

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

I. Occurrence in AKR/J and BALB/c Mice of Hapten-Augmentable, Anti-TNP Plaque-Forming Cells and Their Accelerated Appearance in Recipients of Immune Spleen Cells*

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Jerne (1) has proposed that the immune system functions as an interacting network of inducible elements that normally exist in a steady state. In this network, the immune response is regulated by idio-anti-idiotypic interactions. Antigen administration stimulates some elements of the network, and the subsequent alterations to restore the perturbed steady-state condition represent the cellular and molecular reactions that occur during the immune response.

In many experimental systems that examine idio-anti-idiotypic interactions, the anti-idiotypic antibodies have been induced in species other than the one in which the idiotypic was produced (2-6). Induction of isologous anti-idiotypic antibodies has also been achieved (4-12). Spontaneous, autologous anti-idiotypic-antibody and (or) anti-receptor-antibody production during the course of an immune response has been described for haptens (13-16), sheep erythrocytes (SRBC)¹ (15, 16), and alloantigens (16-19). In addition, the decrease in binding affinity of anti-tobacco mosaic virus antibodies has been attributed to the appearance, during the course of the immune response, of lymphocytes bearing auto-anti-idiotypic receptors (20).

Although cyclic changes in serum-antibody levels and affinity have been observed during the immune response to many antigens, they have generally been attributed to variations in the degree of masking, by serum antibody, of antigenic determinants on persisting antigen (21, 22). Nevertheless, a rapid decrease in affinity of individual plaque-forming cells (PFC) (23) or serum antibody (20, 24) cannot be convincingly explained in this manner. Urbain (25) has suggested that an auto-anti-idiotypic

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¹ *Abbreviations used in this paper:* C, complement; DNP, 2,4-dinitrophenol; DNP-EACA, 2,4-dinitrophenyl- ϵ -amino-*n*-caproic acid; DNP-F, 2,4-dinitrophenyl-lys-Ficoll; F, Ficoll; PC, phosphorylcholine; PFC, plaque-forming cells; RAMB, rabbit anti-mouse brain antisera; SRBC, sheep erythrocytes; TNP, 2,4,6-trinitrophenol; TNP-EACA, 2,4,6-trinitrophenyl- ϵ -amino-*n*-caproic acid; TNP-F, 2,4,6-trinitrophenyl-lys-Ficoll; TNP-SRBC, trinitrophenylated sheep erythrocytes.

response is responsible for such affinity changes. The decrease in the PFC response to *Pneumococcus* R36A vaccine and the subsequent increase in the number of anti-idiotypic-specific PFC may also reflect idiotypic-anti-idiotypic interactions (13, 14, 26).

In recent studies on the immune response of AKR/J mice to 2,4,6-trinitrophenyl-Ficoll (TNP-F), a precipitous decrease in PFC numbers, as well as a decrease in antibody affinity and heterogeneity, were noted after the peak of the response (27). This decrease in the magnitude of the PFC response occurred much more rapidly and was more marked than that observed during the response to haptens on T-dependent (28), or T-independent, carriers (29–31) in other strains. In a further analysis of this phenomenon, it was observed that under certain circumstances, contrary to expectation, the addition of hapten to a PFC assay resulted in an increase in the number of anti-hapten PFC detected, suggesting the displacement, by hapten, of bound auto-anti-idiotypic antibody from the surface of antibody-synthesizing cells. The data to be reported here and in the companion paper (32) support the hypothesis that auto-anti-idiotypic antibody, produced during the course of the immune response, combines with cell-surface antigen receptors and inhibits antibody secretion.

Several observations that can be readily interpreted by this hypothesis will be presented. They include: (a) the very rapid decline in the number of PFC that occurs early in the immune response of AKR/J mice to TNP-F; (b) the disappearance of high-affinity PFC associated with a decrease in average affinity during the primary response to TNP-F; (c) the occurrence after day 4 of the primary response to TNP-F of blocked anti-2,4,6-trinitrophenol (TNP) antibody-secreting cells that can be detected as PFC only in the presence of hapten; and (d) the observation that both cells and serum from TNP-F immune mice can suppress the response of normal mice to this antigen by blocking a large percentage of antibody-forming cells.

Materials and Methods

Animals. Adult male BALB/c and AKR/J mice were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. and The Jackson Laboratory, Bar Harbor, Maine, respectively.

Antigens and Haptens. TNP-F was prepared by coupling TNP-lysine (Sigma Chemical Co., St. Louis, Mo.) to Ficoll 400 (F, Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) (33), and quantified by optical-density measurement at 348 nm (34) and dry weight. The haptens, 2,4-dinitrophenyl- ϵ -amino-*n*-caproic acid (DNP-EACA) and 2,4,6-trinitrophenyl- ϵ -amino-*n*-caproic acid (TNP-EACA), were prepared and quantified as described (34, 35).

Immunization. Mice were immunized intravenously with 10 μ g TNP-F. Animals were bled, were killed by cervical dislocation, and their spleens were assayed for anti-TNP PFC at various intervals.

