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# REGULATION OF THYMUS-INDEPENDENT RESPONSES: UNRESPONSIVENESS TO A SECOND CHALLENGE OF TNP-FICOLL IS MEDIATED BY HAPTEN-SPECIFIC ANTIBODIES[1]

PETER H. BRODEUR[2] AND HENRY H. WORTIS

*From the Department of Pathology, Tufts University School of Medicine, Boston, MA 02111*

**CBA mice immunized with the TI-2 antigen TNP-Ficoll are unresponsive to a second challenge of the same antigen. In addition, spleen cells from unresponsive mice fail to respond to any dose of TNP-Ficoll *in vitro*. This hapten-specific unresponsive state is T cell independent and is due to hapten-specific IgM-mediated inhibition of B cell triggering. The mechanism of inhibition, although unknown, is unlikely to be antigen masking.**

***In vitro* responses to the TI-1 antigen TNP-LPS are not inhibited by concentrations of anti-TNP antibody, which completely suppress responses to TNP-Ficoll.**

Regulation of humoral responses can result from a variety of distinct pathways and mechanisms (1). Although mature T lymphocytes play a major role in regulating thymus-dependent (TD)[3] antibody responses, the major mechanism(s) involved in regulating thymus-independent (TI) antigen responses is not clear. In addition, it is possible that different mechanisms of regulation are important for different TI antigens. TI antigens have been classified as TI-1 or TI-2 based on their ability to stimulate B cells from mice with an X-linked immunodeficiency (CBA/N) (2). Although the classification of TI antigens into two "types" is useful, antigens within each "type" can be distinguished by a number of criteria. For example, it has been shown that TI antigens can vary greatly in terms of B cell subpopulations that are triggered (3), adherent accessory cell dependence (4), mitogenicity (5), C3 activation and splenic localization (6), and the degree of T cell independence (7).

TI-antigens have been shown to generate little or no quantitative or qualitative memory. In fact, several workers have observed that mice previously immunized with a TI-2 antigen are unresponsive to a second challenge with the same antigen (8–10). Recent reports, however, have demonstrated that cells from TI-antigen immunized mice can respond as well or better than normal spleen cells when adoptively transferred to irradiated recipients before a second antigenic challenge (11, 12). It seems likely, therefore, that some form of regulation prevents the TI-antigen triggering of functional B cells after a primary response and that adoptive transfer protocols can circumvent the mechanisms that cause unresponsiveness.

Utilizing the TI-antigen TNP-Ficoll, we have tested the hypothesis that functional B cells are prevented by a humoral mechanism from responding to a second challenge. We then determined the nature, specificity, and T cell dependence of the inhibitory factor. Finally, we asked whether spleen cells can respond to a different TI-antigen, TNP-LPS,[3] under conditions in which responses to TNP-Ficoll are inhibited.

## MATERIALS AND METHODS

*Mice.* CBA/Tufts mice were bred in our colony and used in most experiments unless otherwise noted. Mice were between 3 and 6 mo of age and within each experiment were sex and age matched. CBA/Tufts athymic (nu/nu) and normal littermates (+/?) were from 5th or 12th backcross generation bred under specific pathogen-free conditions.

*Antigens and immunization.* *N*-(2-aminoethyl) carbamylmethylated-Ficoll (aecm-Ficoll) was prepared by the method of Inman (13) using Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, NJ) with an average m.w. of 400,000. Haptenated derivatives of aecm-Ficoll were prepared by reaction with 2,4-dinitrobenzene sulfonic acid, 2,4,6-trinitrobenzene sulfonic acid, or fluorescein isothiocyanate (FITC) isomer 1 (Sigma Chemical Co., St. Louis, MO) to yield $DNP_{93}$-aecm-Ficoll (DNP-Ficoll), $TNP_{60}$-aecm-Ficoll (TNP-Ficoll), and $FL_{21}$-aecm-Ficoll (FL-Ficoll), respectively. The molar substitution ratios were determined by dry weight and optical density (14, 15). TNP-LPS and DNP-LPS[3] were prepared as described by Jacobs and Morrison (16) using LPS W *Escherichia coli* 0127:B8 (Difco Laboratories, Detroit, MI).

Mice were immunized via an i.p. injection with 10 μg haptenated Ficoll or 1.0 μg haptenated LPS in Hanks' balanced salt solution (HBSS).

*Adoptive transfers.* Either $2 \times 10^7$ or $5 \times 10^7$ viable spleen cells were injected i.v. into irradiated (800 R, $^{137}Cs$ source at 590 R/min) recipients. Recipients were immunized within 1 hr after cell transfer.

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[2] To whom correspondence should be directed. Present address: The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111.

