

EXPRESSION OF AN IDIOTYPE (Id-460) DURING
IN VIVO ANTI-DINITROPHENYL ANTIBODY RESPONSES

I. Mapping of Genes for Id-460 Expression to the
Variable Region of Immunoglobulin Heavy-Chain Locus and
to the Variable Region of Immunoglobulin
 κ -Light-Chain Locus

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Unique antigenic determinants found on antibodies of a particular specificity are called idiotypes (Id); inbred mouse strains have been shown to inherit the ability to express a particular Id during responses to the same antigen in a reproducible manner. In general, these studies have shown that the genes controlling the expression of an Id are linked to the immunoglobulin heavy-chain gene complex (Ig-H).¹ Where crossing over between the genes coding for constant portion of the Ig-H complex (CH) and variable portion of the Ig-H complex (VH) have occurred, the Id is inherited in linkage with VH (1). More recently, light-chain polymorphisms have been described, and these genes have also been demonstrated to affect the expression of the Id (2, 3). One of these polymorphisms is located in the genes of variable region of immunoglobulin κ -light chains (V κ) (2, 4). This was first detected by tryptic peptide mapping of cysteine-containing peptides from V κ by Edelman and Gottlieb (4), and it was subsequently demonstrated to be linked to a genetic locus on chromosome 6 that controls the T cell differentiation antigen Lyt-3.1 (5). Studies by Claflin et al. (6) and by Gibson (7) have defined similar κ -chain polymorphisms. Mice of strains that bear the Lyt-3.1 allele (AKR, C58, PL, and RF) express one pattern of V κ -gene products, whereas all Lyt-3.2 strains express a different pattern. More recently, Gibson and MacLean (8) have detected an electrophoretic difference between certain κ -light

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¹ Abbreviations used in this paper: BSA, bovine serum albumin; C.AKR, BALB/AKR-Lyt-2^a,3^a; C.C58, BALB/C58-Lyt-2^a,3^a; CH, constant portion of the immunoglobulin heavy-chain complex; DNP, 2,4-dinitrophenyl; Id, idio(s); Id-460, idio(s) on the DNP-binding myeloma protein MOPC 460; Ig-H, immunoglobulin heavy chain, M315, MOPC 315; M460, MOPC 460; MHC, major histocompatibility complex; OVA, ovalbumin; PBS, phosphate-buffered saline; VH, variable portion of the immunoglobulin heavy-chain complex; V κ , variable region of immunoglobulin κ -light chains; Xid, X-linked immune deficiency.

chains from strain C58 and the other Lyt-3.1 strains of mice, which they have termed Ef-2 (8). They propose that this difference involves a single V κ group.

Previous work in our laboratories has enabled us to define a set of reagents detecting an idiotype on the BALB/c 2,4-dinitrophenyl (DNP)-binding myeloma protein MOPC 460 (Id-460) (9, 10) that is inherited by strains of mice that carry the Ig-H complex derived from BALB/c mice (11). Id-460 is expressed during conventional anti-DNP antibody responses *in vivo*. In these experiments, a different set of reagents has been prepared that define a related Id-460 determinant. We have determined the time-course of expression of Id-460 during primary and secondary *in vivo* anti-DNP antibody responses. In this paper, we demonstrate that the expression of Id-460 during *in vivo* anti-DNP antibody responses is controlled by genes mapping in VH. We further demonstrate that V κ genes present in strains BALB/c and AKR, but missing or unexpressed in strain C58, are required for the expression of Id-460. This is the first Id to clearly differentiate the V κ repertoire of strains C58 and AKR.