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 (36) as modified for slide assay by Dresser and Greaves (37). Trinitrophenylated sheep erythrocytes (TNP-SRBC) were used as indicator cells (28). Normal guinea pig serum, absorbed with SRBC, was the complement (C) source. On occasion, indirect PFC were also enumerated (38).

Assay of Affinity of Anti-TNP PFC. The affinity distribution of anti-TNP PFC was assayed by the inhibition of plaque formation as described previously (39–41). Concentrations of DNP-EACA or TNP-EACA in the agarose and C ranged from 1×10^{-9} to 1×10^{-5} M in half-log increments (42). The Shannon heterogeneity index (42) was used to describe the degree of heterogeneity of affinity of the PFC population from individual mice.

Cell and Serum Transfer. $1.2\text{--}4.0 \times 10^7$ viable spleen cells from normal or immune mice were

transferred, intravenously, together with 10 μg TNP-F into syngeneic, nonirradiated recipients. For serum transfers, normal mice received 1 ml of normal serum, or day-7 immune anti-TNP-F antiserum, intraperitoneally, 2-3 h before the intravenous injection of 10 μg TNP-F. Recipients were killed 3-4 d later and splenic anti-TNP PFC were determined. In some experiments, irradiated recipients were also included (Gammator Irradiator, Radiation Machinery Corp., Parsippany, N. J., 520 rad/min, Cs source). T cells were depleted from the donor cells by treatment with anti-Thy 1.1 antisera plus nontoxic rabbit C (43). Anti-Thy 1.1 antisera was prepared 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). On some occasions, rabbit anti-mouse brain antisera (RAMB) was used for T-cell depletion of the donor population (44). Both anti-Thy 1.1 antisera and RAMB gave similar results in the cell transfers. Sera from donor mice were titrated for anti-TNP antibody by passive hemagglutination with TNP-SRBC (average titer on day 7, 1:5,000).

Results

Hapten Augmentation of Plaque Formation. During a study of the immune response of AKR/J and BALB/c mice to TNP-F, an unusually rapid decline in the number of PFC was observed between days 4 and 7 after antigen injection, particularly in the AKR/J strain (Fig. 1). A switch to 7S PFC was not responsible for this phenomenon because the number of indirect PFC (or 7S PFC detected in the presence of anti- μ chain antibody) was generally lower than the number of direct PFC. To determine if specific immune suppression was responsible for this decline, nonimmune spleen cells or spleen cells taken 7 d after immunization were transferred into normal, nonirradiated, syngeneic mice together with TNP-F. Recipient spleen cells were assayed for anti-TNP PFC 3 or 4 d later. The magnitude of the response was determined and the heterogeneity of the PFC with respect to the affinity of the antibody produced by individual cells was assayed by hapten inhibition of plaque formation. AKR/J recipients of immune spleen cells showed significantly lower PFC responses than did control mice (Table I). However, when the assay for antibody affinity was performed, the addition of hapten had an unexpected effect. Although the recipients of normal cells showed the usual progressive inhibition of plaque formation in the presence of increasing hapten concentrations, the patterns obtained with spleen cells from the experimental group showed a marked augmentation of PFC at intermediate hapten concentrations (Fig. 2). Although the percentage of augmentation was higher on day 3 than on day 4, the total number of hapten-augmentable PFC was higher on day 4 (Table I).

This augmentation of plaque formation was seen over a limited range of hapten concentrations (3×10^{-9} - 10^{-7} M TNP-EACA). As indicated in Table I, the peak number of PFC observed in the presence of hapten averaged two-three times that observed in the absence of hapten. In individual recipients from four different experiments, the percentage of augmentation ranged from 41 to 445%. Augmentation of PFC by free hapten (10^{-7} - 10^{-6} M) was also seen with spleen cells from BALB/c recipients of day-14 immune syngeneic spleen cells, although the degree of augmentation was less than that observed with AKR/J mice (data not shown). No hapten augmentation of PFC was observed in BALB/c recipients of day-7 immune cells. In a similar series of experiments, hapten augmentation of PFC was seen in irradiated AKR/J recipients of day-7 syngeneic immune spleen cells (2,500 PFC in the absence of hapten, 8,600 PFC in the presence of optimal hapten concentrations). As the magnitude of the PFC response was much lower than that of nonirradiated recipients,