[3] Abbreviations used in this paper: TI, thymus-independent: TI-1, thymus-independent type 1; TI-2, thymus-independent type 2; aecm-Ficoll, *N*-(2-aminoethyl) carbamylmethylated-Ficoll; DNP-Ficoll and TNP-Ficoll, 2,4-dinitrophenyl and 2,4,6-trinitrophenyl derivatives of aecm-Ficoll, respectively; DNP-LPS and TNP-LPS, 2,4-dinitrophenyl and 2,4,6-trinitrophenyl derivatives of LPS, respectively; FL-Ficoll, fluoresceinated aecm-Ficoll; TNP-SRBC, SRBC conjugated with trinitrophenyl groups; C3, third component of complement; NMS, normal mouse serum; TD, thymus-dependent; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TNBS, trinitrobenzene sulfonic acid; FL-SRBC, fluoresceinated SRBC; FITC, fluorescein isothiocyanate; FL, fluorescein; DNP-lys-Sepharose, cyanogen bromide-activated Sepharose 4B reacted with DNP-lysine.

*Spleen cell culture.* Culture conditions for generating *in vitro* antibody responses were similar to those described by Hodes and Singer (17). Spleen cells ($5 \times 10^5$) were cultured in microtiter plates (Micro Test II flat-bottom No. 3040, Falcon, Oxnard, CA) in 0.2 ml. Iscove and Melchers' (18) modified Dulbecco's medium (formula 78-5220, GIBCO, Grand Island, NY) supplemented with 100 μg/ml streptomycin sulfate (GIBCO) and 100 U/ml penicillin G (Pfizer, New York, NY), $5 \times 10^{-5}$ M 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (FBS; Lot 40551105, Flow Laboratories, Rockville, MD). Cultures were incubated in a humidified 37°C, 5% $CO_2$-air incubator for 3 to 4 days and then harvested and washed in HBSS containing 0.1% bovine serum albumin, fraction V, and 10 mM HEPES[3] buffer (Sigma Chemical Co.). Three to 4 microtiter wells were pooled as a culture group, and 3 such groups were assayed for PFC.

*PFC assay.* Direct PFC were assayed using the slide modification (19) of the Jerne hemolytic plaque assay (20). SRBC were obtained in Alsever's solution every 2 wk from a local source. TNP-SRBC were coupled by a slightly modified method of Rittenberg and Pratt (21) in which 15 mg TNBS[3] were reacted with 1.0 ml packed SRBC for 10 min in 7 ml 0.28 M cacodylate buffer, pH 6.9. FL-SRBC[3] were coupled as described by Möller (22), except that 4 mg of FITC was reacted with 1.0 ml packed SRBC in 4 ml of 0.12 M carbonate buffer, pH 9.2, for 25 min. Coupled SRBC were washed 5 times in HBSS containing 1% FBS. Guinea pig serum was used as the C source.

*Immunoabsorbents.* An anti-IgM column was prepared by coupling goat anti-MOPC 104E (μ, λ) (affinity purified on a TEPC 183 [μ, κ] column) to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals). Five-tenths milliliter of CBA anti-TNP-Ficoll serum was passed over a 3-ml column containing 10 mg of goat anti-IgM. The material bound to the column was eluted with 0.1 M glycine buffer, pH 2.3. The eluted fractions were brought to pH 7.4 with Trizma base (Sigma Chemical Co.), and both unbound and bound protein were dialyzed against serum-free culture medium. All TNP-specific hemagglutination activity of the anti-TNP-Ficoll serum was bound and eluted from the column.

DNP-lys-Sepharose, a generous gift from Suzan Friedman, was prepared by reacting 75 g cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals) with 90 mg DNP-lysine. A 99% coupling efficiency was determined by spectrophotometry. Five-tenths milliliter of anti-TNP-Ficoll serum was passed over a 1.0-ml DNP-lys-Sepharose column or a control of uncoupled Sepharose 4B. The bound material was eluted with 0.2 M acetic acid, pH 2.7. Bound and unbound fractions were dialyzed against serum-free culture medium. By micro-hemagglutination analysis with TNP-SRBC, all anti-TNP activity was found to be bound by DNP-lys-Sepharose, whereas no anti-TNP activity bound to the control (Sepharose 4B) column.

*Sera.* Mouse sera were obtained by tail-bleeding heated mice, removing the contracted clot, and centrifuging 5 min at 1000 × G. Sera were sterilized by passage through 0.20-μ membranes (Gelman Instrument Co., Ann Arbor, MI). All sera (mouse and FBS) were heat-inactivated at 56°C for 30 min.

## RESULTS

*What is the specificity of induced unresponsiveness?* Control mice or mice immunized 15 days previously with 10 μg TNP-Ficoll were challenged with either 10 μg TNP-Ficoll or a mixture of 10 μg TNP-Ficoll + 10 μg FL-Ficoll. Anti-TNP and anti-fluorescein (anti-FL) splenic PFC were enumerated 5 days after the second immunization (day 20). Mice previously immunized with TNP-Ficoll failed to respond to a second challenge of TNP-Ficoll, but responded normally to the noncross-reactive hapten FL coupled to Ficoll (Fig. 1).

These data demonstrate that unresponsiveness after a TNP-Ficoll or FL-Ficoll response is specific for the haptenic determinant and not for the carrier molecule (aecm-Ficoll). The results also rule out the hapten-specific generation of nonspecific suppression.