Materials and Methods

Mice. Mice of strains BALB/cByJ and B10.D2/oSn were purchased from The Jackson Laboratory, Bar Harbor, Maine. The major histocompatibility complex (MHC)-congenic strains BALB.B (H-2^b) and BALB.K (H-2^k) were provided by Donal Murphy from the Comprehensive Cancer Center, Yale University, New Haven, Conn. Strain C.B20 was obtained from Litton Bionetics under contract to Michael Potter (National Cancer Institute [NCI] contract NO1-CB-92142) and strains BAB-14 and B.C9 were gifts of Roy Riblet and Mel Bosma at the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pa.; these Ig-congenic strains were maintained at Yale from stock provided. Strains BALB/C58-Lyt-2^a,3^a (C.C58) and BALB/AKR-Lyt-2^a,3^a (C.AKR) were bred at the Massachusetts Institute of Technology, Cambridge, Mass. by introgressively backcrossing the Lyt-3.1 marker onto BALB/cAnN mice (12). The generations of backcrossing and inbreeding of these mice was N19F3 for C.C58 and N18F3 for C.AKR. Mice of both strains bear the Lyt-3^a allele and the V κ I_B peptide marker associated with this allele in the parental strains. The (CBA/N \times BALB/c)F₁ male mice bred from CBA/N maintained at Yale and BALB/cByJ express the X-linked immune deficiency (Xid) found in CBA/N mice whereas the reciprocal (BALB/c \times CBA/N)F₁ male mice are phenotypically normal.

Myeloma Proteins. MOPC 460, MOPC 31C, FLOPC 21, and MOPC 104E plasmacytomas along with proteins XRPC-25 and TEPC-15 were obtained from Michael Potter (Litton Bionetics under NCI contract NO1-CB-92142). The plasmacytomas were carried in BALB/cByJ mice as ascites tumors. Ascites fluid was precipitated in 45% saturated ammonium sulfate at 4°C. MOPC 460 and MOPC 315 proteins were purified by affinity chromatography using DNP-lysyl-glycine-Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.). MOPC 31C and FLOPC 21 were further purified by DEAE-ion-exchange chromatography. MOPC 104E was purified on Sephacryl S-300 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.). MOPC 511 protein was a gift from Rose Liebermann, National Institutes of Health (NIH) and affinity-purified HOPC-8 was a gift from Stuart Rudikoff (NIH). G-1 is a monoclonal antibody from BALB/c mice; it binds both DNP and phosphorylcholine (P. Gearhart. Unpublished observations.).

Anti-Id Antisera. Anti-Id-460 antiserum was produced by immunization of New Zealand White rabbits with 500 μ g MOPC 460 (M460) emulsified in complete Freund's adjuvant and given intraperitoneally. Rabbits were blocked for production of antibody to the 315 Id by intravenous injection of 20 mg MOPC 315 (M315) protein immediately before immunization. 3 wk after primary immunization, the rabbits were boosted with 500 μ g of M460 protein in incomplete Freund's adjuvant intraperitoneally. Again, rabbits received 20 mg of M315 protein intravenously. A further boost of 2 mg of an alum precipitate of M460 protein was given 16 d later as three successive intravenous injections. After immunization, rabbits were bled at 2-wk intervals by cardiac puncture. Antibodies specific for Id-460 were detected by Ouchterlony

double-diffusion analysis using M315, HOPC-8, and MOPC 511 proteins and normal mouse serum for comparison. Purification of rabbit antibodies directed against the hapten-binding site of the Id-460 was by a modification of the procedure of Claflin and Davie (13). To remove shared public specificities, 6 ml of antiserum was passed through a 10-ml column of M315-Sepharose. The excluded volume of this column was then absorbed to a 10-ml column of M460-Sepharose equilibrated in phosphate-buffered saline (PBS). After a wash of five times the column volume with PBS, elution of antibody directed to the hapten-binding site of M460 was performed with 6 ml of 0.05 M DNP-L-glycine in PBS. The DNP eluate was collected and dialyzed exhaustively against PBS. Antibody yields averaged $\sim 300 \mu\text{g/ml}$ serum. The purified anti-Id-460 Id antibody was stored in PBS at -20°C .

Antigens and Immunization of Mice. Ovalbumin (OVA), bovine serum albumin (BSA) and their DNP-conjugates, DNP₈-OVA and DNP₂₆-BSA, were prepared as previously described (14).

