CARRIER-DIRECTED ANTI-HAPTEN RESPONSES BY B-CELL SUBSETS*

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Previous investigations in this (1, 2) and other (3–6) laboratories have demonstrated that distinct subpopulations of B cells in the mouse, distinguished by the membrane receptor for C3, respond to T-dependent and T-independent antigens. In essence, only complement receptor (CR)[1]− B cells make antibody responses to weak polyclonal mitogenic T-independent antigens such as trinitrophenyl (TNP)-Ficoll (1). Although the major share of the response to T-dependent antigens is mounted by CR+ B cells, a component of this response was found in the CR− compartment. The requirements for activation of CR+ and CR− B cells by T-dependent antigens differ sharply in that C3 is essential for responses by the former but not by the latter type of B cell (2).

This report addresses the question of whether the same or different precursors in the functionally complex CR− B cell compartment respond to T-dependent and T-independent antigens. For this purpose, we have employed limiting dilution techniques in vitro to analyze responses to the trinitrophenyl hapten on T-dependent and T-independent carriers. In addition, evidence is presented that T-independent carriers with different biological properties activate different subpopulations of B cells.

Materials and Methods

Mice. Inbred female C57BL/6S mice, 6–8 wk of age, were purchased from Simonsen Laboratories, Gilroy, Calif.

Antigens and Immunization. Ficoll (mol wt 400,000) and dextran (mol wt 500,000) were purchased from Pharmacia Fine Chemicals, Piscataway, N. J. Aminoethylcarbamylmethyl (AECM) Ficoll and AECM-dextran were prepared according to Inman (7). The polymers were carboxymethylated for 1 h at 42°C. TNP conjugates of AECM-Ficoll and AECM-dextran were prepared by reacting 300 mg of the respective conjugate dissolved in 15 ml of 0.28 M cacodylate, pH 11.0 (8) with 180 mg of trinitrobenzene sulfonate (TNBS) previously dissolved in 15 ml of cacodylate. The reaction was allowed to proceed for 3 h at room temperature with constant stirring, followed by exhaustive dialysis against water and lyophilization. The conjugation ratios were TNP_{24}-AECM-Ficoll and TNP_{34}-AECM-dextran. TNP-LPS, containing approximately 1.02×10^{-7} mol of TNP per mg of LPS, was prepared according to Jacobs and Morrison (8) by using LPS 055:B5 (Difco Laboratories, Detroit, Mich.). Horse erythrocytes (HRBC) were obtained biweekly

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[1] *Abbreviations used in this paper*: AECM, aminoethylcarbamylmethyl; CR, complement receptor; FCS, fetal calf serum; HRBC, horse erythrocytes; LPS, bacterial lipopolysaccharide; PFC, plaque-forming cell; SRBC, sheep erythrocyte; TNBS, trinitrobenzene sulfonate; TNP, trinitrophenyl.

from Colorado Serum Co., Denver, Colo. Mice were primed with 0.1 ml of a 10% suspension of thrice washed HRBC 5–7 days before use. For use as an in vitro immunogen, TNP-HRBC were prepared according to Kettman and Dutton (9), except that HRBC were used instead of sheep erythrocytes (SRBC).

Culture Conditions. Spleen cell suspensions were prepared in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) with 2 mM glutamine, 50 U of penicillin, and 50 μg of streptomycin per ml. The suspension medium did not contain bicarbonate. The spleen cell suspension was washed once and resuspended in RPMI-1640 (Grand Island Biological Co.) supplemented as above but also containing 8% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, and bicarbonate. In preliminary experiments to establish the immunogenicity of the various antigens, cultures were prepared with 10^7 cells in 1 ml of medium by using Falcon 3004 dishes. (Falcon Plastics, Division of Bioquest, Oxnard, Calif.) These cultures were rocked and fed according to Mishell and Dutton (10) in an atmosphere of 7% O_2, 10% CO_2, and 83% N_2. After 4–5 days of incubation the cultures were harvested and assayed on lightly coupled TNP-SRBC (11) according to Jerne et al. (12).

