# High Frequency of Autoantibodies Bearing Cross-reactive Idiotopes among Hybridomas Using $V_H$7183 Genes Prepared from Normal and Autoimmune Murine Strains

**Blanche Bellon, Audrey Manheimer-Lory, Marc Monestier, Thomas Moran, Alexandra Dimitriu-Bona, Frederick Alt, and Constantin Bona**

*Departments of Microbiology and Pathology, Mount Sinai School of Medicine, New York 10029; and Department of Biochemistry, College of Physicians and Surgeons of Columbia University, New York 10032*

## Abstract

**Hybridomas obtained by in vitro stimulation with lipopolysaccharides (LPS) of BALB/c, MRL/lpr, and NZB splenocytes were selected for expression of $V_H$7183 by hybridization using slot blotting. Northern blot analysis showed that the majority of hybrids produce a full length message complementary to the $V_H$7183 probe. The frequency of $V_H$7183 hybridomas was significantly higher in NZB mice as compared with BALB/c mice. Using multiple binding assays, 60% of the total antibodies encoded by $V_H$7183 were specific for self-epitopes. Finally, the vast majority express cross-reactive idiotypes borne by autoantibodies of various specificities.**

## Introduction

Molecular studies of the heavy chain of autoantibodies have shown that the variable (V),[1] diversity, and joining segments used to encode the variable region are similar to those used by antibodies against foreign antigens (1–3). Also, our previous studies on antibodies displaying various self-specificities have clearly indicated that a restricted set of the variable region, heavy chain ($V_H$) gene repertoire is utilized, particularly $V_H$ J558, the largest $V_H$ gene family, and the two 3′ families, $V_H$ QPC52 and $V_H$7183 (3). The $V_H$7183 family is of particular interest for several reasons. First, there is preferential utilization of a germline gene ($V_H$ 81X) in pre–B cells and immature cells (4), and the presumed autoantibody-producing B cells, Ly1$^+$ B cells, display the phenotype of immature B cells (5). Second, there may be overrepresentation of the $V_H$7183 family by autoantibodies in spite of the small size of this family (12 members), $V_H$ J558 (60 members), and $V_H$ QPC52 (15 members) (6).

The goal of our study was to investigate the frequency of antibodies specific for self-epitopes among $V_H$7183$^+$ hybridomas obtained from normal mice vs. autoimmune mice. We found that more than half of these antibodies could bind to self-antigens independent of their origin. In addition, we identified a cross-reactive idiotype among these antibodies that was shared by autoantibodies of various specificities.

Address correspondence to Dr. Constantin A. Bona, Department of Microbiology, Annenberg Bldg. 16-60, Mount Sinai School of Medicine, 1 Gustave Levy Place, New York, NY 10029.

*Received for publication 8 July 1986 and in revised form 17 December 1986.*

1. *Abbreviations used in this paper:* H, heavy chain; Id, idiotype; IdX, cross-reactive idiotype; LPS, lipopolysaccharide; MOPS, 2-(4-morpholino) propanesulfonic acid; RF, rheumatoid factor; V, variable region.

J. Clin. Invest.

0021-9738/87/04/1044/10 $1.00
Volume 79, April 1987, 1044–1053

## Methods

### *Animals*

3-mo-old BALB/c, MRL/lpr, and NZB mice obtained from the Jackson Laboratory, Bar Harbor, ME were utilized in the preparation of hybridomas.

### *Preparation of monoclonal antibodies*

Splenocytes from BALB/c, MRL/lpr, and NZB mice were cultured in RPMI (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum, 2-mercaptoethanol, pyruvate, nonessential amino acids, and glutamine. Cells were stimulated with E. coli lipopolysaccharide (LPS) (50 μg/ml) for 3 d. Lymphocytes were then fused with the nonsecretory tumor line Sp2/0 using standard protocol (7). All monoclonal antibodies were purified on a Sepharose 4B-rat anti-murine kappa column except Z318, which was purified on a Sephacryl column.

### *Selection of hybridomas positive for $V_H$7183 gene expression*

*(a) RNA slot blotting.* RNA was prepared from cytoplasmic lysates of $1–3 \times 10^7$ hybridoma cells and was applied to nitrocellulose with a Minifold II apparatus (Schleicher and Schuell, Inc., Keene, NH). The filters were baked, hybridized to a $^{32}$P-labeled $V_H$7183 specific probe ($V_H$ 81X probe donated by G. Yancopoulos, Columbia University, New York), washed, and autoradiographed (8).

*(b) Northern blot analysis of total RNA.* To confirm results obtained by slot blotting, total RNA was prepared from $3–5 \times 10^7$ cells and assayed in a Northern blot analysis as previously described (9).

Briefly, 5 μg of RNA were resuspended in 50% deionized formamide, 2.2 M formaldehyde, 20 mM 3-(4-morpholino) propanesulfonic acid (MOPS)/5 mM sodium acetate buffer, pH 7.0, and denatured by heating at 65°C for 5 min. After quick chilling on ice for 5 min, the RNA was fractionated on a 1% agarose gel containing 2.2 M formaldehyde in a running buffer of 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7. The electrophoresis was carried out in a wick-type apparatus at 4°C, 115 V, while continuously circulating the buffer between the anodic and cathodic buffer compartments. The RNA was blotted onto BA85 nitrocellulose paper (Schleicher and Schuell, Inc.) and hybridized with $^{32}$P nick-translated $V_H$ probes and washed with 0.1 M SSC at 68°C before exposure for autoradiography. Under these conditions, we did not observe cross-hybridization.

### *Study of antigen-binding properties*

*(a) Antigens.* Myelin basic protein was a kind gift from Dr. G. Lewis, Institute for Basic Research Development Disabilities, Staten Island, NY, and Sm antigen was kindly donated by Dr. H. Dang, University of South Texas, San Antonio. Thyroglobulin and cardiolipin were purchased from Sigma Chemical Co., St. Louis, MO. $IgG_3$, $IgG_1$, $IgG_{2b}$, and $IgG_{2a}$, were purified from supernatants produced by hybridomas, Y5606(γ3λ), 88-C692(γ1λ), BA6(γ2bλ), HOPC1(γ2aλ), respectively.

*(b) Study of antigen-binding properties.* This was carried out using three techniques: radioimmunoassay to assess the binding activity to IgG, cardiolipin, DNA, Sm, thyroglobulin, myelin basic protein, and thymocytes, enzyme-linked immunosorbent assay (ELISA) to assess the binding activity for murine collagen type II, and immunofluorescence to study the binding activity for cellular antigens.

In a sandwich radioimmunoassay, microtiter plates were coated overnight at 4°C with 10 μg/ml antigen in 1 M sodium carbonate buffer, pH 9, washed with phosphate-buffered saline (PBS), and postcoated with PBS–bovine serum albumin (BSA) (1%) and sodium azide (0.02%). After several washings, the plates were incubated for 2 h at room temperature with 10 μg/ml chromatographically purified monoclonal antibodies in PBS-BSA. After extensive washing, $^{125}$I-rat anti–mouse kappa light chain (50,000 cpm) was added for 2 h at room temperature as the developing reagent. The ability of our panel of monoclonal antibodies to bind to DNA was kindly tested by Dr. B. Diamond, Albert Einstein School of Medicine, New York, NY, according to a previously described technique (10).

