CELLULAR BASIS OF REGULATION OF
EXPRESSON OF IDIOTYPE

II. Immunity to Anti-MOPC-460 Idiotype Antibodies Increases
the Level of Anti-Trinitrophenyl
Antibodies Bearing 460 Idiotypes

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It has recently been shown that an idiotypic determinant(s) (460-Id)\(^1\) found on the
2,4-dinitrophenyl (DNP) and 2,4,6-trinitrophenyl (TNP)-binding myeloma protein,
MOPC-460, is expressed on some anti-TNP antibodies produced by BALB/c mice
after immunization with certain thymus-independent (TI) (1) and thymus-dependent
(2) TNP antigens. In vitro studies indicate that the 460-Id-bearing component of the
anti-TNP response to the TI antigen, TNP-Nocardia water-soluble mitogen (NWSM),
is regulated by a 460-Id-specific suppressor T cell found in the spleen of normal
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In this communication, we report the results of experiments in which we studied
the effect of anti-460-Id antibodies and of anti-[anti-460-Id] antibodies on the response
to two TI TNP antigens. Mice actively immunized with MOPC-460 or acutely
pretreated with anti-460-Id antibodies made an anti-TNP response to TNP-NWSM
and to TNP-levan which lacked a 460-Id\(^+\) component. By contrast, mice actively
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460-Id] antibodies showed an increase in the 460-Id\(^+\) component of their anti-TNP
response. Furthermore, T cells from mice immunized to anti-460-Id antibodies failed
to suppress, in vitro, the 460-Id\(^+\) component of the response of normal B cells to TNP-
NWSM. These results suggest (a) that 460-Id-specific suppressor T cells normally
regulate in vivo responses to TI TNP-antigens; (b) that anti-[anti-460-Id] antibodies
can eliminate 460-Id-specific suppressor T cells; and (c) that 460-Id-specific suppressor
T cells and anti-460-Id antibodies share idiotypic determinants.

Materials and Methods

\(^{1}\) Abbreviations used in this paper: AECM, aminoethylcarbamylmethyl; C, complement; HA, hemagglutination
C, complement; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; NWSM, Nocardia water
mitogen; PFC, plaque-forming cell; RIA, radioimmunoassay; SRBC, sheep erythrocytes; TI,
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thymus-independent; TNP, 2,4,6-trinitrophenyl.

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BALB/cAnN mice, 8–12 wk old, were obtained from the Division of Research
Services, National Institutes of Health, Bethesda, Md.

Antigens. 2,4,6-trinitrophenyl (TNP)-Nocardia water soluble mitogen (NWSM) was pre-
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pared as previously described (1). An aminoethylcarbamylmethyl (AECM) derivative of levan, derived from *Aerobacter levanicum*, was the kind gift of Dr. C. Glaudemans, National Institute of Arthritis, Metabolic and Digestive Diseases, National Institutes of Health, Bethesda, Md. It contained 1 AECM group per 21 sugars. TNP-levan, containing 1 TNP per 24 sugars, was prepared by the reaction of trinitrobenzenesulfonate with AECM-levan.

**Myeloma Proteins.** MOPC-460, an IgA, κ-myeloma protein with DNP- and TNP-binding activity, and EPC-109 and UPC-61, IgA, κ-myeloma proteins with β(2-6), β(2-1) fructosan-binding activity were kindly donated by Dr. Michael Potter, National Cancer Institute, National Institutes of Health, Bethesda, Md.

