PRODUCTION OF AUTO-ANTI-IDIOPTYPIC ANTIBODY DURING
THE NORMAL IMMUNE RESPONSE TO TNP-FICOLL

III. Absence in nu/nu Mice: Evidence for T-Cell Dependence
of the Anti-Idiotype-Antibody Response*

By A. FAYE SCHRATER, EDMOND A. GOIDL, G. JEANETTE THORBECKE, AND
GREGORY W. SISKIND

From the Department of Pathology, New York University Medical School, New York, 10016; and the
Division of Allergy and Immunology, Department of Medicine, Cornell University Medical College, New
York, 10021

In previous papers (1, 2) in this series we have presented evidence that an auto-
anti-idiotypic-antibody response occurs during the normal immune response to a T-
independent antigen. The evidence also suggested that this auto-anti-idiotypic re-
sponse is involved in the regulation of the immune response. It was established that
hapten-augmentable plaque-forming cells (PFC), defined as antibody-producing cells
which could not be detected as PFC in the Jerne assay unless hapten was present
during the assay, represented cells whose secretion of antibody was inhibited by bound
auto-anti-idiotypic antibody. Furthermore, it was shown that anti-idiotypic antibody
can be assayed by its ability to cause hapten-reversible inhibition of plaque formation.

In these earlier studies (1, 2) we noted that the magnitude of the response to
trinitrophenyl-lys-Ficoll (TNP-F) falls precipitously between days 4 and 7 after
immunization in both AKR/J and BALB/c mice (1, 2) and that serum from AKR/
J mice taken 7 d after injection of TNP-F contained anti-idiotypic antibody to anti-
trinitrophenyl (TNP). An important role for anti-idiotypic antibody in the down
regulation of the anti-TNP response was suggested by the presence of hapten-
augmentable PFC in spleens taken more than 4 d after immunization. The percentage
of hapten-augmentable (anti-idiotype-blocked) PFC arising spontaneously during the
immune response to TNP-F in AKR/J mice was 5% on day 4 and 25% on day 5.
Moreover, normal AKR/J recipients of syngeneic TNP-F-immune spleen cells plus
antigen had a far higher incidence of hapten-augmentable PFC 3 and 4 d after cell
transfer (298 and 122%, respectively). The PFC response by such recipients, as
detected in the absence of hapten, was lower than that of recipients of normal cells;
however, the results of the assay in the presence of hapten showed this apparent
decrease to be a result of the blocking of large numbers of PFC by anti-idiotypic

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Abbreviations used in this paper: C, complement; DNP, dinitrophenyl; DNP-EACA, DNP-e-amino-n-
caproic acid; NMS, normal mouse serum; PFC, plaque-forming cells; TNP, trinitrophenyl; TNP-EACA,
TNP-e-amino-n-caproic acid; TNP-F; TNP-lys-Ficoll; TNP-SRBC, trinitrophenylated sheep erythrocytes;
TxBM, irradiated, thymectomized, bone marrow-reconstituted.
antibody. This exaggerated hapten-augmentable PFC response could be elicited by transfer of T-depleted immune spleen cells and by immune serum, but it was most marked when T cells were included in the transferred cell population. The role of T cells in the production of auto-anti-idiotypic antibody was therefore investigated and the results will be presented.

The response to TNP-polysaccharide conjugates is thymus independent (3, 4) and, in fact, is frequently higher and more heterogeneous with respect to affinity in athymic, as compared with normal, mice (4-6). It has been suggested that this is a result of the absence of T suppressor cells in athymic mice (5, 6). The results in this paper will show that the anti-idiotypic-antibody response, and its resulting regulation of the anti-TNP response, are absent in athymic mice. The response to TNP-F of nude mice lacks hapten-augmentable PFC and shows a much less precipitous decline in number of PFC during the first week of the response. Similar findings were obtained with irradiated, thymectomized, bone marrow-reconstituted mice. Thus, the data strongly support the hypothesis that this auto-anti-idiotypic antibody response is thymus dependent and that it is involved in the normal regulation of the immune response to a T-independent antigen.

