CELLULAR REQUIREMENTS FOR THE PRIMARY IN VITRO ANTIBODY RESPONSE TO DNP-FICOLL

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Antibody formation to most antigens requires collaboration between T lymphocytes and B lymphocytes, but certain antigens (e.g., polymerized bacterial flagellin, bacterial lipopolysaccharides, polyvinylpyrrolidone, levan [1–5]) induce antibody synthesis in the absence of detectable numbers of T lymphocytes, and hence are termed "T independent". We have examined the in vitro response of mouse spleen cells to ε-2,4 dinitrophenyl (DNP)-lysyl-substituted Ficoll (a synthetic polymer of sucrose and epichlorhydrin) and find that nanogram quantities of DNP-Ficoll stimulate a primary anti-DNP response of considerable magnitude which appears to require neither T lymphocytes nor splenic adherent cells for its generation. The wide availability of Ficoll and ease of preparation of hapten derivatives makes this powerful T-independent antigen of particular value in the study of direct activation of precursors of antibody-forming cells.

Materials and Methods

Mice.—8- to 12-wk old female BALB/c mice obtained from the Animal Production Section of the NIH were used in most experiments. Outbred congenitally athymic nu/nu mice or heterozygous nu/+ (or +/+) mice were obtained from the same source. C57BL/10 Sn, B10.A, B10.Br, B10.D2, DBA/2, C3H/J, AKR, and SJL mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

Cell Culture.—Mouse spleen cells were cultured by the method of Mishell and Dutton (6) as previously modified (7). Briefly, single cell suspensions at a concentration of 10×10^6 cells per ml were cultured in modified Eagle's minimal essential medium in 35-mm plastic culture dishes on a rocking platform at 37°C in an atmosphere of 7% O_2, 10% CO_2, and 83% N_2. 50 μl of nutritional cocktail and fetal calf serum were added to cultures daily. Altering these conditions by adding 2-mercaptoethanol (10^{-3}M) to the medium, omitting the daily nutritional cocktail and fetal calf serum, and leaving the cultures stationary did not significantly change the response to DNP-Ficoll but did reduce parallel control responses to sheep red blood cells (SRBC) or trinitrophenyl-substituted horse red blood cells (TNP-HRBC).

Antigens.—ε-dinitrophenyl-substituted Ficoll was prepared as described elsewhere.[1] Briefly, the substitution procedure involved reaction of Ficoll with cyanuric chloride at 4°C, followed by the addition of ε-DNP-lysine and subsequent reaction at room temperature. The commonly used preparation had a molar ratio of DNP-Ficoll of 32:1 (DNP_{32}Ficoll). Other substitution ratios ($DNP_{1.2}$Ficoll, DNP_{85}Ficoll) were used in some experiments. A copolymer of D-glutamic acid and D-lysine was dinitrophenylated (DNP_{44}D-GL) as previ-

[1] McMaster, P. R. B., manuscript in preparation.

ously described (8) and used as an in vivo or in vitro tolerogen (9). DNP_{32}bovine serum albumin ($DNP_{32}BSA$) was used to inhibit DNP-specific PFC in some experiments.

Erythrocytes from either a single sheep or horse were collected sterilely in citric acid-dextrose solution (ACD solution, Abbott Laboratories, North Chicago, Ill.) and washed three times with Hank's balanced salt solution before use. TNP-SRBC or TNP-HRBC were prepared by the method of Rittenberg(10).

Assay of Antibody-Forming Cells.—Spleen cells cultured with DNP-Ficoll or TNP-HRBC were assayed for cells releasing anti-DNP antibody by a modification of the Jerne technique (7) using TNP-SRBC as indicator erythrocytes. Plaque-forming cells (PFC) releasing IgG antibodies were detected as previously described (11) by inhibiting IgM PFC with goat anti-mouse μ-chain antibody and developing IgG PFC with rabbit polyvalent antimouse γ-chain antibody (both antisera were kindly provided by Dr. R. A. Asofsky).

Cell Separation.—Adherent and nonadherent spleen cells were separated by three successive incubations in plastic culture dishes as previously described (12).

RESULTS

Normal mouse spleen cells consistently gave rise to large numbers of anti-DNP PFC when stimulated in vitro with DNP-Ficoll. The time-course and dose requirements for the primary in vitro response to DNP_{32}Ficoll were studied. In several replicate experiments, the peak IgM and IgG responses to DNP-Ficoll occurred on the fourth day of culture. The largest increment in DNP-specific PFC's was between 48 and 72 h of culture. This increase was prevented by the addition of the mitotic inhibitor vinblastine (0.1 μg/ml; Velban, Eli Lilly & Co., Indianapolis, Ind.) for the final 24 h of culture. The optimal dose of DNP_{32}Ficoll in three replicate experiments was 5 ng per ml when the concentration of antigen was varied from 0.1 to 10,000 ng per ml. (part of the dose-response curve to DNP_{32}Ficoll is illustrated in Table I). DNP_{32}Ficoll stimulated significant numbers of PFC's over a wide range of low concentrations, but failed to cause a PFC response at concentrations greater than 1 μg per ml. All strains of mice tested (see Materials and Methods) gave significant in vitro IgM PFC responses against DNP-Ficoll.