The binding specificity for various antigens was further studied by a competitive inhibition assay. In these experiments, 10 μg/ml of monoclonal antibodies (deemed positive by radioimmunoassay [RIA]) were incubated for 2 h at 37°C with increasing amounts of antigen (0, 10, 50, 100 μg/ml) and then added to microplates coated with antigens. The binding of the antibody was studied with RIA as above.

Binding to collagen type II was kindly carried out by R. Holmdahl (Uppsala University, Sweden) by ELISA, according to a previously described technique (11).

To test the binding properties to cellular antigens, three techniques were used: RIA for anti–thymocyte antibodies, agglutination tests for anti–red blood cell antibodies, and immunofluorescence for anti–tissue antibodies. Binding to thymocytes was tested with a cell surface assay as previously described (12). Binding to red blood cells was tested with indirect anti-Ig (13) and polybrene tests (14). Binding to tissue antigens was studied using cryostat prepared sections from kidney and stomach. These were incubated for 1 h at 4°C with various amounts of antibodies (0.1, 5, 10 μg/ml), washed extensively, and then incubated for 1 h at 37°C with fluoresceinated rat anti–mouse Ig as the developing reagent.

### *Study of idiotypy*

A competitive RIA as previously described (15) was used to test the expression of cross-reactive idiotypes.

Briefly, microtiter plates were coated overnight at 4°C with 5 μg/ml chromatographically purified antiidiotype antibodies, washed, and then incubated for 1 h at 4°C with 1% BSA in PBS. After extensive washing, the microtiter plates were incubated for 3 h at room temperature with 5, 50, or 500 ng purified antibodies, washed, and then incubated for 2 h with $^{125}$I-labeled corresponding idiotype (Id). In these experiments, we have used several idiotype–antiidiotype systems described elsewhere (15). These systems are comprised of chromatographically purified polyclonal rabbit antibodies against LPS10-1, a rheumatoid factor (RF) monoclonal antibody obtained from in vitro LPS-stimulated splenocytes and 129-48, a monoclonal RF obtained from 6-mo-old 129/Sv mice. The hybridomas producing these RFs express a $V_H$ gene from $V_H$7183 family (3). As an idiotypic control system, we used antibodies specific for Py102-Id expressed on Py102, a monoclonal antibody specific for PR8 influenza virus hemagglutinin (16). The hybridoma secreting this antibody expresses a $V_H$ gene from the 7183 family.

The specificity of anti-Id antibodies used in this study was previously described (3). Thus, anti-129/48 antibodies bind to 14 of 20 IgM RFs, anti-LPS 10-1 Id antibodies to 14 of 20 IgM RFs and anti-PY102Id antibodies bind to only 4 of 12 antibodies specific for influenza virus. Furthermore, these anti-Id antibodies do not exhibit binding activity for other IgM monoclonal proteins specific for α1-3 dextran (MOPC104E), β2-6 fructosan (1-5-1) or β2-1 fructosan (2-9-17). By all the criteria used, i.e., extensive adsorption on IgM columns and then purification by elution from Id columns and the pattern of binding activity to a large panel of IgM, anti-LPS10-1, -129-48, and -Py102 antibodies can be considered anti-Id antibodies.

### *Chi square analysis*

Chi square statistical analysis was calculated according to Zar (17).

## Results

### *Selection of $V_H$7183 positive hybridomas*

Hybridomas from LPS-stimulated lymphocytes that express a variable region gene from the $V_H$7183 family have been selected using a slot-blotting technique. A $^{32}$P–nick-translated $V_H$ probe prepared from the most diverse member of the $V_H$7183 family, $V_H$ 81X, was hybridized to nitrocellulose previously fixed with RNA from cytoplasmic lysates of these cells. Hybridomas positive for $V_H$7183 expression were cloned and 5–10 clones have been retested using the same probe. One positive clone from each was then used for further study. By using this technique, we obtained 14/356 BALB/c, 16/101 NZB, and 5/65 MRL clones positive for $V_H$7183 expression (Fig. 1). The vast majority of these hybridomas are μ,κ except for Z14 (γ2a,κ) and Z318 (μ,λ) (Table I). To confirm that hybridization to the $V_H$7183 probe was from the expressed allele, total RNA was prepared and analyzed in a Northern blot analysis under high stringency conditions (0.1 M SSC at 68°C) using a $^{32}$P–nick-translated probe $V_H$ 129-48 (Figs. 2–4). $V_H$ 129-48 was isolated from a genomic library prepared in Charon 16A (3). This hybridoma secretes a μ,κ RF. The sequence of $V_H$ 129-48 is very homologous to a germline gene $V_H$ 37.1, a member of the $V_H$7183 family (18). Under these conditions, the majority of RNA from 7183$^+$ hybridomas did not hybridize with other $V_H$ probes prototypes for

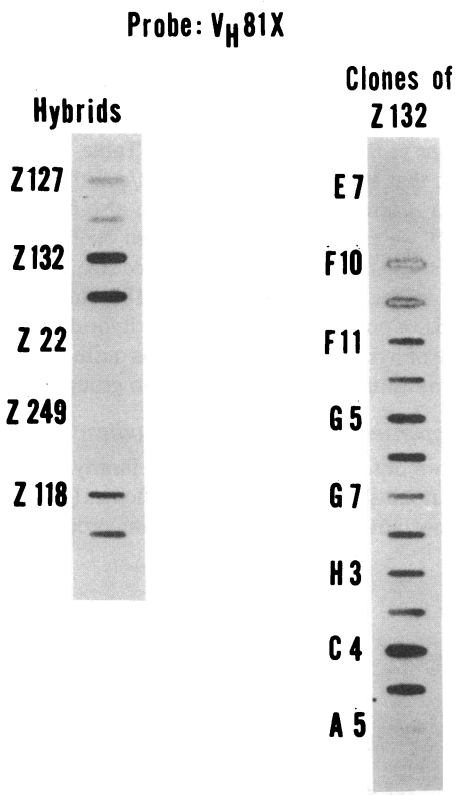


*Figure 1.* Selection of $V_H$7183 hybridomas. Autoradiogram composite of slot-blot analysis of cytoplasmic RNA lysates from NZB hybridomas. (*Left*) Screening of hybrids after fusions. (*Right*) Screening of clones obtained from Z132 hybrid.

