III. Properties of Sequential Anti-Idiotypic Antibodies to Heterologous Anti-γ Globulins. Detection of Reactivity of Anti-Idiotypic Antibodies with Epitopes of Fc Fragments (Homobodies) and with Epitopes and Idiotopes (Epibodies)*

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The immune response against antigenic determinants of immunoglobulin (Ig) molecules (i.e., isotypes, allotypes, and idiotypes) has been extensively studied. In mice, it has been shown that the antibody response against allotypic determinants is under Ir gene control (1, 2).

The study of homologous and autologous anti-Ig responses has suggested that these responses may be uniquely similar among genetically distinct individuals. Thus, autologous anti-IgG antibodies in humans (rheumatoid factors) show a considerable degree of homogeneity, with many of those studied bearing a cross-reactive idiotype (IdX)1 (i.e., Wa) and others bearing other cross-reactive idiotypes (Id) (3, 4). In mice and in rabbits, it has been shown that anti-allotype antibodies bear cross-reactive Id (2, 5, 6), indicating that the genes encoding the anti-allotype immune response are highly conserved. Similarly, syngeneic and homologous anti-Id antibodies share IdX in some cases. Because of these findings, the purpose of this investigation was to study the Id of the anti-Ig response in a heterologous system (murine/human), in which the immunogen was a human monoclonal IgM anti-γ-globulin bearing the IdX Wa (7).

Study of the idiotypic of murine anti-human V region of GI antibodies produced by various strains of mice has shown that they share an IdX, despite the fact that the fine specificity of these antibodies is different from one strain to another. In addition, we found that A/J anti-Id antibodies recognize an epitope related specificity borne by the Fcγ fragment and shared by various human IgM monoclonal proteins. We have called these anti-Id antibodies epibodies. Finally, a fraction of anti-anti-Id antibodies bound to the antigen and therefore behaved as homobodies.

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1 Abbreviations used in this paper: Ab1, anti-epitope antibody; Ab2, anti-Id antibody; Aba, anti-anti-Id antibody; Ab4, anti-anti-anti-Id antibody; C region, constant region; epitope, Ab2 antibody that is able to recognize similar “structures” or “shapes” borne by epitope and Ab1; epitope, antigenic determinant; FCS, fetal calf serum; HA, hemagglutination; H1, hemagglutination inhibition; homobody, Ab2 antibody that mimics the antigen and binds to Ab1 and cell receptor for the antigen; Id1, individual idiotype; idiotope, individual antigen of the idiotype; IdX, cross-reactive idiotype; K, kappa chain; MHC, major histocompatibility complex; RIA, radioimmunoassay; SRBC, sheep erythrocytes; V region, variable region.
Materials and Methods

Mice. BALB/c, C.B20, C58/J, DBA/2, A/J, PL/J, P/J, CE/J, C57BL/6, and AKR/J mice, 6–8 wk old, were used in this study.

Human Proteins. IgM$_{G1}$ GI and PG and IgM$_{G1}$ Lay with anti-IgG activity and IgM$_{G1}$ Dau, Ch, Ga, and Sal with no known specificity were used in this study. They are all monoclonal IgM proteins obtained from patients with macroglobulinemia or mixed cryoglobulinemia. IgM GI was highly purified from the cryoglobulin of patient GI by zone electrophoresis as the first step followed by G200 chromatography. The final preparation showed no detectable IgG by hemagglutination.

Preparation of Human Ig Fragments. Fab fragments of IgM GI and PG, Fcγ fragments of IgG, and Fcα fragments of IgM G1 were prepared according to previously described techniques (8).

Immunization of Mice. Various strains of mice were immunized intraperitoneally according to the method of Tung and Nisonoff (9) with 0.1 ml of an emulsified mixture of 10 mg/ml myeloma protein and complete Freund's adjuvant (1:9 ratio). Ascitic fluid was harvested after the fourth immunization.

Separation of Anti-V Region GI Antibodies. An ammonium sulfate fraction of ascitic fluid was successively absorbed on Sepharose 4B coated with human IgG and IgM$_{G1}$ Ch columns. The effluent was precipitated with ammonium sulfate, dialyzed, and passed over an IgM$_{G1}$ GI-Sepharose 4B column, and antibodies were eluted from this column with glycine-HCl buffer (pH 2.8) as previously described (2).

Preparation of Anti-Id Antibodies. Anti-V-region GI antibodies separated from the ascitic fluid from single BALB/c mice were coupled with keyhole limpet hemocyanin (KLH), as previously described (2), and then used as immunogens to prepare syngeneic anti-Id (anti-V-region) antibodies, as previously described (4). Monoclonal anti-anti-VH antibodies were prepared by fusion of Sp2/0 myeloma cells with spleen cells from CB6 F1 mice immunized with syngeneic affinity-purified anti-VH GI antibody-KLH conjugate. Hybrids producing anti-anti-VH GI antibodies detected by the ELISA technique, as previously described (10), were cloned on thymocyte filler, and the clones were expanded in pristane-primed CB6 F1 mice.