Limiting dilution experiments were performed according to Lefkovitz (13), except that the cultures were fed only on day 1. Cell cultures were established with 1×10^4, 2×10^4, 4×10^4, and 8×10^4 cells per well. In some experiments, mitomycin-C treated (25 μg ml^{-1} for 30 min at 37°C), carrier-primed spleen cells were added to insure T-cell excess. The addition of filler cells did not change either the dose-response curve or the precursor frequency for any of the antigens. A minimum of 60 cultures were established for each cell dilution. At the end of the culture period, individual supernates were transferred to plates containing TNP-SRBC, incubated overnight, and developed at 37°C for 30 min with guinea pig serum diluted 1/10. When the clonal burst size was determined, each of the 60 wells from the plate containing 8×10^4 cells per well was harvested into a common tube, washed once, and plaqued.

Statistical Interpretation of Limiting Dilution Data. By using the Poisson statistic, the probability of finding a culture well which does not contain a precursor cell is given by the following relationship:

(*1*) $P(0) = e^{-n\lambda}$ – where P(0) is the proportion of negative culture wells, n the number of cells per well, and λ the precursor frequency. For two cell types at limiting dilution, relationship (1) is modified as follows to account for the precursor frequency of each cell type.

(*2*) $P(0) = 1 - (1\text{-}e^{-n\lambda}1)\,(1\text{-}e^{-n\lambda}2)$ – where P(0) is the proportion of negative culture wells, n is the number of cells per well, and λ_1 and λ_2 are the respective precursor frequencies of precursor type 1 and precursor type 2. This type of analysis is not possible when $\lambda_1 >> \lambda_2$ because the term $(1\text{-}e^{n\lambda}2)$ becomes negligible and equation (*2*) reduces to equation (*1*). It should be pointed out that in our experiments with two different antigens, the precursor frequencies never differed by more than a factor of 3; therefore, equation (*2*) was valid for analysis of the results.

Results

Immunologic Properties of T-Dependent and T-Independent Antigens Employed in this Investigation. The immunologic properties of the antigens employed in this investigation, drawn from numerous published reports, are presented in Table I. It can be seen that two of the T-independent antigens, TNP-Ficoll, and TNP-dextran, are indistinguishable by the listed criteria, but are readily distinguished from TNP-LPS, the third T-independent antigen. There are also gross differences between TNP-HRBC, a T-dependent antigen, and any of the T-independent antigens. Thus, the four antigens can be classified into three groups on the basis of their requirements for T cells and accessory cells, and their capacities to stimulate polyclonal Ig synthesis and DNA synthesis.

Dose-Response Curves for T-Independent Antigens. To use limiting dilution experiments to study B-cell subpopulations, it was necessary to include two antigens in the same culture vessel. Thus, the possibility existed that if the two antigens stimulated the same B-cell subset, then the sum of the concentrations

TABLE I
Immunological Properties of Antigens

Antigen	Requires T cells	Requires A cells	Stimulates polyclonal Ig synthesis	Stimulates polyclonal DNA synthesis
TNP-Ficoll	–	++++	±	±
TNP-dextran	–	++++	+	+
TNP-LPS	–	±	++++	++++
TNP-HRBC	++++	++++	–	–

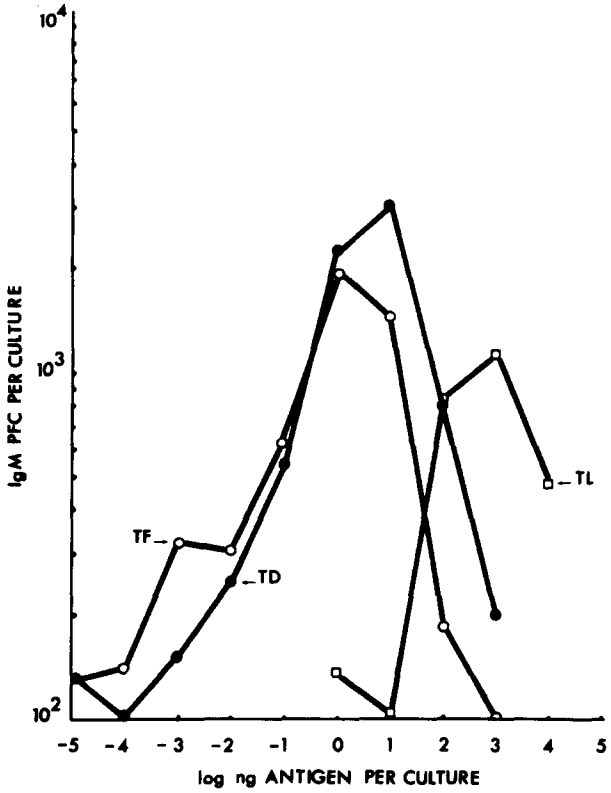

FIG. 1. Dose-response curve for a 4-day primary in vitro IgM response to TNP-Ficoll (TF), TNP-dextran (TD), and TNP-LPS (TL). Standard deviations were less than or equal to 10% of the mean plaque-forming cell (PFC) per culture.