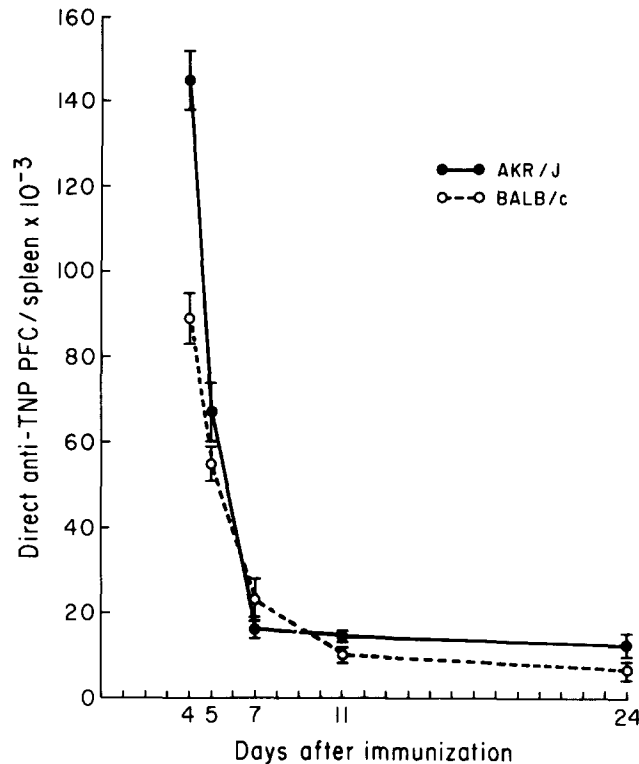


FIG. 1. Kinetics of the PFC response to TNP-F in AKR/J and BALB/c mice. Animals were immunized by the intravenous injection of 10 μ g TNP-F. Data are reported as mean \pm SE for groups of 8-30 mice.

it was concluded that the majority of the PFC in the cell-transfer experiments with normal recipients were probably of recipient origin.

If one calculates the magnitude of the response in terms of the maximum number of PFC detected in the presence of hapten, it is clear that the decrease in the number of antibody-synthesizing cells observed in recipients of immune cells (Table I) was only apparent. Thus, in the presence of hapten, the average number of PFC/spleen in the recipients of immune cells was equal to, or higher than, control values.

The fine specificity of hapten augmentation of anti-TNP PFC was examined by comparing the effect of TNP-EACA with that of the cross-reactive hapten, DNP-EACA (Fig. 3). The degree of augmentation was less with DNP-EACA than with TNP-EACA, and higher concentrations of DNP-EACA were required for augmentation of PFC. Thus, the cross-reactive hapten, 2,4-dinitrophenol (DNP), was less effective than TNP in releasing the PFC from suppression.

B-cell Role in the Regulatory Effects of Immune Spleen Cells. The treatment of immune spleen cells with RAMB or with anti-Thy 1.1 antisera plus C, before transfer, failed to eliminate the hapten augmentation of the recipients' PFC. Furthermore, rather than a decrease in the magnitude of the PFC response, there was an increase over the control response by recipients of normal cells (Fig. 2 and Table II). These observations implied that this form of regulation, and, in particular, the appearance of hapten-augmentable PFC, was not mediated solely by T cells, although they did appear to

TABLE I
Apparent Inhibition of the Primary Response to TNP-F in Normal Recipients of Syngeneic Immune Cells and Reversal by the Presence of Hapten in the Agarose during the PFC Assay

Donor cells*	Assay day	Direct anti-TNP PFC per spleen		Percentage augmentation by hapten‡
		Assayed with hapten absent	Assayed with hapten present	
Normal spleen	3	39,800 \times 1.11 (10)	46,800 \times 1.13	17
Immune spleen (day 7)	3	11,200 \times 1.30 (7)	44,700 \times 1.14	298
Normal spleen	4	112,000 \times 1.26 (11)	120,200 \times 1.26	7
Immune spleen (day 7)	4	67,600 \times 1.29 (11)	149,900 \times 1.17	122

* 4×10^7 spleen cells from normal or immune AKR/J mice were transferred, together with 10 μ g TNP-F, into normal AKR/J mice (day 0). Immune donors had been injected with 10 μ g TNP-F 7 d previously. Direct anti-TNP PFC were assayed 3 or 4 d after cell transfer as indicated. The data are presented as geometric means \times SE (number of animals studied). Results summarize the data obtained from five independent experiments.

‡ Percentage augmentation of PFC by optimal hapten concentration (3×10^{-9} – 10^{-7} M TNP-EACA) is calculated as: $100 \times (\text{PFC in presence of hapten} - \text{PFC in absence of hapten}) / \text{PFC in absence of hapten}$.

have an important role. T-depleted, primed B cells apparently had two effects on the response of recipients: (a) they increased the anti-TNP PFC response to TNP-F, and (b) they controlled the appearance of hapten-augmentable PFC. Because of these findings and of the rigorous hapten specificity (Fig. 3), it seemed probable that appearance of hapten-augmentable PFC was mediated by a B-cell product, most likely, under T-cell control.

Therefore, it was determined whether serum from day-7 immune AKR/J mice could also affect the response of normal recipients to TNP-F. Injection of immune serum into normal mice at the time of immunization caused a decrease in the number of PFC/spleen (Table II). This depression was similar to the apparent decrease in PFC observed after immune-cell transfer, in that it was reversible by hapten. Because the response of the immune-serum-treated mice was low for a day-3 response even after hapten augmentation, an additional inhibition of the immune response due to the high anti-TNP-antibody titer of the serum was likely (45, 46).

