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

III. Detection of Id-460 in Normal Serum That Does Not Bind Dinitrophenyl*

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In Jerne's network theory (1), the immune system is envisaged as a web of interactions between immunoglobulin variable domains. In this schema, regulation (amplification and suppression) of the immune response occurs when anti-idiotype antibody (Ab2) reacts with idiotypes (Ab1) induced by foreign antigenic stimulation. Ab2 also induces and reacts with a nonspecific parallel set of antibody (Ab3)-bearing idiotypes shared with Ab1 but not binding the immunizing antigen. Induction, amplification, and suppression of the immune response thus result from the regulatory network of idiotype-anti-idiotype interactions.

To probe the importance of idiotypic regulation on the heterogeneous response to the hapten 2,4-dinitrophenyl (DNP),1 we have studied the expression of an idiotype (Id-460) associated with the DNP-binding, BALB/c IgA,κ myeloma protein MOPC 460 during in vivo anti-DNP antibody responses (2). Id-460 is expressed in a transiently dominant manner both in serum and by splenic anti-DNP plaque-forming cells; dominant expression occurs early in the secondary in vivo response to DNP-ovalbumin (OVA) in mice having Ig-Vκ and able to express light chains of the Vκ group (2, 3). We have previously demonstrated (4-6) the existence of two helper T cells (Th) acting synergistically early in the course of in vivo secondary anti-DNP responses. One of these two Th was shown by adoptive transfer experiments to be lacking in T cells derived from mice treated from birth with anti-μ antibody (7). This suggested the need for immunoglobulin (Ig) in the differentiation of this T cell. This cell was also shown to be Lyt-1+,2+ and antigen specific (8). We have proposed that this cell recognizes idiotype and is responsible for the dominant expression of Id-460 in the anti-DNP response. This is consistent with findings of Ig-recognizing T cells in other major idiotypic systems (9-11). However, in these other systems idiotype is associated with antibody directed against bacterial antigens. If inherited idiotypes are primarily directed at common environmental antigens and are important in resistance to common pathogens (12), the existence of an inherited, dominant idiotype associated

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Abbreviations used in this paper: Ab1, idiotypic antibodies; Ab2, anti-idiotype antibody; Ab3, nonspecific parallel set of antibodies; BSA, bovine serum albumin; DNP, 2,4-dinitrophenyl; DNP-lysyl-glycine; LPS, lipopolysaccharide; M1g, mouse immunoglobulin; OVA, ovalbumin; RIA, radioimmunoassay; Th, helper T cell; Th1g, immunoglobulin-dependent Th.
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with antibody specific for the synthetic hapten DNP seems anomalous. Since DNP is an artificial antigen that the immune system of the mouse is unlikely to encounter in nature, it seems more likely that the DNP-binding Id-460 we have previously measured should be regarded as a member of the nonspecific parallel set of antibody (Ab3) in the Jerne network theory. This interpretation is supported in the present study by the detection of a non-DNP-binding Id-460 component in preimmune mouse serum that appears to be normal and perhaps inherited form of Id-460, or Ab1.

In our studies of Id-460 expression, we have noted the presence of idiotype (10–100 μg/ml) in the preimmune serum of mice. Original studies of idiotype expression in immune responses used anti-idiotype antibody absorbed on preimmune serum (13). However, Leibman (14) has clearly demonstrated the presence of the TEPC 15 (T15) idiotype in preimmune serum when the anti-idiotype is absorbed with a mixture of myeloma proteins rather than with normal serum.

In this paper, we demonstrate that serum from nonimmunized mice has significant amounts of Id-460 and that this Id-460 is present on non-DNP-binding immunoglobulin. We further report three hybridoma proteins derived by fusion of SP2/0 Ag14 myeloma cells with lipopolysaccharide (LPS)-stimulated normal BALB/c spleen cells that also possess these characteristics. We propose that this Id-460-positive, non-DNP-binding Ig is the biologically relevant form of Id-460, and that this material induces the anti-Id-460 regulatory interactions that lead to the transiently dominant expression of Id-460 in antigen-induced anti-DNP responses.

