IN VITRO INDUCTION OF A PRIMARY RESPONSE TO THE DINITROPHENYL DETERMINANT*

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Induction of a primary antibody response in vitro to a variety of antigens has been obtained in cultures of spleen or lymph node explants (1–3), as well as in cell suspensions (4–6). All these systems, however, are concerned with complex antigens, in which interaction between the antigenic determinants of the immunogen and the reactive cells cannot be adequately analyzed. The present study, therefore, aimed at an induction of a primary immune response to a chemically defined determinant, the 2,4-dinitrophenyl (DNP)[1] group, attached either to a protein or to a synthetic polypeptide carrier. We employed for this purpose an organ culture system, previously used for primary production of antibodies to red blood cells in vitro (1), and in which a secondary response to DNP has already been studied (7). Antibodies were detected by the sensitive technique of the inactivation of chemically modified bacteriophage (8–11).

Materials and Methods

Mice.—Female (Balb/c × C57Bl/6) F_1 mice, 2 months old, were used throughout these experiments.

Culture Technique.—The Millipore filter well technique for antibody response in vitro was employed as previously described (1).

Immunogens.—Hemocyanin of *Callinectes sapidus* to which dinitrophenyl groups were attached (DNP-Hcy), at a ratio of 8 molecules DNP per each molecule of hemocyanin, was used in most of the experiments. α-DNP-poly-L-lysine (DNP-PLL), of an average mol wt of 5000 (12), was obtained by the courtesy of Dr. Arieh Yaron.

Immunization.—Immunization of the explants in vitro was carried out by culturing the tissue in medium containing 5 μg/ml of DNP-Hcy or 50 μg/ml of DNP-PLL for 48 hr. After this period the medium was replaced by antigen-free medium, which was subsequently collected at different time intervals, replaced by fresh medium, and assayed for the presence of antibodies to DNP.

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[1] The following abbreviations are used: DNP, 2,4-dinitrophenyl; DNP-Hcy, 2,4-dinitrophenyl-hemocyanin; DNP-PLL, 2,4-dinitrophenyl-poly-L-lysine; DNP-T4, 2,4-dinitrophenyl bacteriophage T4; 2-ME, 2-mercaptoethanol.

2,4-Dinitrophenyl Bacteriophage T4 (DNP-T4).—The conjugation of the DNP group to the bacteriophage was performed by reacting bacteriophage T4 (10^{11} plaque-forming units/ml) and 2,4-dinitrobenzenesulfonate (50 mg/ml) in 0.3 M sodium carbonate buffer, at pH 9.5 for 20 hr at 24°C. The modified bacteriophage was then extensively dialyzed against 0.05 M phosphate buffer, pH 6.8, and stored at 4°C. 95% of the phage population were inactivated during the coupling process. Immunospecific inactivation of the surviving modified phage was not affected by the large proportion of the inactive phage in the mixture (8, 9). Inactivation of DNP-T4 by a goat antiserum to DNP-Hcy (the serum contained 3 mg/ml of anti-DNP antibodies as determined by the quantitative precipitin analysis) proceeded as first order reaction up to at least 95% inactivation with a first order rate constant of 25,000 min^{-1}.

Antibody Assay.—Samples of the culture medium diluted three-fold were incubated with DNP-T4 (except for the experiments with 2-mercaptoethanol (2-ME), in which the final dilution of the medium was 1:9), and allowed to react for 3 hr at 37°C. Reaction mixtures were then plated by the double agar method (13). The effect of 2-ME on the capacity of the media to inactivate DNP-T4 was determined by preincubating the medium with 2-ME (0.1 M) for 30 min prior to the addition of bacteriophage.

TABLE I

Incidence of Antibody-Forming Cultures to the DNP Group After In Vitro Immunization with DNP-Hcy

Experiment no.	No. of spleens tested	Antigen treated cultures		Untreated cultures	
		Incidence	Percentage	Incidence	Percentage
1	4	20/24	83	5/20	25
2	3	9/18	50	2/18	11.1

EXPERIMENTAL

Experiments were designed to test whether formation of antibodies to DNP could be initiated in vitro. Spleens removed from unimmunized mice and cultured in medium containing DNP-Hcy were compared to controls cultured in medium free of this antigen. Samples of medium were assayed on days 4, 6, and 8 for the presence of antibodies to DNP.

The results (Table I) show that, after treatment with antigen, the percentage of cultures containing antibodies was in the range of 50–83%. Some samples of medium from the control cultures also manifested activity, although at an incidence significantly lower than that obtained in the experimental group (11.1–25%). The difference between the experimental and control cultures was manifested not only in the higher incidence of cultures forming antibody after treatment with antigen, but also in the extent of inactivation of DNP-T4 phage. As seen in Fig. 1, most of the experimental cultures inactivated the phage to the extent of 80–100%, whereas most of the control samples inactivated DNP-T4 to a much lower extent (0–20%).

When samples of medium were pooled from cultures of each of these groups and assayed for antibodies, a remarkable difference in activity was noticed between the experimental and the control cultures (Fig. 2).