*Table I. Origin and Isotypes of Monoclonal Antibodies Produced by Hybridomas Selected with $V_H$7183 Probe*

| Origin | Number of hybridomas studied | Fusion | Designation and isotypes |
|---|---|---|---|
| 3-mo-old BALB/c | 356 | 1 | B57 (μk), B6 (μk), B93 (μk) |
| | | 2 | B56 (μk), B34 (μk), B36 (μk), B68 (μk), B61 (μk), B76 (μk) |
| | | 3 | B28 (μk), B64 (μk), B48 (μk), B38 (μk), B53 (μk) |
| 3-mo-old NZB | 101 | 1* | Z121 (μk), Z250 (μk), Z51 (μk), Z818 (μλ), Z317 (μk), Z39 (μk), Z26 (μk), Z59 (μk), Z218 (μk), Z49 (μk), Z14 (γ2bk), Z232 (μk), Z41 (μk), Z113 (μk) |
| 3-mo-old MRL/lpr | 65 | 1 | M16 (μk), M13 (μk) |
| | | 2 | M56 (μk), M93 (μk), M88 (μk) |

* Z244 and Z132, nonsecretory hybridomas.

other $V_H$ families. Total RNA from two BALB/c hybrids and one MRL hybrid that did not hybridize with the $V_H$7183 probe hybridized with other V probes, indicating that they actually express a different $V_H$ gene. For example, the MRL hybridoma M15 selected by slot blotting as $V_H7183^+$ actually hybridized with $V_H$ 441-4 probe in Northern analysis (data not shown). These two probes cross-hybridize. Two of the hybridomas from the NZB fusion did not secrete Ig, although they hybridized to the $V_H$7183 probe. Southern blot analysis performed 6 mo later showed only the Sp2/0 parental $J_H$ Eco RI 4.9-kb band was present, indicating that there may have been a loss of chromosomes in culture during this period. These results show that slot blotting can only be used as a screening method, and confirmation of $V_H$ expression by Northern analysis is necessary. Our results also show that the frequency of $V_H$7183 usage was significantly higher among hybridomas produced by NZB splenocytes than in BALB/c, whereas no significant difference was observed for MRL/lpr vs BALB/c (see summary, Table V).

Since it is known that $V_H$ genes used by BALB/c antibodies specific for Influenza A strain PR8 hemagglutinin also derives from this family (18), we carried out a fusion from NZB mice immunized with this virus. Among 15 antigen-positive hybridomas, 2 antibodies utilize a $V_H$ gene from the 7183 family as compared with 9 of 51 of our collection of BALB/c hybridomas. Although our sample of hybridomas from NZB was quite small, there was no significant difference between the two groups.

### *Antigen-binding properties of monoclonal antibodies*

In previous studies, we observed that the $V_H$7183 family is frequently utilized by autoantibodies of various specificities (3, 14). Therefore, it was important to determine the binding properties of antibodies that had been selected by a $V_H$7183 probe. This was done with multiple techniques: RIA, ELISA, and immunofluorescence. Although there are a large variety of potential self antigens, we have selected this particular panel of antigens because it is presumed that they are likely to be involved in autoimmune pathology.

#### ANTIGEN-INHIBITABLE ANTIBODIES (TABLE II)

Our panel of antibodies can be divided into five major groups.

*(a) Rheumatoid factors.* Four antibodies obtained from BALB/c (B56, B57, B61, B68) and one from MRL/lpr (M88) exhibited RF activity. BALB/c antibodies exhibited specificity for the Fc fragment of three IgG subclasses ($IgG_3$, $IgG_1$, and $IgG_{2a}$), whereas the antibody from MRL/lpr bound to the Fc of $IgG_1$. This binding activity was inhibited by heat-aggregated Ig.

*(b) Thyroglobulin.* One antibody from NZB (Z51) bound to thyroglobulin and the inhibition of this binding by antigen was weaker compared with a monoclonal antibody (84D1) obtained from CBA/J mice immunized with thyroglobulin.

*(c) Sm.* One monoclonal from BALB/c (B36), two from NZB (Z26, Z318), and four of five (M13, M16, M56, M93) from MRL bound to Sm. However, only four of them (Z26, Z318, M16, M93) exhibited a level of inhibition similar to that obtained with a monoclonal antibody specific for Sm (Y2).

*(d) DNA.* Four antibodies from NZB (Z49, Z317, Z41) showed various degrees of binding to double-stranded DNA and cardiolipin, but only one, Z121, bound as strongly as H241, an anti-DNA antibody obtained from MRL/lpr mice (Table II).

The inhibition of binding of autoantibodies by the corresponding antigens was carried out with a constant concentration of antibodies (10 μg/ml) giving 50–70% of the binding with higher

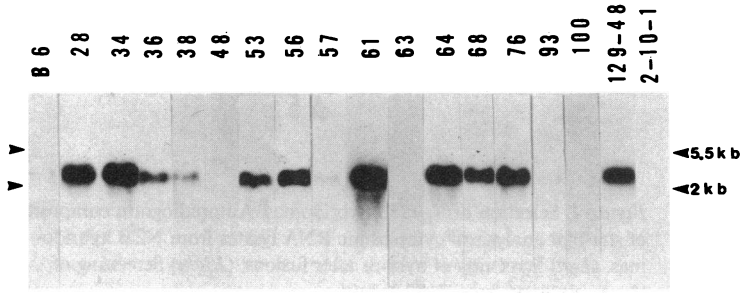


*Figure 2.* Northern blotting analysis of BALB/c hybridomas. 5 μg RNA was electrophoresed through a 1.2% agarose gel (6% formaldehyde) blotted and hybridized to the $^{32}P$ nick-translated $V_H$ 129-48 probe.

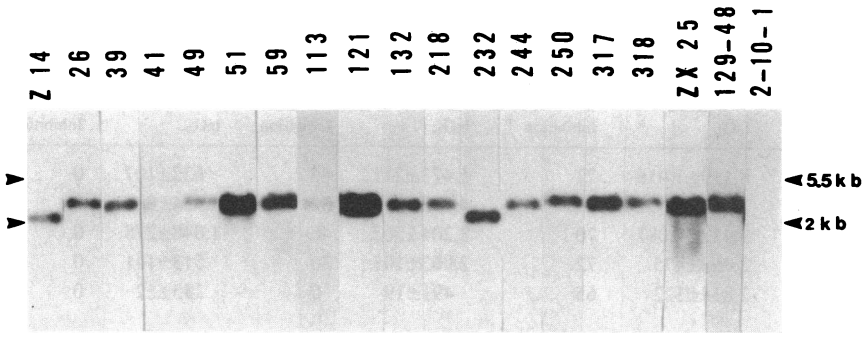


*Figure 3.* Northern blotting analysis of NZB hybridomas.

amounts (i.e., 30 or 100 μg/ml). The percentage of inhibition illustrated in parentheses in Table II represents the inhibition observed at the ratio of 50 μg antigen:10 μg purified antibody (majority IgM). However, it should be mentioned that > 50% inhibition was observed with lower concentrations of antigens for some antibodies.

*(e) Glomerular basement membrane.* Finally, one monoclonal from BALB/c bound to the glomerular basement membrane of human kidney (data not shown).

