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

II. Hapten-Reversible Inhibition of Anti-TNP Plaque-Forming Cells by Immune Serum as an Assay for Auto-Anti-Idiotypic Antibody*

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In the accompanying paper (1), we have shown that after day 4 of the immune response of AKR/J mice to 2,4,6-trinitrophenyl-lys-Ficoll (TNP-F)[1], the addition of free hapten to a plaque-forming-cell (PFC) assay increased the number of observed splenic anti-trinitrophenol (TNP) PFC. Immune spleen cells, taken 7 d after immunization, transferred this property of the immune response to normal recipients; spleen cells from such recipients, assayed 4 d after cell transfer and TNP-F injection, manifested an exaggerated form of this phenomenon. It was hypothesized that the increase in PFC was the result of the displacement, by hapten, of auto-anti-idiotypic antibodies that were synthesized during the course of the normal immune response. If this hypothesis were correct, it should be possible to obtain auto-anti-idiotypic antibodies by hapten elution from appropriate immune spleen cells. Results in the accompanying paper (1) also suggested that the putative auto-anti-idiotypic-antibody response was involved in the downward regulation of the immune response of AKR/J mice to TNP-F. One might, therefore, expect to find auto-anti-idiotypic antibody in the serum of AKR/J mice immediately after the abrupt decrease in the number of detectable splenic PFC: i.e., 7 d after antigen injection.

In the present paper, evidence is presented to support these hypotheses in that hapten-reversible inhibition of PFC in vitro can be demonstrated with hapten eluates from immune cells and with immune serum. The factor responsible for inhibition has immunoglobulin-like determinants, lacks anti-TNP-antibody activity, and is absorbable by an AKR/J anti-TNP-antibody immunoadsorbent. Hapten-reversible inhibition of PFC represents a simple in vitro assay for anti-idiotypic antibody. In this

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[1] *Abbreviations used in this paper:* BAC, bromoacetylcellulose; CFA, complete Freund's adjuvant; DNP, 2,4-dinitrophenol; DNP-HSA, dinitrophenylated human serum albumin; HSA, human serum albumin; MGG, mouse gamma globulin; NMS, normal mouse serum; nu/nu, congenitally athymic, homozygous for the recessive gene *nu*; PBS-D, phosphate-buffered saline; PFC, plaque-forming cells; SRBC, sheep erythrocytes; $TNBSO_3$, 2,4,6-trinitrobenzene sulfonic acid; TNP, 2,4,6-trinitrophenol; TNP-F, 2,4,6-trinitrophenyl-lys-Ficoll; TNP-HGG, TNP-human gamma globulin.

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paper, the conditions for such an assay are defined. In addition, we present evidence to support the view that the increase in PFC observed in the presence of free hapten (1) represents an assay for cells whose secretion of antibody has been inhibited, in vivo, by the binding of auto-anti-idiotypic antibodies to cell-surface receptors. The data are consistent with the interpretations of others that auto-anti-idiotypic antibodies are formed during the course of the normal immune response (2–7).

Materials and Methods

Mice and Immunization. AKR/J male mice, 6–8 wk of age, were obtained from The Jackson Laboratory, Bar Harbor, Maine. AKR/J congenitally athymic, homozygous for the recessive gene, nu (nu/nu) mice were obtained by breeding heterozygote (+/nu) AKR/J mice purchased from The Jackson Laboratory. Mice were immunized by the intravenous injection of 10 μg TNP-F. Pools of antisera were obtained 7 d after immunization.

Reagents and Antigens. Preparation of TNP-F has been described (1). TNP-human gamma globulin (TNP-HGG) was prepared by the reaction of 2,4,6-trinitrobenzene sulfonic acid ($TNBSO_3$; Sigma Chemical Co., St. Louis, Mo.) with human gamma globulin, and dinitrophenylated human serum albumin (DNP-HSA) was prepared by the reaction of 1-fluoro-2,4-dinitrobenzene (Eastman Chemical Products, Inc., Eastman Kodak Co., Rochester, N. Y.) with human serum albumin (HSA) (8). Conjugated proteins were purified by extensive dialysis and their concentrations were determined by dry-weight analysis. TNP-ε-amino-*n*-caproic acid (TNP-EACA) was prepared by the reaction of $TNBSO_3$ with ε-amino-*n*-caproic acid (9). Mouse gamma globulins (MGG) were prepared by two sequential precipitations, at 40% cold saturated ammonium sulfate, followed by dialysis against phosphate-buffered saline (PBS-D, Grand Island Biological Co., Grand Island, N. Y.). Mouse Fab fragments were kindly provided by Dr. A. Nisonoff (Brandeis University, Waltham, Mass.).