*Are T lymphocytes required to induce unresponsiveness?* To determine whether mature T cells are required to induce unresponsiveness, CBA nude mice and normal littermate controls were tested. As shown in Figure 2, both euthymic and athymic mice failed to respond to a second challenge of DNP-Ficoll. These results rule out the necessity for mature T cells or T cell products in the induction and maintenance of the unresponsive state.

*Do TNP-Ficoll-unresponsive mice have functional, TNP-Ficoll-responsive B cells?* Spleen cells from mice immunized 14 days previously with an immunogenic dose (10 μg) of TNP-Ficoll or from normal mice were used to reconstitute irradiated (800 R) syngeneic recipients. Figure 3 demonstrates that $2 \times 10^7$ spleen cells from normal or unresponsive mice were equally effective in reconstituting a TNP-Ficoll response.

A similar experiment was performed using (CBA/N × CBA/Tufts)$F_1$ mice. Male $F_1$ progeny have the X-linked immunodeficiency (xid) defective phenotype and are nonresponders to TI-2 antigens, whereas female $F_1$ mice respond normally to TI-2 antigens.

Male $F_1$ (TI-2 nonresponders) mice were injected with either normal $F_1$ female spleen cells or with cells from $F_1$ female mice immunized 14 days previously with 10 μg DNP-Ficoll. The results of this experiment were essentially identical to the irradiated recipient experiments, thus showing that spleen cells from previously immunized mice are capable of responding upon adoptive transfer to nonirradiated nonresponder hosts (data not shown).

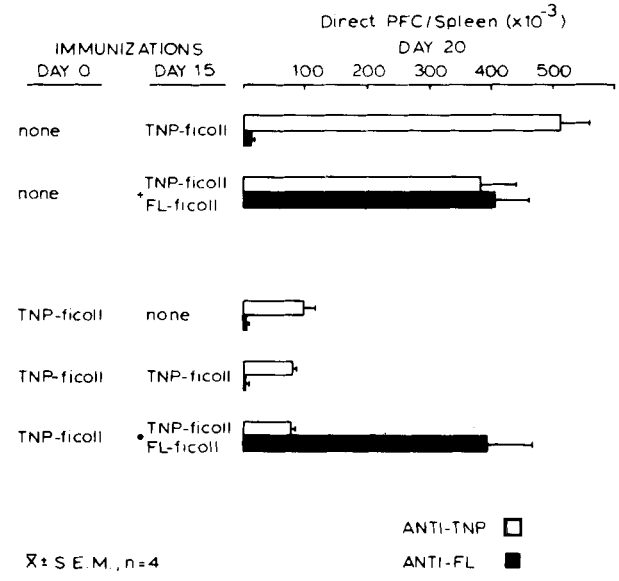


*Figure 1.* Hapten specificity of induced unresponsiveness *in vivo*. Normal and TNP-Ficoll-immunized mice were challenged (on day 15) with 10 μg FL-Ficoll. Spleens were assayed on day 20 for anti-TNP and anti-FL PFC.

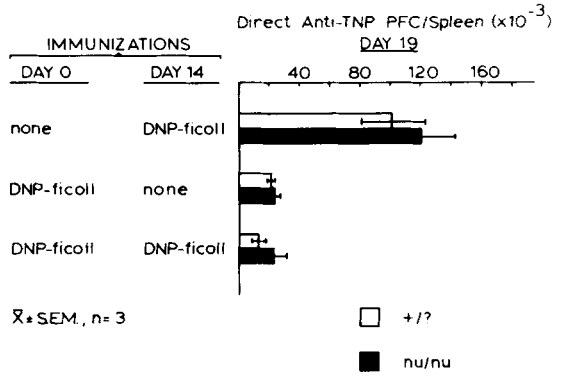


*Figure 2.* Unresponsiveness to a 2nd challenge of 10 μg DNP-Ficoll in nude and normal littermate CBA mice.

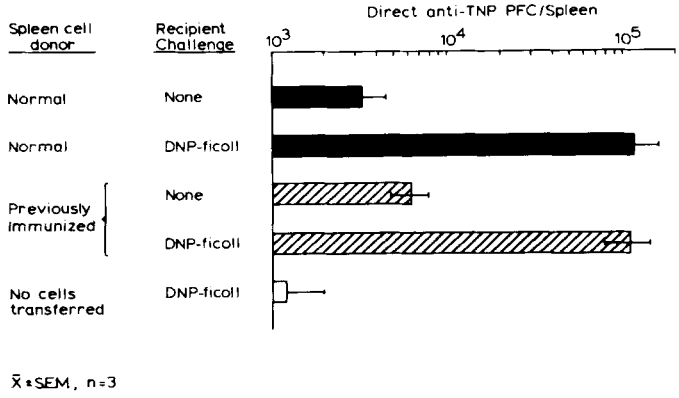


*Figure 3.* Response of spleen cells from normal and DNP-Ficoll-immunized mice to DNP-Ficoll in irradiated syngeneic recipients. Recipients received 2 × $10^7$ viable spleen cells i.v. before i.p. injection with 10 μg DNP-Ficoll. Anti-TNP PFC responses were measured 5 days after adoptive transfer.

