A MAJOR ANTI-MYOGLOBIN IDIOTYPE

Influence of *H-2*-Linked *Ir* Genes on Idiotype expression

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Idiotypic determinants are the antigenically unique sites of the variable regions of immunoglobulin molecules, which distinguish antibodies of different specificities, and are defined by antiidiotypic antibodies (1–5). Because the variable region sequence determines both antibody specificity and idiotype, it is expected that antibodies of different specificity should have different idiotypes (1–5). Therefore, even antibodies that are specific for one antigenic molecule are expected to have different idiotypes when they are specific for different epitopes (6), although several cases in which antibodies of different specificity share idiotypes have been reported (7–12). In addition, it has been shown that *H-2*-linked *Ir*[1] genes can independently control the antibody response to different sets of epitopes on the same antigenic molecule (13–16). Such *Ir* genes would be expected to influence also the idiotype of antibodies (6). However, this prediction has not been tested experimentally.

We have been studying the *H-2*-linked *Ir* gene control of the antibody and T cell responses to sperm whale myoglobin (Mb), a model globular protein antigen, the primary sequence and the three-dimensional structure of which are well known (17–19). Both T cell proliferative response and the antibody response to Mb are controlled by two *H-2*-linked genes mapping in different *I* subregions, *I-A* and *I-C* of the *H-2* complex, and controlling the response to different epitopes (15, 20–22). Therefore, we had the opportunity to test the prediction that epitope-specific *Ir* genes might influence idiotype expression. To study this form of regulation, we prepared monoclonal antibodies to sperm whale Mb in a high responder strain of mice and raised antiidiotypic antibodies in rabbits and guinea pigs by immunization with several of those monoclonal antibodies. Although previous studies with the guinea pig antiidiotypic antibodies revealed interesting

sharing of idiotypes among monoclonal antibodies, the guinea pig antibodies detected largely private idiotypes that were not found in immune anti-Mb antisera (11). We now report a common or cross-reactive idiotype that is detected by rabbit antiidiotypic antibodies made against one of the monoclonal anti-Mb antibodies. This is expressed in the anti-Mb antisera of all strains of mice tested, regardless of allotype, but not in antisera to other antigens.

The widespread expression of this common idiotype allowed us to investigate the relationships between the H-2-linked Ir genes for Mb and the regulation of idiotypic expression. Interestingly, the fraction of antibodies expressing this common idiotype depends on H-2-linked Ir high or low responder status, and correlates with a fine specificity parameter that appears to be under similar control. The best explanation for these results seems to be that Ir gene control of idiotype expression is a consequence of Ir gene control of antibody fine specificity (6, 13–16), which is reflected in the structure of the variable regions of the antibodies with these fine specificities.

Materials and Methods

Animals.   C57BL/10Sn, B10.BR/SgSn, B10.D2/nSn, A/J, A.SW/Sn, DBA/2J, (B10.D2 × B10)F₁, and BALB/cJ mice were obtained from The Jackson Laboratory, Bar Harbor, ME. C.AL-20 mice were generously provided by Dr. David H. Sachs (National Cancer Institute, National Institutes of Health). All mice were between 8 wk and 6 months of age at the first immunization. A rabbit was obtained from National Institutes of Health animal facilities, FCRC, Frederick, MD.

Antigens.   Mb was obtained from the Accurate Chemical and Scientific Co. (Hicksville, NY). The major chromatographic component, IV, purified as described previously (20) by the method of Hapner et al. (23), was used throughout the studies. S-methyl Mb (24, 25), apo-Mb, and the CNBr cleavage fragments 1–55 and 132–153 (15) were prepared as described previously. Fowl gamma-globulin (chicken) (FγG) from United States Biochemical Corp. (Cleveland, OH) was used. Ferritin (horse) was a six-times crystallized preparation from Miles Laboratories (Pentex 96-027-2, lot 16; Elkhart, IN). Staphylococcal nuclease was purified according to published methods (26). Preparation of Mb-coupled-FγG (Mb-FγG) was performed by a modification of the method by Schroer et al. (27) as described in detail in a previous paper (28).

Antibodies.   Rabbit anti-mouse immunoglobulin (Ig) antiserum was obtained from Accurate Chemical and Scientific Co (Westbury, NY). Alkaline-phosphatase-conjugated rabbit anti-goat IgG antiserum and IgG fraction of goat anti-mouse immunoglobulin were from Cappel Laboratories, Inc. (West Chester, PA).

Monoclonal Anti-Mb Antibodies.   The preparation, properties, and fine specificity of the monoclonal antibodies were described previously (29, 30). All those used in the current study derived from the fusion of hyperimmune A.SW spleen cell populations with the NS-1 nonsecreting hypoxanthine-guanine phosphoribosyl transferase negative variant of the MOPC 21 plasmacytoma. All monoclonal antibodies were of the IgG₁-κ subclass except HAL32, which was IgG₂ₐ-κ. For the current studies, the monoclonal antibodies were purified from culture supernatant fluids or ascites by affinity chromatography on Mb bound to Aff-Gel 10 (Bio-Rad Laboratories, Richmond, CA) or to Sepharose. In earlier papers (11, 29, 30), the monoclonal antibodies were designated by a simplified notation as follows: HAL19, clone 1; HAL32, clone 2; HAL38, clone 3.4; HAL39, clone 4; HAL43, clone 5.

Antiidiotypic Antisera.   A rabbit was immunized with the monoclonal IgG₁-κ anti-Mb antibody, HAL19, in the same way as to make guinea pig antiidiotypic sera, which was described previously (11). Antiidiotypic antiserum from the rabbit was absorbed six times by affinity chromatography with excess Sepharose-coupled pooled normal mouse Ig from several mouse strains, and once with MOPC 21, which was coupled to CNBr-activated

Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). Rabbit anti-14-4-4 idiotype, specific for a monoclonal anti-I-E antibody, absorbed three times on the myeloma protein LPC-1 and twice on normal C3H.SW immunoglobulin attached to Sepharose, was the kind gift of Drs. Suzanne L. Epstein and David H. Sachs, NCI, NIH (31). It was absorbed an additional time on a pooled normal mouse Ig column to be a more comparable control.

Conjugation of Alkaline Phosphatase to Antibodies. The antibodies purified by affinity chromatography were conjugated with alkaline phosphatase (from bovine intestine, type VII-S; Sigma Chemical Co., St. Louis, MO), using glutaraldehyde as described previously (11), by the method described by Voller et al. (32).

Immunizations and Antimyoglobulin Antisera. Mice were immunized with 100–200 μg of protein antigen in complete Freund's adjuvant (CFA) and bled 3 wk later. The concentrations of anti-Mb antibody binding sites in the resulting sera were determined by saturation with radiolabeled Mb in a solution radiobinding assay as described previously (20).