Anti-MGG was prepared by immunization of a goat with 100 μg MGG, in complete Freund's adjuvant (CFA), injected intramuscularly into four dorsal sites. Rabbit anti-mouse Fab was prepared by immunization with 1 mg Fab, in CFA, injected into multiple sites. The antiserum gave a single line against Fab fragments and against whole mouse serum in Ouchterlony plates. Rabbit anti-TNP antiserum was obtained after immunization with 500 μg TNP-HGG in CFA. Gamma globulin fractions of the rabbit antisera were prepared (10).

Immunoadsorbents. An anti-mouse-immunoglobulin immunoadsorbent was prepared as described by Wigzell (11). Briefly, MGG was adsorbed onto glass beads followed by the addition of goat anti-MGG. The columns were washed extensively with PBS-D before use. A dinitrophenol, DNP-HSA-BAC, (DNP) immunoadsorbent was prepared by coupling DNP-HSA to bromoacetylcellulose (BAC) (12). For absorption of sera, 1 ml of packed DNP-HSA-BAC was used per 0.5 ml of serum. A mouse anti-TNP-antibody immunoadsorbent was prepared by the addition of 0.5 ml of primary day-7 anti-TNP-F antiserum, obtained from nu/nu AKR/J mice, to a 1-ml DNP-HSA-BAC column. After 1 h at room temperature, the anti-TNP antibody immunoadsorbent was washed extensively with PBS-D, and was used for absorption of sera as described above for DNP-HSA-BAC.

Cell Transfers. Spleen cells from TNP-F-immunized, or nonimmunized, mice were transferred, together with antigen, into normal, nonirradiated, syngeneic recipients as described (1).

Preparation of Eluate from Immune Spleen Cells. 4 d after cell transfer and antigen injections (10 μg TNP-F), recipients of immune (primary d-7 anti-TNP-F), or normal, spleen cells were killed. Spleen-cell suspensions (5×10^7/ml), in Hanks' balanced salt solution (Grand Island Biological Co.), were incubated, for 30 min at 37°C in the presence of 10^{-8} M TNP-EACA. Supernates (eluates) were recovered by centrifugation (1,500 rpm) at 4°C for 15 min, and were then dialyzed against PBS-D, at 4°C, overnight.

In Vitro Assay for Hapten-Reversible Anti-TNP PFC Inhibitory Factor(s). Primary day-4 anti-TNP-F spleen cells from AKR/J mice were used as target cells in this assay. Cells from pooled spleens or from individual donors were washed once and were resuspended at $1–2 \times 10^6$ cells/ml. 1-ml aliquots were incubated with various dilutions of cell eluates or with pools of anti-TNP-F antisera, for 5 min, at room temperature. The cells were centrifuged (1,000 rpm, 10

min, room temperature) and, after one wash, were resuspended to the original concentration. 50 μl was assayed, in duplicate, for anti-TNP PFC. The haptenreversibility of the inhibition was determined by addition of free hapten (1×10^{-7}–1×10^{-8} M TNP-EACA) to the agarose and the complement solution. Further details of the PFC assay have been described (1).

Results

In Vitro Inhibitory Activity of Anti-TNP-F Serum on PFC. Serum from AKR/J mice, obtained 7 d after the intravenous injection of 10 μg TNP-F, was tested for its ability to inhibit anti-TNP plaque formation by spleen cells from mice immunized with TNP-F 4 d previously. As indicated in Table I, a 24–99% decrease in the number of plaques was observed with three independent pools of antisera, and three pools of immune cells. With a single pool of cells, the degree of inhibition varied from 24 to 43%. Results obtained with pools of spleen cells, and with spleen cells from individual donors were comparable. The suppressive effect of the anti-TNP-F antiserum could be reversed by hapten in all cases. A single lot of antiserum suppressed (blocked) the PFC from individual mice to different extents (11–56%; Table I). In addition, the concentration of free hapten which caused maximal augmentation of PFC was different in individual animals and ranged from 1×10^{-8} to 1×10^{-7} M TNP-EACA. The inhibitory activity was specific for anti-TNP PFC, in that it failed to inhibit anti-sheep-erythrocyte (SRBC) PFC (Table II).