Hemagglutination Assay (HA). HA titers of murine anti-V region GI or BALB/c anti-(BALB/c anti-V region) antisera were measured in microtiter plates using sheep erythrocytes (SRBC) coated with human IgG myeloma protein GI, PG, Ch, Ga, or BALB/c anti-V region GI antibodies, respectively. The proteins were coated onto SRBC using Cr Cl$_2$ (2). In each case, the titer recorded as 1/log$_2$ of the highest dilution of antisera that caused agglutination.

Determination of Serum IdX Titer. A hemagglutination inhibition (HI) method described previously (2) was used to test sera for IdX in these experiments, specifically purified BALB/c or P/J anti-V region GI antibodies were coupled with SRBC. The capacity of various sera to inhibit hemagglutination of these cells by BALB/c anti(anti-V region) antibodies was determined. The titer reported as 1/log$_2$ of the highest dilution that caused inhibition of hemagglutination.

Radioimmunoassay (RIA). Purified BALB/c anti-anti-V region antibodies and goat antimouse Ig antibodies were labeled with $^{125}$I, as described by Hunter (11).

The ability of anti-V region GI antibodies to bind to various human IgM myeloma proteins was determined in microtiter plates that had been incubated for 18 h with 50 µg/ml myeloma proteins, followed by three washings in saline. The plates were incubated for 1 h with 50% fetal calf serum (FCS) and, after three washings, were incubated for 3 h with mouse anti-V region GI antibodies. After three washings, the microplates were incubated for 3 h with 50 µl of $^{125}$I-labeled anti-mouse Ig (New England Nuclear, Boston, MA) (~50,000 cpm/50 µl). After incubations, the microplates were washed extensively, and the radioactivity on plates was measured in a γ-spectrophotometer. The titer of BALB/c anti-anti-V region GI antibodies was measured with the use of microplates that had been incubated with 50 µg/ml purified anti-V region GI antibodies.

ELISA Technique. Affinity-purified CB6 F1 and anti-VH GI antibodies were labeled with alkaline phosphatase (grade I; Boehringer Mannheim, West Germany), as previously described (10). The specific activity of labeled antibodies was 930 international units of alkaline phosphatase per mg of enzyme-antibody conjugate. Labeled anti-VH GI antibodies were used in ELISA to test the reactivity of monoclonal anti-anti-VH GI antibodies as follows: microtiter
plates (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA) were coated with IgM GI (10 μg/ml) for 18 h at 4°C. After three washings with phosphate-buffered saline containing 5% Tween, the microplates were incubated for 3 h at 4°C with labeled anti-VH GI antibodies (5 μl/ml). The labeled anti-VH GI antibodies were preincubated or not during 30 min at 23°C with various amounts of unlabeled monoclonal anti-anti-VH GI antibodies before being added to the microtiter plates. Finally, the microtiter plates were reincubated for 1 h at 37°C with p-nitrophenyl phosphate (1 tablet Sigma) in 5 ml of 10% (wt/vol) diethanolamine buffer (pH 9.8). The enzymatic reaction was stopped with 25 μl of 3 M NaOH, and the absorbance at 405 nm was carried out in an ELISA microreader (Dynatech Laboratories, Inc., Dynatech Corp.).

Results

Anti-V Region GI Antibodies. Anti-V region antibodies were obtained by immunization of various strains of mice with IgMκIII monoclonal protein GI displaying IgG-binding activity and belonging to the human WaldX subgroup. These were purified by elution from an IgM GI-Sepharose 4B column after previous absorption and elution on an IgM Ch-Sepharose 4B column.

The agglutinating activity as well as the binding activity of these antibodies toward various human IgM monoclonal proteins was studied initially by HA and RIA assays, respectively. The HA assay results depicted in Table I show that antibodies produced in various strains of mice not only have a high agglutinating titer for IgM GI and PG of the WaldX subgroup, but that these antibodies, excepting those produced by C57BL/6, AKR/J, and C58/J, also agglutinated SRBC coated with IgMκIII Wa- Dau monoclonal protein. Thus, in further studies (Table II), we investigated with RIA the ability of anti-VH GI antibodies produced in various strains of mice to interact with several IgMκIII monoclonal proteins. This study was performed after absorption of anti-V region GI antibodies on human IgG and polyclonal IgM obtained from one individual.

The results depicted in Tables I and II indicate that anti-V region GI antibodies produced by various strains of mice can be classified into several groups according to their specificity, as follows: (a) antibodies produced in certain strains (BALB/c, for example) were specific for IdI and IdX of human IgM myeloma protein GI. The titer

| Anti-V region GI antibodies (100 μg/ml) | HA titer (log 2 units) | SRBC coated with |
|----------------------------------------|------------------------|-----------------|
|                                        | IgMκIII Wa⁺ Gl      | IgMκIII Wa⁺ PG  | IgMκIII Wa⁺ Dau | IgMκI Po⁺ Lay | IgMκIII Wa⁺ Ch |
| BALB/c                                 | >12                    | 6               | 2              | 1             | 0              |
| DBA/2                                  | >12                    | 10              | 2              | 2             | 0              |
| PL/J                                   | 7                      | 7               | 5              | 0             | 0              |
| C57BL/6                                | >12                    | 2               | 4              | 1             | 0              |
| C58/J                                  | >12                    | 2               | 0              | 0             | 0              |
| AKR/J                                  | 7                      | 2               | 0              | 0             | 0              |
| RIII                                   | 8                      | 2               | 0              | 5             | 0              |
| CE/J                                   | 10                     | 10              | 5              | 0             | 0              |
| P/J                                    | 7                      | 6               | 7              | 1             | 0              |
| BALB/c anti-Ch                         | 0                      | 0               | 6              | 5             | >12             |
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**Table II**