The optimal substitution ratio of DNP-Ficoll for stimulating an in vitro anti-DNP PFC response was determined by stimulating unprimed BALB/c mouse spleen cells with three preparations with the molar ratio of DNP to Ficoll varied from 1.2 to 85. The numbers of DNP-specific PFC's releasing IgM antibody was determined after 4 days of culture and the results of one such experiment are shown in Table I. DNP_{32}Ficoll stimulated the most DNP-specific PFC and was used in subsequent experiments. $DNP_{1.2}$Ficoll was non-immunogenic and DNP_{85}Ficoll gave an intermediate response.

The antibody response to DNP_{32}Ficoll was DNP-specific, as shown by the following two experiments: (*a*) BALB/c spleen cells rendered tolerant to DNP by in vivo or in vitro exposure to DNP-D-GL failed to respond to in vitro stimulation by DNP-Ficoll, (*b*) plaque formation by cells cultured 4 days with DNP_{32}Ficoll was completely inhibitable by $DNP_{32}BSA$ added to the plaquing medium, and (*c*) DNP-Ficoll failed to nonspecifically increase PFC against SRBC in vitro (see Table II).

TABLE I

Effect of Substitution Ratio on DNP-Ficoll in Vitro Response

Cells cultured	Antigen	Dose	4d. DNP-specific IgM PFC/culture ± SE*	4d. DNP-specific IgG PFC/culture ± SE‡
		ng/ml		
10×10^6 BALB/c spleen cells	$DNP_{1.2}$Ficoll	0	220 ± 20	ND§
		0.1	380 ± 30	"
		1	300 ± 15	"
		10	330 ± 40	"
		100	385 ± 35	"
		1,000	325 ± 25	"
	DNP_{32}Ficoll	0	290 ± 35	40 ± 10
		0.1	2,350 ± 210	410 ± 40
		1	2,765 ± 185	645 ± 35
		10	3,370 ± 200	720 ± 45
		100	3,330 ± 400	180 ± 20
		1,000	690 ± 40	65 ± 15
	DNP_{85}Ficoll	0	146 ± 35	40 ± 10
		0.1	1,170 ± 80	240 ± 25
		1	1,090 ± 120	310 ± 30
		10	940 ± 110	160 ± 20
		100	455 ± 45	65 ± 10
		1,000	255 ± 35	80 ± 15

* Mean ± SEM of three replicate cultures in one representative experiment.

‡ Indirect PFC were developed with rabbit polyvalent anti-γ serum following suppression of direct PFC with goat anti-μ serum.

§ ND, not done.

T-cell-deficient mice have been found to make normal in vivo responses to DNP-Ficoll, suggesting that DNP-Ficoll is a "T-independent" antigen.[2] Accordingly, the effect of depleting T lymphocytes on the in vitro response to DNP-Ficoll was examined. Table II shows the results of culturing spleen cells deprived of T lymphocytes by three different methods. In each case, T-deficient spleen cell populations were able to respond to DNP_{32}Ficoll in vitro but not to SRBC. DNP-Ficoll thus may be classified as a "T-independent" antigen ("T-independent" is placed in quotation marks since a contribution of a small number of T lymphocytes surviving these depletion procedures is difficult to exclude. Moreover, even if helper T lymphocytes are not required for the activation of B lymphocytes by DNP-Ficoll, recent reports that T lymphocytes may have suppressor functions in other putatively T independent systems suggests that the term "T-independent" soon may need to be defined more rigorously).

The in vitro antibody response to polymerized flagellin has been reported to be independent of both T cells and adherent cells (13), while the in vitro response to SRBC requires both cell types. Since DNP-Ficoll was not dependent upon T lymphocytes for the generation of antibody-forming cells, the possibility that adherent cells also were not required for the response was examined.

[2] Sharon, R., McMaster, P. R. B., Kask, A. M., Owens, J. D., and Paul, W. E., manuscript in preparation.

TABLE II

Thymus "Independence" of the In Vitro Response to DNP-Ficoll

Exp.	Cells	Antigen	4d. IgM PFC/culture ± SE*	
			vs. TNP-SRBC	vs. SRBC
1	10×10^6 BALB/c spleen cells	None	675 ± 120	170 ± 12
		DNP_{32}Ficoll	2,385 ± 326	160 ± 15
		SRBC	—	2,220 ± 215
	10×10^6 anti-θ + C treated BALB/c spleen cells‡	None	530 ± 45	80 ± 10
		DNP_{32}Ficoll	1,845 ± 190	90 ± 15
		SRBC	—	315 ± 40
2	10×10^6 nu/nu (BALB/c) spleen cells§	None	30 ± 3	25 ± 5
		DNP_{32}Ficoll	610 ± 55	20 ± 5
		SRBC	—	50 ± 15
	10×10^6 nu/+ (BALB/c) spleen cells	None	150 ± 22	130 ± 15
		DNP_{32}Ficoll	1,165 ± 120	100 ± 10
		SRBC	—	1,020 ± 90
3	10×10^6 sham-NTx BALB/c spleen cells‖	None	245 ± 20	125 ± 11
		DNP_{32}Ficoll	5,045 ± 475	—
		SRBC	—	3,535 ± 320
	10×10^6 NTx BALB/c spleen cells	None	190 ± 20	70 ± 9
		DNP_{32}Ficoll	4,500 ± 310	—
		SRBC	—	105 ± 12

* Mean ± SEM of three replicate cultures.