of the two antigens might be sufficient to induce high zone unresponsiveness. As shown in Fig. 1, the three T-independent antigens could induce high zone unresponsiveness at concentrations of TNP-Ficoll or TNP-dextran greater than 10 ng ml^{-1} and of TNP-LPS greater than 1 μg ml^{-1}. The optimal stimulating concentration range for TNP-Ficoll and TNP-dextran was 1–10 ng ml^{-1}, whereas the optimal concentration for TNP-bacterial lipopolysaccharide (LPS) was 100–1,000 ng ml^{-1}. Therefore, in all subsequent experiments TNP-Ficoll and TNP-dextran were used at a concentration of 5 ng ml^{-1} while TNP-LPS was employed

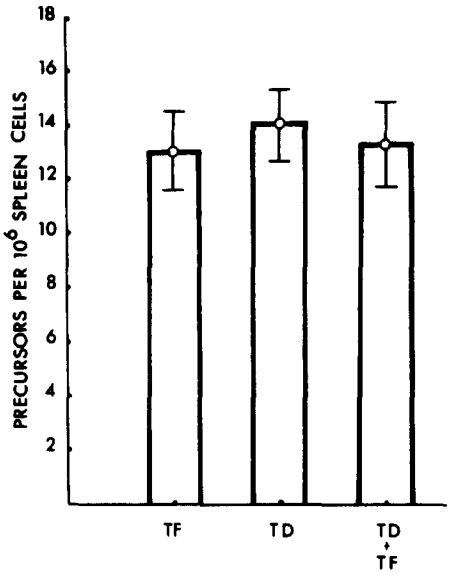

FIG. 2. Splenic precursor frequencies from limiting dilution experiments comparing TNP-Ficoll (TF) and TNP-dextran (TD).

at 500 ng ml^{-1}. Under these conditions, the total antigen concentration would not be expected to lead to high zone unresponsiveness in the event that the same B-cell subset responded to both antigens.

B-Cell Precursors for the TNP Hapten on T-Independent Carriers. To compare the precursor frequencies of anti-TNP IgM PFC for responses to TNP-Ficoll and TNP-dextran, 0.05 ng of each antigen was added separately or in combination into cultures containing 1×10^4–8×10^4 normal spleen cells from C57BL/6S mice. Precursor frequencies per 10^6 cells, determined after four days of culture, were 13.2 ± 1.2 for TNP-Ficoll, 14.0 ± 1.3 for TNP-dextran, and 13.7 ± 1.3 for the two antigens in the same cultures (Fig. 2). Since the precursor frequency was the same whether cells were cultured with the antigens individually or together, the data clearly suggest that the same B cells respond to TNP-Ficoll and TNP-dextran.

For a similar comparison of cells responsive to TNP-Ficoll and to TNP-LPS, cultures were established containing 0.05 ng of TNP-Ficoll, 5 ng of TNP-LPS, or both, and the precursor frequency was determined in the usual manner. In this set of experiments, the frequencies per 10^6 spleen cells were 16.1 ± 1.5, 31.3 ± 2.5, and 45.5 ± 3.6 for TNP-Ficoll, TNP-LPS, and the combination, respectively (Fig. 3). The strict additivity of the responses to these antigens suggests that different B cells are activated by TNP-Ficoll and TNP-LPS. Since TNP-Ficoll and TNP-dextran stimulate the same population of B cells, it follows that TNP-LPS and TNP-dextran also trigger independent subsets of cells.

B-Cell Precursors for the TNP Hapten on T-Dependent and T-Independent Carriers. To determine if the ability to respond to TNP-Ficoll, a T-independent antigen, and to TNP-HRBC, a strictly T-dependent antigen, is shared by a common pool of B cells, varying numbers of spleen cells serving as a source of B cells were cultured with an excess of carrier-primed cells in the presence of one