**Preparation of Anti-Idiotype Antibodies.** Anti-460-Id antibodies were prepared by immunization of BALB/c mice with 75 µg of MOPC-460 myeloma protein emulsified in complete Freund's adjuvant. This was followed 5 d later by a similar dose of myeloma protein in incomplete Freund's adjuvant and then by six weekly injections of 75 µg of protein in saline. Anti-460-Id antibodies were also obtained from a hybridoma [No. 77-14-11-F6(51)] derived by Buttin et al. (3). These antibodies were used in the radioimmunoassay for 460-Id. Antibodies specific for the idiotype determinants of anti-460-Id antibodies (anti-[anti-460-Id] antibodies) were produced by immunization of BALB/c mice with purified anti-460-Id antibodies coupled to keyhole limpet hemocyanin (KLH). Anti-460-Id antibodies were purified by absorption of an ammonium sulfate fraction of BALB/c anti-460-Id serum to a Sepharose 4B-MOPC-460 column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) followed by elution with a glycine-HCl buffer (0.1 M; pH 2.8). The purified antibodies were conjugated to KLH by mixing purified antibody with KLH, at a final concentration of 0.5 mg/ml, in the presence of 0.05% glutaraldehyde. The reaction was allowed to proceed until the solution was opalescent; it was then stopped by the addition of lysine to a final concentration of 0.05 M. This was followed by extensive dialysis. The KLH-anti-460-Id conjugate was then used to immunize BALB/c mice according to the same schedule as that described for immunization with MOPC-460. Anti-E109-Id antibodies were produced in A/He mice as previously described (1).

**Antibody Assays.** TNP-coated sheep erythrocytes (SRBC) were prepared according to the technique of Rittenberg and Pratt (4). Levan-SRBC were prepared using O-steroyl levan as described by Hammerling and Westphal (5). MOPC-460-, EPC-109-, and UPC-61-coated SRBC were prepared using the chromic chloride method (6). Anti-TNP, anti-levan, and anti-460-Id serum antibody titers were determined by agglutination of coated SRBC. The hemagglutinin (HA) titer reported is the log₂ of the reciprocal of the highest dilution giving agglutination. Serum 460-Id titers were determined by the capacity of serum and serum dilutions to inhibit the agglutination of MOPC-460-coated SRBC by anti-460-Id antibodies. The hemagglutination inhibition titer reported is log₂ of the reciprocal of the highest dilution of serum which caused inhibition of hemagglutination.

Serum levels of 460-Id were also measured by a radioimmunoassay in which the capacity of serum and serum dilutions to inhibit the binding of ¹²⁵I-labeled F(ab) fragments of MOPC-460 myeloma protein was determined according to the techniques of Kuetter et al. (7) and labeled with ¹²⁵I through the use of chloramine T (8). Rabbit anti-mouse Fc antibodies were used to precipitate complexes of MOPC-460 F(ab) fragments and anti-460-Id antibodies. These anti-Fc antibodies were prepared by immunization of rabbits with two injections of 1 mg each of purified H chains followed by absorption of the serum on an AH-Sepharose column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) to which F(ab) fragments of A/J IgG had been linked (9). The H chains were purified from pooled A/J IgG by reduction and alkylation, followed by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) equilibrated with 5 M guanidine (10). F(ab) fragments of A/J IgG were prepared as described by Porter et al. (11).

**Separation of T and B Lymphocytes.** Thymus-dependent (T) lymphocytes were purified by passage over nylon wool columns. Of the cells obtained, 95-98% could be killed by anti-Thy 1.2 and complement (C) (12). These cells failed to proliferate in vitro to lipopolysaccharide (LPS) or NWSM. Cell preparations enriched in thymus-independent (B) lymphocytes were obtained by treating spleen cells with anti-Thy 1.2 serum (Litton-Bionetics, Bethesda, Md.) and C as previously described (1). These cells gave in vitro thymidine-incorporation responses.
to concanavalin A of less than twice background in experiments in which unseparated cells
gave responses which were 50–120 times background. The B-cell preparations responded well
to LPS and NWSM. Such responses could be abolished by treating B cells with rabbit anti-
mouse Ig and C (13).

In Vitro Responses to TNP-NWSM. B lymphocytes (5 × 10^5) were cultured alone or with T
lymphocytes (5 × 10^5) in microtiter plates (tissue culture, cluster 36, Costar, Data Packaging,
Cambridge, Mass.) in a modified Mishell-Dutton medium containing 10% fetal calf serum and
10^{-5} M 2-mercaptoethanol in the presence or absence of TNP-NWSM (3 µg/ml) for 3 d.