These experiments demonstrate that TNP-specific B cells are not irreversibly tolerized or clonally deleted. Therefore, their failure to respond must be due to some reversible inhibitory mechanism.

*Will normal spleen cells respond in an unresponsive environment?* To test whether normal spleen cells can respond to TNP-Ficoll in the environment of an unresponsive mouse, 5 × $10^7$ normal spleen cells were transferred into irradiated (800 R) recipients that had been immunized with 10 μg TNP-Ficoll 21 days before the adoptive transfer. The transferred normal cells responded with 81,000 PFC/spleen in normal recipients but gave no significant response above background in unresponsive recipients (Fig. 4). The finding that normal spleen cells fail to respond to TNP-Ficoll in recipients previously immunized with TNP-Ficoll further emphasizes the suppressive nature of the unresponsive state.

*Can spleen cells from TNP-Ficoll unresponsive mice respond to TNP-Ficoll in vitro?* Normal spleen cells or spleen cells from mice immunized 17 days previously with 10 μg TNP-Ficoll were challenged *in vitro* with various concentrations of TNP-Ficoll or FL-Ficoll. Figure 5 shows the anti-TNP and anti-FL direct PFC responses on day 4 of culture. Spleen cells from TNP-Ficoll immunized mice were unresponsive to *in vitro* challenge with any dose of TNP-Ficoll tested (0.01 ng to 10 μg/culture) but responded normally to FL-Ficoll.

Kinetic studies of the *in vitro* response demonstrated that spleen cells from TNP-Ficoll-immunized mice did not respond to TNP-Ficoll at any time during the 4 days of culture (data not shown). As predicted from *in vivo* experiments using athymic mice, we have found that spleen cells from CBA nude mice previously immunized with TNP-Ficoll respond poorly to *in vitro* challenge with TNP-Ficoll while responding vigorously to FL-Ficoll (data not shown).

*Can serum antibody from TNP-Ficoll unresponsive mice inhibit in vitro TNP-Ficoll responses?* The possibility that serum from TNP-Ficoll unresponsive mice could specifically inhibit the *in vitro* TNP-Ficoll response was examined. Figure 6 shows that 0.5% (final concentration) of serum from mice immunized 14 days previously with 10 μg TNP-Ficoll completely inhibited the *in vitro* TNP-Ficoll response without affecting the FL-Ficoll response. Normal mouse serum (NMS)

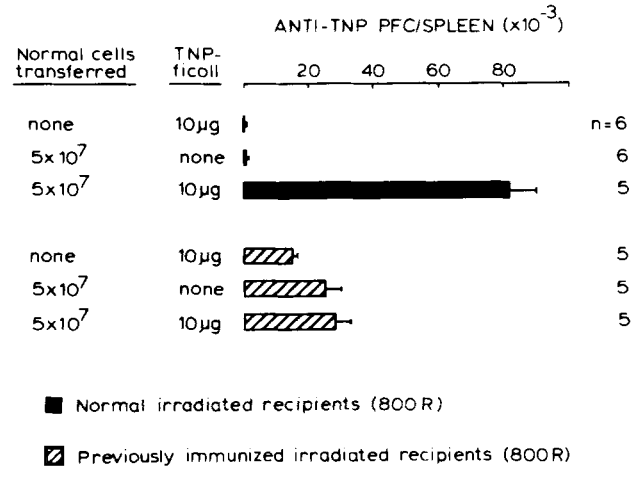



*Figure 4.* Response of spleen cells from normal donors to TNP-Ficoll in normal or TNP-Ficoll immune irradiated syngeneic recipients.

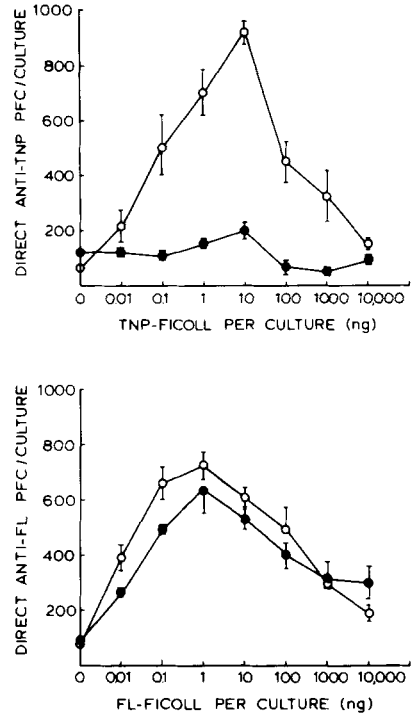


*Figure 5. In vitro* responses of spleen cells from normal (○——○) or TNP-Ficoll immune (●——●) mice. *Upper*, anti-TNP response to TNP-Ficoll; *lower*, anti-FL response to FL-Ficoll.