Mice were immunized by injecting 2×10^9 *Bordetella pertussis* organisms intraperitoneally followed by 100 μg of an alum precipitate of DNP₈-OVA (14). A boosting immunization of 10 μg DNP₈-OVA (in PBS-D or as an alum precipitate) was given intraperitoneally 21 d later. These mice were bled from the orbital plexus at the time of immunization and at various days after both the primary injection and the boost. Individual serum samples were stored at -20°C until assayed.

Radioimmunoassays. Id-460. The radioimmunoassay used to quantitate Id-460 in mouse serum was a modification of the solid-phase tube assay of Askenase and Leonard (15). In a typical experiment, 50 μl of rabbit anti-Id-460 antibody (20 $\mu\text{g/ml}$) in PBS was used to coat individual wells of flexible polyvinyl microtiter plates. The plates were incubated overnight at 4°C . After recovery of the anti-Id-460 antibody, the plates were washed with PBS and coated with 200 μl of 1% BSA for 30 min at room temperature. After three further washes with PBS, 50 μl of dilutions of test serum or M460 standard was added to each well along with 10 μl of ^{125}I -M460 (6,000 cpm/well). The plates were incubated overnight at 4°C , and then washed six times with distilled water. Individual wells were cut, and the counts per minute ^{125}I bound were counted in a gamma counter. The concentration of Id-460 for each serum sample was determined by plotting the percent ^{125}I -M460 bound versus the various dilutions of the serum sample. The reciprocal of the serum dilution giving 50% inhibition of binding of the ^{125}I -M460 was then multiplied by the concentration of M460 standard giving 50% inhibition of ^{125}I -M460 to yield the concentration of Id-460 in the serum. Background counts were $<1\%$. Table I shows the specificity of this assay for various myeloma proteins. This assay does not yield parallel lines for the M460 as compared to serum Id-460 material (11). Thus, the values given for serum Id-

TABLE I
Characteristics of BALB/c Myeloma Proteins and Anti-Id-460 Specificity Controls

| Tumor | Hapten specificity | Isotype | Light chain type | Nanograms necessary to inhibit 50% of ^{125}I -M460 from binding in radioimmunoassay |
|-----------|-------------------------------------|---------------|-------------------|---|
| M460 | Nitrophenyl | α | κ | 50 |
| M315 | Nitrophenyl | α | λ | 3,500 |
| XRPC 25 | Nitrophenyl | α | κ | 32,000 |
| TEPC 15 | Phosphorylcholine | α | κ | 43,000 |
| MOPC 31C | Unknown | γ_1 | κ | 80,000 |
| FLOPC 21 | Unknown | γ_{2B} | κ | 375,000 |
| MOPC 104E | α -1 \rightarrow 3-dextran | μ | λ | Not done |
| HOPC 8 | Phosphorylcholine | α | κ | Not done |
| MOPC 511 | Phosphorylcholine | α | κ, λ | Not done |
| G-1 | DNP, phosphorylcholine hybridoma | γ_1 | λ | $>600,000$ |

460 levels can not be taken as absolute. Nevertheless, all the lines obtained using serum Id-460 are parallel, and thus our distinction of high- and low-producer strains is clearly valid. Furthermore, the fact that immunization with DNP leads to dramatic changes in Id-460 levels, coupled with our finding that the induced Id-460 is all removed by passage of serum over DNP-lysyl-Sepharose (data not shown), provide strong support for our contention that Id-460 is produced by certain strains as a component of their anti-DNP antibody response.

