

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

II. Transient Idiotypic Dominance*

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We have previously demonstrated that the in vivo adoptive secondary antibody response to 2,4-dinitrophenyl-ovalbumin (DNP-OVA)¹ in mice involves the synergistic activity of two distinct helper T cells (1-4). One of these helper T cells is specific for antigen and is thought to be major histocompatibility complex (MHC) restricted in its activity. It requires a hapten-carrier bridge for its function and is absolutely required for in vivo anti-hapten antibody responses in our system (5). The other helper T cell is also specific for antigen, but it does not require a hapten-carrier bridge (5). The second cell is not found in mice treated from birth with anti- μ chain antibody, which led us to propose that this cell actually recognizes B cells directly via their immunoglobulin (Ig) receptor (4). Both helper T cells are Lyt-1⁺,2⁻ (5). The action of the Ig-recognizing helper T cell is transient in secondary antibody responses (1-4). Thus, its major effects are seen by 5-7 d after boosting in adoptive transfer experiments, whereas by 10-12 d, its effects are no longer apparent.

Experiments examining the expression of a variety of Ig idiotypes expressed in response to different antigens are consistent with these findings (6-8). In particular, experiments of Bottomly and co-workers (8, 9) have shown similar results in response to phosphorylcholine (PC), using the TEPC15 idiotypic as a marker for the function of Ig-recognizing T cells. Furthermore, T cells have been implicated in regulation of the isotype (10), allotype (11), charge (12), and affinity (13, 14) of antibody responses.

We have proposed (15) that all of these regulatory effects of helper T cells upon antibody quality might be explained by Ig-recognizing helper T cells. Furthermore, we have suggested that the regulation of isotype might reflect the combined action of idiotypic-specific helper T cells and a restricted expression of idiotypes within certain isotypes, rather than regulation of Ig-isotype per se by such helper T cells.

To study such questions further in the response to the hapten DNP, we have here examined the time-course of expression of a major idiotypic during an in vivo anti-DNP antibody response. The genetics and immunochemistry of this idiotypic (Id-460), found in the hapten-binding site of the DNP-binding myeloma protein MOPC 460,

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¹ *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; DNP, 2,4-dinitrophenyl; MHC, major histocompatibility complex; MIg, mouse immunoglobulin; OVA, ovalbumin; PBS, phosphate-buffered saline; PC, phosphorylcholine; PFC, plaque-forming cells; SRBC, sheep erythrocytes; TNP, 2,4,6-trinitrophenyl.

have been previously characterized in our laboratories (16-19). The present experiments demonstrate several interesting points about Id-460. First, expression of Id-460 in serum varies markedly with time after immunization, often peaking very shortly after boosting, and then falling rapidly. Second, the anti-DNP response does not parallel the expression of Id-460 in serum or in splenic plaque-forming cells (PFC). Third, Id-460 expression shows no apparent restriction to any of the isotypes (IgM, IgE, IgG2a, and IgG1) that we have measured. Rather, it would appear that Id-460⁺ clones are selectively activated early in secondary anti-DNP antibody responses, possibly reflecting the activity of Id-460-specific helper T cells. Later, Id-460⁻ antibody predominates in this response. The genetic and cellular basis of this complex regulatory process will be examined in subsequent experiments.

Materials and Methods

Methods Previously Described. Mice, antigens, myeloma proteins, antisera, immunizations, and radioimmunoassays for Id-460 and for anti-DNP antibody are all as previously described (19).

Hemolytic Plaque Assay. Spleen cells were assayed for direct, indirect, and specific subclass anti-TNP PFC and for Id-460⁺ PFC by the Cunningham and Szenberg modification of the Jerne plaque technique (20). Trinitrophenylated sheep erythrocytes (TNP-SRBC) were prepared as follows: 1 ml packed SRBC (Colorado Serum Co., Denver, Colo.) were mixed with 4 ml 10 mM trinitrobenzenesulfonic acid in phosphate-buffered saline (PBS), pH 7.4, at 37°C for 12 min and washed extensively with PBS (21). TNP-SRBC were used as 1% suspension in RPMI 1640 medium buffered with 10 mM Hepes. Guinea pig serum, as a source of complement, was absorbed on TNP-SRBC to remove hemolytic antibody. All dilutions were done in RPMI 1640.