Determination of Idiotype. Competitive inhibition experiments for characterization of the idiotype expression on anti-Mb antibodies were performed by the enzyme-linked immunoabsorbent assay (ELISA) by a modification of the techniques described by Voller et al. (32) and Murphy et al. (33). The basic procedure was as follows: disposable polystyrene plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated by incubation with 100 μl of a 100 μg/ml solution of protein in phosphate-buffered physiological saline (PBS) overnight at 4°C. Unoccupied sites on the plastic were saturated by 30-min incubation with 200 μl per well of 100 mg/ml bovine serum albumin (BSA) in PBS. The serum samples, which were diluted to the equivalent concentration of anti-Mb antibodies, were preincubated overnight with inhibitor at 4°C before being added to the wells. Then, 100-μl aliquots of samples were transferred to wells coated with antigen and incubated in the wells for 1 h at room temperature, and unbound material was washed out. 100 μl of alkaline-phosphatase-conjugated rabbit anti-mouse Ig were added to each well. The plates were incubated for 3 h at room temperature. Excess conjugated antibody was washed out and the amount of alkaline phosphatase bound to the well was determined by measuring the hydrolysis of *p*-nitrophenyl phosphate (NPP) (Sigma) to the yellow product, *p*-nitrophenolate, which was quantitated by absorbance at 405 nm. The percent of inhibition was calculated according to the equation:

% inhibition = [1 − (Inhibited antibody bound

− Background)/(Uninhibited antibody bound − Background)] × 100,

in which background was determined by using the detecting reagent on antigen-coated wells but omitting the intermediate reagents or test antibodies. The background obtained with a plate coated with BSA instead of antigen was not significantly different from the above background.

Analysis of Fine Specificity Anti-Mb Antibodies. Binding of antibodies to native Mb or S-methyl Mb was assayed also by ELISA similarly to the competitive inhibition assay discussed above. The serum samples, which were diluted to the equivalent concentration of anti-Mb antibodies, were incubated in the wells, coated with Mb or S-methyl Mb for 2 h at room temperature, and unbound material was washed out. 100 μl of goat anti-mouse Ig antibodies (IgG fraction) was added to each well. The plates were incubated for 2 h at room temperature. After additional washes, 100 μl of the alkaline-phosphatase-conjugated rabbit anti-goat IgG was incubated in the wells for another 2 h at room temperature, and the amount of alkaline phosphatase bound to the well was measured. The ratio of anti-Mb antibodies binding to S-methyl Mb to anti-Mb antibodies binding to native Mb was calculated.

Results

Specificity of Rabbit Antiidiotype HAL19 Antiserum. Antiidiotypic antiserum was raised in a rabbit against an affinity-purified monoclonal A.SW anti-Mb antibody of clone HAL19-201-A$_{10}$, abbreviated HAL19 or simply clone 1 (see references

29, 30), as described in Materials and Methods. The immunogen contained no detectable MOPC 21 light chain from the NS-1 parent, as determined by gel electrophoresis. The resulting antiserum was absorbed with Sepharose-coupled MOPC 21 and normal mouse immunoglobulin a total of seven times to remove anti-species, anti-isotypic, and anti-allotypic antibodies. The ability of the absorbed antiidiotypic serum to inhibit the binding of anti-Mb antibodies to Mb attached to the surface of polystyrene microtiter wells was studied. This anti-HAL19 antiserum inhibited the binding of HAL19 to Mb completely (Fig. 1). More interestingly, initial experiments (Fig. 1) showed that it also inhibited the binding to Mb of B10.D2 anti-Mb antiserum, although the inhibition reached a plateau at ~45%. (Note that the dilution titer for plateau inhibition of the B10.D2 antibodies and of the monoclonal HAL19 was the same, 2^{-9}, suggesting that the subpopulation of anti-Mb antibodies in the B10.D2 immune serum that reacted with the rabbit anti-HAL19 at all reacted with most of the anti-HAL19 antibodies with an affinity comparable to that of HAL19.) No contaminating anti-Mb activity that could account for the inhibition was detectable in the antiidiotypic antiserum (data not shown). As a control, we used a rabbit antiidiotype against an unrelated monoclonal antibody, clone 14-4-4 specific for the histocompatibility antigen I-E^k, similarly absorbed three times on the myeloma

DETECTION OF HAL19 IDIOTYPE
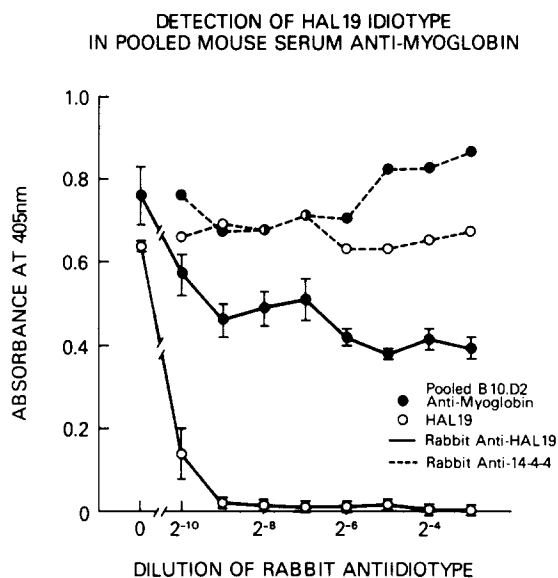IN POOLED MOUSE SERUM ANTI-MYOGLOBIN

DILUTION OF RABBIT ANTIIDIOTYPE

FIGURE 1.   Detection of HAL19 idiotype in pooled mouse serum anti-Mb. The monoclonal anti-Mb antibody, HAL19 (○) or pooled B10.D2 anti-Mb antiserum (●), both diluted to give 10 ng/ml of antibody, final concentration, were preincubated with the titrated rabbit anti-HAL19 idiotypic antiserum (solid line) or control rabbit anti-14-4-4 idiotype serum similarly absorbed (see Materials and Methods) (dashed line) at 4°C overnight. These mixtures were incubated in Mb-coated wells at room temperature for 1 h and unbound material was washed out. Then alkaline-phosphatase-conjugated rabbit anti-mouse Ig was added to each well and incubated for 3 h at room temperature. The amount of alkaline phosphatase bound to the wells was determined by using p-nitrophenylphosphate as a substrate and liberated yellow p-nitrophenolate was measured spectrophotometrically at 405 nm. Results represent the arithmetic means and standard errors of duplicate samples.

protein LPC-1, twice on normal C3H.SW immunoglobulin and once on pooled normal mouse Ig attached to Sepharose (31). This antibody had no inhibitory effect on Mb binding by either HAL19 or serum anti-Mb (Fig. 1), and only a minimal nonspecific enhancement effect was seen. This slight enhancing effect might be due to enhancing antibodies specific for Fc (34), since it was diminished by absorption on pooled normal mouse Ig that probably contains immune complexes (data not shown). No enhancement could be detected with the anti-HAL19 serum, which had been absorbed six times with the pooled normal mouse Ig.