The kinetics of PFC inhibition at 4°C and at room temperature (22°C) were studied (Table III). At both temperatures, maximum inhibition was observed at the shortest incubation period studied (5 min). With increased incubation periods, there was a progressive decrease in inhibition of PFC which was more pronounced at room temperature than at 4°C. All further studies were therefore carried out with a 5-min incubation period.

The nature of the interaction leading to inhibition of antibody secretion was studied. Neither rabbit anti-TNP-HGG antibody nor rabbit anti-mouse Fab inhibited anti-TNP PFC (Table IV). Addition of TNP-F to the immune spleen cells clearly decreased their capacity to produce anti-TNP plaques. It should be emphasized, however, that this in vitro inhibition, by polyvalent antigen, was not hapten reversible under the conditions of the present assay and, therefore, differed fundamentally from the effect of immune serum (Table IV).

Characterization of the PFC Inhibitory Activity in Anti-TNP-F Antiserum. To test the hypothesis that the hapten-reversible PFC inhibitory activity present in the immune sera was due to anti-idiotypic antibodies, the inhibitory factor was characterized by absorption studies. Passage of the antiserum through a DNP-HSA-BAC immunoadsorbent did not alter its inhibitory activity (Table V). It was shown by a solid-phase radioimmunoassay (13), that after passage through this immunoadsorbent column, the serum contained <10 ng/ml anti-TNP antibody. Thus, the inhibitory activity was present in spite of the absence of anti-TNP antibody and, furthermore, remained reversible by addition of hapten to the PFC assay. Passage of the antiserum through an anti-MGG column (11) eliminated PFC inhibitory activity (Table V). Based upon these absorption studies, it was concluded that the inhibitor was a molecule with immunoglobulin-like determinants but was not anti-TNP antibody.

Finally, the serum was passed through an anti-TNP-F·DNP-HSA-BAC column (that is, an anti-TNP-antibody immunoadsorbent). This adsorbent was prepared by

TABLE I

*Inhibition of Anti-TNP-F PFC by Antiserum from Mice Immunized with TNP-F 7 d Previously and Reversal of the Inhibition by Addition of Hapten**

PFC source‡	Control response (direct PFC/spleen)	Percentage of control response after incubation with							
		NMS		Pool I		Pool II		Pool III	
		Hapten absent	Hapten present§	Hapten absent	Hapten present	Hapten absent	Hapten present	Hapten absent	Hapten present
		%							
Pooled spleens									
1	82,000	100	99	1	105	—	—	—	—
2	19,000	100	126	63	147	76	179	57	131
3	164,000	100	101	—	—	—	—	59	102
Individual spleens									
1	164,000	100	102	82	102	75	98	—	—
2	100,000	100	102	68	106	74	100	—	—
3	202,000	100	99	67	101	73	107	—	—
4	182,000	100	101	64	98	61	99	—	—
5	99,000	100	101	44	113	63	101	—	—
6	37,000	100	103	89	103	—	—	—	—
7	22,000	100	105	109	100	—	—	—	—

* Immune cells (1–2 × 10^6/ml) were incubated with immune serum or normal mouse serum (NMS), usually at a 1:20 final dilution, for 5 min at room temperature. NMS was obtained from a pool of 20 mice. Pools of immune serum were obtained from groups of 5–20 AKR/J mice immunized with 10 μg TNP-F, intravenously, 7 d before bleeding.

‡ Spleens from 2–5 AKR/J mice immunized 4 d earlier with 10 μg TNP-F, intravenously, were pooled. Individual mice were immunized similarly.