The use of various plaque-developing or -inhibiting antisera was required for quantitation of direct, indirect (specific subclass), and Id-460⁺ plaques. Direct plaques were inhibited by goat anti-mouse IgM antiserum. Anti- μ chain antibody was produced in a goat twice immunized with 100 μ g MOPC 104E in complete Freund's adjuvant (CFA) given immediately after intravenous injection of 5 mg DEAE-purified mouse Ig (MIg). Immunoelectrophoresis has shown this antiserum to be specific for mouse IgM. Nonspecific developing activities were eliminated by passage of the antiserum over a Sepharose immunoadsorbent column consisting of an IgA and a variety of IgG myeloma proteins (McPC 603, MOPC 31c, MPC 11, FLOPC 21, and MOPC 173).

Developing antiserum for indirect PFC was obtained from rabbits immunized intraperitoneally with 1 mg of DEAE-purified MIg in CFA, followed 2 wk later by an intravenous boost of 4 mg alum-precipitated MIg. To remove nonspecific activity, the antiserum was absorbed on TNP-SRBC. For development of individual IgG subclass PFC, Richard Asofsky (National Institute of Allergy and Infectious Diseases, National Institutes of Health) kindly provided rabbit anti-mouse IgG₁ and anti-IgG_{2a} antisera. Titrations were performed on all antisera to determine the dilution at which maximum activity was attained.

Quantitation of Id-460⁺ PFC was accomplished through inhibition of anti-TNP PFC by rabbit anti-Id-460 antiserum. Immunization procedures and characterization of this antiserum are as previously described (19). Genetic studies performed on immune sera by competitive inhibition radioimmunoassays have shown this antiserum to be specific for Id-460 expression. Mice of the IghV^a haplotype that expresses V_KI light chains can make Id-460. For use in plaquing studies, the antiserum was absorbed on a mixed myeloma sepharose column (consisting of McPC 603, MOPC 31c, MPC 11, FLOPC 21, and MOPC 173) and on McPC 603-Sepharose. A titration of anti-Id-460 antiserum was performed at each time point plaqued to confirm specific inhibition of Id-460⁺ PFC. This antiserum did not inhibit PFC specific for SRBC or PC (K. Bottomly, personal communication). Also, it was found to inhibit the plaque formation of MOPC 460 myeloma cells (40-90%) but not MOPC 315 cells (4%).

The accurate computation of IgM, IgG, IgM-Id-460⁺ and IgG-Id-460⁺ TNP-PFC/spleen necessitated various controls to identify background plaque formation. Table I illustrates a sample calculation of data obtained from a BALB.K mouse immunized with DNP-OVA.

TABLE I
Calculation of PFC Data and Percent Id-460⁺ PFC

Test	Goat anti-IgM, 1:75	Rabbit anti-IgG ₁ , 1:400	Rabbit anti-Id-460, 1:300	PFC/10 ⁶	Component of response measured
1	—	—	—	86	Direct PFC
2	+	—	—	6	Suppression of direct PFC by anti-IgM; residual PFC due to contaminating developing antibody
3	+	—	+	22	Development of PFC due to contaminating developing antibody in anti-Id-460
4	—	—	+	10	Inhibition of direct PFC by anti-Id-460; includes PFC developed by anti-Id-460
5	+	+	—	690	Indirect IgG ₁ PFC
6	+	+	+	60	Inhibition of IgG ₁ PFC by anti-Id-460