To determine whether the inhibition of the binding of anti-Mb to Mb by this rabbit anti-HAL19 antiserum was idiotype specific, the inhibition experiment was performed with different monoclonal anti-Mb antibodies as well as HAL19, and in antigen-antibody reactions unrelated to Mb (Fig. 2). Since the rabbit anti-HAL19 did not inhibit reactions of unrelated antigens and antibodies of the same Igh allotype, Igh-1^e, as HAL19, such as FγG and A.SW anti-FγG antibodies, horse ferritin and A.SW anti-horse ferritin antibodies, and staphylococcal nuclease and A/J anti-staphylococcal nuclease antibodies, it could be concluded that the anti-HAL19 antiserum was not contaminated with detectable antibodies against constant region determinants. Furthermore, the rabbit anti-HAL19 antiserum did not inhibit monoclonal anti-Mb antibody HAL38 (clone 3.4), although it inhibited monoclonal anti-Mb antibodies HAL32 (clone 2), HAL39 (clone 4), and HAL43 (clone 5), as well as HAL19. The failure of the anti-HAL19 antiserum to inhibit HAL38, which has the same IgG$_1$ and κ subclass as

SPECIFICITY OF RABBIT ANTI-Id$_{HAL19}$

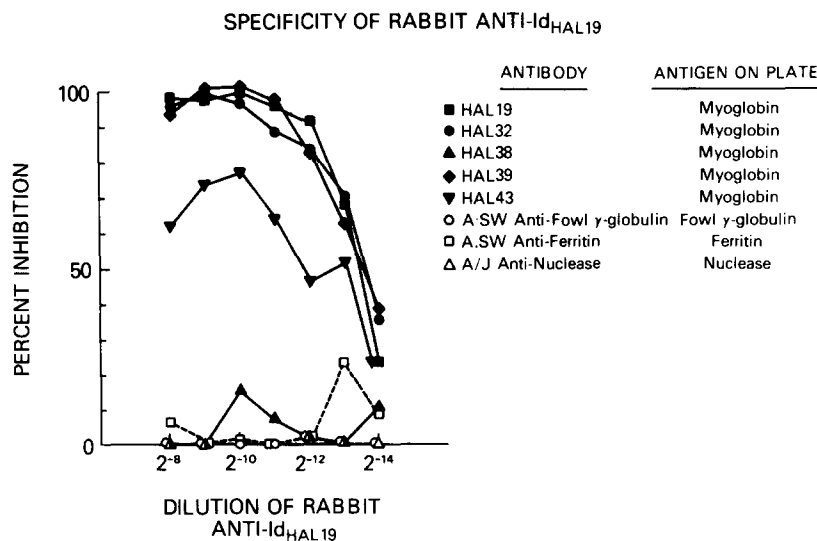| ANTIBODY	ANTIGEN ON PLATE
■ HAL19	Myoglobin
● HAL32	Myoglobin
▲ HAL38	Myoglobin
◆ HAL39	Myoglobin
▼ HAL43	Myoglobin
O A·SW Anti-Fowl y-globulin	Fowl y-globulin
□ A.SW Anti-Ferritin	Ferritin
△ A/J Anti-Nuclease	Nuclease

FIGURE 2. Specificity of rabbit anti-HAL19 antiserum. The monoclonal anti-Mb antibodies, A.SW anti-FγG antiserum, A.SW anti-ferritin antiserum, or A/J anti-staphylococcal nuclease antiserum were preincubated with the titrated anti-HAL19 antiserum at 4°C overnight. These mixtures were then incubated in wells coated with the corresponding antigens (right column) at room temperature for 1 h and processed as in Fig. 1. The percent inhibition was calculated by the formula described in the Materials and Methods. Test materials were all diluted to give 10 ng/ml of antibody as the final concentration, to compare at equal concentration.

HAL19, and its ability to inhibit HAL32, which is of a different subclass (IgG_{2a}), confirmed the specificity for idiotypic determinants of HAL19 (which we will call Id_{HAL19}), rather than for a particular subclass. Note that the failure to inhibit HAL38 could not have been due to a higher affinity of HAL38 for Mb, since HAL38 had a lower affinity than HAL19 and other monoclonals that were inhibited. Also, goat and sheep anti-Mb antisera did not express Id_{HAL19} (data not shown).

Effect of Igh Allotype on the Expression of Id_{HAL19}. As the antiidiotypic antiserum detected Id_{HAL19} on ~45% of B10.D2 anti-Mb antiserum, we studied other strains of mice having different Igh allotypes for expression of idiotype (Fig. 3). The anti-Mb immune serum from each mouse was tested separately, so the results show the statistical variation of mice within each strain. The inhibitory effect of the antiidiotypic antiserum on anti-Mb antiserum from all five strains representing five Igh allotypes was similar. All strains carried high-responder $H-2^s$ (A.SW) or $H-2^d$ (the other strains) haplotypes. BALB/c and C.AL-20 are allotype-congenic. Thus, idiotypic determinants on the anti-Mb antibodies that are recognized by anti-Id_{HAL19} antiserum showed no association with any particular Igh allotype, but were expressed by all allotypes tested. The gene(s) for Id_{HAL19} may still be Igh linked, but without a difference in phenotype, genetic mapping is impossible. The presence of Id_{HAL19} on ~40–50% of the anti-Mb antibodies from strains of five different allotypes indicates that Id_{HAL19} is a major or common idiotype among anti-Mb antibodies, perhaps analogous to the cGAT idiotype found on anti-GAT antibodies of many strains (35).