Materials and Methods

Mice, Immunizations, and Serum. Mice of strains BALB/cByJ, AKR/J, and DBA/2J were purchased from The Jackson Laboratory, Bar Harbor, Maine. BALB.K mice were kindly provided by Donal Murphy of the Comprehensive Cancer Center, Yale University, New Haven, Conn. Other mice, myeloma proteins, immunization procedures, and radioimmunoassays (RIA) are all as previously described (2). Rabbit, goat, guinea pig, and rat sera were obtained from animals maintained at Yale University. Serum from germ-free DBA/2 and AKR mice was a generous gift of Dr. Morris Pollard (Lobund Laboratories, University of Notre Dame, Notre Dame, Ind.).

Anti-μ-treated Mice. BALB/cByJ mice were injected intraperitoneally with rabbit anti-mouse μ chain antibody, starting within 24 h of birth. A total of 200 μl/wk of a 2 times ammonium sulfate concentrate of rabbit anti-μ was given in three weekly injections until the time of the experiment. Such mice have no detectable B cells, little serum Ig, but relatively normal T cell numbers and function (7, 15, 16). Serum Ig levels were determined by radial immunodiffusion on commercially prepared plates (Meloy Laboratories, Inc., Springfield, Va.). Dr. Dean Manning (University of Wisconsin, Madison, Wis.) kindly provided serum from his colony of anti-μ-treated mice to confirm our findings.

Hybridoma and Screening Procedures. Individual spleens from BALB/cByJ mice were stimulated with 100 μg/ml LPS for 2 d in 10 ml RPMI 1640 medium supplemented with 20% fetal calf serum. The activated cells were washed and fused with the nonsecreting cell line, SP2/0 Ag14, according to the procedures of Oi and Herzenberg (17), Kennet et al. (18), and Lerner et al. (19). Spleen cells and SP2/0 cells were mixed in a ratio of 1:2.

Supernates were screened for Id-460 by competitive inhibition RIA and were tested for hemagglutinating activity on 2,4,6-trinitrophenyl-sheep erythrocytes prepared according to the procedure of Shearer (3, 20). After screening, cells were cloned on agar and grown as ascites tumors in pristane primed BALB/c mice or in tissue culture. The Ig fraction of ascites fluid was purified using 45%-saturated ammonium sulfate precipitation. Aliquots of protein were stored at 4°C.

The isotype of each monoclonal antibody was determined by the radial immunodiffusion method on commercially prepared plates.
**Sepharose Preparations.** Mouse Ig (MIg), DNP-lysyl-glycine (DNP-L-GL), preimmune guinea pig Ig, and bovine serum albumin (BSA) were conjugated to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N. J.) by standard procedures. The MIg was obtained from normal BALB/c mice, precipitated in 45% ammonium sulfate, and absorbed on DNP-L-GL Sepharose before conjugation. Guinea pig serum was also precipitated in 45% ammonium sulfate before conjugation. Aliquots of BSA-Sepharose were dinitrophenylated using 2,4-dinitrofluorobenzene in dioxane to produce substitution ratios of 2 or 20 DNP groups per BSA.

**Absorption Analysis.** In a typical study, DNP-L-GL Sepharose and MIg Sepharose were washed extensively in 1 M acetic acid and equilibrated in phosphate-buffered saline until the OD_{280} of the effluent was <0.020. Either 800 or 500 µl of packed Sepharose beads was used for absorption of 200 or 500 µl samples, respectively, in small conical microfuge tubes. Samples of preimmune serum, hyperimmune serum, and MOPC 460 protein were mixed and incubated 30 min at room temperature. After a 30-s spin in the microfuge, effluents were decanted and assayed for Id-460 by competitive inhibition RIA. Dilution during absorption was considered to be 1:5 or 1:2. Various controls showed that by this method anti-DNP antibody is specifically absorbed. MIg-Sepharose was used as a measure of nonspecific binding, and studies with MOPC 460 protein demonstrated the efficiency of the DNP-Sepharose absorption.