*Reaction of Anti-V Region Antibodies Produced by Various Strains of Mice against Different Monoclonal IgM Proteins*

| Origin of anti-V region Gl antibodies (100 µg/ml) | IgM Gl* | IgM PG* | IgM Dau | IgM Sa | IgM Ga | IgM Ch |
|--------------------------------------------------|---------|---------|---------|--------|--------|--------|
| BALB/c                                           | 1,643 ± 40 | 1,174 ± 20 | 143 ± 23 | 166 ± 29 | 134 ± 46 | 42 ± 18 |
| AKR/J                                            | 770 ± 27  | 249 ± 13  | 80 ± 23  | 104 ± 4  | 25 ± 23 | 0      |
| DBA/2                                            | 2,641 ± 28 | 2,079 ± 24 | 230 ± 25 | 1,242 ± 28 | 1,420 ± 88 | 186 ± 36 |
| C.B20                                            | 1,395 ± 115 | 543 ± 21  | 67 ± 6   | 330 ± 27 | 302 ± 9  | 152 ± 26 |
| RIII                                             | 3,418 ± 36 | 2,164 ± 294 | 594 ± 32 | 521 ± 48 | 534 ± 44 | 324 ± 103 |
| PL/J                                             | 2,834 ± 43 | 1,186 ± 32 | 1,716 ± 293 | 913 ± 51 | 510 ± 47 | 0      |
| P/J                                               | 1,434 ± 199 | 893 ± 105 | 494 ± 42 | 562 ± 105 | 126 ± 62 | 105 ± 3  |
| CE/J                                             | 1,935 ± 54  | 1,236 ± 59 | 1,106 ± 24 | 793 ± 76 | 1,043 ± 50 | 0      |
| A/J                                               | 2,942 ± 528 | 2,734 ± 200 | 2,114 ± 62 | 2,494 ± 10 | 1,824 ± 52 | 209 ± 52 |

* Anti-γ-globulin.
† Counts per minute ± SD.

Microtiter plates were coated for 18 h at 4°C with 50 µg/ml of various myeloma proteins and washed three times and incubated 1 h with 50% FCS. After three washings, they were incubated for 3 h at 4°C with anti-V region Gl antibodies washed three times and then incubated for 3 h at 4°C with 125I-goat anti-mouse antibodies (10,000 cpm). The nonspecific binding to microplates coated with 50% FCS was subtracted. In the experiment illustrated in this table, the background was 385 ± 52. Each value represents the average of triplicates. SD was calculated on values after subtraction of average of background.

and the specific binding of these antibodies were much higher for IgM Gl than for IgM PG. PG is an IgM<sub>κ</sub> myeloma protein with anti-γ-globulin activity that also belongs to the WalDX subgroup. (b) Antibodies produced in other strains (DBA/2 for example) were mainly specific for WalDX because the HA titers or the specific binding activity were roughly equal for Gl and PG WalDX<sup>+</sup> monoclonal proteins. (c) Antibodies produced in PL/J mice, for example, recognized WalDX and also framework determinants shared by certain IgM monoclonal proteins, which lack anti-γ-globulin activity.

**Id of Anti-V Region Gl Antibodies.** BALB/c anti-Gl antibodies that are primarily specific for WalDX and Gl Idl were used as immunogens to prepare syngeneic anti-Id (i.e., BALB/c anti-BALB/c anti-V region Gl antisera). These antisera were compared with BALB/c anti-anti-V region Ch antibodies, Ch being an IgM protein without anti-γ activity. They agglutinated BALB/c and DBA/2 anti-Gl antibodies at high titers and the control BALB/c anti-V region Ch antibodies at a very low titer. They did not interact with murine anti-Id (i.e., A48Id) or anti-allotype (i.e., IgG<sub>κ</sub>) antibodies (Table III). From these results, we concluded that the syngeneic BALB/c anti-anti-V region Gl antibodies showed specificity for Id determinants of anti-V region Gl antibodies.

In further experiments, we used these syngeneic anti-anti-Id antisera to investigate the presence of IdX on anti-V region Gl antibodies produced by various strains of mice. The results presented in Table IV show that anti-V region Gl antibodies produced by various strains of mice share an IdX recognized by BALB/c anti-anti-V region Gl antibodies. The expression of this IdX is independent of the MHC or the I<sub>gh</sub> gene complex.