Plaque-Forming Cell (PFC) Assay. The number of cells secreting antibodies specific for TNP
and levan was determined by a hemolytic plaque assay, as previously described (1, 14). The
percentage of anti-TNP PFC-secreting anti-TNP antibodies carrying the 460-Id and of anti-
levan PFC-secreting anti-levan antibodies carrying E109-Id was determined by adding BALB/
c anti-460-Id or A/He anti-E109-Id antisera to the agarose, as previously described (14). The
difference between the number of PFC obtained in the absence and presence of anti-Id
represents the number of PFC-secreting antibody bearing the Id under study. The standard
error of these numbers of PFC-secreting Id-bearing antibodies is \( SE = \sqrt{SE_1^2 + SE_2^2} \), in which
SE_1 is the SE of the number of PFC in absence of anti-Id sera, and SE_2 is the SE of the number
of PFC in the presence of anti-Id sera.

The number of direct and indirect PFC-secreting antibody specific for MOPC-460 was
determined using MOPC-460-coated SRBC.

Results

Production of Syngeneic Anti-460-Id Antibodies in BALB/c Mice. To determine whether
the production of anti-TNP antibodies expressing 460-Id could be influenced by
pretreatment with anti-460-Id antibodies, or by a state of active immunity to 460-Id
determinants, we immunized BALB/c mice with MOPC-460, as described in Materials
and Methods. Such mice developed antibodies which agglutinated MOPC-460-
coated SRBC, but not MOPC-167-coated SRBC (Table I A). Because both MOPC-
460 and MOPC-167 are IgA, κ-myeloma proteins, this result indicates that a syngeneic
anti-460-Id antibody response has occurred and confirms a previous report by Sakato
and Eisen (15). Furthermore, these immune animals have splenic PFC specific for
MOPC-460, but not for U-61 as shown by selective lysis of MOPC-460-coated SRBC
(Table I B) and selective inhibition of this lysis by MOPC-460 (Table I C).

Influence of Anti-460-Id Antibodies on Production of 460-Id\(^+\) Anti-TNP Antibodies. We
have previously shown that a small but significant fraction of anti-TNP antibodies
produced by BALB/c mice in response to immunization with TNP-NWSM carry
460-Id determinants. In the experiment illustrated in Table II, 23% of anti-TNP-PFC
produced in response to TNP-NWSM immunization could be inhibited by incorpo-
ration of anti-460-Id in the agarose, indicating that the anti-TNP antibodies produced
by these cells bore 460-Id. BALB/c mice which had been immunized with MOPC-
460 (Id-2 mice) failed to develop any PFC which could be inhibited by anti-460-Id,
although they made a substantial anti-TNP antibody response. Similarly, normal
BALB/c mice pretreated with three doses of 0.1 µg of anti-460-Id or more failed to
develop anti-TNP PFC which secreted 460-Id\(^+\) antibodies. Pretreatment with three
doses of 0.01 µg of anti-TNP-Id had no effect. These indicate that modest to high
doses of anti-460-Id inhibit the activation of 460-Id\(^+\) anti-TNP B cells, whereas low
doses show neither inhibitory nor stimulatory activity.

Production of Syngeneic Antibodies Specific for Anti-460-Id (Anti-[Anti-460-Id]
Antibodies). BALB/c mice immunized with KLH conjugates of anti-460-Id antibod-
ies produced antibodies which were capable of agglutinating SRBC coated with
purified anti-460-Id (HA titer 3.2 ± 0.3) but not SRBC coated with BALB/c IgG2a or with UPC-10 myeloma protein. To determine whether these serum hemagglutinins were anti-(anti-460-Id) antibodies or anti-TNP antibodies bearing 460-Id determinants, we performed two types of experiments. First, we absorbed the putative anti-[anti-460-Id] antisera on TNP-KLH-Sepharose 4B to remove any anti-TNP antibodies which might have been present. Such treatment did not diminish the HA titer on anti-460-Id-coated SRBC. Secondly, we demonstrated that the binding of 125I anti-460-Id hybridoma by anti-[anti-460-Id] immunoglobulin adsorbed on a plastic surface was not inhibited by TNP-lysine or TNP-glycine. Buttin et al. (3) have shown that both TNP-lysine and TNP-glycine inhibit the binding of MOPC-460 by the anti-460-Id hybridoma. These results indicate that the immunoglobulins which can agglutinate anti-460-Id-coated SRBC lack anti-TNP activity. This strongly suggests that these hemagglutinins are anti-[anti-460-Id] antibodies, although we cannot entirely exclude the possibility that they are 460-Id* immunoglobulins which lack anti-TNP activity.