Anti-DNP. Anti-DNP antibody concentration was also determined by solid-phase radioimmunoassay. Flexible polyvinyl microtiter plates were coated with 10 μ g DNP₂₆-BSA/well (16). The plates were washed with PBS, and then blocked using 1% BSA. Serum samples at various dilutions were added to each well and allowed to react with DNP and washed. ¹²⁵I-anti-mouse Fab (10,000 cpm/well) was allowed to react with bound antibody overnight at 4°C. The concentration of anti-DNP antibody for each serum sample was determined by plotting the percent ¹²⁵I-anti-mouse Fab bound versus the various dilutions of the serum sample. The reciprocal of the serum dilution giving 50% binding of ¹²⁵I-anti-mouse Fab was then multiplied by the concentration of anti-DNP standard at 50% binding of ¹²⁵I-anti-mouse Fab. Standard anti-DNP antibody was affinity purified from a pool of mouse anti-DNP by passage over DNP-lysyl-Sepharose.

Results

The Effect of VH Genes on the Expression of Id-460. To determine the effect of genes in the Ig-H complex on the expression of Id-460, mice of various inbred and congenic strains were immunized with DNP₈-OVA. They were bled at intervals, boosted, and again bled multiple times. The serum was tested for content of Id-460 and total anti-DNP antibody in radioimmunoassay. A typical result of this study is shown in Fig. 1 A. A summary of the results is presented in Table II. It is clear that there are low levels of Id-460 in all strains reacting in our radioimmunoassay; similar material is found also in normal mouse serum. However, if one examines the amount of Id-460 found in secondary anti-DNP responses, high- and low-producer strains can be discriminated. Strains that produce high levels of Id-460 include BALB/c and BAB-14, whereas strains giving low levels include C.B20 and B10.D2. All of these mice make substantial amounts of anti-DNP antibody (>1 mg/ml). The low response in strain C.B20 that has the genetic background of BALB/c and that bears the Ig-H complex of C57BL/6 (Ig^b) demonstrates linkage of a gene required for Id-460 expression to the Ig-H complex. The high response in strain BAB-14, which bears CH genes from C57BL/6 and VH genes derived mainly from strain BALB/c, strongly suggests that a gene(s) required for high levels of Id-460 expression is located in VH. Further studies using a variety of strains have shown that high levels of Id-460 correlate with possession of VH genes derived from BALB/c and low levels of expression with VH derived from C57BL/6 (Table II). However, one exception to this rule is seen; strain C58 does not express high levels of Id-460 even though it possesses the VH genes derived from BALB/c.

The Effect of V κ Genes on the Expression of Id-460. Strain C58 was originally included to determine the possible contribution of V κ genes to the expression of Id-460. The failure of C58 mice to make Id-460 could have been interpreted in several ways. To clearly test the involvement of V κ genes in the expression of Id-460, C.C58 and C.AKR congenic mice—which bear V κ genes from strains C58 and AKR, respectively, on a BALB/c genetic background—were tested. These mice were prepared using the Lyt-3.1 gene product as a selecting marker (12). That they express the κ -chain markers of AKR and C58 associated with Lyt-3.1 was confirmed by determining I_B peptide, Ef-1^a, and Ef-2 marker expression in these mice (W.-T. Hum, D. Gibson, and