§ Maximum number of PFC/spleen observed in the presence of 10^{-8}–10^{-7} M TNP-EACA.

addition, to DNP-HSA-BAC, of day-7 immune antiserum from nu/nu AKR/J mice immunized with 10 μg TNP-F intravenously. It had been shown,[2] that nu/nu AKR/J mice did not produce the PFC inhibitory factor in spite of their high anti-TNP PFC response to the T-independent antigen, TNP-F. These mice, therefore, were a likely source of idiotype-bearing anti-TNP antibody that was not contaminated with auto-anti-idiotypic antibody. The anti-TNP-antibody column removed all detectable PFC inhibitory activity (Table V). Because the DNP-HSA-BAC column itself failed to remove the PFC inhibitory activity, it was concluded that the inhibitory factor had been bound to the anti-TNP antibodies. It should be noted that a similarly prepared anti-dansyl-antibody column (anti-dansyl·dansyl-HSA-BAC) failed to remove the anti-TNP PFC inhibitory activity from the serum of TNP-F immunized mice. The results were thus consistent with the interpretation that the inhibitor was an immunoglobulin-like molecule with anti-idiotype specificity (anti-anti-TNP).

Elution and Characterization of the PFC Inhibitory Activity from Immune Spleen Cells. Based upon the hypothesis that hapten augmentation of plaque formation was due to displacement of bound auto-anti-idiotypic antibodies, it would be predicted that a

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TABLE II

*Inhibition of Anti-TNP-F PFC by Antiserum from Mice Immunized with TNP-F 7 d Previously: Specificity of Inhibitory Activity**

Experiment	Antiserum‡	Direct PFC/slide§			
		Anti-TNP		Anti-SRBC	
		Hapten absent	Hapten present	Hapten absent	Hapten present
1	NMS	164	166	178	184
	Pool IV	96	166	175	180
2	NMS	135	133	220	ND‖
	Pool VI	102	135	225	ND
	Pool VII	79	125	225	ND
	Anti-SRBC	133	135	230	ND

* Immune spleen cells were obtained from groups of five AKR/J mice that had been immunized, intravenously, with 10 μg TNP-F or with 4×10^8 SRBC 4 d before assay.

‡ Pools of immune sera were obtained from groups of 5–20 AKR/J mice that had been immunized with 10 μg TNP-F or 4×10^8 SRBC, intravenously, 7 d before bleeding. All cell suspensions were pretreated with the antisera for 5 min at 4°C, were washed once, and were resuspended and plated.

§ PFC were assayed, after pretreatment, so that 1/100 (anti-SRBC) or 1/1,000 (anti-TNP) of a spleen equivalent was plated per slide.

‖ ND, not done.

factor inhibitory for PFC could be eluted from appropriate immune-spleen-cell populations, i.e., from recipients of immune spleen cells plus antigen. A large proportion of anti-TNP PFC in such recipients are blocked, but can be revealed in vitro by hapten (1). As indicated in Table VI, a hapten eluate prepared from such cells markedly inhibited anti-TNP plaque formation. The inhibition could be reversed by the inclusion of 10^{-8} M TNP-EACA in the assay mixture. Inhibition was specific, in that the material had no effect on anti-SRBC PFC. A similarly prepared eluate from recipients of immune cells from nu/nu AKR/J mice did not inhibit anti-TNP PFC (data not shown).

Results in Table VI also indicate that the eluted factor was similar to the PFC inhibitor in serum. It was not anti-TNP antibody because it was not removed by DNP-HSA-BAC, and it was immunoglobulin-like because it was removed by an anti-MGG immunoadsorbent. After passage through either column, the eluate lacked anti-TNP-antibody activity. Like the serum inhibitor, the factor in the eluate was removed by passage through the anti-TNP-antibody column. These results strongly support the previous conclusion that this factor is an anti-idiotypic antibody that is produced during the normal course of the immune response to TNP-F.

Discussion

In the accompanying paper (1), we demonstrated an in vitro augmentation by hapten of the number of PFC detected in recipients 4 d after transfer of TNP-F immune spleen cells and antigen. It was proposed that this was due to the displacement of auto-anti-idiotypic antibodies from antibody-producing cells. In the present paper

TABLE III

Effect of Varying Incubation Conditions on the Inhibition of Anti-TNP PFC by Serum from Mice Immune to TNP-F

Incubation conditions		Percentage of control response after incubation with*	
Time	Temperature	Antiserum‡	NMS
min	°C	%	
5	4	57§	100
15	4	76	102
30	4	72	102
5	22	66	101
15	22	78	99
30	22	86	106

* Control response (164,000 anti-TNP PFC/spleen) was determined after incubation of day-4 immune spleen cells (1–2 × 10^6/ml) with NMS (1:20) for 5 min at 4°C. PFC were assayed, after pretreatment, such that 1/1,000 of a spleen equivalent was plated per slide.