ANTIBODIES EXHIBITING MULTIPLE BINDING PROPERTIES FOR SELF-ANTIGENS (TABLE III)

Five monoclonal antibodies, three from BALB/c (B28, B34, B93) and two from NZB (Z113, ZX25), exhibit various binding specificities for self-antigens. For example, B28 bound to myelin basic protein, Sm, IgG, and collagen type 2, whereas Z113 bound to thyroglobulin, Sm, cardiolipin, smooth muscle, and glomerular basement membrane. However, the binding of these antibodies to the various antigens was not significantly inhibited by the corresponding antigens.

"STICKY" ANTIBODIES

In this panel of monoclonal antibodies we found two that exhibited an unusually high binding activity to BSA and all autoantigens tested (B76 and Z121). Obviously, the binding activity of these antibodies was not inhibited by antigen (data not shown).

ANTIBODIES LACKING ANTI-SELF ACTIVITY

Antibodies that did not exhibit binding activity for self-antigens were tested for their ability to bind to galactan and PR8 influenza virus. These antigens were chosen because antibodies specific for galactan and PR8 influenza virus are encoded by $V_H$ genes from the $V_H$7183 family (18, 19). It should be mentioned that only one antibody (B38) bound galactan and only one antibody bound to PR8 (B64) (data not shown).

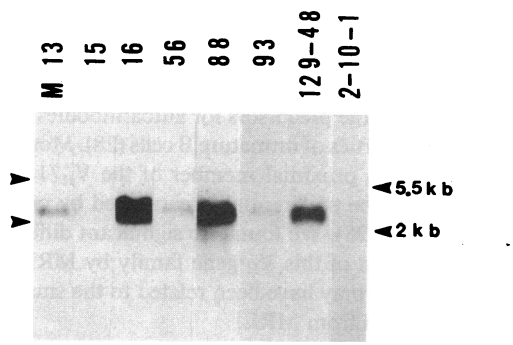


*Figure 4.* Northern blotting analysis of MLR/lpr hybridomas.

ANTI-THYMOCYTE AND ANTI-RED CELL ACTIVITY

No antibody from our panel exhibited anti-thymocyte or anti-red cell activity.

### *Cross-reactive idiotypes*

In previous studies we showed that autoantibodies with various specificities shared a cross-reactive idiotype (IdX) expressed on RF and anti-Sm antibodies (5, 20). We used three IdX systems expressed on $V_H$7183 RFs to investigate the expression of this IdX by our monoclonal antibodies selected for $V_H$7183 expression. As a control in the study, we used an Id system that can detect IdX expressed on PR8 influenza virus anti-hemagglutinin antibodies (Py102, VM202), which are also encoded by $V_H$ genes derived from this family (16). The presence of IdX was studied by competitive inhibition RIA using various amounts of chromatographically purified antibodies (5–500 ng/well). The binding of labeled Y19-10 (a RF) to anti-LPS10-1 Id (anti-RF-Id) antibodies was inhibited in a dose-dependent manner by a vast majority of our monoclonal antibodies except for B28 and Z113 (Fig. 5). A similar study was carried out employing other IdX systems, i.e., $^{125}$I-LPS10-1 and anti-129-48-Id (an anti-RF-Id), $^{125}$I-Y19-10 anti-129-48-Id, $^{125}$I-VM202, and anti-Py102-Id (data not shown).

The results of this study, summarized in Table IV, show that 9 of 35 monoclonal antibodies share 129-48 Id, and 27 of 35 share the LPS10-1 Id.

## Discussion

In this communication, we present results on the properties of antibodies secreted by hybridomas selected for $V_H$7183 expression. This family is relatively small (12 members) and has been mapped to the 3′ end of the murine $V_H$ locus (6). The $V_H$ genes that derive from this family are infrequently used by clones specific for foreign antigens (4). By contrast, this family may be preferentially expressed by autoantibodies of various specificities (3, 15). Because our aim was to compare the frequency of autoreactive B cells among $V_H$7183 antibodies, we used 3-mo-old mice, an age when MRL/lpr and NZB exhibit first autoimmune symptoms.

The hybridomas used in this study have been obtained from spleen lymphocytes stimulated in vitro with LPS before fusion. Recent results reported by Dildrop et al. (21) on LPS-stimulated B cells representing an equal number of IgM and IgG secreting cells showed that 7183 family was under-represented compared with J558 family, indicating that the $V_H$ representation is not unequivocally random in LPS-reactive B cells. However, we used in vitro LPS stimulation for several reasons. First, we had to obtain a high yield of fusion. Second, it was shown that in vivo

*Table II. Antigen Inhibitable Antibodies Secreted by $V_H7183^+$ Hybridomas*

Rheumatoid factors

| Antibody (10 μg) | BSA | $IgG_3$ | % Inhibition | $IgG_1$ | % Inhibition | $IgG_{2a}$ | % Inhibition | $IgG_{2b}$ | % Inhibition |
|---|---|---|---|---|---|---|---|---|---|
| B56 | 971±139* | 14,419±1,667 | 78 | 8,119±1,016 | 21 | 5,473±211 | 43 | 622±107 | 0 |
| B57 | 1,577±26 | 8,301±854 | 34 | 3,753±1,692 | 81 | 4,733±259 | 32 | 1,964±814 | 0 |
| B61 | 533±30 | 3,610±140 | 69 | 9,422±2,087 | 70 | 3,204±562 | 46 | 1,048±278 | 0 |
| B68 | 336±16 | 5,704±49 | 49 | 2,463±475 | 72 | 2,063±105 | 30 | 515±141 | 0 |
| M88 | 160±3 | 1,020±60 | 0 | 2,818±122 | 65 | 491±19 | 0 | 283±52 | 0 |

Thyroglobulin

| Antibody (10 μg) | BSA | TG | % Inhibition |
|---|---|---|---|
| 8.4D1 | 375±12 | 1,362±432 | 49 |
| Z51 | 550±61 | 2,754±587 | 24 |

Sm

| Antibody (10 μg) | BSA | Sm | % Inhibition |
|---|---|---|---|
| Y2 | 576±44 | 10,097±982 | 60 |
| B36 | 601±22 | 7,844±917 | 18 |
| Z26 | 606±146 | 10,107±1,100 | 49 |
| Z118 | 216±27 | 2,183±304 | 51 |
| M13 | 553±35 | 2,534±387 | 19 |
| M16 | 694±117 | 8,897±521 | 58 |
| M56 | 703±165 | 8,887±702 | 29 |
| M93 | 212±150 | 2,176±304 | 44 |

Cardiolipin and DNA

| Antibody (10 μg) | BSA | Cardiolipin | % Inhibition | Double-stranded DNA Exp. 1 | Double-stranded DNA Exp. 2 |
|---|---|---|---|---|---|
| H 241 | 95±7 | 2,643±508 | 45 | 2,377±72 | 4,673±24 |
| Z 121 | 186±24 | 1,743±36 | 65 | 137±11 | 324±15 |
| Z 317 | 71±12 | 823±61 | 28 | 368±51 | 944±22 |
| Z 41 | 85±24 | 714±14 | 40 | 289±26 | 504±44 |
| Z 49 | 88±12 | 1,142±2 | 36 | 313±22 | 520±2 |
| B6 (negative control) | 113±16 | 127±3 | 0 | 53±5 | 102±10 |

Glomerular basal membrane (immunofluorescence)

B53

* cpm, average±SD. Inhibition obtained with 50 μg of antigen. Antibodies were preincubated for 2 h at 37° with antigen before to be added to microtiter plates coated with antigen. 8.4D1 is an anti-TG monoclonal antibody, Y-2 is specific for Sm and H-241 for DNA (positive controls).