**Results**

In previous studies, we have used affinity-purified, site-specific rabbit anti-M460 antibodies to determine the genetics of expression of an idiotype (Id-460) during in vivo responses to DNP-OVA. This antibody was first absorbed with the DNP-binding IgA,λ myeloma MOPC 315, then eluted with DNP-glycine from a column of MOPC 460. This antibody detected a determinant that was expressed in large amounts during in vivo anti-DNP-OVA responses of mice having Ig-h-V genes from BALB/c mice, provided that the responding strain was able to express κ light chains of the Vº1 group (2). Because later studies of this response in collaboration with Dr. David Gibson (personal communication) had demonstrated that Ig-h-V genes determine to some extent κ chain expression in anti-DNP responses, the possibility existed that the reagent was an anti-Vκ1 antibody. To test this, several Vκ1 myeloma proteins were tested for reaction with this antibody in the competition radioimmunoassay of 125I-M460 binding. As can be seen in Fig. 1, these proteins did not block significantly in this assay. In the present experiments, we have used this basic system to determine the expression and antigen specificity of Id-460 in the sera of nonimmune mice.

**Expression of Id-460 in Normal Mouse Serum.** Analysis of preimmune serum from individual BALB/cByJ mice has revealed concentrations of Id-460 ranging from 10 to 100 µg/ml. Unlike the expression of Id-460 during anti-DNP-OVA responses, we find Id-460 in the normal serum of all strains thus far tested (Table 1). In all cases, essentially complete inhibition of the reaction of labeled M460 to anti-Id-460 is obtained. Normal serum from other species (rat, guinea pig, rabbit, and goat) inhibits far less well than normal mouse serum; the best xenogeneic inhibitor was normal rat serum, which had 16-fold less Id-460 than normal mouse serum (Fig. 2), while the other sera had 19- to 1,500-fold less inhibitory activity.

**Evidence that Id-460 in Normal Mouse Serum Is Ig.** We have used several approaches in attempting to demonstrate that the Id-460-bearing material in normal serum is Ig. The material precipitates in 45%-saturated ammonium sulfate, and, when bound to rabbit anti-Id-460, it is bound by affinity-purified radiolabeled goat anti-mouse Ig (data not shown). More convincing is the finding that serum from mice treated from birth with anti-μ chain antibody such that they have no B cells and little or no serum Ig express little or no Id-460 (Fig. 3). Normal BALB/c age-matched mice in this
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Figure 1. Inhibition of $^{125}$I-MOPC 460 binding to anti-Id-460 by various myeloma proteins whose κ chains belong to the VκI group: MOPC 460 (X), FLOPC 21 (●), FLOPC 1 (○), TEPC 821 (■), TEPC 105 (□), TEPC 602 (▲), and TEPC 817 (△).

Table 1

| Strain                        | IgGκ | IgVH | H-2 | Concentration of Id-460* | μg/ml |
|-------------------------------|------|------|-----|--------------------------|-------|
| BALB/cByJ                     | a    | a    | d   |                          | 70.2  |
| BALB.K                        | a    | a    | k   |                          | 28.8  |
| BALB.B                        | a    | a    | b   |                          | 72.4  |
| B.C9                          | a    | a    | b   |                          | 125.1 |
| BAB.14                        | b    | a    | d   |                          | 26.6  |
| CBA/J                         | a−   | a−   | k   |                          | 33.0  |
| (BALB/c × CBA/N)F1J           | a × a−| a × a−| d × k|                          | 73.4  |
| (CBA/N × BALB/c)F1J           | a × a| a × a| k × d|                          | 138.3 |
| CB.20                         | b    | b    | d   |                          | 13.1  |
| B10.D2/oSn                    | b    | b    | d   |                          | 260.2 |
| C57Bl/6                       | b    | b    | b   |                          | 40.4  |
| C3H/J                         | a    | a    | k   |                          | 96.7  |

* See reference 2.

§ Geometric mean (× rel. SE).

§ Id-460 levels in pooled normal serum samples.