To test whether inactivation of the modified phage reflects the presence of antibodies to the DNP group or whether inactivation was caused by a direct effect on the T4 phage per se, each of the samples was followed in parallel for

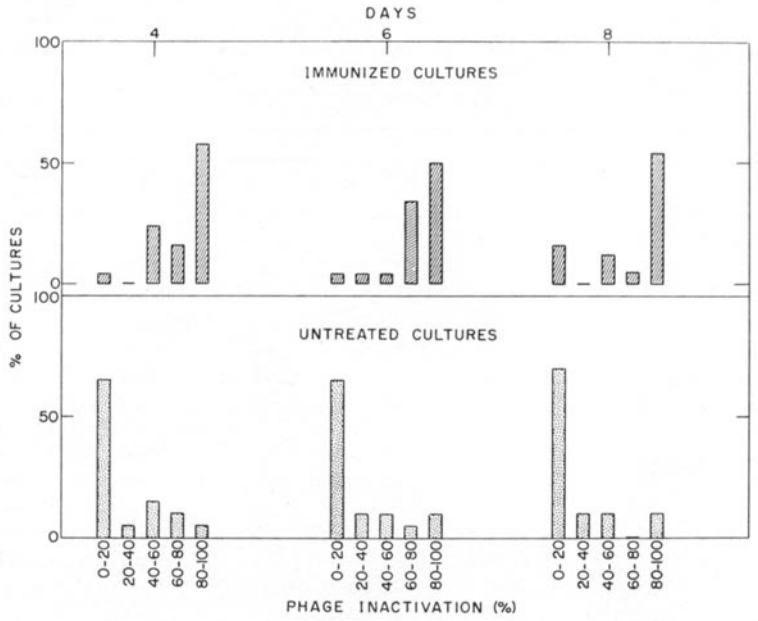

FIG. 1. Incidence of cultures producing anti-DNP antibodies after in vitro immunization with DNP-hemocyanin (24 cases), as compared to untreated cultures (20 cases). Media were tested on days 4, 6, and 8 of culture.

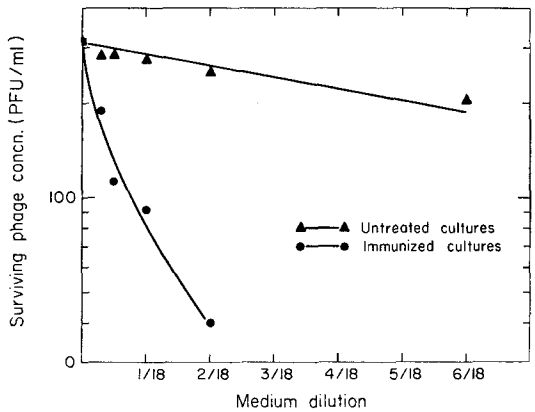

FIG. 2. Inactivation of DNP-T4 phage by media pooled from immunized as compared to untreated cultures. Equal volumes from the media of all cultures described in Table I, experiment No. 1 were pooled.

activity against T4 phage. No inactivation of the unmodified phage could be detected in any of the samples tested. It was thus concluded that the antibodies were directed against the DNP group.

TABLE II

Inhibition by DNP-Lysine of the Inactivation of DNP-T4 Phage by Media of Cultures Immunized with DNP-Hcy

Culture No.	Inactivation					
	Day 4		Day 6		Day 8	
	With DNP-Lys	Without DNP-Lys	With DNP-Lys	Without DNP-Lys	With DNP-Lys	Without DNP-Lys
	%	%	%	%	%	%
1	27	27	12	43	23	23
2	29	60	33	67	22	65
3	28	35	40	41	0	0
4	51	91	13	61	16	22
5	0	0	0	42	17	68
6	9	9	23	74	52	89
7	0	51	1	67	24	24
8	8	34	23	23	—	—
9	30	50	23	23	—	—
10	30	74	0	27	—	—
11	34	62	0	56	0	38
12	24	51	7	24	62	87
13	11	34	42	81	—	—
14	13	38	—	—	—	—

TABLE III

Sensitivity of Anti-DNP Antibodies to 2-ME

Age of culture	Number of reactive cultures			
	Antigen-treated		Untreated	
	Total	2-ME treated	Total	2-ME treated
days				
4	19	0	6	0
6	19	5	6	0
8	21	19	6	6

To determine the extent of specificity of the antibodies detected to the DNP component, the capacity of DNP-lysine (10^{-3} M) to inhibit the inactivation of DNP-T4 phage was tested. Samples of media from 14 reactive cultures were tested on days 4, 6, and 8 after immunization. In most cases the reaction was inhibited in the presence of DNP-lysine, although the extent of inhibition varied in different cultures (Table II). This variation could be attributed to the difference in the amount and affinity of the antibodies in the different cultures.

To characterize the type of antibodies produced, samples of medium were incubated with 2-ME. As shown in Table III and Fig. 3, gradual change in sensitivity of antibodies to 2-ME was detected in various individual cultures.