LPS injection accelerates the onset of autoimmune diseases (22, 23) and we can assume that LPS-activated autoreactive cells include those producing pathogenic autoantibodies (23). Third, it is known that there is only slight variation in the frequency of LPS-reactive B cells in various strains of mice (24) (1 of 4 or 6, with the exception of $C_3H$/HeJ, a LPS-hyporesponsive strain in which the frequency is 1 of 10,000).

Finally, it was shown that the same 460IdX is equally expressed on antibodies elicited following in vivo immunization with TNP conjugates or in vitro stimulation with LPS (25).

In this study, we demonstrated that there is a significant difference in the usage of the $V_H7183$ gene family of NZB mice vs. BALB/c mice. The high frequency of 7183 expression in NZB may be related to the high frequency of the Ly1.B subset of B cells in these mice (26) as in the autoimmune motheaten mouse strain where almost all B cells are $Ly1^+$ (27). Apparently, this lineage is comprised of the precursors for autoantibodies in NZB and exhibits the properties of immature B cells (28). Moreover, $V_H$ 81X, the most $J_H$ proximal member of the $V_H7183$ family, has been shown to be preferentially rearranged by pre-B and immature B cells (4, 28). We found no significant difference between the utilization of this $V_H$ gene family by MRL/lpr and BALB/c mice. This may have been related to the small number of hybrids obtained from MRL.

The most striking aspect of this study is the high percentage of autoantibodies produced by $V_H7183^+$ hybridomas indepen-

*Table III. Antibodies Secreted by $V_H$7183 Hybridomas Exhibiting Multiple Binding Properties for Self-Antigens*

| Method | Antigens | Antibodies (10 μg) B28 | B34 | B93 | Z113 | ZX25 |
|---|---|---|---|---|---|---|
| RIA | BSA | 476±26* | 170±26 | 365±39 | 681±43 | 213±19 |
| | Myelin | 3,090±454 | 557±36 | 522±63 | 822±42 | 453±15 |
| | Basic protein | (17%) | (0%) | (0%) | (0%) | (0%) |
| | Thyroglobulin | 763±131 | 441±3 | 1,276±56 | 5,242±717 | 674±58 |
| | | (0%) | (0%) | (0%) | (15%) | (0%) |
| | Sm | 4,982±517 | 684±150 | 978±284 | 1,014±322 | 5,295±93 |
| | | (0%) | (0%) | (0%) | (0%) | (0%) |
| | Cardiolipin | 866±202 | 383±49 | 1,267±11 | 3,528±185 | 647±17 |
| | | (0%) | (0%) | (0%) | (0%) | (0%) |
| | $IgG_{2a}$ | 2,468±149 | 298±24 | 488±33 | 2,028±373 | 428±142 |
| | | (0%) | (ND) | (ND) | (0%) | (ND) |
| ELISA | BSA | 0.025‡ | 0.025 | 0.025 | 0.025 | 0.025 |
| | Type II collagen | 1.800 | 0.327 | 0.240 | 0.109 | 0.102 |
| | | (ND) | (ND) | (ND) | (ND) | (ND) |
| | Mitochondria | + | − | − | − | − |
| | Smooth muscle | − | − | − | + | − |
| | Nucleus | − | − | − | − | + |
| | Glomerular basement membrane | − | + | − | + | + |

In this experiment $F_2$, a monoclonal specific for collagen type 2, gave an optical density of 1.9. * cpm, average±SD; % of inhibition with antigen. ‡ Optical density at 405 nm. ND, not done.

dent of their strain origin. No significant differences were found between $V_H7183^+$ autoantibodies produced by BALB/c vs. autoimmune strains (Table V), suggesting that autoreactive B cells, like pre-B cells (4) or young lymphocytes (28), may preferentially use genes from the $V_H$7183 family. This was unexpected because antibodies specific for a foreign antigen, such as influenza virus

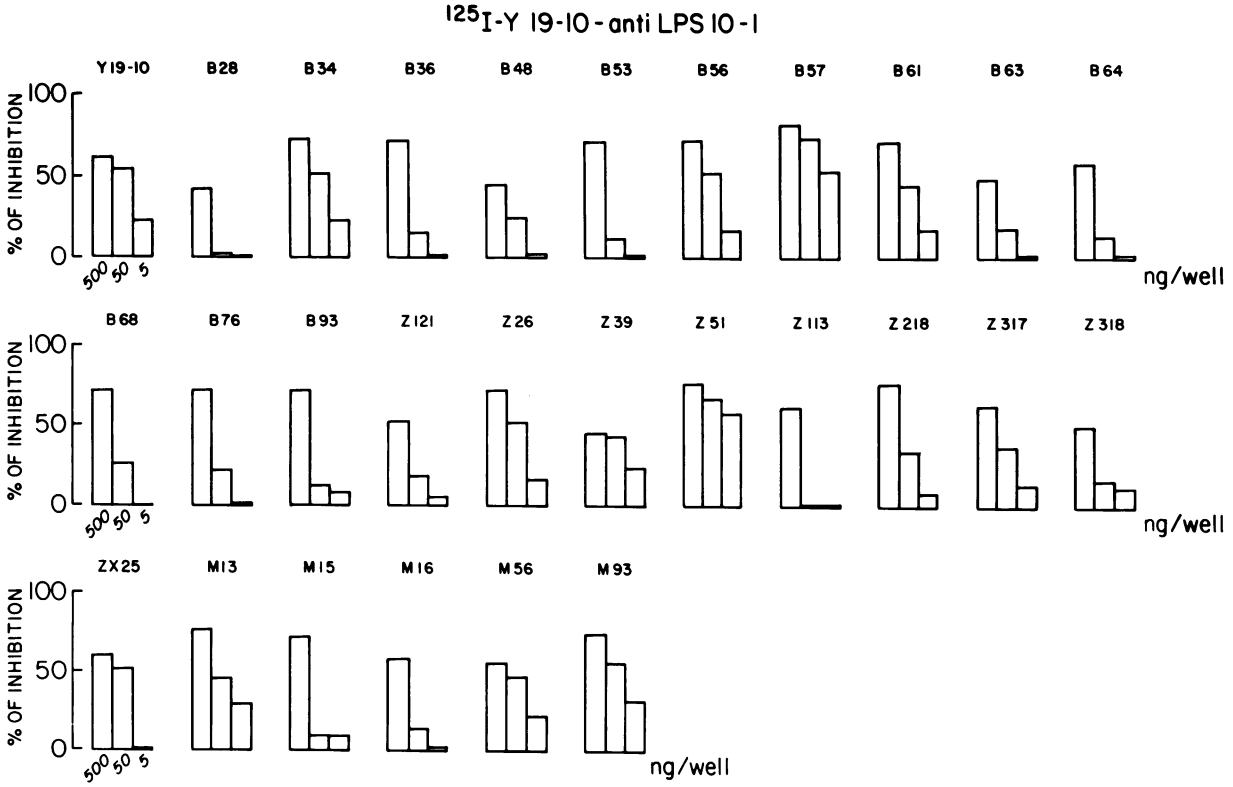


*Figure 5.* Competitive inhibition of the binding of $^{125}$I-Y19-10 to anti-LPS10-1 idiotypic antibodies by various amounts (5–500 ng) of monoclonal antibodies. Microtiter plates coated with 5 μg chromatographically purified antibodies and then 1% BSA in PBS were incubated for 3 h at room temperature with $^{125}$I Y19-10 (50,000 cpm). After extensive washings the radioactivity was counted in a γ-counter.