‡ Day-4 immune spleen cells (1–2 × 10^6/ml) were incubated with a 1:20 final dilution of a pool of immune serum taken from AKR/J mice 7 d after intravenous injection of 10 μg TNP-F. All the data in the table are derived from PFC determinations in the absence of hapten in the assay.

§ In the presence of 10^{-8} M TNP-EACA, PFC returned to 101% of control.

we have demonstrated the existence of a factor in the serum of AKR/J mice, immunized with TNP-F 7 d earlier, that inhibits, in vitro, anti-TNP PFC, but not anti-SRBC PFC. The inhibitory factor was specific for anti-TNP PFC, was antigenically immunoglobulin-like, but was not anti-TNP antibody. Inhibition of PFC was reversible by TNP-hapten. A factor with similar characteristics was eluted, by hapten, from spleen cells of AKR/J mice that had a high proportion of hapten-augmentable PFC. The factor present in the eluate as well as in the serum was removed by passage over an anti-TNP antibody column but not by a TNP-HSA-BAC column. Thus, the results are consistent with the view that the PFC inhibitory factor is an auto-anti-idiotypic antibody that is specific for the idiotypes of some of the AKR/J anti-TNP antibodies. Because the eluates were prepared by incubation with hapten, at 37°C, it is possible that the factor in the eluates was, at least in part, actively secreted during the incubation rather than simply dissociated from the cell surface. However, it seems unlikely that significant amounts of inhibitor could be secreted during the relatively short (30 min) incubation period.

It has been proposed by Jerne (14) that the immune response is regulated by a network of idiotype-anti-idiotype interactions. The data presented here offer direct support for the in vivo operation of such an idiotype-specific regulatory network. It is interesting to recall the classic observation that the increase in immunoglobulin concentration during immunization is often too great to account for in terms of antibody specific for the immunizing antigen (15–17). Some of this immunoglobulin may be auto-anti-idiotypic antibodies.

Several lines of evidence support the conclusion that the PFC inhibitory factor is not anti-TNP antibody: (*a*) inhibitory activity is not removed by absorption with a TNP immunoadsorbent; (*b*) rabbit anti-TNP antibody fails to inhibit TNP-specific plaque formation, and (*c*) anti-TNP-F antiserum from nude mice does not inhibit

TABLE IV

Lack of Specific Hapten-Reversible In Vitro Inhibition of Day-4 Anti-TNP PFC by Various Antisera and Antigens

Experiment	Pretreatment of immune spleen cells*	Direct anti-TNP PFC/slide‡	
		Hapten absent	Hapten present
1	NMS	101	106
	Rabbit anti-TNP-HGG	106	102
	Rabbit anti-mouse Fab	112	120
	AKR/J anti-TNP-F (Pool III)	55	103
	TNP-F (1.0 μg/ml)	54	54
	TNP-F (0.1 μg/ml)	52	53
2	NMS	135	133
	AKR/J anti-TNP-F (Pool VI)	102	135
	TNP-F (1.0 μg/ml)	38	50
	TNP-F (0.001 μg/ml)	43	42

* Immune spleen cells were obtained from groups of five AKR/J mice that had been immunized with 10 μg TNP-F intravenously 4 d before assay. The cells (1–2 × 10^6/ml) were pretreated for 5 min at 4°C with the antigens and antisera indicated. Gamma globulin fractions of rabbit anti-TNP-HGG and rabbit anti-mouse Fab were used at a final concentration of 12.5 μg protein/ml. The anti-TNP hemagglutination titer at this dilution was 1:500. Anti-TNP-F antiserum was obtained from a pool of AKR/J mice 7 d after intravenous injection of 10 μg TNP-F.

‡ After pretreatment the cells were washed once and were resuspended for PFC assay so that 1/1,000 of a spleen equivalent was plated per slide.

anti-TNP plaque formation.[3] This latter point deserves special emphasis because TNP-F is a T-independent antigen and elicits an excellent response in nude mice (18). Thus, anti-TNP antibody is probably not responsible for the PFC inhibition. It is to be expected that the anti-idiotypic-antibody response to anti-TNP antibody would be T-dependent as are responses to most protein antigens.