Experiment had 11.0 ± 1.14 μg/ml Id-460, whereas anti-μ treated mice had 0.8 ± 1.42. On radial immunodiffusion, these mice had no IgM and low levels of IgGκ1. These results were confirmed by testing sera from more completely suppressed mice.
Id-460 in Normal Mouse Serum Does Not Bind DNP. Id-460 levels increase markedly during in vivo anti-DNP-OVA responses in mice that have VH genes derived from BALB/c, but not in mice that have VH genes derived from C57BL/6; also, to express high levels of Id-460 during anti-DNP responses, mice must be able to express light chains of the VK1 group (2) (Table I). When sera from BALB.K mice bled 10 d after secondary immunization with DNP-OVA are absorbed with DNP-sepharose, most of the Id-460 material is removed (Fig. 4). However, Id-460 in normal mouse serum is not removed by absorption with the same DNP-sepharose (Fig. 4). M460 is also prepared by Dr. Dean Manning and kindly provided to us (Fig. 3). From this, we conclude that the expression of Id-460 in normal serum is dependent on normal B cell function, and most likely represents immunoglobulin rather than an idiotype-bearing T cell product, although the latter cannot be conclusively ruled out by these experiments (see below).
absorbed by DNP-sepharose. Therefore, Id-460 exists in two forms, DNP-binding and non-DNP-binding; the form found in normal serum is largely or entirely non-DNP-binding. It is interesting to note that the non-DNP-binding form does not significantly increase, or may even decrease, during the anti-DNP-OVA response. In the example
in Fig. 4, preimmune serum from BALB.K mice had 51.7 \(\mu g/ml\), whereas in immune serum the level is 25.4 \(\mu g/ml\).

LPS-activated Normal BALB/c Spleen Cells Given Rise to Id-460-positive, Non-DNP-binding Hybridomas. To study the Id-460-positive, non-DNP-binding Ig component of normal serum in pure form, normal BALB/c spleen cells were activated in vitro for 2 d with LPS and fused to the nonsecreting myeloma SP2/0 Ag14. Supernates were screened for Id-460 production by competition RIA. From one such fusion, 60 wells were plated with \(10^6\) cells per well, and all wells had growth; supernatant fluid from one well gave >50% inhibition in the RIA. From this well, hybridoma LB10 was cloned. The IgM protein produced did not bind to DNP-Sepharose (Fig. 5). In a second fusion, 86% of 104 wells were positive for growth, and three supernates inhibited >50%. Two of these were cloned and further tested (LF4 and LB8b). LB8b was also non-DNP-binding. LF4 shows marginal binding to DNP. The third positive hybrid was lost (Fig. 6).

Serum from Germ-free Mice Has Decreased Amounts of Id-460. To test the hypothesis that Id-460 in normal mouse serum represents antibody produced in response to environmental pathogens, Id-460 levels were measured in the serum of germ-free normal mice. As seen in Fig. 7, normal mouse serum from germ-free DBA/2 and AKR/J mice contained about five times less Id-460 than did serum from age-matched, conventionally reared mice of the same two strains.

Discussion

In previous studies, we have characterized the genetic control and time-course of expression of an idiotype, Id-460, during in vivo anti-DNP responses. Expression of this idiotype in anti-DNP responses is controlled by genes mapping to Igh-V and to Vk (2). During secondary responses, Id-460 shows transient dominance of the anti-DNP serum antibody and splenic plaque-forming cell response (3). Its time-course of expression correlates with previous studies on the time-course of expression of a second,
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Fig. 6. Absorption analysis of MOPC 460 (50 μg/ml) (upper left) and ascites produced by hybridomas LB10 (upper right), LB8b (lower left), and LF4 (lower right) with BSA-Sepharose (▲), DNP2-BSA-Sepharose (X), DNP20-BSA-sepharose (●), or tested unabsorbed (■). Assay as in Fig. 1.

Fig. 7. Comparison of Id-460 levels in normal serum from conventionally reared (X) or germ-free, age-matched (●) mice of strains AKR/J (---) and DBA/2 (→). Mean percent binding for groups of five sera each. Assay as in Fig. 1.