It was concluded that a serum factor, as well as immune cells, could cause a spurious inhibition of the PFC response. It was also concluded that although B cells were a major contributor to the regulatory effect, the presence of immune T cells in the transferred-cell population increased the degree of apparent suppression.

Hapten-Augmentable PFC Occur Spontaneously in Spleens of Normal Mice during the Immune Response to TNP-F. It was argued that if auto-anti-idiotypic interactions were responsible for the suppression observed after immune-spleen-cell and serum transfers, a similar apparent suppression of PFC might occur during the normal immune response in the donor animals. A temporal study was therefore carried out on the ability of hapten to inhibit or augment the apparent number of anti-TNP PFC present at

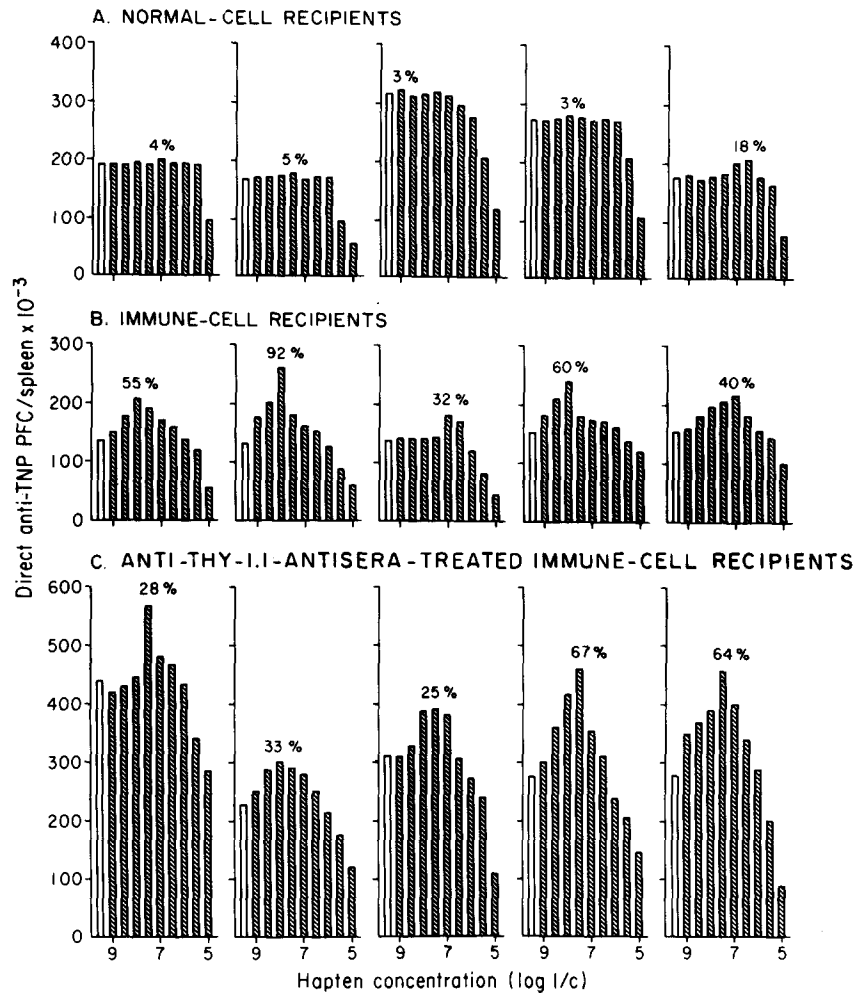


FIG. 2. The splenic PFC response and presence of hapten-augmentable PFC in AKR/J recipients of anti-Thy 1.1 antisera plus C treated syngeneic immune donor spleen cells. The anti-TNP PFC responses of all recipients were determined 4 d after intravenous injection of donor cells plus $10 \mu\text{g}$ TNP-F. The number of PFC/spleen is indicated on the abscissa. The effect of TNP-EACA on the number of PFC is indicated by the hatched bars. Hapten concentration increases to the right. Maximum percentage augmentation of PFC by hapten is written at the top of the relevant bar in the histogram. The number of PFC in the absence of hapten is indicated by the open bar. Panel A: recipients of 4×10^7 normal spleen cells; Panel B: recipients of 4×10^7 immune spleen cells; Panel C: recipients of 2.6×10^7 anti-Thy-1.1 antisera-treated immune spleen cells. 10^7 cells/ml were incubated with C (1:18) alone or together with anti-Thy 1.1 (1:60), for 45 min at 37°C . The number of cells transferred is the number of viable cells present (out of an initial 4.5×10^7 cells/recipient) after treatment with these reagents. Immune donor cells were obtained from AKR/J mice 7 d after intravenous injection of $10 \mu\text{g}$ TNP-F.

various times after the injection of TNP-F. The frequency and the degree of augmentation of PFC are reported in Table III. In both strains of mice studied, there was a low incidence (17–22%) of animals having detectable hapten-augmentable PFC on day 4, the peak of the PFC response. The incidence increased to 60% by day 5 and, essentially, all mice tested on days 11 and 14 showed hapten-augmentable PFC. The

