and L chains of 11E7-1 are identical to the germline sequence of the prototype T15 idiotype. Furthermore, monomeric and dimeric T15 IgA were shown to bind to insolubilized T15 and other T15⁺ antibodies including 11E7-1. Thus, the self-binding activity is an inherent property of the T15 germline sequence. The self-binding is highly dependent on the polymeric state of the binding antibody since the IgM pentamer of 11E7-1 is about three fold more effective than the T15 dimer and 50 times more than the T15 monomer.

These data suggest that the self-binding activity of a germline-encoded idiotype may play an important role in the biology of its expression, and more specifically, may be responsible for the establishment of its dominant expression.

We thank Dr. A. Sood for help in nucleotide sequencing, and Mrs. C. Zuber and Mrs. D. Ovak for preparation of the manuscript.

Received for publication 16 December 1986.

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