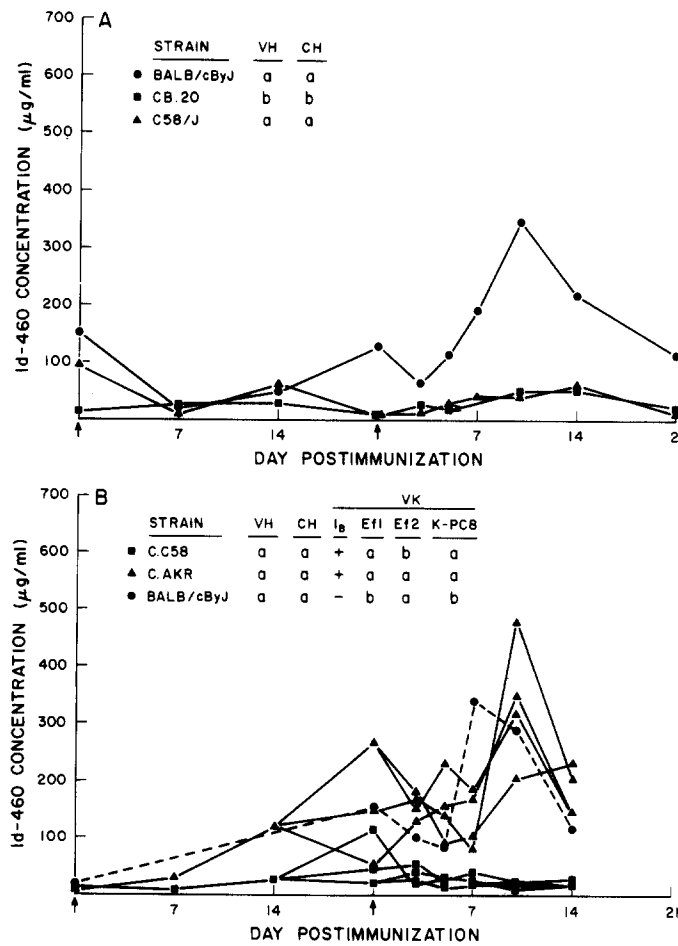


FIG. 1 A AND B

FIG. 1. Mice of various strains were immunized with 100 μ g DNP₈-OVA on alum plus *B. pertussis* on day 0 and boosted with 10 μ g DNP₈-OVA on day 21. Groups of four mice were bled, and levels of Id-460 and of anti-DNP antibody determined. Values represent geometric mean values, except in B, where individual mice are shown with a pool serum from 10 BALB/c mice. SE < 1.4, and are omitted. All mice made > 1 mg anti-DNP antibody during the secondary response. (A) Effect of Igh genes. (B) Effect of V κ genes. (C) Effect of H-2. (D) Effect of the Xid gene.

P. D. Gottlieb. Unpublished results.). The mice were immunized along with BALB/c mice, and the Id-460 levels determined at several times. The results are shown in Fig. 1 B. It is clear that mice having V κ genes derived from strain C58 do not express significant amounts of Id-460 in this response, whereas mice that bear V κ genes derived from AKR do indeed make normal levels of Id-460. Thus, expression of Id-460 depends on genes mapping to V κ as well as to VH, and expression of Id-460 discriminates between the V κ genes carried by AKR and the V κ genes carried by C58.

The Effect of MHC Genes on Id-460 Expression. The effect of MHC genes on the expression of Id-460 in our system was examined by immunizing strains BALB/c (H-2^d), BALB.B (H-2^b), and BALB.K (H-2^k). These strains are congenic mice differing

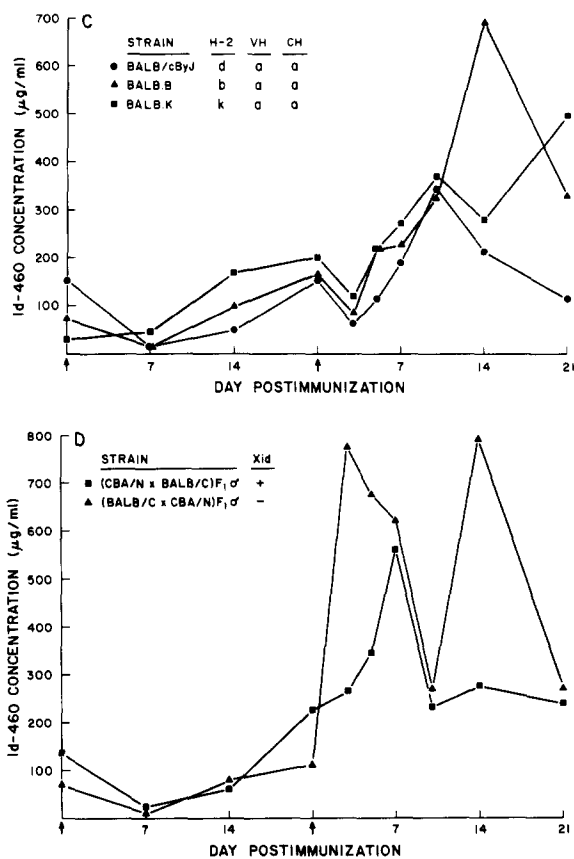


FIG. 1. C AND D

only for MHC-linked genes. The results of one of two experiments are given in Fig. 1 C. It is clear that MHC-linked genes may have a quantitative effect on the expression of Id-460, but do not in any absolute way control Id-460 expression. All strains make strong anti-DNP responses in this system. Ir genes that have been described for the immune response to OVA do not operate with the high doses of antigen we have used, and would, in any case, predict higher responses in H-2^d than in H-2^k mice.