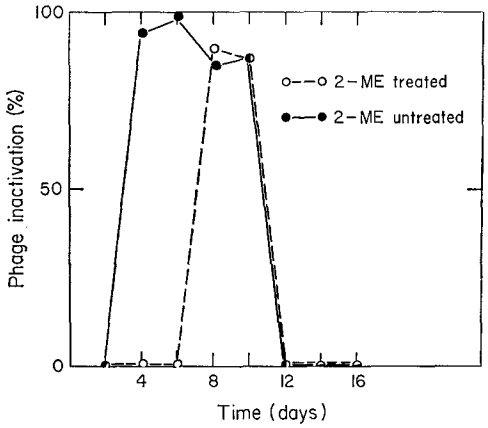

FIG. 3. Inactivation of DNP-T4 phage by medium collected at different time intervals after the reaction of the culture with antigen, before and after the treatment with 0.1 M 2-ME

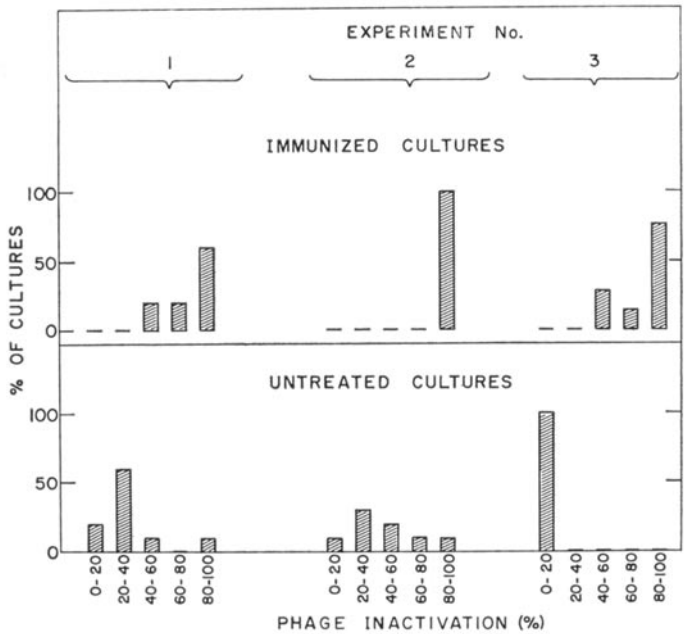

FIG. 4. Incidence of cultures producing anti-DNP antibodies after in vitro immunization with α-DNP-poly-L-lysine as compared to untreated cultures. Media were tested on the 4th day of culture.

Antibodies detected on days 4 and 6 were sensitive to 2-ME, whereas resistant antibodies were found on days 8 or 10 in most of the cultures, similar to the results from other systems of a primary antibody response in vitro (1, 2).

In view of the results described, it was of interest to find out whether induction of a primary response to the dinitrophenyl determinant can be obtained also when the latter is coupled to a chemically defined synthetic carrier. We have used α-DNP-poly-L-lysine in an experimental system identical to that employed for the DNP-Hcy. Three repeated experiments were performed in which a total of 27 cultures were employed for each of the experimental groups. The result was that α-DNP-poly-L-lysine elicited the production of antibodies, as shown in Fig. 4.

DISCUSSION

The purpose of this study was to establish a system in which a primary antibody response to a chemically defined haptenic group can be induced in vitro. The data presented show clearly that a primary antibody response to a haptenic group can indeed be initiated in vitro in spleen explants. This was achieved by application of an appropriate organ culture system (1), as well as by a sensitive method for the detection of antibodies, namely, the inactivation of chemically modified bacteriophage (8, 9) which was previously used for the detection of antibodies at low concentrations (7, 14–17). The specificity of the response was proved both by the lack of the capacity of the culture medium to inactivate the unmodified bacteriophage T4 and by the inhibition of the antibody activity with DNP-lysine.

The finding that cultures from normal mice which were not treated with antigen in vitro also showed specific anti-DNP activity, although to a much lower extent, may raise the question as to whether indeed a primary response was initiated in our experiments. It should, however, be noted that such low levels of antibodies have been found to quite a number of different antigens in normal, unimmunized animals. In fact, anti-hapten antibodies in the sera of non-immunized organisms have been reported previously (16, 18). These may represent a basal noninduced antibody synthesis, without invalidating the primary nature of the induced production of antibodies.

The fact that a primary immune response to a haptenic determinant can be induced in vitro by a synthetic antigen presents a system which may be applied in studies aimed at the clarification of the cellular and molecular aspects of the antibody response.

SUMMARY

Primary antibody response against the dinitrophenyl group has been elicited in vitro after the stimulation of normal mouse spleen explants with 2,4-dinitrophenyl (DNP)-hemocyanin or α-DNP-poly-L-lysine (PLL). Antibodies were detected in the culture medium by the inactivation of DNP-T4 phage.

The specificity of the reaction was manifested by the lack of the capacity of the medium to inactivate the unmodified bacteriophage and by the inhibition of the inactivation of DNP-T4 with DNP-lysine.

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