It thus appears that anti-idiotypic antibody can reversibly inhibit secretion by B cells. Inhibition of plaque formation by anti-idiotypic antibody has been seen by several previous workers (19, 20). The mechanism of this inhibition is not known, but there is precedence for reversible inhibition of antibody secretion by the cross-linking of cell-surface antigen receptors. Effector-cell blockade (21, 22), in which polyvalent antigens inhibit antibody secretion, appears to represent such a situation. Inhibition of secretion is induced rapidly and is reversible if the antigen is removed from the cell surface by treatment with appropriate enzymes. It is of interest that although TNP-F did inhibit plaque formation, the inhibition was not hapten reversible, and thus is operationally distinct from the inhibition by anti-idiotypic antibody. The difference in ease of hapten reversibility might lie in the extent of cross-linking involved in the two phenomena. It is probable that with the highly polyvalent antigen, TNP-F, more bonds to cell-surface antigen receptors are formed than with the bi- or oligovalent anti-idiotypic antibodies. Increasing the number of bonds formed would markedly decrease the ease with which displacement by hapten would occur. Antigen-antibody complexes might also inhibit plaque formation (23). Thus, it was important to

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TABLE V

Characterization of the PFC Inhibitory Activity of Anti-TNP-F Antiserum

Experiment	Serum source*	Absorption of serum‡	Percentage of NMS-incubated control§			
			Pool	Mouse 1	Mouse 2	Mouse 3
			%			
1	None	Not absorbed	109	—	68	88
	NMS (1:10)	Not absorbed	100	100	100	100
	Anti-TNP-F (1:100)	Not absorbed	36	30	41	50
	Anti-TNP-F (1:200)	Not absorbed	—	64	78	83
	Anti-TNP-F (1:300)	Not absorbed	—	84	76	83
	Anti-TNP-F (1:10)	DNP-HSA-BAC	39	25	37	30
	Anti-TNP-F (1:100)	DNP-HSA-BAC	54	60	59	50
	Anti-TNP-F (1:200)	DNP-HSA-BAC	83	64	67	68
	Anti-TNP-F (1:300)	DNP-HSA-BAC	105	84	102	80
	Anti-TNP-F (1:10)	Anti-MGG	100	84	100	87
2	NMS (1:10)	Not absorbed	100	—	—	—
	Anti-TNP-F (1:10)	Not absorbed	11	—	—	—
	Anti-TNP-F (1:100)	Not absorbed	31	—	—	—
	Anti-TNP-F (1:10)	Anti-TNP	98	—	—	—
	Anti-TNP-F (1:100)	Anti-TNP	100	—	—	—
3	NMS (1:10)	Not absorbed	100	—	—	—
	Anti-TNP-F (1:10)	Not absorbed	39	—	—	—
	Anti-TNP-F (1:100)	Not absorbed	64	—	—	—
	Anti-TNP-F (1:10)	Anti-dansyl	26	—	—	—
	Anti-TNP-F (1:100)	Anti-dansyl	47	—	—	—

* NMS was a pool from 20 AKR/J mice. A pool of immune serum was taken from 20 mice 7 d after intravenous immunization with 10 μg TNP-F.

‡ DNP-HSA-BAC and anti-MGG immunoadsorbent columns were prepared as described in Materials and Methods. Anti-TNP antibody columns were prepared by incubation of primary (10 μg TNP-F intravenously) day-7 anti-TNP-F serum from AKR/J nu/nu mice, with DNP-HSA-BAC, at room temperature, for 1 h, followed by extensive washing with PBS-D. An anti-dansyl antibody immunoadsorbent (anti-dansyl·dansyl-HSA-BAC) was prepared and used in a manner similar to the anti-DNP-antibody immunoadsorbent.