Materials and Methods

Mice and Immunization. Adult AKR/J male mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. AKR/J nu/nu mice were obtained by breeding heterozygote (+/nu) AKR/J mice obtained through the courtesy of Dr. E. Eicher, The Jackson Laboratory. Adult male BALB/c mice were purchased from the Charles River Breeding Laboratories, Wilmington, Mass. Nude BALB/c female mice were supplied by the Division of Cancer Treatment, Animal Program, National Cancer Institute, Bethesda, Md.; they were a 10th backcross generation to Charles River BALB/c mice. Animals were immunized by the intra-venous injection of 10 μg TNP-F. Pools of anti-TNP-F antisera were obtained 7 d after immunization. These sera were titrated for anti-TNP antibody by passive hemagglutination with trinitrophenylated sheep erythrocytes (TNP-SRBC). All animals used were <3 mon of age.

Preparation of Irradiated, Thymectomized, Bone Marrow-reconstituted (TxBM) Mice. AKR/J mice were thymectomized at 4 wk of age. After a 2-mon rest, the mice were irradiated (750 rad, Cs source, Gammaor Irradiator; Radiation Machinery Corp., Parsippany, N. J.) and repopulated with 5 × 10⁶ bone marrow cells from 6- to 8-wk-old AKR/J donors. The bone marrow cells were treated with anti-Thy-1.1 antisera plus nontoxic rabbit complement (C) before cell transfer. TxBM mice were used 1–2 mon after irradiation and repopulation.

Antigens and Reagents. Preparation of TNP-F has been described (1). TNP-e-amino-n-caproic acid (TNP-EACA) was prepared and quantified by established methods (7, 8). Anti-Thy-1.1 antisera was obtained by repeated immunization of C3H mice with thymocytes from AKR/J mice (titer on AKR/J thymocytes and spleen cells was 1:600 and 1:60, respectively).

Cell Transfers. Spleen cells from TNP-F-immunized mice or from nonimmunized mice were transferred intravenously together with 10 μg TNP-F into normal, nonirradiated, syngeneic recipients. Splenic anti-TNP PFC were determined 4 d later.

Plaque Assay. Cell suspensions were prepared from individual spleens, were washed once, and were resuspended in Hanks’ balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.). Direct splenic anti-TNP PFC were assayed by the method of Jerne and Nordin (9) as modified for slide assay by Dresser and Greaves (10). TNP-SRBC were used as indicator cells (11). Normal guinea pig serum, absorbed with sheep erythrocytes, was the C source.

In Vitro Assay for Anti-Idiotypic Antibody. As previously described, hapten-reversible inhibition of plaque formation was used to assay anti-idiotypic antibody (2). Primary day-4 anti-TNP-F spleen cells from AKR/J or BALB/c mice were used as target cells in this assay. Cells from pooled spleens or from individual donors were washed once and were resuspended at 1–2 × 10⁶
cells/ml. 1-ml aliquots were incubated with a 1/10 or 1/20 dilution of anti-TNP-F antisera for 5 min at 4°C. The cells were centrifuged at 1,000 rpm, 4°C, and after one wash, were resuspended to the original concentration. 50 μl were assayed in duplicate for anti-TNP PFC. The hapten reversibility of the inhibition was determined by addition of free hapten (1 × 10^-8 to 1 × 10^-6 M TNP-EACA) to the agarose and the C solution (2, 12).

Assay of Affinity of Anti-TNP PFC. The distribution of anti-TNP PFC with respect to affinity was assayed by the hapten inhibition of plaque formation as described previously (12-14). Concentrations of dinitrophenyl (DNP)-EACA or TNP-EACA, in the agarose and C, ranging from 1 × 10^-9 to 1 × 10^-5 M in half-log increments were used. The Shannon heterogeneity index (15) was employed to describe the degree of heterogeneity of affinity of the PFC population from individual mice.