‡ T lymphocytes depleted by treatment with undiluted AKR anti-θ^{C_3H} serum followed by 1:20 guinea pig complement and 10 μg/ml DNase.

§ Homozygous and heterozygous nude mice (on a BALB/c genetic background).

‖ BALB/c mice were thymectomized or sham-thymectomized at less than 24 h of age.

TABLE III

Adherent Cell "Independence" of the In Vitro Response to DNP-Ficoll

Exp.	Antigen	4d. IgM PFC/culture ± SE* of:	
		Normal BALB/c spleen cells	Nonadherent BALB/c spleen cells‡
1	DNP_{32}Ficoll	2,625 ± 90	2,685 ± 90
	SRBC	4,955 ± 290	480 ± 60
2	DNP_{32}Ficoll	3,655 ± 140	5,040 ± 260
	SRBC	4,115 ± 150	580 ± 120
3	DNP_{32}Ficoll	2,980 ± 80	4,490 ± 190
	SRBC	5,950 ± 230	804 ± 40

* Numbers are the mean ± SEM of duplicate assays on three replicate cultures. DNP-specific PFC's were assayed against TNP-substituted SRBC.

‡ Adherent cells were removed by three successive incubations of nonadherent spleen cells in plastic culture dishes. Macrophages were >95% depleted.

BALB/c spleen cells were depleted of adherent cells by three successive incubations in plastic culture dishes and DNP-Ficoll or SRBC added to the resulting nonadherent cells or to the original unseparated spleen cells. The results of three such experiments are shown in Table III. Whereas the response to SRBC

is greatly diminished by removing adherent cells, the response to DNP-Ficoll is unaffected or slightly enhanced by this procedure. In further experiments with nonadherent spleen cells (data not shown), nonadherent (macrophage-depleted) cells developed greater numbers of DNP-specific PFC than did unseparated spleen cells, but the concentration of antigen required to generate the optimal PFC response was increased 10–100 times to 50–500 ng per ml DNP_{32}Ficoll. The generation of DNP-specific antibody-forming cells by stimulation with DNP-Ficoll in vitro thus appears to require neither adherent cells nor T lymphocytes, or so few of such cells as to elude our methods of cell separation.

DISCUSSION

DNP-Ficoll has been demonstrated to initiate primary anti-DNP antibody responses in vitro. The response is independent of both thymus-dependent lymphocytes and adherent cells, or requires such small numbers of these cells as to escape detection by our procedures. DNP-Ficoll thus appears to stimulate B lymphocytes directly. The in vitro response consists mainly of IgM antibody but significant amounts of IgG are produced. The response is DNP-specific since DNP-specific tolerance induced by DNP-D-GL abolishes the subsequent in vitro response to DNP-Ficoll.

The association of T-lymphocyte and adherent cell independence repeats the experience with bacterial flagellin and suggests that the function of adherent cells is closely associated with the activity of T lymphocytes in culture.

The requirements for direct stimulation of antibody synthesis by "T-independent" antigens are not fully understood. Several concepts have been advanced to explain B-lymphocyte activation. One states that immunoglobulin synthesis is activated by a nonspecific signal provided by mitogenic portions of "thymus-independent" antigens and that the specific interaction of antigenic determinants and surface immunoglobulin serves only as a high-energy bond to concentrate mitogen/antigen (14). Another hypothesis suggests that two signals, one provided by antigen-specific binding and a second by nonspecific binding of antigen or antigen-activated molecules such as C-3b are required for stimulation (15). A third relevant concept suggests that "thymic-independent" antigens all have structural similarities that may lead to presentation of antigenic determinants in a two-dimensional array or matrix and that multideterminant binding by specific receptors leads to efficient cell activation (16). While evidence exists to support each of these possibilities, preliminary experiments with DNP-Ficoll have failed to demonstrate substantial mitogenicity by concentrations from 1 pg to 1 mg per ml, nor does DNP-Ficoll activate C3 at 100 μg per ml. Ficoll is a large molecule consisting of cross-linked sucrose subunits to which DNP-lysyl groups were randomly coupled. It is thus possible that presentation of DNP to the B cell in a two-dimensional matrix may lead to efficient cross-linking of receptors and cell activation. DNP-Ficoll is easily prepared and promises to be a useful antigen for studying B-cell activation in vitro.

SUMMARY

The cellular requirements for the primary in vitro IgM and IgG response to dinitrophenyl-substituted Ficoll were examined. Neither thymus-derived lymphocytes nor macrophage-rich splenic adherent cells were required for anti-DNP antibody synthesis. DNP-Ficoll is therefore tentatively classified as a "thymic-independent" antigen.

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