Enhancement of the 460-Id* Component of the Anti-TNP Response in Id-3 Mice and in Normal Mice Pretreated with Anti-[Anti-460-Id] Antibody. Mice which had been immunized with KLH-conjugates of anti-460-Id antibodies and which developed an HA response
TABLE III
Proportion of Anti-TNP-Antibodies Carrying 460-Id in Normal BALB/c and BALB/c Mice Producing Anti-[Anti-460-Id] Antibodies (Id-3 Mice)

|                     | TNP-NWSM | | TNP-levan | |
|---------------------|----------|----------------|----------|----------------|
|                     | Normal   | Id-3           | Normal   | Id-3           |
| Number of mice      | 5        | 3              | 5        | 4              |
| Anti-TNP PFC/10⁶ cells | 100 ± 14 | 84 ± 8         | 276 ± 15 | 239 ± 64       |
| Percent 460⁺        | 27 ± 12  | 54 ± 5         | 12 ± 1   | 34 ± 5         |
| Anti-levan PFC/10⁷ cells | ND*     | ND             | 203 ± 130 | 284 ± 41       |
| Percent E109⁺       | ND       | ND             | 42 ± 5   | 45 ± 20        |
| Anti-TNP HA titer   | 6 ± 0.5  | 8 ± 2          | 12 ± 0.3 | 12 ± 0         |
| 460-Id HI titer     | 0.5 ± 0.5| 2 ± 1          | 1 ± 0.5  | 3.5 ± 0.5      |
| μg 460-Id/ml (RIA)‡ | 22 ± 6   | 57 ± 21        | 14 ± 6   | 60 ± 23        |

Normal BALB/c or Id-3 BALB/c mice were immunized with 30 μg of TNP-NWSM or 50 μg of TNP-levan. These were sacrificed 5 d later and humoral and spleen PFC responses tested.
* Not done.
‡ Radioimmunoassay.

specific for anti-460-Id-coated SRBC (Id-3 mice) were immunized with either TNP-NWSM or TNP-levan. These mice produced an anti-TNP antibody response in which the 460⁺ component was substantially increased as compared to normal BALB/c mice immunized with the same TNP antigens (Table III). Thus, of the anti-TNP PFC produced by Id-3 mice immunized with TNP-levan, 34% could be inhibited with anti-460-Id, whereas only 12% of the anti-TNP PFC produced by normal BALB/c mice immunized with TNP-levan were inhibitable. The P value for the difference of these values was < 0.005. A similar difference was observed in the anti-TNP response to TNP-NWSM, although it did not reach levels of statistical significance. In addition, serum concentration of 460-Id molecules were also higher in Id-3 mice than in normal mice, as judged both by the 460-Id HI titer and by radioimmunoassay. On the other hand, the total anti-TNP response in normal and Id-3 mice was essentially the same and Id-3 mice immunized with TNP-levan displayed a proportion of E109⁺ anti-BL PFC which was similar to that of normal mouse.

Furthermore, when normal BALB/c mice were pretreated with three doses of 100 μg of an ammonium sulfate fraction of anti-[anti-460-Id] antiserum, previously absorbed on a TNP-lysine-Sepharose-4B column, both the proportion of the 460⁺ anti-TNP PFC and the serum 460-Id concentration produced in response to TNP-NWSM were significantly increased (Table IV). This result and that of the previous experiment indicate that anti-[anti-460-Id] antibodies enhance the number of anti-TNP PFC which secrete 460-Id⁺ antibodies.