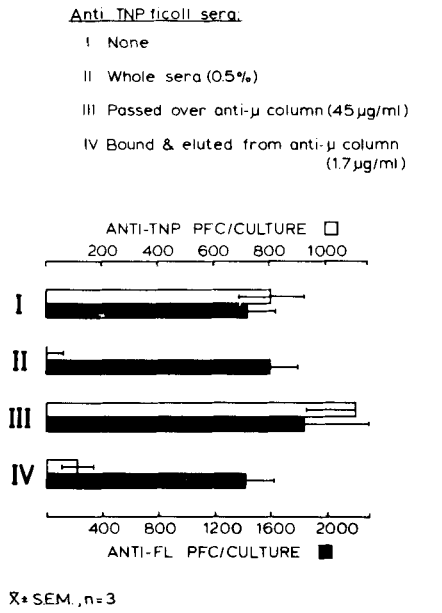


*Figure 6. In vitro* responses of normal spleen cells to TNP-Ficoll and FL-Ficoll in the presence of: 1) no mouse serum; 2) 0.5% TNP-Ficoll immune serum; 3) TNP-Ficoll immune sera passed over (unbound) a goat anti-mouse IgM column; and 4) material from TNP-Ficoll immune serum bound and eluted from the anti-IgM column.

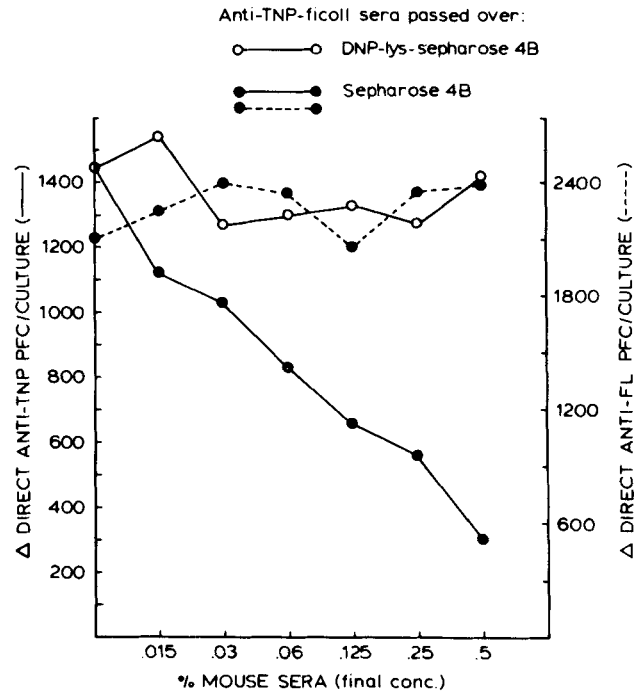


*Figure 7. In vitro* responses of normal spleen cells to TNP-Ficoll in the presence of various concentrations of TNP-Ficoll immune serum passed over DNP-lys-Sepharose column or uncoupled Sepharose 4B column. Included as specificity control is the FL-Ficoll response in the presence of TNP-Ficoll immune serum passed over uncoupled Sepharose 4B.

had no effect on either response. Figure 6 also shows that the inhibitory factor is removed by passing the immune serum over a column of Sepharose-coupled goat anti-mouse $\mu$ antibody and that the specific inhibitory factor can be eluted (with acid) from an anti-$\mu$ column. In this experiment the same cultures were challenged with TNP-Ficoll and FL-Ficoll and plaqued against both TNP-SRBC and FL-SRBC. We have found that TNP-Ficoll and FL-Ficoll placed in the same culture do not influence the responses to one another.

These data demonstrate that the inhibitory serum factor is antibody (IgM) and strongly suggests that the mechanisms of *in vivo* unresponsiveness is antibody mediated.

*What is the specificity of the inhibitory antibody?* Serum from mice immunized 14 days previously with TNP-Ficoll was passed over either a DNP-lys-Sepharose column or an uncoupled Sepharose 4B column as a control. Serum passed over the uncoupled column specifically inhibited the *in vitro* TNP-Ficoll response without affecting the FL-Ficoll response of the same cultures. In contrast, serum passed over the DNP-lys-Sepharose column no longer inhibited the TNP-Ficoll response (Fig. 7). Material eluted with 0.2 M acetic acid from the DNP column could inhibit the TNP-Ficoll response without affecting the FL-Ficoll response of normal spleen cell cultures challenged with both 10 ng TNP-Ficoll and 10 ng FL-Ficoll (Fig. 8).

These data indicate that the inhibitory antibody in sera from TNP-Ficoll-unresponsive mice is hapten specific.

*Can antigen masking explain unresponsiveness in vitro?* Since antibody specific for TNP can inhibit the response to TNP-Ficoll, we asked whether determinant masking by antibody could explain the inhibition. That is, would partial inhibition by a low dose of antibody be overridden by high doses of TNP-Ficoll? To test this, normal spleen cells were cultured with various concentrations of anti-TNP-Ficoll serum. Figure 9 shows that increasing concentration of anti-TNP-Ficoll serum decreases the PFC response to TNP-Ficoll without shifting the antigen dose-response curve to the right. Since antibody-mediated inhibition could not be overridden by increasing the antigen concentration, simple determinant masking cannot explain the observed phenomenon. It is important to note that high concentrations of TNP-Ficoll (10 $\mu$g/culture) are not nonspecifically inhibitory, since the *in vitro* response to FL-Ficoll is not affected by 10 $\mu$g/culture of TNP-Ficoll (data not shown).