Therefore, our results indicate that murine anti-V region Gl antibodies share an
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Table III

| Agglutinating antisera | BALB/c anti-anti-V region GI | BALB/c anti-anti-V region Ch |
|------------------------|-----------------------------|-----------------------------|
| BALB/c anti-V region GI | 8                           | 1                           |
| BALB/c anti-V region Chr | 0                           | 5                           |
| DBA/2 anti-V region GI | >8                          | 0                           |
| RIJI anti-V region GI  | 3                           | 0                           |
| BALB/c anti-A48Id*     | 0                           | 0                           |
| BALB/c anti-IgG63 allotype‡ | 0             | 0                           |

* ABPC48 is murine IgAκ β2-6 fructosan-binding myeloma protein that expresses A48Id shared with UPC10 myeloma protein.
‡ These antibodies were obtained by immunization of BALB/c mice with CBPC 101 myeloma protein and purified on Sepharose 4B-C57BL/6 Ig columns.

Table IV

Expression of Cross- Reactive Id of BALB/c Anti-V Region Gl Antibodies in Various Strains of Mice

| Anti-V region Gl antibodies from* | H2 | IghC | Number of mice expressing IdX | BALB/c | P/J | RIA |
|----------------------------------|----|------|-------------------------------|--------|-----|-----|
|                                  |    |      |                               |        |     |     |
| BALB/c                           | d  | a    | 9                             | 9      | 8/11| 5,467 ± 121 |
| DBA/2                            | d  | c    | 8                             | 5      | 2/4 | 4,629 ± 70  |
| PL/J                             | u  | j    | 4                             | 4      | 2/4 | 3,422 ± 283 |
| C.B20                            | d  | b    | 8                             | 2      | 2/4 | 3,840 ± 417 |
| C57BL/6                          | h  | b    | 8                             | 5      | 4/4 | 4,203 ± 60  |
| AKR/J                            | k  | d    | 9                             | 6      | 3/4 | 3,784 ± 720 |
| C58/J                            | k  | a    | 3                             | 5      | 5/5 | 2,896 ± 707 |
| RIJI                             | r  | g    | 3                             | 5      | 4/4 | 1,134 ± 351 |
| P/J                              | p  | h    | 6                             | 8      | 2/4 | 4,417 ± 603 |
| CE/J                             | k  | q    | 2                             | 2      | 2/6 | 1,704 ± 12  |
| A/J                              | a  | e    | 0                             | 0      | 0/6 | 380 ± 88   |
| Anti-A48Id(BALB/c)               | d  | a    | 0                             | 0      | —  | 197 ± 21   |

* In HI assay, the purified anti-V region Gl antibodies enumerated in column 1 were used as inhibitors, whereas in RIA they were used to coat the microtiter plates (at 50 μg/ml concentration).
‡ Two systems were used to investigate the expression of IdX on anti-V region Gl antibodies as follows: BALB/c anti-anti-V region Gl antiserum and SRBC§ coated with BALB/c anti-V region Gl or P/J anti-V region Gl antibodies.

interstrain IdX. It should be mentioned that this IdX was not identified on two murine monoclonal antibodies specific for the human μ chain, kindly provided by J. Kearney, University of Alabama, for this study.

Polyclonal Syngeneic Anti-Anti-V Region Gl Antibodies Contain a Subset That Binds to Human Fc Fragment. In further studies, we investigated the ability of anti-anti-V region Gl
antibodies to bind to the human Fcγ fragment. The rationale of this investigation follows from several reports that suggest that anti-Id antibodies represent the internal image of antigen and from the fact that human IgM with anti-γ globulin activity was the initiator of the anti-anti-V region GI antibody pathway. A comparative study of the binding of these anti-anti-V region GI antibodies to plates coated with various human IgM myeloma proteins and Fcγ fragments demonstrated that a subset representing ~10% of this population of antibodies bound to Fc. This binding was inhibited by preabsorption of anti-anti-V region GI antibodies with the Fcγ fragment but not with the Fab GI fragment. No binding of heterogeneous 3H-syngeneic anti-anti-A48Id antibodies to Fcγ fragments was observed in control experiments (Table V). These results suggest that a fraction of syngeneic (BALB/c) anti-anti-V region GI antibodies displayed a binding activity similar to IgM GI, the anti-γ monoclonal protein that was the initiator of this network pathway. In these terms, this subset of antibodies that bind to Fcγ fragments can be considered as homobodies (12).

Monoclonal Syngeneic Anti-Anti-V Region GI Antibody That Bind to Human IgM Monoclonal Proteins. Several syngeneic CB6/F1 monoclonal anti-anti-V region GI antibodies were obtained by fusion of SP2/0 myeloma cells with spleen cells from CB6/F1 mice immunized with anti-V region GI antibodies. The majority of these monoclonal anti-anti-V region antibodies bound labeled anti-V region GI antibodies as well as inhibited the binding of anti-V region GI antibody to IgM GI monoclonal protein.