§ Pooled immune spleen cells from five AKR/J mice, immunized intravenously 4 d earlier with 10 μg TNP-F, were incubated at 1–2 × 10^6 cells/ml with the various serum preparations, for 5 min, at room temperature. The cells were washed once, and were then assayed for anti-TNP PFC. The 100% values for PFC determined in the presence of NMS were: experiment 1: Pool, 76,000 PFC/spleen; mouse 1, 88,000 PFC/spleen; mouse 2, 63,000 PFC/spleen; mouse 3, 60,000 PFC/spleen; experiment 2: Pool, 154,000 PFC/spleen; experiment 3: Pool, 170,000 PFC/spleen. In the presence of 10^{-8}–10^{-7} M TNP-EACA, all PFC values returned to 100% of the NMS control determined in the absence of hapten.

establish, as was done in the present studies, that the inhibitory activity was not removed by absorption with antigen. Furthermore, the presence of immune complexes in antigen excess was considered unlikely in that the serum contained a high anti-TNP-antibody titer.

The observations reported here suggest that hapten-reversible inhibition of plaque formation by immune serum represents a convenient assay for auto-anti-idiotypic antibodies. The approach to assaying anti-idiotypic antibodies described here is equally applicable to the detection of the spontaneous production of auto-anti-idiotypic antibody. The short incubation period used in the assay is essential, as

TABLE VI

*Characterization of the PFC Inhibitory Activity Eluted by Hapten from Immune Spleen Cells**

Inhibitor‡	Source	Dilution	Inhibitor absorbed with§	Percentage of NMS control‖
				%
NMS	Nonimmune AKR/J mice	1:10	Not absorbed	100
Anti-TNP-F serum	Day-7 primary immune AKR/J mice	1:10	Not absorbed	4
Anti-TNP-F serum	Day-7 primary immune AKR/J mice	1:100	Not absorbed	33
Anti-TNP-F serum	Day-7 primary immune AKR/J mice	1:200	Not absorbed	90
Spleen-cell eluate	Normal-cell transfer recipients	1:10	Not absorbed	85
Spleen-cell eluate	Immune-cell transfer recipients	1:10	Not absorbed	27
Spleen-cell eluate	Immune-cell transfer recipients	1:10	DNP-HSA-BAC	34
Spleen-cell eluate	Immune-cell transfer recipients	1:10	Anti-MGG	89
Spleen-cell eluate	Immune-cell transfer recipients	1:10	Anti-TNP-F	100

* Immune spleen cells from AKR/J mice (pool of five) immunized 4 d earlier with 10 μg TNP-F, intravenously, were incubated at $1–2 \times 10^6$ cells/ml with the various inhibitors for 5 min at room temperature. The cells were washed once and were then assayed for anti-TNP-PFC.

‡ NMS was obtained from a pool of 20 AKR/J mice. Pools of immune serum were obtained from groups of 5–20 AKR/J mice immunized, intravenously, with 10 μg TNP-F 7 d before bleeding. Spleen cell eluates were prepared in the following manner: 4 d after intravenous cell transfer and antigen injection (10 μg TNP-F), recipients of immune (primary day-7 anti-TNP-F) or normal spleen cells were killed. Spleen-cell suspensions (5×10^7 cells/ml) were incubated for 30 min at 37°C in the presence of 1×10^{-8} M TNP-EACA. Supernates (eluates) were recovered by centrifugation (1,500 rpm) at 4°C for 15 min followed by overnight dialysis against PBS-D at 4°C.

§ The anti-TNP-F (anti-TNP·DNP-HSA-BAC) immunoadsorbent column was prepared by incubation of primary day-7 anti-TNP-F (10 μg intravenously) serum from AKR/J mice, with DNP-HSA-BAC at room temperature for one h, followed by extensive washing with PBS-D.

‖ In the presence of $10^{-8}–10^{-7}$ M TNP-EACA, all PFC values returned to 100% of the NMS control determined in the absence of hapten.

prolonged incubation, especially at room temperature, yielded less inhibition of plaque formation. Presumably, this progressive loss of inhibition upon prolonged incubation reflects the capping and disappearance from the surface of idiotype-anti-idiotype complexes.

Pools of anti-idiotypic-antibody-containing antisera inhibited anti-TNP plaque formation to varying degrees when tested on d-4 PFC from individual mice of the same strain. This suggests that the assay may detect variability in idiotype expression. Variation in the representation of major idiotypes in sera from individual mice of the same strain has been noted by previous workers (24, 25).