Absence of 460-Id-Specific Suppressor T Cells in Id-3 Mice. In a previous study, we have shown that naturally occurring 460-Id-specific suppressor T cells regulate the activation of those cells potentially capable of producing 460-Id⁺ anti-TNP antibodies. In the following experiment, we compared the suppressive effect of nylon wool-enriched T lymphocytes from normal and Id-3 mice. Data presented in Table V show that T lymphocytes from normal BALB/c mice inhibited the 460-Id⁺ component of the in vitro anti-TNP response of normal and of Id-3 B lymphocytes to TNP-NWSM.
TABLE IV
Proportion of Anti-TNP Antibodies Carrying 460-Id in BALB/c Mice Pretreated with Anti-[Anti-460-Id] Antibodies*

| Pretreatment of mice | TNP-NWSM | Anti-TNP-PFC | Anti-TNP HA titer | µg 460-Id/ml (RIA) |
|----------------------|----------|--------------|-------------------|------------------|
|                      |          | Anti-TNP-PFC | Percent 460-Id*   |                  |
|                      |          | PFC/10^6 cells |                  |
| − −                  | 3        | 35 ± 3       | 0                 | 4 ± 0.5          | <0.1 |
| − +                  | 8        | 519 ± 49     | 32 ± 3            | 6.3 ± 0.7        | 12.5 ± 7.3 |
| + −                  | 3        | 27 ± 4       | 43 ± 8            | 2 ± 0.7          | <0.1 |
| + +                  | 5        | 597 ± 24     | 52 ± 4            | 7.8 ± 1.1        | 48.5 ± 7.8 |

*Mice were pretreated three times, at 3-d intervals, with 100 µg of an ammonium sulfate fraction of BALB/c anti-[anti-460-Id] antibodies and immunized 1 d after completion with 30 µg TNP-NWSM. Mice were sacrificed 5 d later and humoral and spleen PFC responses tested. The anti-[anti-460-Id] antibody used in the experimental group had been passed over a TNP-KLH-Sepharose-4B column.

TABLE V
Proportion of PFC-Secreting Anti-TNP Antibodies Carrying 460-Id in Cultures of T and B Lymphocytes from Normal and Id-3 BALB/c Mice

| Donor of lymphocytes | Anti-TNP-PFC |
|----------------------|--------------|
|                      |              |
| Normal               |              |
| Normal               |              |
| Id-3                 |              |

| B       | T       | PFC/culture | 460-Id* PFC/culture | Percent 460-Id* |
|---------|---------|-------------|---------------------|-----------------|
| Normal  | —       | 191 ± 26    | 73 ± 27             | 38              |
| Normal  | Normal  | 105 ± 6     | 12 ± 7              | 11              |
| Id-3    | —       | 134 ± 13    | 55 ± 13             | 41              |
| Id-3    | Normal  | 432 ± 15    | 224 ± 18            | 52              |
| Id-3    | Id-3    | 440 ± 18    | 42 ± 26             | 10              |

B lymphocytes (5 × 10^5) were cultured alone or with 5 × 10^5 T lymphocytes in microtiter wells with 5 µg/ml of TNP-NWSM for 4 d. Anti-TNP PFC in the absence or presence of BALB/c anti-460 antiserum (1/100 dilution) were measured using TNP-SRBC.

By contrast, T cells obtained from Id-3 mice failed to show any detectable inhibition of the 460-Id+ anti-TNP response of either normal or Id-3 B cells. This result indicates that Id-3 mice lack, or are deficient in, 460-Id-specific suppressor T cells. The absence of such cells may be a major factor in the heightened 460-Id+ anti-TNP response of Id-3 mice.