Among these monoclonal anti-anti-V region antibodies, we found one, P1A2.3, that did not bind labeled anti-V region GI antibodies but was able to inhibit the binding of anti-V region GI antibodies to IgM GI monoclonal protein (Table VI). Surprisingly, we found that this monoclonal antibody significantly bound to IgM GI, PG, Sal, and Ga (IgMKII), but not to IgM Dau, Ch (IgMKIII), Lay (IgMKI), and human IgG (Table VII). The binding of P1A2.3 monoclonal antibody to human monoclonal proteins GI and Ga was inhibited with Fab GI and Fab PG, but not with Fcγ GI (Fig.

| Table V |

**Binding of BALB/C Anti-BALB/C Anti-V Region (GI) Antibodies to Human Fc Fragment**

| Plates coated with 50 μg/m of | Absorption of antibodies | 125I-BALB/c anti-anti-V region | 3H-BALB/C anti-anti-A48Id* |
|-------------------------------|--------------------------|-------------------------------|---------------------------|
| BALB/c anti-V region GI       | --                       | 5,467 ± 121                  | 332 ± 25^{1}               |
| BALB/c anti-V region Ch       | --                       | 196 ± 10                     | 356 ± 18                   |
| BALB/c anti-A48Id             | --                       | 197 ± 21                     | 14,685 ± 2,135             |
| IgM Wa^a GI                   | --                       | 289 ± 112                    | 342 ± 24                   |
| IgM Wa^a PG                   | --                       | 342 ± 65                     | 333 ± 24                   |
| IgM Po^b Lay                  | --                       | 153 ± 42                     | 342 ± 35                   |
| IgM Wa^a Da                   | --                       | 220 ± 63                     | 335 ± 26                   |
| Human IgG                     | --                       | 484 ± 112                    | 305 ± 38                   |
| Fcγ                           | --                       | 651 ± 38                     | 336 ± 28                   |
| Fcγ Fab GI                    | Fab GI^{b}               | 648 ± 26                     | 388 ± 122                  |
| Fcγ Fcγ                       | Fcγ^{c}                  | 305 ± 18                     | 312 ± 16                   |
| Fcγ Fcγ (50%)                 | --                       | 328 ± 33                     | 289 ± 22                   |

* This antibody used as a control is specific for the A48 Id of murine ABPC48 monoclonal protein (IgA kappa with B2-6 fructosan-binding activity).

^{1} 50 μg/ml.

^{b} Counts per minute minus mean of triplicates ± SD.
### Table VI

**Binding and Inhibitory Activity of Two Monoclonal Anti-Anti-V Region Gl Antibodies (ELISA)**

| Microtiter plates coated with | Binding of labeled anti-V region antibodies (5 μg/ml)* |
|------------------------------|--------------------------------------------------------|
| BSA                          | 0                                                      |
| IgM (GI)                     | 0.52†                                                  |
| IgM (PG)                     | 0.13                                                  |
| PIA5.6                       | 0.16                                                  |
| PIA2.3                       | 0.02                                                  |

Microtiter plates were coated for 18 h with proteins mentioned in column 1 and then incubated with alkaline phosphatase-labeled anti-V region Gl antibodies.

### Table VII

**Binding of P1A2.3 Monoclonal Anti-Anti-V Region Gl Antibody to Various Human IgM Monoclonal Proteins**

| Microtiter plates coated with | Binding of |
|------------------------------|------------|
|                              | BALB/c anti-human Ig | CB6 F1 anti-V region Gl | P1A2.3 | P4D2.7 |
| IgM Gl                       | 18,392 ± 1,184*     | 12,575 ± 2,600           | 6,059 ± 799 | 1,544 ± 1,146 |
| IgM PG                       | 20,236 ± 2,619      | 11,792 ± 3,191           | 11,068 ± 18 | 252 ± 257 |
| IgM Dau                      | 23,586 ± 989       | 2,898 ± 1,782            | 1,678 ± 373 | 2,363 ± 408 |
| IgM Sal                      | 16,639 ± 1,629     | 403 ± 385                | 6,610 ± 193 | 0 |
| IgM Ga                       | 16,357 ± 57        | 601 ± 635                | 2,744 ± 470 | 0 |
| IgM Lay                      | 12,926 ± 870       | 1,088 ± 202              | 0        | 0 |
| IgM Ch                       | 18,643 ± 1,038     | 85 ± 43                  | 905 ± 147 | 115 ± 16 |
| IgG                          | 18,046 ± 521       | 354 ± 300                | 959 ± 958 | 0 |

Microtiter plates were coated with 50 μg/ml of human IgM monoclonal proteins mentioned in column 1 and then with 50 μg/ml of purified heterogeneous BALB/c anti-human Ig or CB6 F1 anti-V region Gl antibodies or with P1A2.3 or P4D2.7 monoclonal CB6 F1 anti-anti-V region Gl antibody. Their binding was estimated in RIA by incubating the plates with 50,000 cpm 125I-goat anti-mouse Ig. The binding is expressed as mean ± SD of cpm after the subtraction of the background (i.e., the binding 125I-goat anti-mouse Ig to human IgM proteins).