H-2 Influence on Id_{HAL19} Expression. We studied the effect of *H-2* genes on the expression of Id_{HAL19}, based on our previous studies which have shown that *H-2*-linked *Ir* genes control the fine specificity of the antibody response to Mb
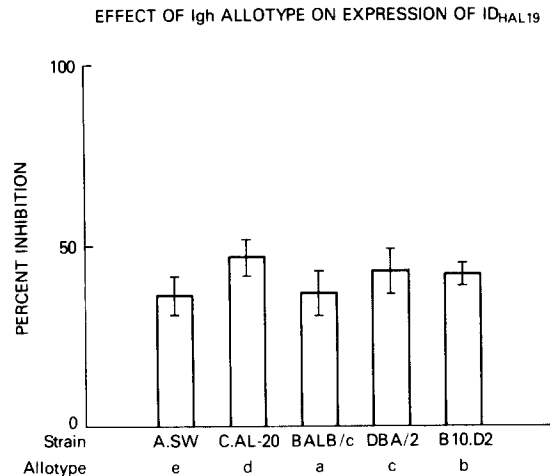

EFFECT OF Igh ALLOTYPE ON EXPRESSION OF ID_{HAL19}

FIGURE 3. Effect of Igh allotype on expression of Id_{HAL19}. Each anti-Mb antiserum from three to five mice of the indicated strains was tested independently for the expression of Id_{HAL19} by competitive inhibition assay. Every antiserum was diluted to give 7.5 ng/ml of antibody as the final concentration and preincubated with the rabbit anti-Id_{HAL19} antiserum diluted to 1:256 at 4°C overnight and processed as in Figs. 1 or 2. Results represent the arithmetic means and standard errors of three to five mice of each strain.

(15, 22). Even the low responder mice can produce significant quantities of anti-Mb antibodies. Fig. 4 shows the results of the competitive inhibition by anti-Id_{HAL19} of equivalent amounts of antibodies from low and high responder mice binding to Mb. The serum from each mouse was tested separately, and was diluted to give equivalent control (uninhibited) binding in order to be able to compare sera of high and low responders in an unbiased way. There was a large and statistically significant difference between anti-Mb sera from congenic low and high responder mice in their fractional level of idiotype expression. ~80% of anti-Mb antibodies from low responder mice, namely B10.BR (H-2^k) and B10 (H-2^b), were inhibited, whereas <50% of anti-Mb antibodies from high responder mice, B10.D2 (H-2^d), were inactivated. The (high responder × low responder)F_1, which is phenotypically a high responder (20), displayed the same idiotype expression pattern as the high responder parent (Fig. 4). Therefore, the higher relative expression of Id_{HAL19} in the low responder was not due to the presence of genes enhancing the expression in the low responder, but rather due to genes diminishing the fraction of antibodies bearing idiotype in the high responder (see below). This result showed that H-2-linked genes influence the fraction of antibodies expressing Id_{HAL19} idiotype. In the four haplotypes and F_1 hybrid studied, this influence correlates with Ir gene phenotype.

Fine Specificity of Id_{HAL19}-positive Anti-Mb Antibodies. Several studies have shown that H-2-linked Ir genes control the relative amounts of antibodies
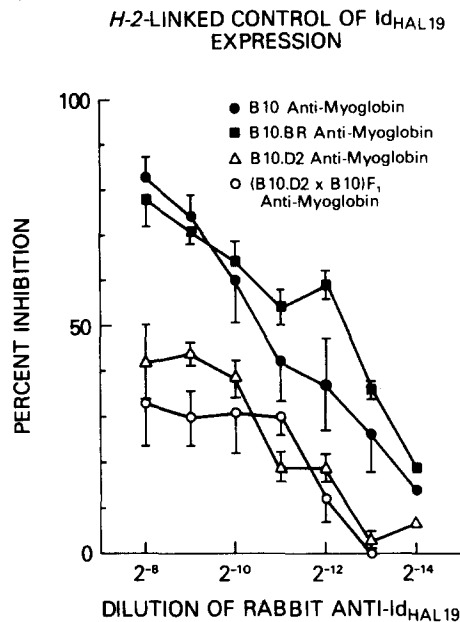

FIGURE 4. H-2 linked control of Id_{HAL19} expression. Each anti-Mb antiserum from five mice of the B10 congenic strains, B10 (●), B10.BR (■), B10.D2 (△) or (B10.D2 × B10)F_1 (○), was diluted to give 5 ng/ml of antibody as the final concentration and tested individually for the expression of Id_{HAL19} by competitive inhibition assay with rabbit anti-Id_{HAL19} antiserum. B10 and B10.BR are low responder strains and B10.D2 is a high responder strain to sperm whale Mb. Results represent the arithmetic means and standard errors at each point for five mice of each strain. See Figs. 1 and 2.

produced against different antigenic determinants of the same molecule (13–16). If the rabbit antiidiotypic antiserum were preferentially recognizing antibodies produced against some epitopes, to which low responder mice made relatively more antibodies, the low responder *H-2* haplotype would result in an increase in the relative level of expression of such an idiotype, as the results showed.

To explore the possibility of such a mechanism, at first we tried to analyze the fine specificity of anti-Mb antibodies that were detected with the antiidiotypic antiserum. Anti-Mb antibodies, which had been fractionated on fragment 1–55 or 132–153 affinity columns, were tested for inhibition by the antiidiotypic antiserum in a binding assay with native Mb on the plate. Both the 1-55-binding fraction and the 132-153-binding fraction were inhibited only minimally compared to the unseparated serum, so that the idiotype was depleted, not enriched, by the fractionation (Fig. 5A). This result was not unreasonable, since HAL19 itself does not bind to any of the three CNBr-cleavage fragments of Mb, but only to native Mb (30). We do not have pure middle fragment (56–131), in order to determine whether the antiidiotypic antiserum might recognize anti-Mb antibodies specific for middle fragment of Mb.
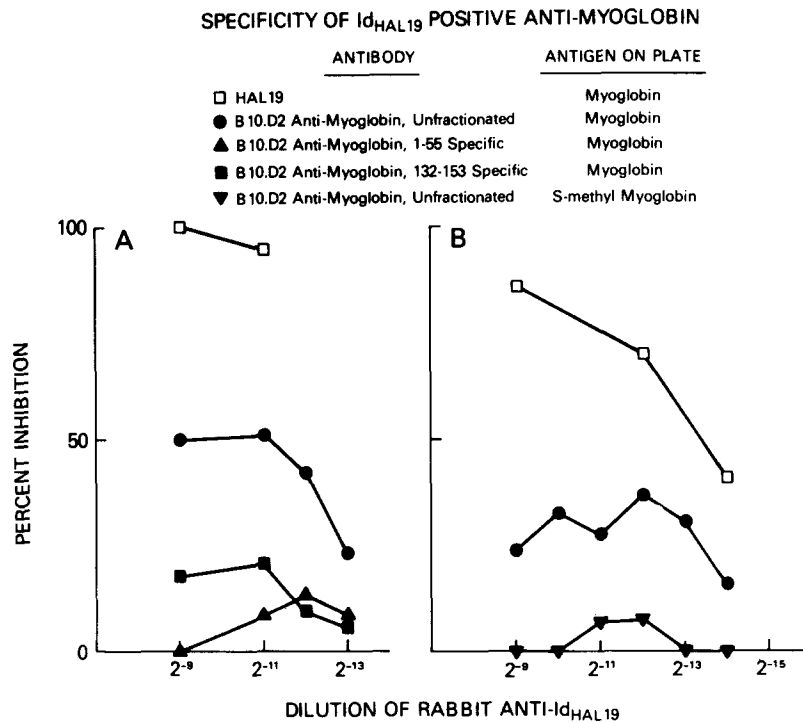
SPECIFICITY OF Id$_{HAL19}$ POSITIVE ANTI-MYOGLOBIN