Results

Comparison of Immune Responses to TNP-F in AKR/J and BALB/c ++/+ and nu/nu Mice. The results in Table I show that the numbers of anti-TNP PFC during the immune response to TNP-F was higher in nu/nu mice than in ++/+ mice. This difference was already suggested by day 4, and became more striking at day 7 and thereafter (BALB/c, day 7: P < 0.001; AKR/J, day 7: P < 0.001). Thus, the precipitous fall in the magnitude of the anti-TNP PFC response between day 4 and 7 previously noted in both strains (1), was not present in nude mice from either strain (Table I). That is, between 4 and 7 d after antigen injection, euthymic mice showed an 80-90% decrease in the number of anti-TNP PFC, whereas nude mice showed only a 40-50% decrease. Because additional observations (data not shown) indicated that the decrease in direct PFC between days 4 and 7 of the response to this dose of TNP-F in ++/+ mice was not accompanied by the appearance of large numbers of indirect PFC, the prolonged presence of direct PFC in nude mice was not a result of an impaired switch from IgM- to IgG-antibody formation.

An early indication of the autoregulation by anti-idiotypic antibody in both AKR/J and BALB/c mice is the appearance, after day 4 of the primary response to TNP-F, of anti-idiotypic-antibody-blocked anti-TNP PFC which can be detected as PFC only in the presence of hapten. The results in Table II show that such specific hapten-augmentable PFC are totally lacking on days 5 and 7 of the immune response in athymic (nu/nu and TxBM) mice. The absence of hapten-augmentable PFC was especially meaningful because in normal mice (++/+) the peak incidence of such anti-idiotypic-antibody-blocked PFC was at this time in the primary immune response (1).

Absence of Anti-Idiotypic Antibody in the Sera of Athymic Mice Responding to TNP-F. In previous studies (2) an assay for anti-idiotypic antibody was described that made use of the ability of such antibody to inhibit plaque formation by idiotype-producing cells. This inhibition was reversible by the hapten TNP-EACA. Results on the second and third lines of Table III show that pools of immune sera from AKR/J ++/+ mice cause a hapten-reversible block of plaque formation by spleen cells from TNP-F immune AKR/J ++/+ or nu/nu mice. The degree of inhibition of plaque formation varied from 14 to 46% when tested on spleen cells from different donors. In only 1 out of 13 cases was no inhibition observed. In every case the inhibition was completely reversible by low concentrations of hapten. The results indicate that nu/nu and ++/+ mice have a comparable, although variable, representation of the assayed idiotypes among their anti-TNP PFC during the initiation of the response to TNP-F. Data in Table III also show that sera from nu/nu or TxBM AKR/J mice, obtained 7 d after immunization with TNP-F, totally failed to inhibit PFC from either ++/+
TABLE I

| Strain       | Day 4  | Day 7  | Day 11 | Day 14 |
|--------------|--------|--------|--------|--------|
| BALB/c +/+   | 89,500 | 18,600 | 8,700  | 7,800  |
| BALB/c nu/nu | 115,000| 55,200 | 29,500 | 37,000 |
| AKR/J +/+    | 141,200| 15,000 |        |        |
| AKR/J nu/nu  | 245,000| 95,500 |        |        |

* Mice were immunized by the intravenous injection of 10 μg TNP-F.

§ Data are presented as the geometric mean ×/+ SE. The numbers in parentheses represent the number of animals studied.

§ Arithmetic mean of two determinations: 300,000; 190,000.

TABLE II

| Strain          | Assay day | PFC per spleen* | Incidence of >10% augmentation| Average percentage of augmentation§ |
|-----------------|-----------|-----------------|------------------------------|-------------------------------------|
| AKR/J +/+       | 5         | 88.3            | 4/4                          | 35                                  |
| AKR/J +/+ ; TxBM| 5         | 115.0           | 0/4                          | 6                                   |
| AKR/J +/+       | 7         | 15.8            | 2/4                          | 18                                  |
| AKR/J nu/nu     | 7         | 95.5            | 0/7                          | 6                                   |
| AKR/J +/+ ; TxBM| 7         | 29.5            | 0/3                          | 2                                   |
| BALB/c +/+      | 11        | 10.9            | 6/6                          | 36                                  |
| BALB/c nu/nu    | 11        | 29.5            | 0/8                          | 4                                   |

* Geometric means.