*Table IV. Fraction of Antibodies Produced by Hybridomas Selected with $V_H$7183 Probe Expressing IdX*

| Idiotype systems | BALB/c | NZB | MRL/lpr | BALB/c antibodies specific for influenza virus ($V_H$7183$^+$) PR8 | X31 |
|---|---|---|---|---|---|
| LPS10-1 anti-129-48 | B34 (49.3%)* | Z218 (51.8) | M88 (43.8%) | | |
| | B36 (51.3%) | | | | |
| | B53 (50.7%) | | | | |
| | B57 (50.2%) | | | | |
| | B61 (58.5%) | | | | |
| | B64 (47%) | | | | |
| | B76 (52%) | | | | |
| | 7/16‡ | 1/14 | 1/6 | 0/12 | 0/4 |
| Y19-10 anti-LPS10-1 | B93 (70.9%) | Z218 (66%) | M15 (71%) | VM114 (48%) | |
| | B66 (71%) | Z113 (61%) | M56 (57%) | | |
| | B68 (73%) | Z318 (50%) | M16 (59%) | | |
| | B36 (71%) | Z317 (62%) | M93 (61%) | | |
| | B57 (81.8%) | Z39 (47%) | M13 (77%) | | |
| | B76 (76%) | Z51 (75%) | | | |
| | B56 (74%) | Z121 (53%) | | | |
| | B34 (75%) | Z26 (71%) | | | |
| | B28 (66%) | | | | |
| | B38 (76.6%) | | | | |
| | B48 (78.5%) | | | | |
| | B53 (81%) | | | | |
| | B63 (57%) | | | | |
| | B64 (75%) | | | | |
| | 14/16 | 8/14 | 5/6 | 1/12 | 0/4 |
| Y19-10 anti-129-48 | B28 (62%) | | | VM114 (51%) | |
| | B38 (62.1%) | | | | |
| | B48 (64.6%) | | | | |
| | B53 (67.9%) | | | | |
| | B63 (71.3%) | | | | |
| | B64 (72%) | | | | |
| | 6/16 | 0/14 | 0/6 | 1/12 | 0/4 |
| VM202 anti-PY102 | | | | VM202 | XY101 |
| | | | | PY102 | |
| | | | | VM113 | |
| | | | | PT109 | |
| | 0/16 | 0/14 | 0/6 | 4/12 | 1/4 |

* In parentheses, percentage of inhibition at concentration of 500 ng of chromatography-purified antibody. ‡ Fraction of antibodies expressing IdX.

hemagglutinin, randomly use genes derived from all seven families. Furthermore, several antigen specificities are encoded by V genes derived from the smallest $V_H$ family (i.e., $V_H$ 441-4, composed of only two members), which in association with various $V_L$ chains codes for $\beta$2-6 fructosan (18), $\beta$1-6 galactan (18), ACHO polysaccharide of *Streptococcus* A (6), egg white lysozyme (29), and influenza virus antigens (30).

Among 11 antibodies selected with $V_H$ ×24 probe out of 534 hybridomas obtained from spleen cells of BALB/c mice stimulated in vitro with two mitogens (LPS and nocardia water-soluble mitogen), none exhibited binding activity to our panel of autoantigens (data not shown). Our results do not preclude that a high frequency of self-reactive antibodies or pathogenic autoantibodies will be found in $V_H$ J558 or $V_H$ QPC52 families that can also be used by autoantibodies (15). A study is in progress to elucidate this point. Our data show that among antibodies randomly selected with a $V_H$ 7183 probe from BALB/c mice and two autoimmune strains, an important fraction of these antibodies exhibits self-binding activity supporting our previous data (15) that this $V_H$ gene family contains genes used by autoantibodies.

The second important result of this study is the heterogeneity of autoantibodies produced by $V_H$7183$^+$ hybridomas. The first group comprises antibodies specific for a single self antigen. Thus, four of six monospecific autoantibodies obtained from BALB/c mice displayed RF activity. This frequency is in agreement with the observation of Dresser et al. (31), who showed that half of the PFC obtained from mice not prone to autoimmune disease

*Table V. Summary of Results and Statistical Significance of the Usage of $V_H7183$ Gene Family in Autoimmune Strains and Autoantibodies*

| | Hybridomas obtained from LPS-stimulated lymphocytes | | |
|---|---|---|---|
| | BALB/c | NZB | MRL |
| Number of hybridomas tested | 356 | 101 | 65 |
| Number of $V_H7183^+$ | 14 | 16 | 5 |
| Chi square test | BALB/c vs-NZB, $P<0.0001$; BALB/c vs-MRL, $P<0.18$; MRL vs-NZB, $P<0.11$ | | |
| Number of hybridomas secreting Ig | 14 | 14 | 5 |
| Number of hybridomas secreting monospecific autoantibodies | 5 | 7 | 5 |
| Chi square test | BALB/c vs-NZB, $P<0.62$; BALB/c vs-NZB, $P<0.20$; MRL vs-NZB, $P<0.37$ | | |
| Number of hybridomas secreting multispecific antibodies | 3 | 2 | 0 |
| Number of hybridomas secreting sticky antibodies | 1 | 1 | 0 |
| Number of hybridomas secreting antibodies specific for foreign antigens | 2 | 0 | 0 |
| | Hybridomas obtained from PR8 influenza virus immunized mice | | |
| Number of hybridomas tested | 51 | 15 | N.D. |
| $V_H7183^+$ | 9 | 2 | N.D. |
| Chi square test | BALB/c vs-NZB, $P<0.69$ | | |

injected with LPS had RF activity. The majority of autoantibodies produced by $V_H7183^+$ hybridomas obtained from MRL/lpr mice were specific for Sm. This is not surprising because these mice characteristically produce high amounts of anti-Sm antibodies after the onset of lymphoproliferative disease (2). However, we did not find any $V_H7183$ antibodies derived from NZB mice that could bind to red blood cells, in spite of the fact that this mouse strain develops an autoimmune hemolytic anemia later in life. These results suggest that other mechanisms such as the alteration of the balance between helper and suppressor cells and the breakdown of self-tolerance could be responsible for the activation of the anti–red blood cells and autoreactive B cells that could occur before the onset of the disease in NZB mice.