Calculation of Id-460⁺ direct and indirect PFC

$$\begin{aligned}
 \text{A. IgM PFC} &= 1 = 86 \text{ PFC}/10^6 \\
 \text{B. IgM Id-460}^+ \text{ PFC} &= 1 - (4 - [3 - 2]) = 92 \text{ PFC}/10^6 \\
 \text{C. IgG}_1 \text{ PFC} &= 5 - 2 = 684 \text{ PFC}/10^6 \\
 \text{D. IgG}_1 \text{ Id-460}^+ \text{ PFC} &= (5 - 2) - (6 - 3) = 646 \text{ PFC}/10^6
 \end{aligned}$$

$$\text{Percent Id-460}^+ \text{ IgM PFC} = \frac{B}{A} \times 100\% = \frac{92}{86} \times 100 = 100\%$$

$$\text{Percent Id-460}^+ \text{ IgG}_1 \text{ PFC} = \frac{D}{C} \times 100\% = \frac{646}{684} \times 100 = 94\%$$

Data from BALB.K day 5 secondary anti-TNP PFC response to DNP-OVA.

Results

Levels of Id-460 and Anti-DNP Antibody Are Independently Regulated. In the previous paper (19), responses to DNP-OVA in several strains of mice were shown. The time-course of such a response in BALB/c and BALB.K mice is shown in Fig. 1. Low levels of Id-460 are formed in the primary response to DNP-OVA. However, the secondary response is marked by a rapid and early rise in Id-460 levels, which is paralleled by a rapid and early rise in total anti-DNP antibody. Subsequently, Id-460 levels fall sharply and may increase again (BALB.K), whereas the anti-DNP antibody response reaches and maintains high levels. Thus, it would appear that Id-460 levels and anti-DNP levels in the serum may not be regulated in precisely the same fashion. Rather, there would appear to be idiotype-specific regulation of Id-460 levels in this response. However, serum anti-DNP antibody and Id-460 levels represent the complex outcome of production and catabolism; a more detailed analysis of this response was performed at the antibody-forming cell level.

Transient Dominance of the Anti-DNP PFC Response by Id-460. Because of the dramatic regulatory events apparent from assays of serum Id-460 levels in the secondary anti-DNP-OVA response (19; Fig. 1), we have carefully analyzed splenic PFC for isotype and Id-460 according to the techniques outlined in Materials and Methods (Table I). Using various inhibitory and developing antisera, we can readily identify PFC producing anti-DNP of three isotypes as well as the production of Id-460⁺ anti-DNP antibody of each isotype. The results in Fig. 2 are typical of this response, and show experiments in two strains of mice typed as Id-460⁺ in our previous genetic analysis

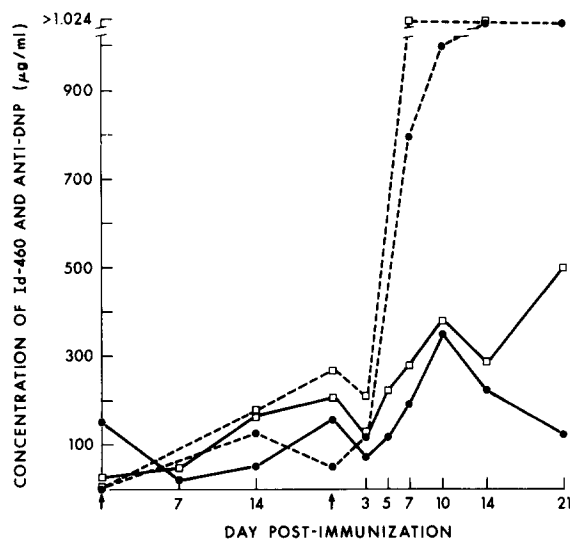


FIG. 1. BALB/cByJ and BALB.K mice were immunized with 100 μg DNP₈-OVA in alum plus pertussis intraperitoneally on day 0, and boosted with 10 μg DNP₈-OVA in saline intraperitoneally on day 21. Groups of four mice were bled, and levels of Id-460 and of anti-DNP antibody determined by radioimmunoassay. Values represent geometric means with standard errors all <1.4 . ●, BALB/cByJ; □, BALB.K; —, Id-460; - - -, anti-DNP.