‡ Number of mice showing >10% increase of PFC at optimal hapten concentration per total number of mice tested. Optimal TNP-EACA concentration: AKR/J, 10^{-8}-10^{-7} M; BALB/c, 10^{-7}-10^{-6} M.

§ Calculated as: 100 × (PFC in presence of hapten − PFC in absence of hapten)/PFC in absence of hapten. Data for all animals were included in the calculation.

or nu/nu mice. Thus, the sera from TNP-F-immunized athymic mice lacked detectable anti-idiotypic antibody.

Absence of Idiotype-blocked PFC in Recipients of Spleen Cells from Nude Mice Immune to TNP-F. Because the most striking manifestation of regulation by auto-anti-idiotypic antibody was observed after cell transfers from immune +/+ donors into normal recipients (1), it was of interest to determine whether or not cells from nu/nu immune mice could manifest such a regulatory effect. Spleen cells from AKR/J or BALB/c donors taken 7 or 14 d after injection of TNP-F were transferred, together with TNP-F, into normal (+/+, nonirradiated) syngeneic recipients. The response of these recipients was determined 4 d later (Table IV). An apparent suppression, which was reversed by the presence of hapten in the assay, was seen after transfers of cells from
Absence of Anti-Idiotype Antibody Activity in Immune Sera from Athymic AKR/J Mice as Assayed by Hapten-reversible Plaque Inhibition

| Serum source†‡ | Anti-TNP titer (reciprocal)§ | Percentage of control response* |
|----------------|-----------------------------|---------------------------------|
|                |                            | Spleen pool 1 | Spleen pool 2 | Spleen pool 3 | Spleen 4 | Spleen 5 | Spleen 6 | Spleen 7 |
| NMS +/+ (pool) | <2                         | 100          | 100          | 100          | 100      | 100      | 100      | 100      |
| Immune +/+ (pool I) | 1,280           | 34           | 69           | 74           | 73       | 86       | 67       | 82       |
| Immune +/+ (pool II) | 2,560          | ---          | 64           | 71           | 72       | 115      | 59       | 77       |
| Immune nu/nu (pool III) | 5,120         | 100          | 99           | 92           | 102      | 100      | 102      | 99       |
| Immune TxBM (pool IV) | 2,560         | ---          | ---          | 96           | ---      | ---      | ---      | ---      |

* Immune spleen cells were obtained from groups of five AKR/J +/+ mice or from individual AKR/J nu/nu mice that had been immunized intravenously with 10 µg TNP-F 4 and 7 d, respectively, before assay. The control values (PFC determined in the presence of normal mouse serum [NMS]) ranged from 59,000 to 243,000 anti-TNP PFC/spleen. Immune cells at 1–2 × 10⁶ cells per ml were incubated with immune sera or NMS at a 1:20 final dilution for 5 min at 4°C. The cells were washed once, resuspended to the original volume, and plated. PFC were assayed after pretreatment so that 1/1,000 of a spleen equivalent was plated per slide. In all cases inhibition of plaque formation was completely reversible by the addition of hapten. Pools of immune spleen cells were used in the experiments with +/+ mice.

† The NMS pool was obtained from 20 AKR/J mice. Pools of immune sera were obtained from groups of 5–20 AKR/J mice, which had been immunized with 10 µg TNP-F intravenously 7 d before collection of sera.

§ Determined by passive hemagglutination.

Discussion

The evidence in this paper demonstrates the absence, in nu/nu and in TxBM mice,
Table IV

Failure of Immune Spleen Cells from Athymic Mice to Suppress the Anti-TNP Response or to Cause the Appearance of Anti-Idiotype-blocked PFC in Normal Recipients Immunized with TNP-F*