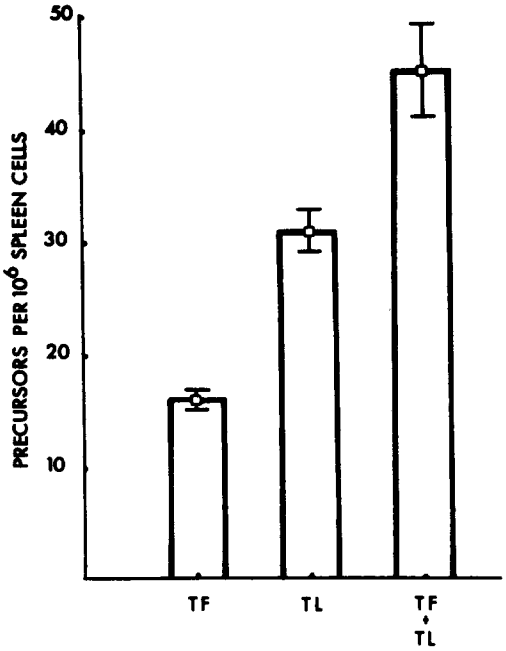

FIG. 3. Splenic precursor frequencies from limiting dilution experiments comparing TNP-Ficoll (TF) and TNP-LPS (TL).

or both antigens. T-helper cell excess in these cultures was established by the ability to obtain responses in 100% of the cultures when 10^5 normal spleen cells were cultured with the carrier-primed cells. On the other hand, carrier-primed cells cultured alone did not yield anti-TNP producing cultures, establishing that the responses observed in co-cultures were derived from the normal spleen cells.

The precursor frequency for TNP-Ficoll in this series of experiments was 8.3 ± 0.8 per 10^6 spleen cells, that for TNP-HRBC was 14.7 ± 1.3, and 26.3 ± 2.1 were found for TNP-Ficoll plus TNP-HRBC (Fig. 4). The additivity of these frequencies indicates that different B cells respond to the two antigens. It is worth noting, though perhaps obvious, that precursor frequencies for a particular antigen will vary from experiment to experiment, due primarily to variation between individuals in a population. Thus, it is only valid to compare data within an experiment, not between different experiments.

Since TNP-Ficoll (or TNP-dextran) appears to activate a subset of anti-TNP precursors distinct from those reactive to either TNP-LPS or TNP-HRBC, it was clearly of interest to determine if the latter two antigens, one T-independent and the other T-dependent, activated the same or different subsets. To address this question, cultures were established with a constant number (3.5×10^4) of nylon wool-passed spleen cells from mice primed with HRBC plus from 1×10^4 to 8×10^4 normal spleen cells as a source of B cells. The cultures contained 5.0 ng of TNP-LPS, 3×10^4 TNP-HRBC, or both antigens. In this series of experiments, TNP-LPS stimulated 83.3 ± 7.4 precursors per 10^6 spleen cells, TNP-HRBC generated a response by 31.3 ± 2.4 cells/10^6, and the two antigens together triggered 71.4 ± 6.4 precursors (Fig. 5). These figures provide strong evidence

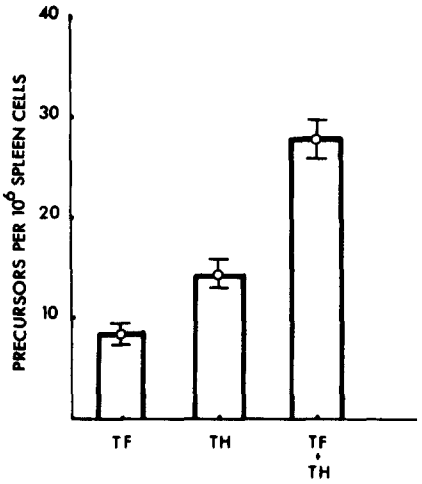

FIG. 4. Splenic precursor frequencies from limiting dilution experiments comparing TNP-Ficoll (TF) and TNP-HRBC (TH).

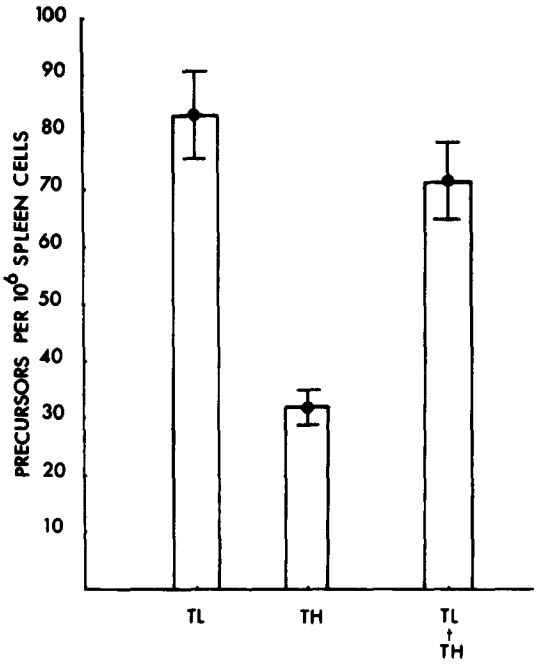

FIG. 5. Splenic precursor frequencies from limiting dilution experiments comparing TNP-LPS (TL) and TNP-HRBC (TH).