The second group of antibodies encoded by the $V_H7183$ gene family exhibits multiple binding properties, and their binding activity is not inhibited by self-antigens. It is important to note that there may have been more binding specificities than we have identified here; however, we were limited by the number of antigens available in our laboratory at that time. In addition, because of the finite number of antigens available, we may not have been able to identify all specificities of those antibodies we classed as "unknown."

Four antibodies obtained from BALB/c and two from NZB mice exhibited multiple binding activities which were not antigen inhibitable. This group of antibodies may be similar to that described by Dighiero et al. (32), obtained from newborn BALB/c mice. Lack of inhibition of the binding by antigens suggests that they have low avidity for the corresponding self-antigens. It should be mentioned that all of the antibodies with multiple-binding specificities are IgM. Their multiple binding property can be related either to a common three-dimensional structure shared by various self-antigens or to a protein–protein interaction between globular domains of variable regions of antibodies and domains bearing the epitopes of self-antigens. We recently proposed this model (33) to explain antigen-binding properties of epibodies we described several years ago (34). An epibody is also a multispecific antibody that recognizes both an idiotope and an antigenic determinant (epitope) on the original immunizing antigen.

Two IgM antibodies (B76 and Z121) strongly bound nonspecifically to self-antigens and a foreign antigen such as BSA. Such antibodies have been reported by other groups (35), and their origin is not yet clearly understood.

The existence of these multispecific autoantibodies is intriguing. They could be antibodies specific for foreign antigens that could bind to various proteins through weak bonds such as hydrophobic or coulombic forces. Alternatively, they could play a role in autoimmune phenomena contributing to formation and deposition of immune complexes.

The pathological implications of antibodies with multiple specificity is best illustrated in the case of cryoglobulins produced by patients with type II mixed cryoglobulinemia. Like epibodies, the cryoglobulins bind to the Fc fragment of IgG and to Fab fragment (36, 37).

Only a small fraction of antibodies produced by BALB/c (and not by NZB or MRL) bound to foreign antigens galactan and PR8.

It is difficult to determine definitely what physiologic role is played by antibodies with autospecificities in BALB/c mice, yet we have ascertained that the precursors are present in these mice as well as in mice that eventually manifest autoimmune pathology. We may speculate that autoreactive cells exist in the repertoire of normal mice and their products may perhaps have some kind of scavenger or housekeeping function, thereby contributing to the clearance of altered self-molecules as proposed by Grabar et al. (38). The function of multispecific antibodies and whether they can exert the same effects as some monospecific autoantibodies are certainly unknown. We are presently investigating whether B28, a multispecific antibody that binds collagen, MBP, Sm, and $IgG_{2a}$, elicits a pathogenic effect in DBA/1 mice as a known autoantibody monospecific for collagen, D3$\mu$ (39).

Finally, we identified the presence of interstrain IdX expressed on RFs but not on anti–influenza virus antibodies among the $7183^+$ antibodies. Three systems have been used to identify Id expressed on two RFs (i.e., LPS10-1 and 129-48) (3). These IdX have been identified on other spontaneous or induced monoclonal autoantibodies (15).

These results taken together indicate that autoantibodies with various specificities encoded by genes from the $V_H7183$ family share IdX that can function as regulatory idiotopes, targets for regulatory cells responsible for self-tolerance in both normal an-

imals and animals prone to autoimmune disease before the onset of the clinical syndrome. The role of regulatory idiotopes in the expansion of autoantibodies was documented in various antigenic systems such as thyroglobulin, DNA, etc. (data reviewed by Zanetti, reference 40).

In conclusion, our data show a significant number of antibodies encoded by genes from the $V_H$7183 family are specific for self-antigens. Furthermore, the anti-self response and cross-reactive idiotypy we observed were independent of mouse strain origin.

## Acknowledgments

We are grateful for the kind help provided by Dr. B. Diamond (Albert Einstein Medical School, New York) for anti-Id DNA assay, Dr. R. Holmdahl (Uppsala School of Medicine, Sweden) for anti-collagen type II assay, Dr. D. Rigal (Centre de Transfusion Sanguine, Lyon, France) for anti-red blood cell assay, and to Dr. H. Dang (Southern Texas Medical School, San Antonio) for Sm-purified antigen.

This study was supported by grant AG/A1271601, National Institutes of Health grant AI-20047, and Program Project grant CA 21112-09 from the United States Public Health Service. Dr. Monestier is supported by the Foundation pour la Recherche Medicale and the French government. Dr. Moran is supported by a Sinsheimer Foundation Fellowship.

## References

1. Andrews, S. W., and J. D. Capra. 1981. Complete amino acid sequence of variable domains from two monoclonal human anti-gammaglobulins of the Wa cross-idiotypic group: suggestion that the J segments are involved in the structural correlates of the idiotypes. *Proc. Natl. Acad. Sci. USA.* 78:3799–3803.

2. Kofler, R., D. J. Noonan, D. E. Levy, M. C. Wilson, N. P. H. Moller, F. J. Dixon, and A. N. Theophilopoulos. 1985. Genetic elements used for a murine lupus anti-DNA autoantibody are closely related to those for antibodies to exogenous antigens. *J. Exp. Med.* 161:805–815.

3. Manheimer-Lory, A., M. Monestier, B. Bellon, F. W. Alt, and C. A. Bona. 1986. Fine specificity, idiotypy and nature of cloned $V_H$ genes of murine monoclonal rheumatoid factor antibodies. *Proc. Natl. Acad. Sci. USA.* 83:8293–8297.

4. Yancopoulos, G. D., S. V. Desiderio, M. Paskind, J. F. Kearney, D. Baltimore, and F. W. Alt. 1984. Preferential utilization of the most $J_H$ proximal $V_H$ gene segments in pre-B cell lines. *Nature (Lond.).* 311: 727–733.

5. Hayakawa, K., R. R. Hardy, M. Honda, L. A. Herzenberg, A. D. Steinberg, and L. A. Herzenberg. 1984. Ly1.B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. *Proc. Natl. Acad. Sci. USA.* 81:2494–2498.

6. Brodeur, P. H., and R. Riblet. 1984. The immunoglobulin heavy chain variable region (Igh-V) locus in mouse I. One hundred Igh-V genes comprise seven families of homologous genes. *Eur. J. Immunol.* 14:922–930.

7. Kohler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.).* 256: 495–497.

8. White, B. A., and F. C. Bancroft. 1982. Cytoplasmic hybridization. Single analysis of relative mRNA levels in multiple small cell or tissue samples. *J. Biol. Chem.* 257:8569–8572.

9. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, New York. 91.

10. Solomon, G., J. Schiffenbauer, H. D. Keiser, and B. Diamond. 1983. Use of monoclonal antibodies to identify shared idiotypes on human antibodies to native DNA patients with SLE. *Proc. Natl. Acad. Sci. USA.* 80:850–854.