* Results expressed as cpm minus mean of triplicates ± SD.
Fig. 1. Inhibition of binding of PIA2.3 monoclonal antibody to human IgM Gl by IgM fragments. Microtiter plates were coated with human IgM Gl (50 μg/ml) overnight, washed three times, and then incubated for 1 h with 50% FCS. PIA2.3 was preincubated for 1 h with human IgM fragments and then added for 3 h to IgM Gl-coated plates. After three washings, the microplate was incubated for 3 h with 125I-goat anti-mouse antibody, washed extensively, and radioactivity was counted in a γ-spectrometer. O, Fab Gl; Δ, Fab Fc; ●, Fcγ Gl.

1. It should be mentioned that 50% inhibition was reached with high amounts of Fab fragments (100 μg/ml). This particular monoclonal anti-anti-V region Gl antibody, which interacts with anti-V region antibodies as well as with antigenic determinants of V region Gl, which we have called epibodies.

Special Properties of A/J Anti-V Region Gl Antibodies. Special studies were carried out on A/J anti-V region Gl antibodies. These antibodies, obtained by the same regimen of immunization used for other strains, exhibited two peculiar properties: (a) they bound in an equal manner to most IgM myeloma proteins independent of anti-IgG activity (Table II); and (b) they did not express the IdX recognized by BALB/c anti-anti-V region Gl antibodies.

Because of these particular properties of A/J anti-V region Gl antibodies, we studied their binding to human IgG. The data presented in Table VIII showed that anti-V region Gl antibodies produced in A/J mice, but not in other strains, exhibited the ability to bind to human IgG. In further experiments, we studied the effects of absorption of A/J anti-V region Gl antibodies with various IgM proteins, Fab Gl, Fcγ, and IgG on their ability to agglutinate IgM Gl-SRBC or to bind to microtiter plates coated with Fcγ fragment. The results presented in Table IX showed that the ability of A/J anti-V region antibodies to agglutinate SRBC was partially inhibited after absorption with IgM Gl, Sal, Dau, Fab Gl fragments, IgG, and Fcγ fragments. Similarly, the binding of these antibodies to Fcγ fragments was inhibited by the same proteins. We explain these results by a possible related epitope between the Fcγ fragment (for which the IgM Gl is specific) and framework determinants of various other IgM proteins. Therefore, these anti-Id antibodies that bind to the V region of human IgM proteins and to the Fcγ fragment can also be considered epibodies.

Discussion

The results presented in this communication indicate that various strains of mice, in response to immunization with a monoclonal human IgM protein with anti-γ
Table VIII

Binding of anti-V Region Gl Antibodies Produced in Various Strains of Mice to Human IgG

| Antibodies          | cpm      |
|---------------------|----------|
| Nil                 | 3,536 ± 349 |
| BALB/c anti-human Ig| 22,959 ± 521 |
| BALB/c anti-V region Ch | 3,673 ± 519 |
| BALB/c anti-V region Gl | 3,826 ± 471 |
| DBA/2 anti-V region Gl | 5,050 ± 318 |
| RIII anti-V region Gl | 6,353 ± 345 |
| P/5 anti-V region Gl | 4,570 ± 533 |
| A/J anti-V region Gl | 17,340 ± 1,463 |
| C.B20 anti-V region Gl | 3,412 ± 240 |
| PL/J anti-V region Gl | 8,732 ± 316 |
| C58/J anti-V region Gl | 2,721 ± 528 |
| AKR/J anti-V region Gl | 1,665 ± 203 |
| CE/J anti-V region Gl | 5,446 ± 164 |

Microtiter plates were coated for 18 h at 4°C with 50 µg/ml human IgM, washed three times, and incubated for 1 h with 50% FCS, washed three times and then coated with 50 µg/ml antibodies mentioned in column 1. After three washings, the plates were incubated for 3 h at 4°C with 50,000 cpm of 125I-goat anti-mouse Ig. Results are expressed in cpm mean ± S.D.

Table IX

Absorption of Human Fc-Binding Activity of A/J Anti-V Region Gl Antibodies

| Absorption of A/J anti-V region Gl antibodies with* | HA(log2 units) titer* | RIA (cpm)† |
|----------------------------------------------------|-----------------------|---------|
| None                                               | 7                     | 8,897 ± 23 |
| Fab Gl                                             | 2                     | 6,667 ± 342 |
| Fc                                                  | 4                     | 5,669 ± 156 |
| IgG                                                 | 3                     | 4,467 ± 156 |
| Polyclonal IgM                                       | 6                     | 7,128 ± 525 |
| IgM Wa* Gl                                          | 3                     | 6,300 ± 394 |
| IgM Wa* Sal                                         | 5                     | 5,836 ± 144 |
| IgM Wa* Ca                                         | 7                     | 8,587 ± 488 |
| IgM Wa* Dau                                         | 5                     | 6,117 ± 1 |
| IgM Wa* Ch                                          | 7                     | 8,767 ± 460 |
| IgM Po* Lay                                         | 7                     | 8,839 ± 485 |

* SRBC coated with IgM Gl.
‡ Microplates coated for 18 h with Fcy (30 µg/ml) washed three times and then incubated further with 50% FCS. After three washings, the microplates were incubated for 3 h with A/J anti-V antibodies washed three times and then incubated for 3 h with 125I-goat anti-mouse antibodies. Results are expressed in cpm, mean of triplicates ± SD. Adsorption was performed by mixing equal volumes (i.e., 100 µg/ml) of anti-A/J anti-V region (Gl) antibodies and various proteins for 30 min at room temperature before incubation in RIA or use in HA assays.

globulin activity and bearing the WaIdX, produced multiple antibodies with specificities for IdI, WaldX, and against framework-associated antigenic determinants of the monoclonal protein used as the immunogen.