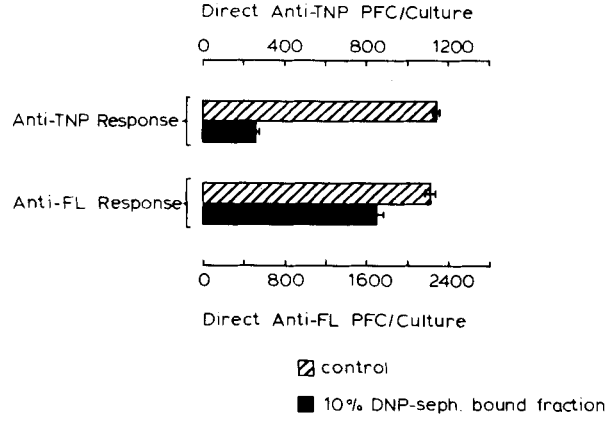


*Figure 8. In vitro* responses of normal spleen cells to TNP-Ficoll and FL-Ficoll in the presence of the TNP-Ficoll immune serum fraction bound and eluted from TNP-lys-Sepharose column. Results are given as the mean ± range of duplicate pooled triplicate cultures.

*Does DNP-LPS induce unresponsiveness for a second DNP-LPS challenge?* To determine whether unresponsiveness follows a primary immunization using a different TI-antigen, mice immunized with 1.0 $\mu$g DNP-LPS or 1.0 $\mu$g LPS were challenged 16 days later with 1.0 $\mu$g DNP-LPS (Fig. 10). In agreement with Fidler (see Reference 35), the secondary response to haptenated LPS is similar to the primary response.

*Is the in vitro response to TNP-LPS inhibited under conditions that inhibit TNP-Ficoll responses?* Finally, the possibility that the TI-2 antigen TNP-Ficoll is particularly suscep

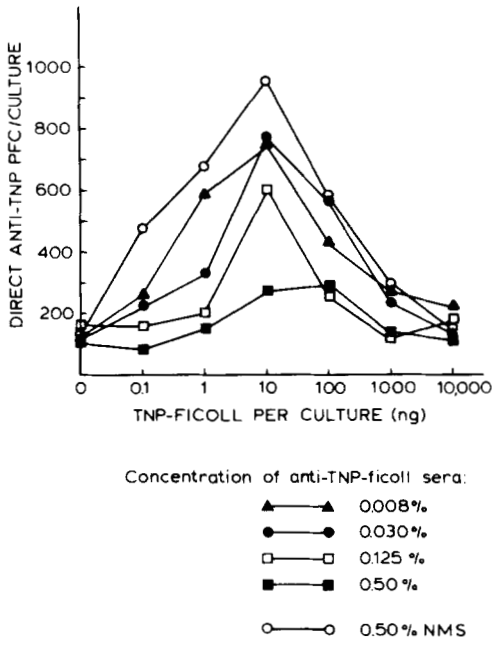


*Figure 9. In vitro* response of normal spleen cells to TNP-Ficoll in the presence of various concentrations of TNP-Ficoll immune serum.

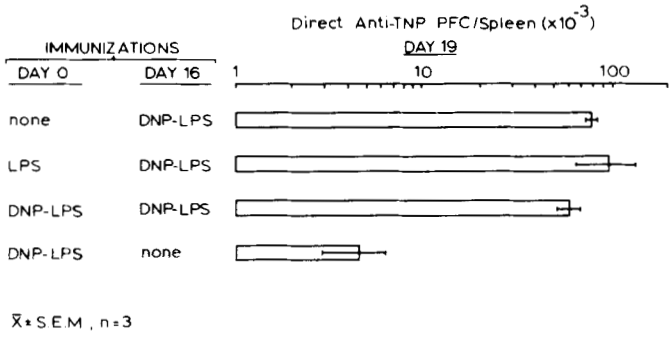


*Figure 10. In vivo* response to 1.0 μg DNP-LPS i.p. after immunization with 1.0 μg LPS or 1.0 μg DNP-LPS.

tible to antibody-mediated suppression compared with the TI-1 antigen TNP-LPS was tested. Normal spleen cells cultured with anti-TNP-Ficoll sera were challenged with TNP-Ficoll or TNP-LPS. Figure 11 shows that the TNP-LPS response was not affected by the anti-TNP antibody, whereas the TNP-Ficoll response was completely inhibited. Figure 11 also demonstrates that spleen cells from mice previously immunized with TNP-Ficoll respond normally to TNP-LPS but do not respond to TNP-Ficoll.

Included in these experiments is the response to sham-coupled LPS, showing that the response generated to TNP-LPS is not due to the polyclonal activation of B cells by LPS alone.