that the subset of B cells responsive to TNP-HRBC resides within the population activated by TNP-LPS.

Size of Clones from Precursors Responsive to T-Dependent and T-Independent Antigens. The relative capacities of the four antigens employed in this investigation to induce antigen-specific proliferation was determined by comparing precursor frequencies to numbers of PFC in spleen cell cultures. As

TABLE II
Burst Size of Clones Responding to T-Dependent and T-Independent Antigens

Experiment	Antigen	Precursors/10^6 ± SD	PFC/10^6 ± SD	Burst Size ± SD
1	TF*	8.3 ± 0.8 (10.8 ± 5.6)‡	223 ± 15	26.8 ± 3.1 (19.7 ± 5.8)
	TH	14.7 ± 1.3 (21.3 ± 8.7)	512 ± 23	34.8 ± 5.8 (42.4 ± 10.7)
2	TF	13.2 ± 1.2	191 ± 20	14.5 ± 2.0
	TD	14.0 ± 1.3 (13.5 ± 1.0)	202 ± 21	14.4 ± 2.0 (14.6 ± 0.5)
3	TF	16.1 ± 1.5	248 ± 16	15.4 ± 1.7
	TL	31.2 ± 2.5 (56.7 ± 30.0)	348 ± 19	11.1 ± 1.0 (8.9 ± 5.4)
4	TL	83.3 ± 7.4	1,099 ± 105	13.1 ± 1.7
	TH	31.2 ± 2.4	1,591 ± 126	50.9 ± 14.0

* TF, TNP-Ficoll; TH, TNP-HRBC; TD, TNP-dextran; TL, TNP-LPS.
‡ Numbers in parentheses are the means for all experiments to date and represent the observed interexperiment variation.

previously reported (6), T-dependent antigens generally engender more vigorous proliferation than T-independent antigens, an observation which held firm in this series of experiments. TNP-HRBC elicited a greater degree of proliferation than either TNP-Ficoll or TNP-LPS (Table II). Comparison of the T-independent antigens with one another disclosed that TNP-Ficoll and TNP-dextran were equally effective mitotic agents, but, surprisingly, TNP-Ficoll induced larger clones than did TNP-LPS (Table II). In a series of five different experiments, TNP-Ficoll provoked the same size (one experiment) or larger (four experiments) clones than TNP-LPS. In no instance did the polyclonal mitogen give rise to larger clones than the weakly-polyclonal mitogenic T-independent antigen.

Discussion

The findings in this investigation provide further support for the existence of distinct subpopulations of B cells responsive to T-dependent and T-independent antigens. The in vitro limiting dilution technique provides independent corroboration of earlier studies which were based on (*a*) differential susceptibility of T-dependent and T-independent antibody responses to C3 depletion in vivo by cobra venom (1) and (*b*) fractionation of lymphocyte populations on the basis of the complement receptor (1, 2). Those studies demonstrated essentially that T-independent antigens with little or no polyclonal mitogenicity stimulated B cells in the complement receptor negative compartment, whereas T-dependent responses were mounted by cells in both compartments. However, the T-dependent responses of CR+ B cells required C3, whereas responses by CR− B cells did not, suggesting the existence of at least two distinct pathways of T-B cooperation.

The limiting dilution technique has permitted a clear segregation of the responses to TNP-Ficoll and TNP-dextran on the one hand, as compared to TNP-HRBC on the other, without the ambiguities introduced by manipulations such as fractionation of spleen cell populations. The anti-TNP precursors responsive to the T-independent antigens with little or no polyclonal mitogenicity were clearly independent of the cells responsive to the T-dependent antigen (Fig. 4).