ANTIBODY		ANTIGEN ON PLATE
□ HAL19		Myoglobin
● B10.D2 Anti-Myoglobin, Unfractionated		Myoglobin
▲ B10.D2 Anti-Myoglobin, 1-55 Specific		Myoglobin
■ B10.D2 Anti-Myoglobin, 132-153 Specific		Myoglobin
▼ B10.D2 Anti-Myoglobin, Unfractionated		S-methyl Myoglobin

DILUTION OF RABBIT ANTI-Id$_{HAL19}$

FIGURE 5. Specificity of Id$_{HAL19}$-positive anti-Mb antibodies. In *A*, B10.D2 anti-Mb antibodies purified by binding to and elution from Mb fragment 1-55 (▲) or 132–153 (■) affinity columns were tested for Id$_{HAL19}$ expression by competitive inhibition assay with rabbit anti-Id$_{HAL19}$ antiserum and compared to unfractionated B10.D2 anti-Mb antibodies (●) or HAL19 (□). In *B*, the effects of the anti-Id$_{HAL19}$ antiserum on the binding of B10.D2 anti-Mb antibodies to native Mb (●) or S-methyl-Mb (▼) were tested. The test samples were diluted to give 7.5 ng/ml of antibody as the final concentration. See legend to Figs. 1 and 2.

However, since one fine specificity property of HAL19 is its recognition of an assembled topographic antigenic determinant that consists of residues far apart in the primary sequence and juxtaposed only on the surface of the native molecule (30, 36), we used the conformational specificity of anti-Mb antibodies as another fine specificity parameter to compare with idiotype. Therefore, we compared the inhibition by the antiidiotypic antiserum of anti-Mb antibodies binding to plates coated with either native Mb or the denatured form, S-methyl Mb. S-methyl Mb has been partially denatured and unfolded by the addition of extra methyl groups to the sulfur atoms of hydrophobic methionine residues at positions 55 and 131, introducing positive charges into the hydrophobic core of the molecule, and thus requiring the molecule to unfold to admit solvent (24, 25). The denaturation and free exposure of these regions to aqueous solvent, in contrast to native Mb, was demonstrated by ^{13}C-NMR, which demonstrated identical sharp resonances for the two S-methyl methionine residues equivalent to free S-methyl methionine, in contrast to the distinct chemical shifts observed for the native molecule (24, 25). We found that the subpopulation of anti-Mb antibodies that was reactive with S-methyl Mb was not inhibited by anti-Id$_{HAL19}$ (Fig. 5B), whereas the population binding native Mb was inhibited ~40%. These results suggested that the antiidiotypic antiserum recognizes only anti-Mb antibodies that are specific for the native conformation and do not bind denatured forms. This was compatible with the fact that all of the monoclonal anti-Mb antibodies we have, including HAL19, HAL32, HAL39, and HAL43, which bear the idiotype (as well as HAL38 which does not) are specific for native Mb, but do not bind to any of the CNBr-cleavage fragments of Mb as reported previously (30). Also, HAL19 does not bind to S-methyl Mb-coated plates (data not shown).

H-2-linked Control of Fine Specificity of Antibody. Since the antiidiotypic antiserum appeared to react with only anti-Mb antibodies specific for the native conformation (Fig. 5, A and B), and we saw a difference in the relative amount of Id$_{HAL19}$ expression between high and low responder mice (Fig. 4), it was of interest to see whether the low responder mice made relatively more anti-Mb antibodies specific only for native Mb than the high responder mice. Using anti-Mb antisera from H-2 congenic low responder strains of mice, B10 and B10.BR, or the congenic high responder strain of mice, B10.D2, we measured the amount of anti-Mb antibodies binding to native Mb or S-methyl Mb, and calculated the ratio of the amount of antibodies binding to the two conformational forms of the antigen (Fig. 6). The results of testing sera from five mice of each strain showed a strong, statistically significant difference between high and low responder mice. The ratio of anti-Mb antibodies reactive with S-methyl Mb to anti-Mb antibodies reactive with native Mb in the antiserum from low responder mice was much lower than in the antiserum from the high responder mice. Again, the high responder phenotype was dominant in the F$_1$ hybrid. Thus, it appears that *Ir* genes, which control the magnitude of antibody response to Mb, also influenced low responder mice to make anti-Mb antibodies specific for conformational antigenic determinants preferentially, compared to the high responder mice. Note that the high responder sera may be compared with goat, sheep, and rabbit
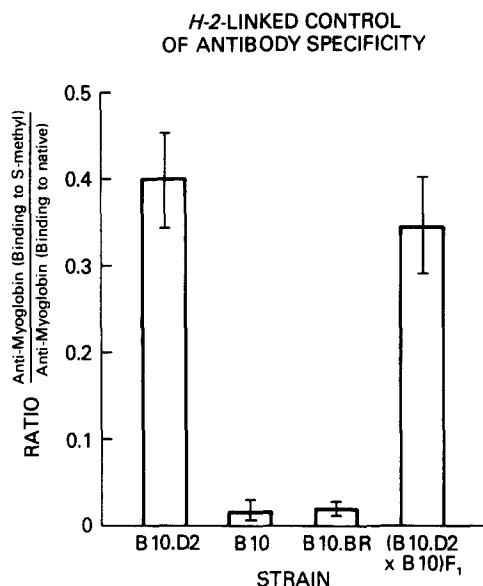
H-2-LINKED CONTROL
OF ANTIBODY SPECIFICITY

FIGURE 6. *H-2*-linked control of antibody specificity. Each anti-Mb antiserum from the same mice as in Fig. 4 was incubated in the wells coated with Mb or S-methyl Mb at room temperature for 2 h, and unbound material was washed out. Then, the IgG fraction of goat anti-mouse immunoglobulin antiserum was incubated in the wells at room temperature for 2 h. After additional washes, alkaline-phosphatase-conjugated rabbit anti-goat IgG was incubated in the wells for another 2 h at room temperature. The amount of alkaline phosphatase bound to the wells was determined and after the background was subtracted the ratio of anti-Mb antibodies reactive with S-methyl Mb to total anti-Mb antibodies was calculated. The antisera were diluted to 10 ng/ml of antibodies. Results represent the arithmetic means and standard errors of five mice of each strain.

anti-Mb antisera, in which 30–40% of the antibodies bound only the native conformation (37).