| Experiment | Strain   | Donor cells       | Direct anti-TNP PFC per spleen† | Percentage of augmentation by hapten |
|------------|----------|-------------------|---------------------------------|------------------------------------|
|            |          |                   | Without hapten                  | With hapten                         |
|            |          |                   | ×10⁶                             | ×10⁷                               | %                                |
| 1          | AKR/J    | Immune +/+ (day 7) | 34.5 ± 1.33 (3)                 | 76.1 ± 1.32                         | 120.4                            |
|            |          | Immune nu/nu§ (day 7) | 103.9 ± 1.12 (8)              | 113.8 ± 1.10                       | 9.5                              |
|            |          | Normal +/+         | 92.6 ± 1.44 (3)                | 96.7 ± 1.44                         | 4.4                              |
| 2          | AKR/J    | Immune +/+ (day 7) | 149.1 ± 1.14 (3)               | 217.2 ± 1.37                       | 45.7                             |
|            |          | Immune nu/nu (day 7) | 247.5 ± 1.13 (3)              | 248.6 ± 1.11                       | 0.5                              |
| 3          | BALB/c   | Immune +/+ (day 14) | 195.8 ± 1.03 (3)               | 246.3 ± 1.11                       | 25.8                             |
|            |          | Immune nu/nu (day 14) | 101.2 ± 1.20 (3)              | 105.5 ± 1.19                       | 4.3                              |

* 4 × 10⁷ (experiments 1 and 3) or 2 × 10⁷ (experiment 2) spleen cells were injected intravenously together with 10 μg TNP-F, into syngeneic, nonirradiated recipients. PFC/spleen were determined 4 d thereafter and the results are presented as geometric means ×± SE. 10⁻⁸–10⁻⁷ M TNP-EACA was used to demonstrate hapten-augmentable PFC.

§ Data pooled from two similar experiments. The numbers in parentheses represent the number of animals studied.

of a humoral anti-idiotype antibody response to anti-TNP idiotype(s) despite the fact that the idiotype is produced during the response to TNP-F by nu/nu as well as by +/+ mice. The evidence for the lack of anti-idiotype antibody production in both AKR/J and BALB/c athymic mice is as follows: (a) absence of splenic anti-idiotype-blocked, hapten-augmentable PFC; (b) absence of anti-idiotype antibody in the sera of nu/nu and TxBM immune mice; (c) absence of hapten-reversible suppression of the anti-TNP PFC response in normal recipients of immune spleen cells from nu/nu mice.

In previous studies of this series it was suggested that auto-anti-idiotype antibody causes a downward regulation, manifested as an acute decrease in the number of anti-TNP PFC between days 4 and 7 after injection of TNP-F (1, 2). The present data confirm this proposed regulatory role for auto-anti-idiotype antibody by showing that the precipitous decline seen in the response of +/+ mice is absent in nu/nu mice of both strains studied. Thus, when as first shown by Najjar (16), antibody induces the formation of anti-antibody, one role of the auto-anti-idiotype antibody in vivo is the regulation of the height and duration of the immune response, as originally postulated by Jerne (17).

Previous studies on the transfer of immune cells and serum from euthymic donors had shown that, whereas purified immune B cells or anti-idiotype antibody-containing serum could induce the appearance of anti-idiotype-blocked PFC, a greater degree of hapten-reversible suppression was obtained when immune T cells were included in the transferred cell population. This suggested an important regulatory role for
immune T cells. The present studies strongly reinforce the suggestion (18) that T cells are needed for the anti-idiotypic antibody response. The phenotype of the T cells involved in this regulation, with respect to their cell-surface markers, is not known. However, it should be noted that there are reports describing Ly1+2− T cells, which are idiotype specific, and are required for production of the major idiotype in the response to azophenylarsonate-keyhole limpet hemocyanin (19) as well as group A streptococcal carbohydrate (20). The possibility exists that such cells are also involved in regulating the production of anti-idiotypic antibody. That is, a T cell that recognizes the idiotype might function as a helper cell for the anti-idiotypic antibody response. It is also possible that helper T cells that recognize antigen or anti-idiotype could be involved. Although the response to TNP-F appears to be largely thymus independent, a positive influence of either antigen-specific or idiotype-specific helper T cells has been noted in vitro (J. Mond and W. E. Paul, personal communication). The data at present do not allow a distinction among these various possibilities.