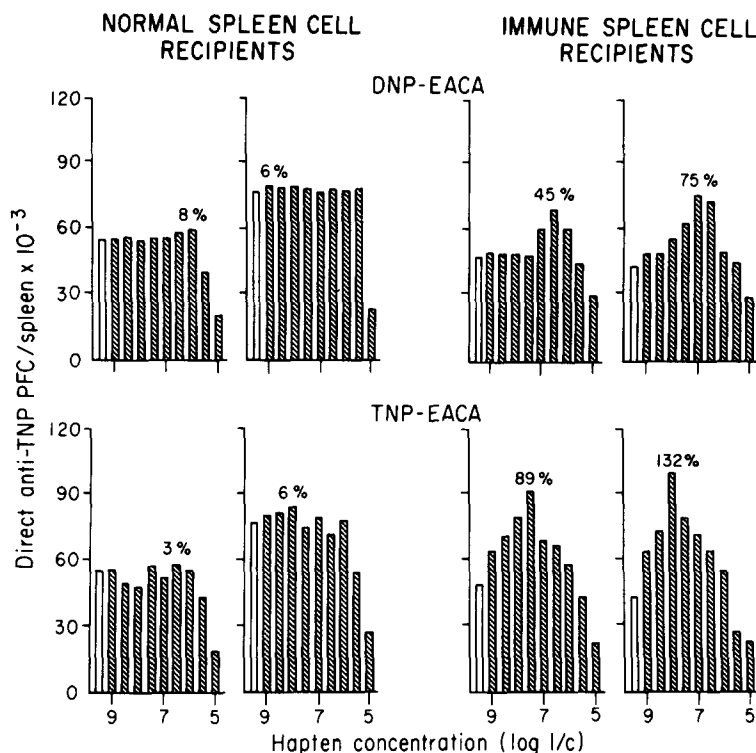


FIG. 3. Specificity of hapten augmentation of plaque formation. Normal AKR/J mice received 4×10^7 spleen cells from normal, or day-7 immune ($10 \mu\text{g}$ TNP-F), syngeneic donors together with $10 \mu\text{g}$ TNP-F. Splenic direct anti-TNP PFC were assayed 4 d later. The effect of DNP-EACA (top panel) or TNP-EACA (bottom panel) on the number of PFC is shown. Hapten concentration increases to the right. The number of direct PFC per spleen is indicated on the ordinate and the log of the reciprocal of the hapten concentration is indicated on the abscissa. The numbers at the top of the histograms indicate the percentage of augmentation of PFC at optimal hapten concentration as compared with the number of PFC in the absence of hapten. In each case, aliquots of the same spleen were used in hapten-augmentation studies with TNP-EACA and DNP-EACA (presented above one another). The number of PFC/spleen in the absence of hapten is indicated by the open bar.

overall average percentage of augmentation of PFC remained relatively low and constant with time after immunization in AKR/J mice. In contrast, the data suggest that there is an increase in the percentage of augmentation of PFC with time after immunization in BALB/c mice.

However, the number of demonstrable auto-anti-idiotypic-antibody blocked PFC cannot account for the decline in total PFC with time after immunization. There is, for instance, on days 11–14 of the response of AKR/J mice, only a 26–30% increase in the number of observed PFC in the presence of hapten, whereas the decrease from the peak (day 4) “observed” PFC response is 90%.

Heterogeneity Indices of PFC during the Response to TNP-F. An attempt was made to relate the above findings on blocked antibody-forming cells to the decrease in apparent heterogeneity of antibody as measured by the conventional plaque inhibition assay (39). In these calculations, any hapten-augmentable PFC were purposely ignored. Within the limitations of this approach, a pattern of affinity maturation quite opposite

TABLE II
Relative Roles of T and B Cells in the Regulation of the Immune Response of Normal AKR/J Recipient Mice to TNP-F

Experiment	Donor cells*	Pretreatment	Number of cells injected‡	Direct anti-TNP PFC per spleen§		Percentage augmentation by hapten
				Assayed with hapten absent	Assayed with hapten present	
			$\times 10^7$			%
1	Normal spleen	C	4	216,900 \times 1.13 (5)	230,600 \times 1.12	6
	Normal spleen	anti-thy 1.1 + C	2.6	243,400 \times 1.15 (5)	249,800 \times 1.15	3
	Immune spleen	C	4	139,600 \times 1.03 (5)	205,800 \times 1.06	47
	Immune spleen	anti-thy 1.1 + C	2.6	299,600 \times 1.12 (5)	426,600 \times 1.11	42
2	Normal spleen	C	2	240,500 \times 1.13 (2)	242,500 \times 1.14	1
	Immune spleen	C	2	189,900 \times 1.06 (3)	321,300 \times 1.08	69
	Immune spleen	RAMB + C	1.2	349,300 \times 1.04 (3)	454,500 \times 1.07	30
3	Normal serum (1 ml)	—	—	31,500 \times 1.20 (5)	32,600 \times 1.20	3
	Immune serum (1 ml)	—	—	3,800 \times 1.21 (5)	10,900 \times 1.23	189

* Spleen cells or serum from normal or immune AKR/J mice were transferred, together with 10 μ g TNP-F, into normal mice (day 0). Immune donors had been injected with 10 μ g TNP-F 7 d previously. The data are presented as geometric means \times / + SE (number of animals studied).