Ig-dependent Th (ThIg) (3, 7). Studies of ThIg activity in this and other systems (7, 10, 11) suggest that ThIg specific for idiotype require expression of the idiotype as Ig for their development. In the present experiments, we have shown that Id-460 is represented in significant amounts in normal mouse serum. This Id-460 does not show detectable binding to DNP. Similar results were obtained with three monoclonal antibodies derived from LPS-activated spleen cells from normal BALB/c mice. Thus,
in the Id-460 system, at least two forms of the idiotype can be distinguished, a DNP-binding form produced in large amounts upon immunization with DNP, and a non-DNP-binding form found in normal mouse serum. This clearly dissociates Id-460 from antigen binding specificity in this system, despite the fact that the anti-idiotypic reagent was eluted from M460-Sepharose with hapten and should be binding-site specific. The antigen binding specificity of the Id-460 found in normal serum is not yet known; the finding that Id-460 is present in reduced amounts in germ-free normal mouse serum suggests that it may be specific for environmental pathogens.

Immune responses to specific antigens are often associated with the production of antibody bearing a particular idiotype. Analysis of such responses suggests a close relationship between the antigen-binding site and idiotype (13, 14), a finding consistent with many studies showing that idiotype-anti-idiotypic interactions may be inhibited with free hapten (21). However, it is clear from a number of other studies (22–26) that idiotypic determinants on an Ig molecule do not always correlate with its specificity for antigen. Oudin and Cazenave (22) first showed that rabbit idiotypic antibody produced in response to OVA does not necessarily bind to OVA. Dissociation of idiotype and antigen-binding specificity was also clearly demonstrated in studies by Eichmann et al. (23), in which LPS-activated B cells produced the A5A idiotype, and only 45% of the idiotype-producing B cells made Ig that bound the Strep A carbohydrate, the defining antigen in the A5A system. Most recently, a dominant idiotypic marker for the mouse response to hen egg lysozyme has been demonstrated on monoclonal antibodies reactive with two completely distinct antigenic determinants on the lysozyme molecule (24). Finally, in the arsonate system, it has been possible to raise cross-reactive idiotype-bearing immunoglobulins that do not bind arsonate by immunization with anti-idiotypic antibody (25).

Jerne’s network theory postulates anti-idiotypic effects that could explain these findings (1). As shown in Fig. 8, antigen induces cells bearing Ab1. Ab1 in turn, either by itself or complexed with antigen (27), activates Ab2 and regulatory cells. Ab2 and anti-idiotypic regulatory cells can either induce or suppress the production of Ab1, and also interact with a further set of immunoglobulins (Ab3) and cells that bear

![Fig. 8](image-url)

FIG. 8. Schematic version of Jerne’s (1) network hypothesis. Three main sets of cells and molecules are shown. An antigen-induced set produces idiotype that triggers a regulatory set of cells and molecules that can act to enhance or suppress cells bearing idiotype. Such cells occur in two sets: the induced, antigen-specific set and a nonspecific parallel set that does not bind the inducing antigen but presumably binds to other antigens.
idiotypic determinants related to Ab1 but not able to bind the antigen. These Ab3 molecules and related cells are referred to as the nonspecific parallel set.

To interpret our findings in the context of Jerne's network hypothesis, we propose that the normal immune response to common environmental antigens generates an antigen-induced set of idiotypic antibodies (Ab1). Among these is the Id-460-positive, non-DNP-binding immunoglobulin that we detect in normal mouse serum. This Ab1 will induce the regulatory set of anti-idiotypic cells and molecules (Ab2). In the DNP system, we have evidence for ThIg (7) that acts to greatly augment the early secondary anti-DNP antibody response in adoptive transfer experiments (4-8). It is antigen specific but does not require a hapten-carrier bridge (8). It is absent in mice that are treated from birth with anti-μ chain antibody so that they have no B cells or circulating Ig (7) or Id-460 (Fig. 2). In studies on the phosphorylcholine-T15 idiotype system, such cells have been termed ThId and act to greatly augment idiotype production (10, 11). We would thus suggest that these Th idiotypes are required for the transient idiotypic dominance we observe for Id-460 in secondary in vivo anti-DNP antibody responses; we propose that ThId are induced by the non-DNP-binding form of Id-460 found in normal mouse serum, and act during anti-DNP antibody responses to greatly increase Id-460 production as anti-DNP. In such responses, Id-460 is DNP specific because its production is driven by DNP-OVA and H-2-restricted OVA-specific helper cells, on which such responses are absolutely dependent (8, 10, 11). Thus, Id-460 binding to DNP would fit as a nonspecific parallel set in this interpretation, and the antigen(s) that Id-460 has evolved to recognize are environmental antigens antigenically unrelated to DNP. This interpretation is outlined schematically in Fig. 9. Thus, we suggest that the induced, biologically relevant form of Id-460 is the non-DNP-binding form, whereas the nonspecific parallel set is the Id-460-positive, DNP-binding set induced by DNP-OVA immunization. The dominance of Id-460 in the secondary anti-DNP antibody response is the result of idiotype-specific amplification, induced initially by the normal serum form of Id-460.