The discovery that the potent T-independent polyclonal activator TNP-LPS was incapable of triggering B cells responsive to TNP-Ficoll (or TNP-dextran)

(Fig. 3) was unexpected. This finding, coupled with the inability to separate responses to TNP-LPS from TNP-HRBC (Fig. 5) and the ability of LPS to evoke proliferative responses from both CR+ and CR− B cells (1), leads to several conclusions. One is that there exist at least three functionally distinct subpopulations of murine B cells: CR+ cells which respond to T-dependent antigens only in the presence of C3 and which also respond to LPS; CR− B cells which make T-dependent and LPS responses; CR− B cells which respond only to true, weakly polyclonal activating T-independent antigens.

A second conclusion which may be drawn from these findings is that LPS, though T independent, is capable of activating only those B cells which mount T-dependent responses. It would be tempting to speculate that LPS substitutes for the T-cell signal, as proposed by Sjöberg et al. (14), and hence is able to activate T-dependent B cells in the absence of T cells; but it should be pointed out that the in vitro response to TNP-LPS is unaffected by removal of C3 (15), in contrast to the response to T-dependent antigens (2, 15). Of course it is possible that the T-cell signal is delivered to CR+ B cells in the form of an activated product of C3 as proposed earlier (1), and that LPS does, indeed, substitute for this signal. However, it is unlikely that it does so by binding to the C3 receptor, since CR− B cells which mount T-dependent responses and apparently have different signalling requirements which do not involve C3 are also activated by LPS, presumably by a common mechanism. The data, therefore, indicate that the activation pathways for TNP-LPS and T-dependent antigens must be different, and, consequently, that not only do there exist distinct B-cell subsets but there are also multiple ways by which a particular subset can be activated.

It is of interest that a correlation may be drawn between the biological properties of the T-independent carriers (Table I) and their capacity to activate particular B-cell subsets. Ficoll and dextran possess similar profiles and activate the same population of B cells. LPS differs from the other two by its strong polyclonal activation and relative independence of accessory cells, and it activates the T-cell-dependent B-cell subpopulations. Although it was initially suggested that TNP-Ficoll did not require the participation of accessory cells to engender antibody responses (16), recent exploration of this question by ourselves (manuscript in preparation) and others (17) indicates a strict requirement for accessory cells in responses to TNP-Ficoll and TNP-dextran. We have also occasionally, but inconsistently, abrogated the response to TNP-LPS by depleting accessory cells. Therefore, it seems plausible that responses to all these T-independent antigens may be dependent on accessory cells to different degrees.

Despite its polyclonal mitogenicity, TNP-LPS elicited less antigen-specific proliferation than TNP-Ficoll or TNP-dextran, based on clonal burst size (Table II). Since macrophages appear to participate to a greater degree in the anti-Ficoll (or dextran) response than in the anti-LPS response, and it has been reported that macrophages possess intrinsic polyclonal activating properties (18), it is attractive to consider that the macrophage may amplify B-cell activation by weakly mitogenic T-independent antigens.

Summary

The capacity of the trinitrophenyl (TNP) haptenic group, coupled to a series of chemically dissimilar carriers, to cross-stimulate putative T-dependent and T-

independent murine B-cell subpopulations was determined by using an in vitro limiting dilution technique to generate primary IgM responses. It was found that TNP-Ficoll and TNP-dextran, two T-independent antigens with little or no polyclonal mitogenicity, stimulate the same population of anti-TNP precursors, which is distinct from the precursor population activated by TNP-bacterial lipopolysaccharide (LPS), a T-independent polyclonal mitogen, or TNP-horse erythrocytes (HRBC), a T-dependent antigen. On the other hand, TNP-LPS and TNP-HRBC activate the same precursor population, indicating that LPS can substitute for the T-cell signal in T-dependent B-cell responses, whereas nonmitogenic T-independent antigens cannot. However, the cumulative evidence from this and other laboratories strongly indicates that LPS and T-dependent antigens activate B cells by different mechanisms. Of particular interest, LPS is incapable of activating B cells responsive to weakly- or nonmitogenic T-independent antigens.

Based on clonal burst size, T-dependent antigens are capable of inducing greater antigen-specific B-cell proliferation than T-independent antigens. However, TNP conjugates of Ficoll and dextran, which are relatively poor inducers of polyclonal B-cell activation, induced larger anti-TNP clones than did TNP-LPS, a strong polyclonal mitogen.

The findings reinforce the evidence favoring existence of multiple B-cell subpopulations with distinctive activation pathways. They also strengthen the proposition that a given B-cell subset can be activated by more than one mechanism.

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