11. Holmdahl, R., T. Moran, and M. Anderson. 1985. A rapid and efficient immunization protocol for production of monoclonal antibodies reactive with autoantigens. *J. Immunol. Methods.* 83:379–384.

12. Waters, S. J., P. R. Luzzatti, and C. A. Bona. 1984. Functional properties of T cell clones with a double specificity for alloantigens and foreign antigens. *J. Exp. Med.* 160:1300–1315.

13. American Association Blood Banks. 1977. Technical Manual. American Association of Blood Banks, Washington. Seventh ed.

14. Lalezari, P. 1968. A new method for detection of red blood antibodies. Transfusion *(Phila.).* 8:372–380.

15. Monestier, M., A. Manheimer-Lory, B. Bellon, C. Painter, H. Dang, N. Talal, M. Zanetti, R. Schwartz, D. Pisetsky, R. Kuppers, N. Rose, J. Brochier, L. Klareskog, R. Holmdahl, B. Erlanger, F. Alt, and C. Bona. 1986. Shared idiotopes and restricted $V_H$ genes characterize murine autoantibodies of various specificities. *J. Clin. Invest.* 78:753–759.

16. Moran, T. M., M. Reale, M. Monestier, R. Mayer, J. L. Schulman, and C. Bona. 1985. Idiotypy of anti-influenza virus immune response. *Concept Immunopathol.* 3:233–252.

17. Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall Inc., Englewood Cliffs, NJ. 41.

18. Hartman, A. B., and S. Rudikoff. 1984. $V_H$ genes encoding the immune response 1-6-galactan: somatic mutation in IgM molecules. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:3023–3030.

19. Clarke, S. H., K. Huppi, L. Ruezinsky, W. Gerhard, and M. Weigert. 1985. Inter and intraclonal diversity in the response to influenza hemagglutinin. *J. Exp. Med.* 161:687–704.

20. Dang, H., M. Fischbach, and N. Talal. 1985. Anti-idiotypic antiserum to monoclonal anti-Sm inhibits the autoantigen-induced proliferative response. *J. Immunol.* 134:3825–3830.

21. Dildrop, R., U. Krawinkel, E. Winter, and K. Rajewsky. 1985. $V_H$-gene expression in murine lipopolysaccharide blasts distributes over the nine known $V_H$ gene groups and may be random. *Eur. J. Immunol.* 15:1154–1156.

22. Fournie, G. J., P. H. Lambert, and P. A. Miescher. 1974. Release of DNA in circulating blood and induction of anti-DNA antibodies after injection of bacterial polysaccharides. *J. Exp. Med.* 140:1189–1206.

23. Izui, S., R. A. Eisenberg, and F. J. Dixon. 1979. IgM rheumatoid factors in mice injected with bacterial lipopolysaccharides. *J. Immunol.* 122:2096–2102.

24. Andersson, J., A. Coutinho, and F. Melchers. 1977. Frequencies of mitogen reactive B cells in the mouse. *J. Exp. Med.* 145:1511–1519.

25. Marion, T. N., E. A. Dzierzak, M. S. Lee, R. L. Adams, and C. Janeway. 1984. Non-dinitrophenyl-binding immunoglobulin that bears a dominant idiotype (Id460) associated with anti-dinitrophenyl antibody is specific for an antigen on Rostewella pneumotropica. *J. Exp. Med.* 159:221–233.

26. Hayakawa, K., R. R. Hardy, D. R. Parks, and L. A. Herzenberg. 1983. The Ly-1 B cell: subpopulation in normal, immunodefective and autoimmune mice. *J. Exp. Med.* 157:202–218.

27. Sidman, C. L., L. D. Schultz, R. R. Hardy, K. Hayakawa, and L. A. Herzenberg. 1986. Production of immunoglobulin isotypes by Ly-$1^+$ B cells in viable motheaten and normal mice. *Science (Wash. DC).* 232:1423–1425.

28. Perlmutter, R. M., J. F. Kearney, S. P. Chang, and L. E. Hood. 1985. Developmentally controlled expression of immunoglobulin $V_H$ genes. *Science (Wash. DC).* 227:1597–1600.

29. Smith-Gill, S. J., C. R. Mainhart, T. B. Lavoie, S. Rudikoff, and M. Potter. 1984. $V_L$-$V_H$ expression by monoclonal antibodies recognizing avian lysozyme. *J. Immunol.* 132:963–967.

30. Reale, M. E., A. J. Manheimer, T. M. Moran, G. Norton, C. A. Bona, and J. L. Schulman. 1986. Characterization of monoclonal antibodies specific for sequential influenza A/PR/834 virus variants. *J. Immunol.* 137:1352–1358.

31. Dresser, D. W., and A. M. Popham. 1976. Induction of an IgM anti-IgG response in mice by bacterial lipopolysaccharide. *Nature (Lond.).* 269:552–554.

32. Dighiero, G., P. Lymberi, D. Holmberg, I. Lundquist, A. Coutinho, and S. Avrameas. 1985. High frequency of natural autoantibodies in normal newborn mice. *J. Immunol.* 134:765–771.

33. Bona, C., C. Y. Kang, and H. Kohler. 1986. Epibody: the image of the network created by a single antibody. *Immunol. Rev.* 90:115–127.

34. Bona, C., S. Finley, W. Waters, and J. H. Kunkel. 1982. Anti-immunogobulin antibodies. III. Properties of sequential anti-idiotypic antibodies to heterologous anti-γ-globulins. Detection of reactivity of anti-idiotype antibodies with epitope of Fc fragments (homobodies) and with epitopes and idiotopes (epibodies). *J. Exp. Med.* 156:986–999.

35. Serban, D., C. Rodorf-Adam, Y.-Z. Sun, and J. Gordon. 1985. Murine polyspecific antibodies. I. Monoclonal and serum anti-DNA antibodies cross-reactive with 2,4,6 trinitrophenyl derivatives. *J. Immunol.* 135:3122–3127.

36. Geltner, D., E. G. Franklin, and B. Fragione. 1980. Anti-idiotypic activity in the IgM fractions of mixed cryoglobulinemia. *J. Immunol.* 125:1530–1535.

37. Renversez, J. C., S. Roussel, M. J. Valle, G. Brighhouse, and P. H. Lambert. 1984. Idiotype interactions in type II mixed cryoglobulins. *Rev. Fr. Transfus. Immuno-Hematol.* 27:737–755.

38. Grabar, P. 1983. Autoantibodies and the physiological role of immunoglobulins. *Immunol. Today.* 12:337–340.

39. Holmdahl, R., K. Rubin, L. Klareskog, E. Larson, and H. Wigzell. 1985. Characterization of the antibody response in mice with Type II collagen-induced arthritis, using monoclonal anti-Type II collagen antibodies. *Arthritis Rheum.* 29:400–410.

40. Zanetti, M. 1986. Idiotypic regulation of autoantibodies production *Crit. Rev. Immunol.* 6:151–183.