In addition, since S-methyl Mb lacks the heme of native Mb, we had to test for the possibility that the distinction was due to antibodies produced by low responder mice specific just for the heme of Mb rather than conformation. Therefore, the ability of antibodies from low responder mice to bind apo-Mb was tested. Apo-Mb also has the heme removed but is not as completely denatured as S-methyl Mb. Anti-Mb antibodies from low responder mice bound to apo-Mb just as much as to native Mb (data not shown). Therefore, it is a higher proportion of conformation-specific, not heme-specific, antibodies that distinguishes the strains.

Effect of Carrier on the Idiotypic Expression and the Fine Specificity of Antibodies. *H-2*-linked *Ir* genes control immune response to Mb through the interactions of antigen-presenting cells, helper T cells, and B cells, (21, 28, 38–40). We tried to change the helper T cells employed and see the effect on idiotype expression, by immunizing low responder B10.BR mice with Mb coupled to FγG. It has been shown previously that coupling to FγG can provide carrier epitopes for FγG-specific T cells, which can help B cells produce antibodies against the coupled Mb (38).

Immunization of B10.BR with Mb-FγG complex decreased the fraction of

anti-Mb antibodies that could be inhibited by the antiidiotypic antiserum from 80% to 45% (means of four mice in each group) (Fig. 7). The effect of FγG carrier coupled to Mb on the ratio of anti-Mb antibodies that reacted with S-methyl Mb versus native Mb was also tested, using the same sera (Fig. 8). Sera from low responder mice immunized with Mb-FγG complex behaved like sera from high responders immunized with Mb, both with respect to the proportion of antibodies expressing Id$_{HAL19}$ (Fig. 7) and with respect to conformational specificity (Fig. 8), as well as in the total level of antibodies produced, as previously shown (38). This effect was unlikely to be due to denaturation of Mb attached to the carrier FγG, since the Mb-FγG complex bound HAL19 (specific for the native conformation) at least as well, per mole of Mb, as free native Mb, whereas S-methyl Mb did not bind HAL19 at all (data not shown).

Discussion

In this study, we raised an antiserum against a monoclonal anti-Mb antibody (HAL19) in a rabbit. This antiserum, after absorption on MOPC 21 and normal mouse globulin affinity columns, was specifically directed to idiotypic determinants of anti-Mb antibodies. This was shown by the following properties: (a) Only anti-Mb antibodies were inhibited. The binding of unrelated proteins such as FγG, ferritin, and staphylococcal nuclease by their specific antisera were not affected (Fig. 2). (b) This antiidiotypic antiserum failed to inhibit a monoclonal
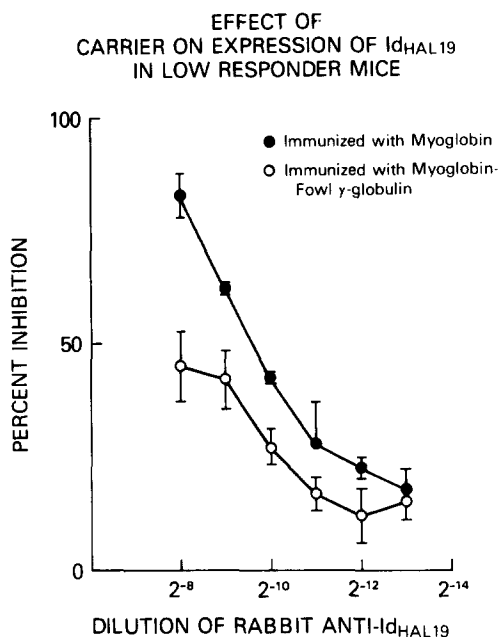

FIGURE 7. Effect of carrier on expression of Id$_{HAL19}$ in low responder mice. Groups of four lower responder B10.BR mice were immunized with Mb (●) or Mb-FγG complex (○), and each antiserum was tested independently for Id$_{HAL19}$ expression by competitive inhibition assay with rabbit anti-Id$_{HAL19}$ antiserum. The results represent arithmetic means and standard errors for four mice in each group. The antisera were diluted to give 7.5 ng/ml of antibody as the final concentration. See legend to Figs. 1 and 2.

EFFECT OF CARRIER
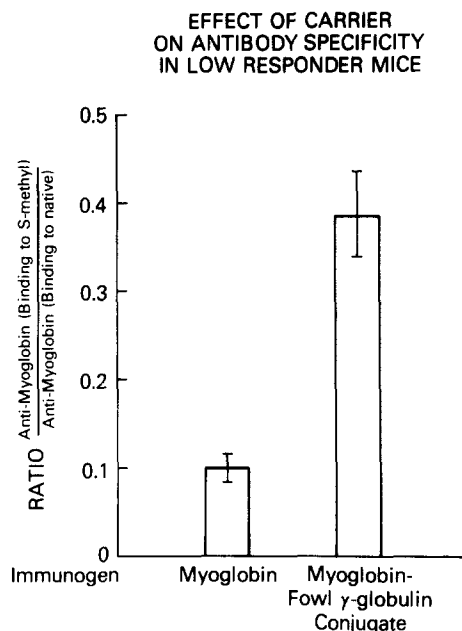ON ANTIBODY SPECIFICITY
IN LOW RESPONDER MICE

FIGURE 8. Effect of carrier on antibody specificity in low responder mice. The ratio of anti-Mb antibodies reactive with S-methyl Mb to anti-Mb antibodies was assayed as in Fig. 6 using the same sera as in Fig. 7, which were from low responder B10.BR mice immunized with Mb or Mb-FγG complex. The data represent the arithmetic means and standard errors for four mice in each group. See legend to Fig. 6.

anti-Mb antibody, HAL38, of the same IgG$_1$ κ subclass as HAL19, whereas one of a different subclass, IgG$_{2a}\kappa$, was inhibited. In addition, since HAL38 has lower affinity for Mb than HAL19 (29), the failure to inhibit the binding of HAL38 to Mb was not because of higher affinity of HAL38. (c) The binding of Mb by antibodies specific for fragment 1–55 and 132–153, and of S-methyl Mb by anti-Mb antibodies, was not inhibited. (d) Cross-reactivity of antiidiotypic antiserum with monoclonal anti-Mb antibodies other than HAL19 is not due to anti-MOPC 21 light chain specificity, since there was not detectable contamination of MOPC 21 light chain from the parental cell, NS-1 derivative of the MOPC 21 plasmacytoma, in HAL19, which was used as antigen to raise antiidiotypic antiserum (29), and the antiidiotype was absorbed with MOPC 21. (e) The antiidiotypic antiserum contained no anti-Mb binding activity, which might be induced with undetectable Mb contaminating the affinity-purified HAL19 and which could have inhibited binding of anti-Mb to the plate.