The Effect of Xid Genes on Id-460 Expression. The immune defective mouse strain CBA/N, which carries the Xid gene, and the F₁ male progeny of (CBA/N x BALB/c) matings do not express the VH-linked Id associated with the phosphorylcholine-binding BALB/c myeloma protein TEPC 15, whereas the F₁ female progeny express normal levels of this Id (17). Reciprocal F₁ male offspring of CBA/N and BALB/c parental strains were immunized with DNP₈-OVA to determine if the expression of Id-460 was controlled or affected by the Xid gene. The results are given in Fig. 1 D. Although it is apparent that the nondefective F₁ males make more Id-460 than do their defective F₁ male counterparts, the differences are at most twofold, and both sets of mice make very high levels of Id-460. Thus, the Xid gene does not preclude Id-460 expression in this response.

TABLE II
Expression of Id-460 During In Vivo Anti-DNP_K-OVA Responses in Various Mouse Strains

| Strain | H-2 | CH | VH | IgV κ | Xid | Geometric mean (\times/\pm relative SE) maximum concen- tration of Id-460 |
|--|-----|-------------------|-------------------|--------------|-----|---|
| | | | | | | $\mu\text{g/ml}$ |
| BALB/cByJ | d | a | a | +* | - | 421.9 \times 1.22 |
| BALB.K | k | a | a | + | - | 497.8 \times 1.31 |
| BALB.B | b | a | a | + | - | 692.9 \times 1.39 |
| B.C9 | b | a | a | + | - | 212.6 \times 1.31 |
| BAB.14 | d | b | a | + | - | 277.2 \ddagger |
| CBA/J | k | a ⁻ | a ⁻ | + | - | 264.0 \ddagger |
| (BALB/c \times CBA/N)F ₁ δ | dxk | axa ⁻ | axa ⁻ | + | - | 741.4 \times 1.22 |
| (CBA/N \times BALB/c)F ₁ δ | kxd | a ⁻ xa | a ⁻ xa | + | + | 561.8 \times 1.25 |
| CB.20 | d | b | b | + | - | 53.9 \times 1.33 |
| B10.D2/oSn | d | b | b | + | - | 49.3 \times 1.28 |
| C57BL/6 | b | b | b | + | - | 18.5 \ddagger |
| C58/J | k | a | a | - | - | 59.5 \times 1.30 |

* +, V κ allele associated with Lyt-3^b allele; -, V κ allele associated with Lyt-3^a.

\ddagger Id-460 levels in pooled serum samples.

Discussion

In these studies, we have examined the effect upon the expression of Id-460 of four different gene complexes that are known to control the quantity or quality of antibody responses. We have succeeded in mapping at least two genes required for Id-460 expression to VH and to V κ . We have also shown that MHC-linked genes and the Xid gene have little effect on Id-460 expression.

Most Id that have been studied demonstrate linkage to VH genes. Thus, the linkage of Id-460 to VH conforms to expectations for an inherited Id (1). Furthermore, after the discovery of V κ polymorphisms, linkage of Id to V κ genes has also been shown for some Id (2). However, Id-460 is the first Id to distinguish between V κ genes in strains C58 and AKR. A recent publication by Gibson and MacLean (3) has defined a marker (Ef2) that distinguishes V κ genes of C58 from those of AKR and BALB/c. The Ef2 marker appears to define a V κ group. More recently, Gibson et al.² have shown that κ -chains from BALB/c myeloma proteins that focus at this position belong to the V κ 1 group, as does the M460 κ -chain. Thus, the finding that Id-460 expression correlates with Ef2 is supported by amino acid sequence data.² The finding of involvement of V κ genes in Id-460 is further strengthened by the failure of the G-1 monoclonal antibody to inhibit our anti-Id-460 assay. This monoclonal antibody binds to DNP and has a VH sequence identical to that of M460 as far as has been determined (45 residues), except at position 31 (P. Gearhart, Personal communication.), but has a λ -light chain.