The anti-Id antibodies produced by various strains of mice also share an interstrain
The expression of this IdX is independent of the MHC or Igh gene complexes. This suggests that the response to idiotypic determinants of antibodies produced across heterologous as well as homologous (2, 5, 6) and syngeneic (3, 13) barriers is highly conserved. The existence of an interstrain IdX that is expressed in the majority of the individuals of several strains strongly suggests that this response is encoded by a germline gene.

The study of the binding activity of anti-V region and anti-anti-V region Gl antibodies, obtained by hetero or syngeneic immunization, respectively, showed that some of these antibodies display special binding activity. Thus, we found that a fraction of anti-anti-V region Gl antibodies exhibited antigenic-binding activity similar to the IgM Gl monoclonal protein that was the initiator of our network pathway. These antibodies, which represent ~10% of total anti-anti-V region antibodies, bound to Fcγ and can be considered homobodies, as defined previously (12).

In our system, Ab1, i.e., human IgM monoclonal protein with anti-γ globulin activity, recognizes epitopes of the Fcγ fragment. Immunization of mice with Ab1 induces the synthesis of anti-V region antibodies, which can be considered as Ab2 or anti-Id antibody. Some idiotopes of the Ab2 antibodies are probably similar to epitope(s) of the Fcγ fragment and represent their internal image. Therefore, immunization of a second mouse with these anti-V region antibodies elicited the production of a family of anti-anti-V region antibodies; among these, a small subset recognizes some epitopes of the Fcγ fragment as well as the idiotopes of Ab2 antibodies. The existence of these homobodies implies, first, a certain degree of similarity of structure between the antigen and anti-V region antibodies and, second, that some anti-Id antibodies represent the "internal image" of the antigen within the immune system. A prediction of the internal image concept is that homobodies might bind to antigen, displacing Ab1, because the same interaction takes place between antibody and antigen as between homobodies and antigen.

There are several examples that suggest that homobodies represent topochemical copies of the antigen. Thus, Ab4 (i.e., anti-anti-anti-Id antibodies), obtained in rabbits and in mice in the M. lysodeikticus or bacterial levan systems, display binding activity similar to Ab2 (i.e., anti-Id antibodies) to idiotypic determinants of Ab1 (14, 15). If the idiotopes of Ab1 are considered as epitopes in these systems, then Ab4 represents the internal image of the Ab2-combining site, because Ab1 and Ab4 share similar idiotopes.

The internal image concept predicts that homobodies can display biological functions of the antigen. Sege and Peterson (16) have shown that anti-Id antibodies against anti-insulin antibodies can mimic some functions of insulin. Schreiber et al. (17) found that anti-Id antibodies against rabbit anti-alprenolol antibodies compete with the binding of dehydroalprenolol to β-adrenergic receptors and can stimulate basal adenylate cyclase activity in the cells bearing these receptors.

A second category of antibodies observed in our system was quite different and exhibited the ability to bind to both the epitopes of the antigen and idiotopes of the antibodies. We found two types of antibodies that exhibited this peculiar property as follows: (a) a monoclonal anti-anti-V region antibody (Ab3) that binds to IgM Gl as well as several IgM proteins. This binding was inhibited by Fab Gl and PG fragments but not by Fcγ Gl fragments; and (b) a polyclonal A/J anti-V region Gl antibody (Ab2) that was able to recognize some epitopes of Fcγ fragments as well as framework determinants of several IgM monoclonal proteins (Abi).
The (a) type of antibody was a clear-cut reaction of a monoclonal antibody from a mouse that had not received human Ig, but where the anti-anti-V region antibody reacted with the human IgM. The (b) type antibody might conceivably have arisen because the original IgM G1 used to give rise to anti-V region antibodies, despite its known purity, was contaminated with small amounts of IgG that produced anti-IgG antibodies. A number of observations argue against this possibility: (a) the reaction of the antiserum with IgM G1 was markedly reduced by absorption with IgG that could only have been the case if there was a large contamination of IgM G1 with IgG. This is known not to be the case (b) The reaction of A/J anti-V region G1 antibodies with IgM G1 was virtually lost by absorption with the Fab fragment of IgM G1. (c) None of the other mouse strains produced such antibodies when injected with IgM G1.

The best explanation of the peculiar binding activities of these antibodies is that they recognize some idiotopes or framework determinants of anti-Id antibodies that mimic the shape of the epitopes of the immunizing antigen, which was the initiator of the idiotypic network pathway. We have called these epibodies. Fig. 2 shows the various antibodies studied and the specific reactions of the two types of epibodies.