In these experiments, each of the antigens was used at its optimal concentration. In experiments in which TNP-Ficoll was added at the super-optimal concentration of 0.1 μg/culture anti-TNP-Ficoll sera suppressed the response by 70% or more (data not shown).

## DISCUSSION

We have shown that after a primary immune response to haptenated Ficoll (TNP-Ficoll), CBA mice are unable to respond to a second challenge with the same antigen, but can respond to the noncross-reactive hapten FL on Ficoll. Similarly, mice primed with FL-Ficoll cannot respond to a FL-Ficoll

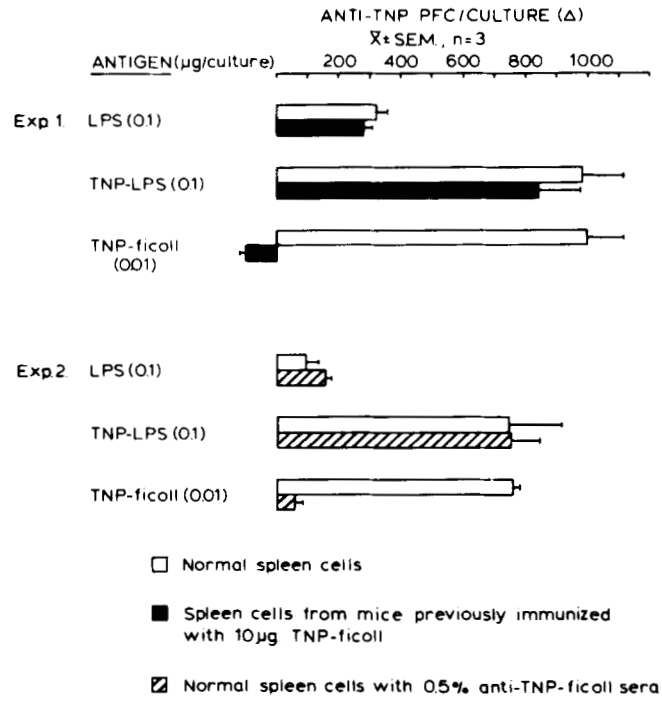


*Figure 11.* Experiment 1: *In vitro* responses to LPS, TNP-LPS, and TNP-Ficoll of normal spleen cells or spleen cells from mice previously immunized with TNP-Ficoll. Experiment 2: *In vitro* responses of normal spleen cells with and without 0.5% TNP-Ficoll immune serum.

challenge but respond normally to TNP-Ficoll (data not shown). We have also found that spleen cells from mice previously immunized (14 to 28 days) with immunogenic doses of TNP-Ficoll will not respond *in vitro* to any dose ($10^{-5}$ to $10^{1}$ μg/culture) of the same antigen. However, since spleen cells from such TNP-Ficoll unresponsive mice can effectively reconstitute 800 R irradiated recipients, we feel that the TNP-Ficoll-reactive B cells are suppressed, not irreversibly inhibited. Furthermore, since unresponsiveness can be generated in nude mice, it appears that B cells are prevented from responding to TNP-Ficoll via some suppressive mechanism that is mature T cell independent. As this model predicts, normal spleen cells do not respond to TNP-Ficoll when adoptively transferred into irradiated mice previously immunized with TNP-Ficoll. Finally, suppression can be induced in primary modified Mishell-Dutton cultures with sera from TNP-Ficoll-immunized mice. The inhibitory factor in such sera can be removed and recovered from either a DNP-lys-Sepharose column or a goat anti-mouse IgM-Sepharose column. We conclude that the failure of mice to respond to a second challenge of TNP-Ficoll is due to anti-TNP antibody-mediated inhibition of functional B cells. We have also shown that inhibition is not due to antigen neutralization.

The finding that spleen cells from previously immunized mice respond poorly *in vitro* to the homologous haptenated Ficoll antigen suggests that either anti-TNP antibody is carried over (e.g., on cell membrane) or that the number of TNP-specific antibody-secreting cells remaining from the primary *in vivo* response can produce sufficient anti-TNP antibody in culture to inhibit *in vitro* responses. Preliminary experiments suggest that inhibition results from secreted antibody, since removal of antibody-forming cells allows spleen cells from unresponsive mice to respond normally to *in vitro* stimulation.

An interesting finding in our studies was that *in vitro* responses to TNP-LPS were not inhibited by concentrations of anti-TNP-Ficoll sera that completely inhibited TNP-Ficoll responses. Three differences between the TI-1 antigen TNP-LPS

and the TI-2 antigen TNP-Ficoll may account for their differential susceptibility to antibody-mediated regulation. First, LPS is a potent mitogen, polyclonal B cell activator and adjuvant (reviewed in 23) and may be capable of overriding an antibody-induced "off" signal. Second, it is possible that antibody-mediated suppression involves accessory cells, as shown for several thymus-dependent antigen systems (24, 25). If so, since responses to TNP-LPS are less macrophage dependent than TNP-Ficoll responses (4), TNP-Ficoll responses would be more affected by this mechanism. Finally, TNP-Ficoll and TNP-LPS trigger antibody secretion in distinct subpopulations of B cells (3). Suicide experiments show that the population of primed B cells responding to TNP-LPS includes, but is not identical to, the population responding to TNP-Ficoll (26). Thus, TNP-Ficoll- and TNP-LPS-responding B cells may be sensitive to different regulatory signals. We do not yet know which, if any, of these distinctions are relevant to antibody-mediated suppression, nor do we know whether the inability of anti-TNP antibody to regulate TNP-LPS responses is absolute or merely quantitative.