Discussion

In the studies presented here, we have demonstrated that BALB/c mice with active or passive immunity to the idiotype(s) of MOPC-460 fail to express 460-Id+ antibodies in their humoral response to TNP-levan and TNP-NWSM. Conversely, mice with active or passive immunity to idiotypic determinants of anti-460-Id antibodies express a heightened 460-Id+ component in their anti-TNP response to TNP-NWSM and to TNP-levan. The mechanism through which immunity to MOPC-460-Id eliminates a 460-Id+ response has not been established. However, based on studies in the phosphoryl choline-T15 system and in the bacterial levan-E109 system (16, 17) elimination or inactivation of precursors of cells capable of secreting 460-Id+ anti-TNP antibodies by anti-Id antibody is a major possibility. In addition, in preliminary experiments we
have shown that such mice possess suppressor T lymphocytes capable of inhibiting a 460-Id⁺ response to TNP-NWSM but we have not yet established whether suppressor activity in mice actively immune to MOPC-460 exceeds that in normal mice. The heightened 460-Id⁺ response of mice with active or passive immunity to anti-460-Id antibodies correlates with the absence of 460-Id-specific suppressor T lymphocytes in these mice, and is very likely a result of the absence of such cells. Furthermore, it seems likely that anti-[anti-460-Id] antibodies have reacted with and eliminated these 460-Id-specific suppressor T cells, suggesting that these suppressor T cells and anti-460-Id antibody share common idiotypic determinants. This would be consistent with a similarity in the structure of the 460-Id-specific receptor on the suppressor T cell and of the binding site of the anti-460-Id antibody.

Furthermore, these results suggest that the in vitro regulation by suppressor T cells of the 460-Id⁺ component of the anti-TNP response also explains the relatively small component of 460-Id⁺ molecules in the in vivo anti-TNP response. This would suggest that a regulatory network consisting of Id-bearing B cells and of T cells specific for idiotypic is a important feature of the normal immune response. Whether spontaneous development of anti-[anti-460-Id] antibodies and T cells also occurs and acts to regulate the natural level of suppression is a provocative but unresolved issue.

Other instances in which an idiotype-anti-idiotype network has been employed to explore the role of such a mechanism in regulation should be mentioned. In one striking example, rabbit anti-Id antibody to anti-ribonuclease obtained from a single donor was prepared. Rabbits immunized to these anti-Id antibodies and then immunized with ribonuclease developed anti-ribonuclease antibodies with an idiotype similar to that expressed by the antibodies used to prepare the initial anti-Id antibody. Because sharing of idiotypes in anti-ribonuclease antibodies of normal rabbits is rarely observed (18), these results suggest that immunization with anti-Id antibody perturbed the existing balance, either by directly activating Id-bearing B or T cells or by eliminating suppressor T cells which normally limited the expression of that idiotype.

A similar observation has been made in response to certain polysaccharide antigens (19).

These examples and our results give clear illustrations of the ways in which regulatory interactions among a network (20) of Id-bearing and Id-specific antibodies and cells might occur, as well as emphasizing that second degree idiotype-anti-idiotype (Id-2-anti-Id-2) interactions could profoundly effect the expression of Id-1 (e.g., 460-Id⁺ anti-TNP antibodies) determinants on the antibodies produced as a result of conventional immunization. The extent to which anti-[anti-460-Id] antibodies occur and to which anti-460-Id T cells are eliminated in normal strains immunized with TNP-NWSM or TNP-levan would shed considerable light on the physiologic relevance of these systems.

Summary

The antibody response of BALB/c mice to trinitrophenyl (TNP)-levan or TNP-Nocardia water-soluble mitogen (NWSM) includes a small but significant fraction of antibodies which share idiotypes (Id) with the 2,4-dinitrophenyl (DNP)- and TNP-binding myeloma protein MOPC-460. Active immunization of BALB/c mice with MOPC-460 or passive administration of anti-460-Id antibodies suppresses the 460-Id⁺ component of the anti-TNP response. By contrast, active immunization of BALB/
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c with anti-460-Id antibodies or passive administration of BALB/c anti-[anti-460-Id] antibodies leads to an enhanced 460-Id+ component in the anti-TNP antibodies produced in response to TNP-levan or TNP-NWSM. This enhanced 460-Id+ response appears to be a result of the elimination of suppressor T lymphocytes specific for the 460-Id as T lymphocytes from such mice are unable to suppress the in vitro 460-Id+ response to TNP-NWSM whereas normal T cells are suppressive. These results indicate that suppressor cells specific for 460-Id normally regulate the activation of precursors of cells capable of secreting 460-Id+ anti-TNP antibodies.

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