‡ 10^7 cells/ml were incubated either with C(1:18), or together with anti-Thy 1.1 (1:60), or together with RAMB (1:50) for 45 min at 37°C. The number of cells transferred represent the number of viable cells present (out of an initial 4.5×10^7 cells per recipient) after treatment with these reagents.

§ Assayed 3 d (experiment 3) or 4 d (experiments 1 and 2) after cell transfer.

|| Calculated as in Table I.

to that usually observed with T-dependent antigens was found. Rather than the expected increase in heterogeneity and in average affinity with time after immunization, a decrease in both parameters was seen (Table IV). This decrease was, at least to some extent, an artifact due to the simultaneous presence of PFC inhibition and augmentation. Inhibition curves obtained with DNP-EACA showed a much greater decrease in apparent heterogeneity than did inhibition curves obtained with TNP-EACA. One would expect that the heterogeneity determinations would be more affected by the augmentation caused by the DNP hapten, because this ligand required higher concentrations to bring about an augmentation of PFC than did the TNP hapten (Fig. 3). Thus, augmentation by DNP-EACA would be observed at a higher hapten concentration, and the observed affinity distribution would be restricted, artificially, to the lower-affinity region of the curve.

Hapten-concentration dependence of plaque inhibition is influenced by both the

TABLE III
Augmentation by Hapten of Observed Anti-TNP Splenic PFC from AKR/J and BALB/c Mice during the Primary Immune Response to TNP-F

Strain	Assay day	Incidence of >10% augmentation*		Overall average percentage augmentation*
		Ratio	Percentage	
AKR/J	4	2/12	17	5 ± 1.2
	5	6/10	60	25 ± 5.4
	7	4/14	29	15 ± 6.14
	11	8/8	100	26 ± 2.47
	14	8/8	100	30 ± 5.3
BALB/c	4	2/9	22	9 ± 3.3
	5	6/10	60	15 ± 4.1
	7	7/14	50	26 ± 8.6
	11	9/9	100	37 ± 5.3
	14	7/9	78	54 ± 15.3

* Number of mice showing >10% increase of PFC at optimal (3×10^{-9} – 10^{-6} M TNP-EACA) hapten concentration/total number of mice tested.

‡ Calculated as in Table I, and reported as mean ± SE.

affinity of the antibody for the inhibitor and the rate of antibody secretion. The conditions employed here (direct haptentation of erythrocytes at a fairly high epitope density and the use of a monovalent inhibitor) probably result in an inhibition curve that can provide thermodynamic information (affinity); however, the conditions of the assay have not been optimized for obtention of affinity data (47). For the purposes of the present studies, the slight uncertainty introduced is not significant because the main concern is not with hapten inhibition or affinity, but, rather, with hapten augmentation of PFC. Within the limits of the techniques used, it appeared that the decreases in affinity and heterogeneity after the peak of the immune response were caused by the *in vivo* inhibition of, and resulting inability to detect, *in vitro*, as PFC, a subset of antibody-synthesizing cells, due to the presence of an auto-anti-idiotypic response.

Discussion

Numerous studies indicate that important immuno-regulatory effects can be obtained by deliberate immunization with idiotypes (4, 16, 17, 19, 48, 49). Although several observations concerning spontaneous, production of auto-anti-idiotypic antibody have been made (13–19, 50), no definitive evidence for their regulatory effect *in vivo* has been obtained. Taken in conjunction with the results reported in the accompanying paper (32), which provide direct evidence for the auto-anti-idiotypic-antibody nature of the inhibitory factor studied here, the present report argues strongly in favor of the auto-regulatory effect of such antibodies, as originally suggested by Jerne (1). In the accompanying paper (32), it has been shown that the auto-anti-idiotypic antibody present in immune sera can block secretion of anti-TNP antibody by PFC *in vitro*. In this paper, it was shown that blocked PFC are present in immune mice at a time when both the magnitude of the PFC response and the apparent heterogeneity of PFC with respect to antibody affinity are decreasing rapidly.

TABLE IV
Comparison of Shannon Heterogeneity Indices Obtained with TNP-EACA and DNP-EACA as Inhibitors of Splenic Direct anti-TNP PFC at Various Days after Primary Immunization of AKR/J Mice with TNP-F

Days after immunization	Heterogeneity index*	
	TNP-EACA	DNP-EACA
4	2.14 ± 0.06 (7)	1.54 ± 0.21 (9)
5	1.79 ± 0.10 (10)	ND‡
7	1.67 ± 0.08 (23)	ND
11	1.52 ± 0.07 (6)	0.02 ± 0.02 (6)

* Hapten inhibition of PFC data obtained at various days after intravenous injection of 10 µg TNP-F. Heterogeneity is expressed as the Shannon index (42) and presented as mean ± SE (number of animals studied). In calculation of these indices any augmentation of PFC by hapten was purposely ignored.