Regulatory mechanisms not involving antibody have been proposed for other TI antigens. Howard and Courtenay (8) concluded that unresponsiveness after optimal immunization with the TI-1 antigen levan was the result of exhaustive differentiation (clonal elimination) of levan-specific B cells. Our results show that clonal elimination does not occur after a primary TNP-Ficoll response. This difference between levan and TNP-Ficoll may result from levan's inability, and TNP-Ficoll's ability, to generate antibody levels great enough to regulate B cell triggering; that is, a critical level of antibody may be required to prevent exhaustive immunization. If this is indeed the case, haptenated levan may generate sufficient anti-hapten antibody to prevent B cell exhaustion at optimal antigen doses (see Klaus, Reference 27).

Baker and colleagues (28) have argued that T-suppressor cells regulate responses to the TI antigen pneumococcal polysaccharide (SIII), since anti-lymphocyte serum (ALS) can enhance responses to SIII and prevent low dose tolerance. Except for polyvinylpyrrolidone (PVP) (29), evidence for T cell regulation of other TI antigens has not been observed (30).

Schrater *et al.* (31) have recently made the interesting observation that certain mouse strains (AKR, BALB/c) produce anti-idiotypic antibodies during a primary response to TNP-Ficoll. The anti-idiotypic antibody was shown to reversibly inhibit the secretion of antibody by antibody-forming cells and was not detectable in nude mice. Similar results have been reported by Fernandez and Möller (32) using the TI-2 antigen dextran. We have found no evidence of inhibitory anti-idiotype antibody in sera from TNP-Ficoll immunized CBA mice. In our hands, anti-TNP-Ficoll serum inhibits *in vitro* B cell triggering when added to cultures within the first 24 hr, but has no effect when added at 72 hr of a 96-hr culture. Therefore, there is no direct inhibitory effect on antibody-secreting cells.

Fernandez and Möller (33) reported that pre-immunization with dextran suppressed the anti-FL response to FL-dextran. These authors showed that a serum factor was responsible for the observed inhibition, although the role of anti-dextran antibody in suppression was not directly tested. These authors concluded that inhibition was the result of antigen clearance by anti-carrier antibody, because high doses of antigen (1 mg) could override partial (42%) suppression in mice passively administered small amounts (200 μl) of serum from dextran immunized mice. Although this experiment demonstrates the reversibility of the suppression, it does not prove that antigen neutralization is the suppressive mechanism, since large antigen doses would be expected to complex with and clear the passively administered inhibitory antibody.

Waldmann and Pope (34) have also suggested that carrier-specific antibody (to LPS) could prevent subsequent anti-TNP responses to TI hapten-carrier conjugates (TNP-LPS). In contrast, we have found that pre-immunization with LPS or DNP-LPS has no effect on a subsequent response to TNP-LPS when mice are given the second challenge 3 to 16 days after the primary immunization (Fig. 10). These results are comparable to those of Fidler (35), who showed that the secondary response to TNP-LPS was similar in magnitude to the primary response.

The mechanism of antibody-induced inhibition has been studied extensively for many years. Although some investigators have concluded that antibody-mediated regulation occurs via antigen masking (36), others have argued that, although simple antigen masking probably occurs, neutralization of the antigenic stimulus is not a sufficient explanation for many experimental observations (37, 38). In fact, Terres and Wolins (39) and others have observed that antigen-antibody complexes are, in some cases, more immunogenic than antibody alone. It appears certain that antibody- or antigen-antibody complex-(40) mediated regulation involves multiple pathways dependent on many factors, including titer, isotype (41), and affinity (42) of the regulating antibody.

We have emphasized here that the type of antigen is also an important factor in determining the outcome of antigen-antibody interactions on immunity; that is, responses to TNP-Ficoll are inhibited dramatically by IgM anti-TNP antibody generated by a single immunization. Such potent regulation by antibody is not observed in responses to TD antigens, which stimulate strong secondary responses even in the presence of high antibody titers directed towards the antigen (43). The importance of antigen type is further emphasized by our finding that *in vitro* TNP-LPS responses are not susceptible to antibody-mediated inhibition in contrast to *in vitro* TNP-Ficoll responses.

It is interesting that, in parallel to our observations of murine responses to TNP-Ficoll, antibody levels to polysaccharides in people (levan, dextran) also show little or no increase upon further immunization (44).

We are currently investigating the relative susceptibility to antibody-mediated inhibition of a number of TD, TI-1, and TI-2 antigens. We are also interested in determining whether there is a requirement for the Fc portion of inhibitory antibody, the cellular target of inhibition, and the possible role of antigen-antibody complexes.

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