The antiidiotypic antiserum inhibited to a comparable extent (40–50%) anti-Mb antisera produced in five responder strains representing five different Igh allotypes, namely Igh[a,b,c,d, and e]. The failure to find an Igh allotype that does not express this idiotype prevents us from mapping it genetically with respect to allotype, but does not exclude the possibility that the idiotype is encoded by Igh-linked genes that are shared by or similar in all of these allotypes. This situation is comparable to that described for the cGAT idiotype of antibodies to the random terpolymer Glu^{60}Ala^{30}Tyr^{10} (35). Alternatively, it is possible that the

idiotype is determined primarily by the immunoglobulin light chain, and might map to the kappa chain locus (41).

A third explanation for an idiotype that is shared by all Igh allotypes tested is that the rabbit antiidiotype is actually the internal image of an epitope on the antigen (42). By this explanation, the rabbit anti-HAL19 antibodies would be postulated to have a variable region that resembles or cross-reacts with the epitope of Mb bound by HAL19. Thus, any anti-Mb antibodies specific for this epitope would bind to the rabbit anti-HAL19 and appear to share an idiotype. This explanation is compatible with the correlation of idiotype with fine specificity. However, we believe it unlikely because neither goat nor sheep anti-Mb antisera, which bind a variety of epitopes of Mb, were inhibited by anti-Id$_{HAL19}$ (data not shown). The postulated internal image antibodies would be expected to be bound by anti-Mb antibodies raised in different species as well as different strains of mice.

The immune responses to sperm whale Mb are under H-2-linked Ir gene control. B10.D2 mice with the H-2^d haplotype are high responders to sperm whale Mb, whereas the congenic B10.BR mice and B10 mice possessing the H-2^k and the H-2^b haplotypes, respectively, are low responders to this antigen (20). Although low responder mice make only about one-tenth as much anti-Mb antibody as high responder mice, concentration of anti-Mb antibodies in the serum of the low responder mice immunized with Mb becomes high enough to be studied for idiotypic pattern and fine specificity. Using the antiidiotypic antiserum, we compared the idiotype expression of high (H-2^d) and low (H-2^b and H-2^k) responder mice. A significantly higher proportion of anti-Mb antibodies from low responder mice was inhibited from binding to Mb than in the case of high responder mice. These results suggest a relationship between anti-Mb idiotype expression and H-2-linked Ir genes. While there are several reports that have shown idiotype expression linked to Igh allotype in the mouse with anti-hapten, anti-polymer, or anti-protein systems, such as azobenzene arsonate (5), GLPhe (43), and staphylococcal nuclease (44), or linked to light chain polymorphisms (41), or idiotype expression controlled by idiotype networks (45, 46), to our knowledge only one case has been reported in which the expression of antibody idiotypic determinants is clearly controlled by the H-2 locus of the mouse, namely, the case of anti-GPhe antibodies (47). Rabbit anti-(SWR/J anti-GPhe) antiserum strongly inhibited the binding of GPhe by anti-GPhe antisera produced only in mice of H-2^q haplotype, but had no effect on the binding of GPhe by anti-GPhe antisera produced in mice of other responder haplotypes. A second case was discovered in the guinea pig, in which MHC-linked Ir genes regulated production of antibodies highly specific for ϵ-DNP-LYS$_{10}$ and concomitantly influenced expression of the corresponding idiotype (48). This case more closely resembles ours than does the case of GPhe in the mouse. Other reports have shown that mice of both high and low responder H-2 haplotypes made less antibody with a certain idiotype, when immunized with immunogens coupled with MBSA carrier, than did high responder H-2 haplotype mice, when immunized with just the immunogen alone (49, 50). In such cases, carrier-induced help, not H-2-linked Ir genes, controlled the idiotypic expression. No previous cases have been reported for idiotypes to natural protein antigens, or for

differences in idiotype expression between high and low responder mice, under control of H-2-linked Ir genes. However, a very recent paper on idiotypes of anti-insulin antibodies did consider H-2-linked control of idiotype as one explanation for the results (51). It has been predicted that Ir gene control should lead to control of idiotype expression (6). For the antigen GAT in the guinea pig (13), and for the antigens of staphylococcal nuclease (14), Mb (15), and hepatitis B (16) in the mouse, strains were found that had Ir genes allowing an antibody response to one set of epitopes but not another set of epitopes on the same molecule. If antibodies against such epitopes expressed unique combining sites and therefore idiotypes, Ir genes would appear to control the level of expression of such idiotypes.

The results in the present paper fulfill the predictions of this hypothesis and thus provide some of the first support for its validity. The rabbit anti-Id$_{HAL19}$ antiserum seemed to be specific for anti-Mb antibodies that reacted with native Mb but not with fragments of Mb or the denatured form, S-methyl Mb. Low responder mice, which made relatively more antibodies with Id$_{HAL19}$, made mostly anti-Mb antibodies that did not react with S-methyl Mb, whereas high responder mice made relatively more anti-Mb antibodies that reacted with S-methyl Mb, and had proportionately fewer Id$_{HAL19}$ positive antibodies. These may be compared with rabbit, goat, and sheep anti-Mb antisera, which were found to contain 30–40% of the antibodies that bound only to the native conformation (37). Not all of the anti-Mb antibodies that fail to bind to S-methyl Mb bear the Id$_{HAL19}$ idiotype. Nevertheless, it is probable that the difference in the idiotype expression between high and low responder mice is due to an H-2-linked difference in fine specificity of anti-Mb antibodies, which is observed as the difference in ratio of anti-S-methyl Mb antibodies to anti-native Mb antibodies. Note that the lower proportion of antibodies expressing Id$_{HAL19}$ idiotype in the high responders compared to the low responders is not due to the production of less idiotype-positive antibody, but rather to the production of more idiotype-negative antibody in the high responders. The dominance of the high responder phenotype in the F_1 hybrid (Figs. 4 and 6) supports this interpretation. If the low responder B10 carried genes that positively enhanced the expression of Id$_{HAL19}$, its phenotype would be expected to be dominant in the F_1, as, for example, in the case of allotype-linked genes for idiotype. On the contrary, however, it is the presence of high responder genes, not the absence of low responder genes, which lowers the fraction of antibodies expression Id$_{HAL19}$ and the corresponding fine specificity. This would be predicted if the Ir high responder phenotype led to the production of a greater diversity of antibodies including many negative for Id$_{HAL19}$. Thus, we do not conclude that the Ir genes control the expression of Id$_{HAL19}$ directly, but rather that they influence the overall repertoire of idiotypes expressed.