‡ ND, not done.

The precipitous decline in the number of PFC/spleen between days 4 and 7 of the primary immune response has not been observed by other investigators who studied the response to intraperitoneally injected DNP-F in other strains of mice (29, 31). TNP-F-anti-TNP-F immune complexes might be responsible for the induction of an auto-anti-idiotypic response (51, 52). Because such complexes are likely to be more prominent after intravenous than after intraperitoneal immunization, there might be an influence of route of antigen injection on this phenomenon. There also might be strain differences in the number of idiotypes specific for the TNP hapten or in their quantitative representation in the immune response that, in turn, could influence the anti-idiotypic response. Such differences are illustrated in the present paper in that the decline in the number of PFC/spleen and in heterogeneity (Fig. 1 and unpublished observations) was less marked in BALB/c than in AKR/J mice. It could be inferred from these observations that the humoral immune response of AKR/J mice to TNP-F involves a major idio type specific for the TNP hapten. It is of interest that the isoelectric-focusing spectra of serum antibody from individual AKR/J mice immunized with 2,4-dinitrophenyl-lys-Ficoll (DNP-F) are severely restricted and display identical patterns as compared to the marked heterogeneity of anti-DNP-F antibody from individual C57BL/6 mice.²

Although the auto-anti-idiotypic response occurs in both AKR/J and BALB/c mice, studies with sera from both strains (unpublished data) indicate that the response occurs earlier and reaches higher serum levels in the AKR/J strain. Hapten-augmentable PFC are clearly present in both strains after the peak of the response to TNP-F (Table III), and may actually be more numerous in BALB/c, possibly due to the more prolonged nature of the auto-anti-idiotypic response and (or) because of the absence of a major idio type to TNP in this strain. Preliminary observations in a number of other strains and with other thymus-independent and thymus-dependent TNP conjugates suggest that the occurrence of hapten-augmentable PFC during the anti-TNP response of mice may be a universal phenomenon.³ Further experiments are needed to determine whether similar (cross-reactive) or different idiotypes are

² Zitron, I. Personal communication.

³ Goidl, E. A. Unpublished data.

involved in the immune responses of AKR/J and BALB/c mice to TNP-F. There are several descriptions of cross-reactive idiotypes (49, 53-56); the majority are linked to genes coding for heavy-chain allotypes (57), although some are not (56, 58, 59).

The unblocking of PFC by displacement of anti-idiotypic antibody was observed with both TNP- and DNP-EACA. The greater effectiveness of the TNP hapten probably reflects the known, fine-specificity differences of anti-TNP antibodies and cell receptors for these haptens (60, 61). Another interesting observation is that the peak hapten augmentation of AKR/J anti-TNP PFC usually occurred between 3×10^{-9} and 1×10^{-7} M hapten, whereas with BALB/c anti-TNP PFC it occurred between 1×10^{-7} and 1×10^{-6} M TNP-EACA. This might reflect subtle differences between these strains in their idiotypic-anti-idiotypic interactions or in the affinity of their antibody for the haptenic determinant.

The results presented here indicate that transfer into normal AKR/J mice of day-7 immune serum, or splenic B cells plus TNP-F, led to an earlier appearance of hapten-augmentable PFC than did immunization with TNP-F alone. However, immune B cells alone did not appear to transfer suppression because, in recipients of T-cell-depleted immune spleen cells, the numbers of PFC detected in the presence or absence of hapten was equal to, or greater than, what was observed in the response of mice that received normal cells plus antigen. Apparently, anti-TNP immune B cells could add to the magnitude of the response of normal recipients only when immune T cells were excluded from the donor population. The inclusion of day-7 immune T cells led to a decrease in the combined response of donor and recipient B cells. T cells could have several roles in this phenomenon. Firstly, they may simply function as helper T cells in the induction and height of the anti-idiotypic-antibody response, which is clearly suggested by the observation that congenitally athymic, homozygous for the recessive gene *nu* (*nu/nu*) AKR/J mice do not make a detectable anti-idiotypic response.⁴ Additional evidence for this supposition comes from work showing that mice depleted of T cells fail to make anti-idiotypic antibody to a myeloma protein (62), indicating that the response to this protein antigen is T-dependent as might be expected. The existence of idiotypic-specific helper T cells has recently been demonstrated (63). It could be postulated that such cells also operate as helper cells for the anti-idiotypic response. Secondly, an idiotypic-specific suppressor T cell may also be involved in the suppression described here, for which there are many precedents (16, 19, 20, 48, 49). Further experiments are needed to determine the exact nature of the T-cell involvement in these studies.