It should be emphasized that two distinctly different interactions are occurring simultaneously in this assay: (*a*) hapten "displacement" of anti-idiotypic antibody, due to the binding of hapten to the cell-surface antigen receptors, leading to the visualization of additional antibody-secreting cells; (*b*) hapten inhibition of plaque formation at the target cell level, as a result of the binding of hapten to the secreted antibody; thereby blocking the interaction of the latter with the hapten-conjugated SRBC. The observed change in the number of PFC is consequently the combined effects of these two factors that act in opposite directions. Therefore, the number of hapten-augmentable PFC detected represents a minimal estimate of the number of anti-idiotype-blocked antibody-synthesizing cells. One must also consider the implications of the phenomenon reported here on the use of hapten inhibition of PFC as an assay of antibody affinity. Clearly the existence of hapten-augmentable PFC will bias the results of affinity measurements by decreasing the number of high-affinity PFC detected as well as reduce the observed heterogeneity. Provided this bias is born in mind, hapten-inhibition data can still provide a useful estimate of PFC affinity. It is necessary to perform studies over a wide range of hapten concentrations to detect any augmentation of the number of PFC. The precise shape of the curve describing the effect of increasing hapten concentration on the number of PFC will be determined by the combination of the two factors noted above, and will be influenced by the

affinity of the anti-idiotypic antibody for the idiotype, and the affinity of the antibody (idiotope) for the hapten.

The ease with which low concentrations of hapten reverse the inhibition and bring about the displacement of anti-idiotypic antibody suggests that such inhibition may operate reversibly in vivo. That is, a steady-state condition may prevail in vivo in which inhibition by auto-anti-idiotypic antibodies, and activation as a consequence of their dissociation, might occur continuously. The number of PFC normally detected (or the serum-antibody level achieved) would reflect the operation of such a homeostatic mechanism. The present work thus supports the view that auto-anti-idiotypic antibody may be spontaneously induced during an immune response to a foreign antigen (1) and suggests that its formation is involved in the normal regulation of immune reactivity.

Summary

Sera taken from AKR/J mice 7 d after the intravenous injection of 2,4,6-trinitrophenyl-lys-Ficoll (TNP-F) caused a specific inhibition of anti-trinitrophenol (TNP) plaque-forming cells (PFC) in vitro. This inhibition was reversed by the incorporation of 10^{-8}–10^{-7} M 2,4,6-trinitrophenyl-ϵ-amino-*n*-caproic acid (TNP-EACA) into the agar during the PFC assay. The factor responsible for the hapten-reversible PFC inhibition was removed from serum by passage through an anti-immunoglobulin column or through a 2,4,-dinitrophenyl-human-serum-albumin-bromoacetylcellulose plus anti-TNP-antibody column, but not by DNP-HSA-BAC alone. It was concluded that this immunoglobulin-like substance, lacking anti-TNP activity but reacting with anti-TNP antibody of AKR/J origin, was most likely an auto-anti-idiotypic antibody that had been produced during the normal course of the response of AKR/J mice to TNP-F. Pools of anti-idiotypic-antibody-containing antisera inhibited anti-TNP plaque formation to varying degrees when tested on d-4 PFC from different mice of the same inbred strain, suggesting a variability in idiotype expression.

4 d after transfer of immune (7 d after 10 μg TNP-F, administered intravenously) AKR/J spleen cells plus 10 μg TNP-F into syngeneic mice, the number of PFC detectable in the recipients' spleens could be markedly augmented by the inclusion of TNP-EACA in the agar during the PFC assay. Incubation of spleen cells containing such hapten-augmentable PFC with TNP-EACA yielded a factor in the supernate that caused a specific, in vitro, hapten-reversible inhibition of anti-TNP PFC. Studies with immunoadsorbents indicated that this PFC-inhibiting factor was antigenically immunoglobulin-like, lacked anti-TNP-antibody activity, but reacted with anti-TNP antibody of AKR/J origin. The results are consistent with the view that this PFC inhibitor is auto-anti-idiotypic antibody that is involved in the normal regulation of the immune response.

It is proposed that hapten-reversible inhibition of plaque formation can be employed as an assay for anti-idiotypic antibody and the conditions for such an assay are described. It is further proposed that the detection of hapten-augmentable PFC suggests the presence of auto-anti-idiotypic antibody.

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