of this response, BALB/c and BALB.K (19). Two points are made by these experiments: first, in both strains of mice there is an early increase in Id-460⁺ PFC, and in some cases, up to 80–100% of the PFC of a given isotype may be Id-460⁺. This dominance of the splenic PFC response correlates well with the data obtained by radioimmunoassay of serum Id-460. Second, the dominance of Id-460⁺ PFC is a transient phenomenon, because by day 7–10 of the response, Id-460⁺ PFC have fallen to low levels. Thus, anti-DNP antibody production and Id-460 production are not regulated in parallel either in serum or as observed with splenic PFC. Subsequent experiments confirm these results in general. Peak Id-460⁺ PFC responses occur early after boosting immunization but do not always occur on the same day from experiment to experiment. Id-460⁺ PFC (direct plus indirect PFC) usually account for 52–91% of anti-DNP PFC seen between days 3 and 5, whereas minimum percentages of these plaques are seen days 7 and 10. A summary of maximum and minimum percentages of Id-460⁺ PFC in several experiments is shown in Table II, and confirms the highly variable nature of Id-460 expression in these responses, as well as the tendency of Id-460 to dominate such anti-DNP antibody responses transiently.

The specificity of the anti-Id-460 antiserum used for inhibition in the PFC assay has been confirmed in several ways. The transience of PFC inhibition by itself demonstrates that anti-Id-460 identifies only some, and not all, anti-DNP PFC. This antiserum does not inhibit anti-PC PFC at any concentration tested (K. Bottomly, personal communication) or anti-SRBC PFC. It does not inhibit plaque formation of MOPC 315 myeloma cells while inhibiting the formation of MOPC 460 PFC. Thus, the anti-idiotypic inhibitor antiserum reveals specific Id-460⁺ PFC in the secondary *in vivo* anti-DNP response.

Representation of Id-460 in Various Ig Isotypes. To test the hypothesis that regulation