The earliest indication of regulation by anti-idiotypic antibody may be the appearance of hapten-augmentable PFC. Regulation by relatively minor anti-idiotypic responses might go undetected without a specific examination for the presence of blocked, hapten-augmentable PFC. Hapten-augmentable PFC were, in fact, present in the primary response of mice to phosphorylcholine (PC) in studies by Cosenza and Köhler (8), although their significance was not discussed. Because day-7 immune B cells greatly accelerated the appearance of hapten-augmentable PFC, the anti-idiotypic response must have occurred almost immediately after the peak of the anti-

⁴ Schrater, A. F., E. A. Goidl, G. J. Thorbecke, and G. W. Siskind. Production of auto-anti-idiotypic antibody during the normal immune response to TNP-Ficoll. III. Absence of regulation by auto-anti-idiotypic antibody in *nu/nu* mice. Manuscript in preparation.

TNP-F response (Table I). This is consistent with data in the accompanying paper showing the presence of anti-idiotypic antibody in day-7 immune serum (32). In contrast, during the response to PC, the auto-anti-idiotypic antibody was detected in serum only after repeated immunizations (13). Cosenza (14) found that the anti-idiotypic (anti-anti-PC) PFC response peaked, in the spleen, 3 d after the peak of the secondary PFC response to *Pneumococcus* R36A vaccine. The rapidity of the auto-anti-idiotypic response in AKR/J mice may be due to their unusually high antibody response to TNP-F. It is also possible that relatively nondegradable polysaccharide antigens might favor the induction of auto-anti-idiotypic responses, because the persisting complexes of antigen and antibody (idiotype) are likely to be more immunogenic than is the idiotype present on cell surfaces, or free in the circulation (51, 52).

These results support the view, proposed by Tasiaux et al. (20) and Urbain (25), that the fall in antibody affinity during the primary and secondary immune responses may be due to regulation by auto-anti-idiotypic. It is likely that in the present studies, at least part of the decrease in PFC affinity and heterogeneity is artifactual, and is the result of the hapten-augmented PFC obscuring the presence of high-affinity PFC that would otherwise have been observed in the low-hapten-concentration end of the inhibition curve. However, others (24, 35) have detected, by equilibrium analysis, similar affinity changes in serum antibody.

Hapten inhibition of plaque formation has been used by many workers as an assay of antibody affinity since it was first described by Andersson (39). Both correlative studies and mathematical analysis (40) indicate that a valid estimate of affinity can be obtained in this manner. When this method has previously been employed in our laboratory, we have often noted a modest (<20%) augmentation of plaque formation at low hapten concentrations. Because, in most cases, the 5 or 10% augmentation observed was within experimental error, it was ignored. Furthermore, augmentation of the minimal degree usually seen in anti-hapten responses does not significantly alter the affinity-distribution curve calculated from the hapten data. When, in the cell-transfer studies described here, hapten augmentation of PFC far exceeded any conceivable experimental error, it became apparent that a real phenomenon existed that required precise explanation. The results reported in this and the accompanying (32) papers provide evidence that hapten augmentation of PFC is due to displacement of auto-anti-idiotypic antibody and can be used as an assay for the presence of such antibodies. Finally, the data point out a potential hazard in the use of hapten inhibition of plaque formation as an assay of antibody affinity. Clearly, when marked hapten augmentation of plaque formation is present, hapten inhibition cannot be directly used as an assay for affinity distributions.

Summary

Attempts were made to elucidate the cause of the downward regulation of the splenic plaque-forming cell (PFC) response in AKR/J and BALB/c mice between days 4 and 7 after a single intravenous injection of 2,4,6-trinitrophenyl-lys-Ficoll (TNP-F). AKR/J spleen cells, taken 7 d after injection of TNP-F, were transferred, together with TNP-F, into normal AKR/J mice. The day-3 or -4 PFC response of the recipients was much lower than that of recipients of normal cells. However, the suppression was only apparent because the presence of 10^{-8} – 10^{-7} M 2,4,6-trinitro-

phenyl- ϵ -amino-*n*-caproic acid (TNP-EACA) (or 10^{-7} – 10^{-6} M 2,4,-dinitrophenyl- ϵ -amino-*n*-caproic acid) in the PFC assay caused a dramatic increase in observed PFC, averaging 298% on day 3 and 122% on day 4. Recipients of normal cells showed no such hapten-augmentable PFC. T-depleted immune spleen cells did not cause any apparent suppression of the response to TNP-F, but hapten-augmentable PFC in recipient spleens were again prevalent. Suppression of the PFC response, as well as hapten-augmentable PFC, were seen after transfer of immune serum. It was postulated that hapten augmentation of PFC was caused by displacement of auto-anti-idiotypic antibody from the surface of blocked antibody-synthesizing cells.

Further studies showed that such hapten-augmentable PFC occurred in the spleens of a large percentage of both AKR/J and BALB/c mice examined after day 4 of the primary response to TNP-F. Thus, it was hypothesized that the downward regulation of the magnitude and, possibly, also of the heterogeneity of the splenic-PFC response was due to an auto-antibody response to one or more major idiotypes of the anti-TNP response.

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