A variant of this interpretation is suggested by a recent paper by Conger et al. (28). They have demonstrated changes in expression of the cross-reactive idiotype in responses to azophenyl arsonate that are similar to those seen with Id-460. They also observe an increase in affinity of idiotype-positive antibody during such responses. It is possible that the marginal DNP binding seen with LF4 represents a primary form of anti-DNP bearing Id-460, whose affinity increases upon antigenic stimulation. We believe this interpretation to be unlikely because the apparent affinity of LF4 for DNP is so low, but it cannot be ruled out.

Our current interest is to define the antigen specificity of Id-460-positive, non-DNP-

![Fig. 9. The Id-460 system as we envision it in the content of Jerne's (1) network hypothesis. An unknown environmental antigen triggers an induced set to produce Id-460 that does not bind DNP. This activates regulatory (anti-idiotypic) cells that, in the presence of DNP-OVA and appropriate Th induce expression of DNP-binding Id-460. We refer to the anti-DNP as the nonspecific parallel set, because the primary antigen in the system appears to be antigenically unrelated to DNP.](image-url)
binding immunoglobulins from normal serum, taking advantage of the hybridomas we have produced. We are testing the hypothesis that these molecules are specific for common environmental antigens, as is true for another idiotype found in large amounts in normal serum, T15 (14). Indeed, it has recently been shown that normal serum anti-phosphorylcholine, the majority of which is T15 idiotype positive, is protective against pneumococcal infection in mice (12). It is striking that most of the inherited idiotypes are in fact directed at bacterial antigens: T15 is anti-phosphorylcholine; A5A is anti-Strep A carbohydrate; MOPC 104E is anti-dextran; UPC 10 is anti-levan. Because germ-free normal mouse serum has less Id-460 than control serum from conventionally reared mice, it seems likely that this form of Id-460 is also antibacterial. We are currently testing our hybridomas against a variety of bacterial antigens.

Summary

Using an anti-idiotypic antibody previously characterized as specific for the hapten binding site of the 2,4-dinitrophenyl (DNP)-binding BALB/c myeloma protein MOPC-460, we have detected substantial amounts of this idiotype (Id-460) in the serum of normal mice. Whereas the idiotypic material in DNP-immune serum binds to DNP, the Id-460-positive material in normal mouse serum is not specific for DNP. The material in normal serum appears to be immunoglobulin. Furthermore, Id-460-positive, non-DNP-binding monoclonal immunoglobulins that completely inhibit our assay for Id-460 are repeatedly isolated when hybridomas are prepared from LPS-activated normal spleen cells. These data are interpreted in the context of Jerne's network hypothesis. It is our conclusion that the non-DNP-binding form of Id-460 is the inherited form and that this form establishes an idiotypic network favoring the production of anti-DNP-bearing Id-460. Thus, the paradox of finding an inherited idiotype in the antibody response to the nonpathogen DNP may be resolved by proposing that the true form of Id-460 is specific for an environmental pathogen and that Id-460 dominance in the anti-DNP response is simply a consequence of idiotype-specific regulatory events preconditioned by Id-460-bearing immunoglobulin specific for antigenic determinants unrelated to DNP.

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