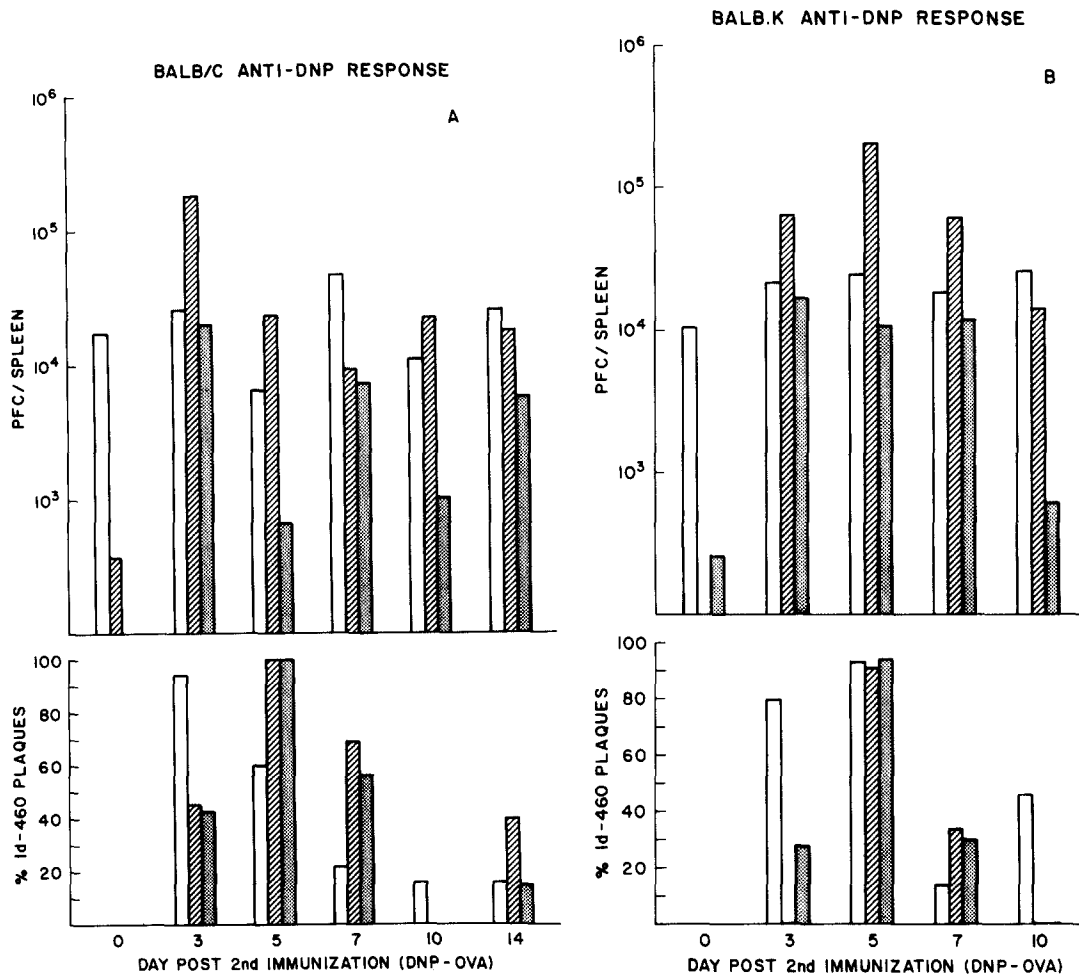


FIG. 2. Individual spleens from: A. BALB/cByJ; B. BALB.K, were plated at each time point after boosting with DNP-OVA (day 0) for: IgM, IgG₁, and IgG_{2a} TNP-PFC. Percent Id-460⁺ PFC was determined by inhibition of TNP-PFC with specific anti-Id-460 antiserum at all time points except day 0. □, IgM; ▨, IgG₁; ▩, IgG_{2a}.

of Ig isotype production is mediated by anti-idiotypic T cells operating on idiotypes that are selectively represented in certain isotypes, we have examined the representation of Id-460 as a function of time in various isotypes during the secondary anti-DNP PFC response to DNP-OVA. The results of two experiments are shown in Fig. 2. The pattern of the response appears to be clear in that Id-460⁺ PFC peak first for IgM PFC, then decline rapidly. Id-460 representation in IgG PFC lags slightly behind that of IgM PFC. This pattern of response has been observed in other experiments, but the maximum Id-460 response in the various isotypes does not always occur on exactly the same day after boosting or represent the same maximum percentage of the response. Therefore, compilation of the isotype time-course data for all experiments averages the highly fluctuating percentages of Id-460⁺ PFC early in the response and results in high standard errors. Individual spleens must be analyzed to obtain accurate

TABLE II
 Maximum and Minimum Levels of Id-460-positive PFC during Secondary
 Anti-DNP Responses*

Isotype of PFC	Maximum percent Id-460 ⁺ PFC		Minimum percent Id-460 ⁺ PFC	
	BALB/c	BALB.K	BALB/c	BALB.K
IgM	68‡ (32-95)	85 (64-98)	16 (0-39)	6 (0-14)
IgG ₁	63 (17-100)	70 (52-91)	11 (0-31)	4 (0-13)
IgG _{2a}	57 (41-81)	81 (53-100)	17 (0-41)	11 (0-20)

* Groups of BALB/c and BALB.K mice were primed and boosted with DNP-OVA, and secondary splenic anti-TNP-PFC were measured on several different days after boosting. For each experiment (BALB/c, seven experiments; BALB.K, three experiments), the maximum and minimum level for the percent Id-460⁺ PFC was determined for PFC of each isotype tested. Responses of <10⁴ PFC/spleen in any isotype were not considered.

‡ Arithmetic mean percent Id-460⁺ PFC and range (in parentheses) are presented.

maximum and minimum percentages of Id-460⁺ PFC. Table II shows the fluctuation and range of Id-460 expression in IgM, IgG₁, and IgG_{2a} PFC in seven plaquing experiments with BALB/c mice and three with BALB.K mice. It is clear that Id-460 is represented in all three isotypes and that Id-460⁺ PFC can represent a major portion of the anti-DNP PFC response in all three isotypes. Minimum percentages of Id-460⁺ PFC for each experiment also reveal the transience of idiotypic dominance in each isotype. Thus, it would appear that the simplest explanation of these findings is that activation of an Id-460⁺ B cell gives rise to Id-460⁺ PFC forming first IgM and then IgG. Sometimes a late increase in Id-460⁺ PFC is also observed. From these data there is no preferential association of idio type and isotype, and we cannot confirm our hypothesis that isotype is regulated via idio type, at least in this system. The explanation for independent regulation of IgM and IgG responses should probably be sought elsewhere.