Although the data do not exclude a supplementary role of conventional suppressor T cells with specificity for either antigen or idiotype, the results presented here cannot be explained solely on such a basis. Thus, our demonstration of a requirement for T cells to generate an anti-idiotypic response, introduces an added level of complexity...
into the interpretation of studies on T-cell-dependent suppression. Unless the specificity of the putative suppressor T cell has been critically determined, the possibility exists that suppression is actually mediated by anti-idiotype antibody, the production of which is highly dependent upon the presence of helper T cells. The possible role of idiotype-anti-idiotype interaction in immunoregulatory T-cell circuits (21) needs to be further examined.

We have previously reported that nude mice immunized with TNP-pneumococcal polysaccharide produced a secondary direct PFC response of greater heterogeneity and higher affinity than that of +/+ littermates (6). When euthymic mice were immunized with T-independent antigens no increase in antibody affinity with time after initial antigen injection was observed (6 and this paper). In fact, after immunization of normal mice with TNP-F, a progressive loss of the relatively high-affinity PFC occurred between days 4 and 11 of the primary immune response (1). In contrast, with nude mice, an increase in high-affinity PFC was noted between days 4 and 7 after immunization with TNP-F (Fig. 1). With T-dependent antigens, a decrease in antibody affinity has been seen late in the immune response of both normal mice (22) and rabbits (23). It is possible that the decrease in affinity observed in all of these examples reflects the operation of an idiotype-anti-idiotype regulatory network. An anti-idiotype response against idio-types of high-affinity antibodies could result in a preferential inhibition of these high-affinity clones. Thus, the failure to observe an increase in affinity and in heterogeneity within the first week after immunization with T-independent antigens might be more apparent than real in that high-affinity clones might be selectively expanded, but their secretion might be inhibited by anti-idiotype antibodies. The ultimate fate of such suppressed cells is at present unclear.

The possibility of a preferential stimulation of anti-idiotype antibodies that are specific for clones producing high-affinity antibodies should be considered. Several possible mechanisms might give rise to such a situation. Firstly, high-affinity antibodies would form complexes with antigen that were more stable than would low-affinity antibodies, and antigen-antibody complexes might be important in stimulating the production of auto-anti-idiotype antibody (16, 24). Secondly, it is reasonable to assume that high-affinity antibodies actually represent the product of relatively few clones, whereas low-affinity antibodies probably represent the product of a large number of clones. Thus, although high-affinity antibodies might represent a small fraction of the total specific-antibody population, the antibody produced by individual high-affinity clones could be present in higher concentration than the individual products of low-affinity antibody-producing clones. This would result in a more marked anti-idiotype-antibody response to high-affinity antibodies which have restricted clonal diversity. Regardless of its affinity, an antibody that is the product of a dominant clone, and is therefore present in relatively high concentration, would be more likely to induce an anti-idiotype response than would antibodies secreted by a heterogeneous population of cells.

It should be emphasized that the assay used here to demonstrate anti-idiotype antibodies does not lend itself to the detection of such antibodies directed against low-affinity idiotypes. This is because, at the relatively high hapten concentration that would be required to reveal low-affinity PFC, hapten inhibition of plaque formation would occur. Therefore the data obtained from this assay provide a minimum estimate of anti-idiotype antibody-blocked PFC.
Summary

Although athymic mice make an excellent immune response to the thymus-independent antigen trinitrophenyl-lys-Ficoll (TNP-F), nude mice of AKR/J and BALB/c strains lack the anti-idiotypic response that occurs in euthymic mice of both of these strains within the first 1–2 wk after injection of more TNP-F. Anti-idiotypic antibody-blocked (hapten-augmentable) anti-TNP splenic plaque-forming cells (PFC) do not occur at any time and serum anti-idiotypic antibody is absent in both congenitally athymic mice, and thymectomized, irradiated, bone marrow-reconstituted mice. Nevertheless, nu/nu mice do have PFC which can be inhibited by exposure to anti-idiotypic antibody produced in +/+ mice. As a consequence of the failure to produce anti-idiotypic antibodies, the anti-TNP PFC response in athymic as compared to euthymic mice is of greater magnitude, declines less precipitously, and shows an increase rather than a decrease in affinity between days 4 and 7 after antigen injection. It is concluded that the anti-idiotypic antibody response is thymus dependent and that athymic mice lack a helper cell required for the induction of anti-idiotypic antibodies.

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