Immunization of low responder mice (H-2^k) with Mb coupled with FγG led to the production of anti-Mb antibodies that were reactive to S-methyl Mb. In parallel, Id$_{HAL19}$ expression was decreased by immunization with Mb-FγG complex. In the Mb-FγG complex, it has been shown that FγG provided carrier epitopes for T cells that help B cells specific for Mb (38). Further, it has been shown that these carrier-specific T cells overcome the Ir-gene-controlled low

responsiveness (38). We have now found that not only the total magnitude of the response, but also the fine specificity and idiotype become similar to those of the high responder.

The most likely explanation is that either changing the quantity of T cell help, or changing the repertoire of helper T cells by coupling of antigen to another carrier, alters the fine specificity of antibodies, leading to expression of alternative idiotypes, as we have shown here. However, the possibility could not be ruled out that the coupling of FγG to Mb changed the expression of epitopes on the Mb molecule, leading to the production of anti-Mb antibodies reactive with S-methyl Mb. We believe this latter explanation unlikely, however, (a) because the Mb-FγG complex bound HAL19, specific for the native conformation, at least as well, per mole of Mb, as free Mb, whereas S-methyl Mb did not bind HAL19 at all, indicating that the Mb attached to FγG is native, and (b) because the parallel changes in idiotype and specificity seen with the use of carrier were the same as those seen with differences in H-2-linked high or low responder status, when only native Mb was the immunogen. It should be noted that, in contrast, in the anti-GPhe system (47), immunization with GPhe coupled with MBSA did not change the idiotype expression. This result suggests the possibility of a fundamentally different mechanism for GPhe from those suggested for Mb.

How do T cells influence the fine specificity of B cells and the idiotype expression, as we are reporting here? We have considered two possible models. 1) In the previous reports, which have described Ir genes for staphylococcal nuclease (14, 52) and sperm whale Mb (15, 22), antibodies were made preferentially to the part of the protein antigen to which proliferative T cells responded. It seemed that T cells that were selected to be stimulated or present under control of H-2-linked Ir genes selected B cells, specific for certain epitopes, to be helped, from among all B cells that are specific for various epitopes on the antigen molecule (40). Likewise, studying anti-lysozyme responses, Cecka et al. (53, 54) suggested that T cell specificity limits B cell specificity because of a requirement to avoid steric hindrance in antigen bridging. Thus, there might be a deficiency in the T cell repertoire in low responder mice of T cells specific for epitopes that are located at the optimal position to help B cells specific for epitopes shared between native Mb and S-methyl Mb.

2) If there are many epitopes on Mb and S-methyl Mb in common, it is difficult to explain by the steric restriction between T and B cell epitopes mentioned above why the T cell repertoire necessary to help B cells specific for epitopes shared between S-methyl Mb and native Mb is selectively lacking in the low responder mice. Bottomly et al. (55) suggested the possibility that B cells that appeared to bind to antigen more efficiently were less dependent on helper T cell signals for activation, based on these and other data (56) in the anti-PC response. T cells from the high responder mice immunized with Mb proliferate to Mb in vitro more than those from the low responder mice (15). If a larger number of helper T cells were required to help B cells producing lower affinity antibodies for Mb, it is possible that high responder mice have sufficient helper activity to help B cells producing antibodies that are specific for S-methyl Mb and have lower affinity for native Mb, whereas low responder mice have only enough help for B cells with high affinity for native Mb. We are currently

studying the affinities of antibodies with these different fine specificities in various strains to test these possibilities. In the preliminary experiments, antibodies from low responder mice have, on the average, higher affinity for native Mb than those from high responder mice, consistent with the above hypothesis. Bekoff et al. (48) also found an influence of T cells on fine specificity and idiotype of antibodies in the guinea pig, but found no influence on antibody affinity. Further work on the quantity and quality of T cell help will be necessary to sort out these possibilities.

Summary

A rabbit antiidiotypic antiserum raised against an A.SW IgG$_1\kappa$ monoclonal anti-sperm whale myoglobin (Mb) antibody, HAL19, and extensively absorbed with normal mouse immunoglobulin and MOPC 21 (IgG$_1\kappa$), was found to detect a common or major anti-Mb idiotype expressed by some but not all anti-Mb monoclonal antibodies, regardless of immunoglobulin G (IgG) subclass, and by 40–50% of the anti-Mb antibodies in immune serum from five high responder strains of mice representing five different Igh allotypes. It did not inhibit antibodies to three unrelated protein antigens. The fraction of antibodies expressing this idiotype, denoted Id$_{HAL19}$, was regulated by H-2-linked genes that correlated exactly in four independent haplotypes and an F$_1$ with the known Mb immune response (Ir) genes and may be identical to these. Whereas <50% of antibodies from high responder mice were inhibitable by anti-Id$_{HAL19}$, >80% of antibodies from low responder mice, tested at comparable final antibody concentration, were inhibitable. This result was true for both low responder haplotypes, H-2^b (B10) and H-2^k (B10.BR). The idiotype was found to be present on antibodies that bound to native Mb but not fragments 1–55 or 132–153 of Mb or a denatured form, S-methyl Mb. This specificity for native Mb paralleled that of the monoclonal idiotype HAL19 itself. Therefore, the production of antibodies specific for native in contrast to denatured Mb was studied in H-2-congenic high and low responder strains. Strikingly, low responders produced antibodies that reacted almost exclusively with the native conformation, whereas a larger proportion of antibodies from high responder mice also reacted with the denatured form, S-methyl Mb. Bypassing of the Ir gene defect by immunization with Mb attached to a carrier, FγG, resulted in low responder antisera resembling higher responder sera in both idiotype expression and conformational specificity.

The simplest explanation of these results is that H-2-linked Ir genes control antibody fine specificity, which is reflected in the idiotypes of the variable regions expressed. We suggest that low responder mice produce a more limited repertoire of antibodies consisting primarily of Id$_{HAL19}$-positive antibodies specific for the native conformation of Mb. High responder mice produce a greater diversity of antibodies to Mb, so that the Id$_{HAL19}$-positive, conformation-specific population represents a smaller proportion of the total. Similarly, the use of carrier-specific helper T cells in low responder mice results in a greater diversity of antibodies, which dilutes out the Id$_{HAL19}$ subset. The possible roles of T cell specificity, the magnitude of T cell help, and antibody affinity are discussed. These results provide some of the first experimental evidence to support the

prediction that *H-2*-linked *Ir* genes that control antibody fine specificity should also influence the repertoire of idiotypes expressed.

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