Discussion

Responses to several antigens have been shown to include expression of particular inherited idiotypic determinants (22). In most systems, these idiotypes make up a stable proportion of the response. Thus, the response to PC in BALB/c mice is >95% T15-Id⁺ (23, 24). Responses to streptococcal A carbohydrate (25) and to *p*-azophenylarsonate (26) in A/J mice each have 20-70% antibody bearing a major, cross-reactive idio type. In contrast, certain idiotypes are found only in individual or small numbers of mice (27) or not at all in immune responses of normal mice (18). The only previous idio type showing transient dominance of a response are those of C57BL/6 (28) and BALB/c (29) mice to the hapten 3-nitro-4-hydroxyphenylacetyl, in which the primary response is dominated by antibody bearing an inherited idio type and λ light chains, but hyperimmune antibodies are κ and idio type negative (29). Id-460 shows a transient dominance in the secondary anti-DNP response and is the predominant idio type expressed in serum early in the response.

In this paper, the phenomenon of transient dominance of Id-460 expression during *in vivo* anti-DNP antibody responses in normal mice is strikingly demonstrated by measuring splenic anti-TNP PFC. The percentage of Id-460⁺ PFC reaches an early maximum about 5 d after boosting, whereas the concentration of serum Id-460 usually peaks around day 7–10. It would appear that there is a preferential production of anti-DNP antibody bearing Id-460 early in the secondary response. The time-course of Id-460 expression correlates well with the time-course of action of a second helper T cell that has been observed shortly after boosting in adoptive transfer experiments and is missing in mice treated with anti- μ chain antibody in the DNP system (4). Such treatment has been shown to deplete idiotype-specific helper T cells in other experiments (8). We would propose that the transient expression of Id-460 reflects the early action of such Ig-dependent helper T cells. This suggests the presence of some type of idiotype-anti-idiotype interaction and the existence of an Id-460 recognizing helper T cell. Furthermore, we have proposed (15) that this same cell also induces suppressor T cells via the heavy-chain variable-region-restricted pathway defined by Eardley et al. (30), and thus its action may be twofold. First, it induces Id-460⁺ B cells selectively, and second, it activates suppressor T cells via an idiotype-anti-idiotype pathway potentially leading to the inactivation of such Id-460-bearing B cells. The result of such a complex interaction would be the transient idiotypic dominance that was in fact observed. The validity of this interpretation is now being tested at the cellular level.

The data presented also show that Id-460 is not restricted in its expression to particular isotypes, although IgM and IgG₁ are the major isotypes represented. Id-460 is found associated essentially equally in the three isotypes, IgM, IgG₁, and IgG_{2a}, that we have studied at the PFC level. We have also found parallel expression of Id-460 levels by radioimmunoassay and IgE anti-DNP antibody by passive cutaneous anaphylaxis. In preliminary experiments we find that some, but not all, of the IgE in this response can be neutralized by anti-Id-460 (unpublished results).

Because Id-460 expression is not restricted to particular isotypes, idiotypic regulation cannot by itself account for isotypic regulation in this system. Rather, the pattern observed is one in which cycling occurs. Id-460⁺ PFC switch from IgM to IgG and are followed by a wave of Id-460⁻ PFC, again switching from IgM to IgG. Late in the response, as the total number of anti-DNP PFC decreases, the proportion of Id-460⁺ PFC are often observed to increase. We have no evidence to suggest that the Id-460⁻ PFC arise from the Id-460⁺ population. Instead, it would appear at present that Id-460⁺ PFC are active early in the response and can be regulated independently of the Id-460⁻ PFC or the total anti-DNP PFC response.