The lack of effect of the Xid gene on the expression of Id-460 is of interest because

² Dzierzak, E. A., R. W. Rosenstein, and C. A. Janeway, Jr. The expression of an idiotype (Id-460) during *in vivo* anti-hapten antibody response. II. Transient idiotypic dominance. Manuscript in preparation.

this gene greatly influences the expression of the T15 Id. It has also been said to control the expression of other Id (R. Woodland. Personal communication.). It is of interest to us because it is our belief that the finding of high levels of Id-460 shortly after boosting in the secondary response reflects the activity of a unique set of helper T cells required for optimal production of anti-DNP antibody early in the response (18, 19). In other systems, this helper T cell would appear to operate by selecting particular sets of B cells for activation by means of an anti-idiotypic receptor (20-22). Because Id-460 appears to be the dominant Id in the response to DNP-OVA expressed early in the secondary response,² and because careful analysis of the functioning of this specialized set of helper T cells in CBA/N mice for the anti-DNP antibody response has not detected any abnormalities (23), the finding of normal expression of Id-460 would be expected. In the T15 system, failure to express the T15 idiotype in (CBA/N × BALB/c)F₁ males is correlated with the absence of the Id-recognizing helper T cell set for the T15 idiotype (20). Further analysis of helper T cells for the Id-460 response would be expected to confirm their presence in (CBA/N × BALB/c)F₁ male mice.

These studies can not resolve the question of whether the genes we have mapped to V_H and V_κ are structural or regulatory genes. The linkage of V_κ genetic markers to Lyt-3 had suggested a possible regulatory effect of this locus on κ-chain expression, and this was of interest because Lyt-3 is expressed on suppressor T cells but not on helper T cells (24). However, gene mapping data have shown that the structural genes for V_κ are indeed on chromosome 6 (25). Furthermore, our results show that Id-460 expression is not controlled by Lyt-3 itself, because mice carrying Lyt-3.1 derived from strain AKR express the Id, whereas mice that carry Lyt-3.1 derived from strain C58 do not express the Id. The data of Gibson et al. (26) strongly support the structural nature of this genetic difference, although activity of an unidentified regulatory gene linked to the structural gene still can not be excluded.

It is clear from other work that Id-460 expression is strongly regulated,² and this can be seen in the dramatic fluctuations observed during the course of an antibody response to DNP-OVA (Fig. 1). Subsequent experiments will focus on the time-course of expression of Id-460 at the antibody-forming-cell level, with particular attention on the regulation of Id within particular isotypes.² The cellular basis of this regulation is now being studied.

Summary

The genetic control of the expression of an idiotype (Id-460) associated with the 2,4-dinitrophenyl (DNP)-binding BALB/c myeloma protein MOPC 460 was studied using congenic strains of mice. It was shown that the expression of high levels of Id-460 during secondary *in vivo* anti-DNP-ovalbumin responses was determined by genes governing immunoglobulin heavy-chain variable and κ-light chain variable regions (V_κ). Appropriate alleles at both loci were required for the expression of Id-460. Genes in the major histocompatibility complex and the X-linked immune deficiency gene found in strain CBA/N did not greatly affect Id-460 expression. The V_κ gene controlling Id-460 expression can be differentiated from Lyt-3, and it is the first instance in which expression of an idiotype subdivides the V_κ genes associated with the Lyt-3^a allele. Although it is likely that the V_κ gene(s) involved are structural, the involvement of a regulatory gene linked to the structural gene can not be excluded.

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