One may speculate about the structural homology and cellular mechanisms that lead to the occurrence of epibodies. There are few data on the 3-dimensional structures of V domains of an antibody molecule that allow it to recognize epitopes and to be recognized by an antibody that generally is defined as an anti-Id antibody (18, 19). There is little knowledge of structural correlates of internal image.

However, from our study, it clearly appears that the mice immunized with a human IgM respond to several epitopes, such as individual antigenic specificities borne by IgM G1 (IdI), WaIdX, and framework determinants of IgM V regions. Therefore, among the B cells of these mice, it is possible that there are clones that bear an "idiotype" that represents the internal image of one of the epitopes and that, subsequent to the binding to IgM G1, can be stimulated to produce antibody that will recognize the epitope. This explanation is supported by the data regarding the properties of monoclonal anti-anti-V region G1 antibodies (Ab3), which can inhibit the binding-V region antibodies (Ab2) to IgM G1 (Ab1), but that has a very low binding activity to Ab2, as assessed in ELISA. Therefore, the epibodies appear to be combining site-induced antibodies, rather than classical anti-Id antibodies induced by Id. The epitopes recognized by epibodies probably do not include the dominant epitope that induces the synthesis of Ab1, and may represent bystander epitopes that resemble idiotopes or framework antigenic determinants of a subset(s) of Ab1 antibodies (i.e., of human IgM G1 monoclonal protein).

Fig. 2. Diagram showing the series of antibodies studied and their specific interactions. The arrows indicate the epibody and homobody reactions.
Wordfang and Rubin (20) reported some results that can be interpreted as epibodies. They prepared in tolerized rabbits a highly specific anti-Id antiserum against B6 anti-CBA (Ia^K) antibodies called 5936 Id. The anti-5936 Id antiserum binds not only to B6 anti-Ia^K cytotoxic antibodies, but also to antibody devoid of cytotoxic activity. Among various explanations provided by the authors, one that they take into account is that 5936 Id-bearing antibodies that lack anti-Ia^K cytotoxic activity may represent anti-(anti-CBA) antibodies that share 5936 Id with B6 and CBA antibodies.

These data, when considered collectively, indicate that anti-Id antibodies represent a heterogeneous population composed of at least four major categories: (a) conventional anti-Id antibodies, directed against idiotopes associated with the combining site (hapten inhibitable); (b) anti-Id antibodies directed against idiotopes associated with the framework of the V region (not inhibited by hapten); (c) epibodies that interact with epitope or idiotope-related structures of antibodies (Ab1) or anti-Id antibodies (Ab2), respectively; and (d) homobodies that may mimic the antigen (Ab2 homobodies).

The existence of epibodies and homobodies strongly supports the concept of an internal image of the antigen within the immune system (21). This concept, wherein the average of the three-dimensional shapes of all V domains could be cross-reactive to the average shapes of epitopes, is significant for the understanding of the genetic, regulatory, and recognition mechanisms of the immune system. The implications of this concept include: (a) each antibody molecule can be regarded as an anti-Id antibody for another Ig; (b) the immune system does not consist of two separate entities (anti-epitope and anti-idiotope antibodies), as has been proposed (21), but a unitary system of multiple antibodies; and (c) that the antigens are not completely foreign because the immune system already contains structures (i.e., Id) that are similar to the antigens (epitopes).

Research in progress is designed to test this concept by studying the binding activity of several monoclonal anti-murine Id antibodies to human myeloma proteins as well as of murine anti-V region Gl antibodies to murine myeloma proteins.

**Summary**

Murine anti-V region antibodies against a human monoclonal protein Gl with anti-γ-globulin activity and bearing the Wa cross-reactive idiotype were prepared in several strains of mice. Antibodies were obtained that were specific for the Gl idiotype, the Wa cross-reactive idiotype, and for various framework antigenic determinants that were distinguished by a variety of procedures. Synthesis of such antibodies were found to be independent of MHC and Ig gene complexes. These anti-V region antibodies, produced by a majority of mouse strains investigated, also share a cross-reactive idiotype recognized by BALB/c anti-anti-V region Gl antibodies. A fraction of BALB/c anti-anti-V region Gl antibodies displayed human Fcγ binding activity and, therefore, can be considered homobodies, as described in other systems.

Two of the anti-idiotype antibodies obtained in this system exhibited a peculiar property: they interacted not only with their own antigen (IgM Gl), but also with the Fc fragment with which IgM Gl reacts. Thus, A/J anti-V region Gl antibodies bind to the human Fcγ fragment in addition to IgM Gl. Similarly, a monoclonal CB6 F1 anti-anti-V region Gl antibody interacted with the V region of IgM Gl as well as with
the syngeneic anti-V region antibodies. We called these anti-idiotypic antibodies epibodies because they interact with epitopes of the primary antigen. These homobodies and epibodies, obtained by immunization across heterologous barriers, represent new examples of recognition of the internal image of the antigen within the immune system.

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