These results would appear to conflict with those obtained previously both in these laboratories (18) and those of Bona and Paul (31) and Bona and co-workers (32). There are several possible explanations for these differences. First, the antigen and schedule of immunization was different; Zeldis et al. (18) used DNP-bovine gamma globulin in CFA, and measured serum Id-460 at day 10 of the secondary response, whereas Bona and Paul (31) and Bona et al. (32) used TNP on various T-independent carriers *in vivo* or *in vitro*, and measured PFC at day 3 of the primary response. Given the extreme variability in Id-460 expression we have observed, it is not surprising that such changes in protocol might influence the percent Id-460⁺ antibody detected. However, the more likely explanation for the differences between the present

results and those of Bona and Paul (31) and Bona et al. (32) lies in the anti-idiotypic reagents used. Bona et al. used mouse anti-Id-460, which is not characterized in terms of light-chain genetics or binding-site specificity, whereas we used a site-specific rabbit anti-Id-460 that detects a determinant, the expression of which is regulated by genes mapping to both Igh-V and IgK-V. Even more significant is the finding² that our anti-Id-460 detects Id-460 in the serum of all strains of normal mice thus far tested, and in significant amounts. Thus, it is unlikely that mouse anti-Id-460 would detect the determinant(s) on MOPC 460 measured in our studies. Indeed, one might almost predict from the serology alone that the results in these studies would differ. Nonetheless, all three sets of studies agree in finding that anti-DNP antibody can express significant amounts of Id-460 in BALB/c mice.

We are currently studying the cellular, genetic, and environmental factors that contribute to this transient idiotypic dominance. Besides idio-type-specific helper T cells, we are testing the role of the Id-460-specific suppressor T cell described by Bona and Paul (31) as regulating in vitro anti-TNP responses in our system. Because of the great variability of Id-460 expression, characterization of this response has proven to be more difficult than with other more stably dominated responses. One critical factor in dominant expression of Id-460 may be the finding of large amounts of Id-460⁺, non-DNP-binding material in serum from nonimmune mice (33).² This material may play a role in establishing the idiotypic cell interactions for the Id-460 response.

Summary

After immunization of mice with 2,4-dinitrophenyl-ovalbumin (DNP-OVA), it was shown previously that strains having Igh-V^a genes and able to express light chains of the V_κ1 group produce high levels of anti-DNP antibody bearing an idio-type (Id-460) associated with the combining site of the BALB/c DNP-binding myeloma protein MOPC 460. Expression of Id-460 in serum is transient; Id-460 levels peak early in the response and are regulated independently of total anti-DNP antibody. In this paper, the transient dominance of Id-460 expression has been confirmed at the cellular level by inhibition of splenic anti-DNP plaque-forming cells (PFC) with rabbit anti-Id-460 antiserum. Id-460⁺ PFC can account for 52–91% of anti-DNP PFC early after secondary challenge with DNP-OVA. Furthermore, Id-460 is represented at these high levels in IgM, IgG₁, and IgG_{2a}, the three isotypes tested in the PFC assay, as well as in IgE, as tested by passive cutaneous anaphylaxis. Thus, there is no preferential association of Id-460 with a given isotype. We conclude from these studies that Id-460 is a dominant idio-type in the anti-DNP antibody response of BALB/c mice to DNP-OVA. This dominance is expressed transiently and is independent of isotype. A further conclusion from these studies is that regulation of isotype expression is independent of the regulation of idio-type expression in this system. We would suggest that regulation of Id-460 expression involves Ig-dependent helper T cells specific for Id-460 that induce Id-460⁺ B cells and also activate suppressor T cells, both events occurring via idio-type-anti-idio-type interactions.

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² Dzierzak, E. A., and C. A. Janeway, Jr. Expression of an idio-type (Id-460) during in vivo anti-dinitrophenyl antibody responses. III. Detection of Id-460 in normal serum that does not bind dinitrophenyl. *J. Exp. Med.* 154:1442.

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