SEPARATION OF ANTIGEN-SPECIFIC LYMPHOCYTES

II. Enrichment of Hapten-Specific Antibody-Forming Cell Precursors* ‡

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Clonal selection theories have postulated that individual immunocompetent cells are precommited to the formation of antibodies of a single specificity (1, 2). There is now experimental evidence that only a distinct subpopulation of lymphocytes can bind and be stimulated by a particular antigen (for review see 3). Furthermore, it has been shown that individual lymphocytes display only one type of receptor, homogeneous in respect to specificity (4). This strongly suggests that the precommitment of lymphocytes is expressed by surface receptors for antigen.

Depletion of antigen-binding cells by adsorption to antigen-coated beads (5–7) and specific inactivation by radiation (8, 9), provided direct evidence for the specific function of antigen-binding cells in humoral immune responses. However, experiments with lymphocytes depleted in functionally active antigen-specific cells do not prove finally the precommitment hypothesis. Furthermore they do not allow studies on the mechanism of the induction of an immune response. However, isolation and enrichment of lymphocytes bearing surface receptors of a single specificity would be an obvious advantage in studies of the interaction of specific lymphocytes with immunogens, tolerogens, or mitogens. Furthermore, purification of antigen-specific cells would be essential to dissect the various specific and nonspecific interactions of lymphocytes in immune responses.

Several attempts have been made to purify antigen-specific lymphocytes and to study their function. Lymphocyte populations from unimmunized mice enriched in sheep red blood cell rosette-forming cells (SRBC-RFC)[1] contained anti-SRBC antibody-forming cell precursors (AFCP), whose capacity to proliferate and differentiate in vitro (10) or in vivo (11) into antibody-forming cells was dependent on the presence of collaborating cells. Lactoside-specific cells from unimmunized mice were retained in a lactoside-coupled polyacrylamide bead column and could be eluted by an excess of free haptens. The eluted cells were highly enriched in antilactoside AFCP as determined in an adoptive transfer

* This work was supported by the Deutsche Forschungs Gemeinschaft (grant no. Ha 723/4), Germany, and the following grants to Professor G. J. V. Nossal: National Health and Medical Research Council and the Australian Research Grants Committee, Canberra, Australia; by NIH grant AI-O-3958, U. S. Public Health Service and by contract NO1-CB-23889 with the National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare; and by the Volkswagen Foundation grant no. 112147.

‡ This is publication no. 2051 from the Walter and Eliza Hall Institute of Medical Research.

§ Holder of a postdoctoral fellowship (no. Ha 723/5) of the Deutsche Forschungs Gemeinschaft, Germany.

[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; AFCP, antibody-forming cell precursors; FCS, fetal calf serum; HEM, Hepes buffered Eagle's minimal medium; NIP-, 4-hydroxy-3-iodo-5 nitrophenylated; POL, polymerized flagellin; PBS, phosphate-buffered saline.

system (16). The higher frequency of AFCP in lymphoid cell populations from immunized mice greatly facilitates the separation of immunocompetent cells. Purified SRBC-RFC from immunized mice included B-memory cells and, early in the response, T-helper cells (12). Small numbers of lymphocytes from immunized mice highly enriched in antigen-specific cells by a fluorescence-activated electronic cell sorter (14) or the nylon fiber method (15), responded to the specific antigen in collaboration with T-helper cells in lethally irradiated adoptive recipients.

In general, the function of purified antigen-specific thymus-independent (B-) lymphocytes has been studied in T-cell-dependent adoptive immune responses (10–16, 26). However, the complexity of such transfer systems restricts their application in studies of more fundamental events in the immune response.

In the accompanying article we have described a new simple method for purification of hapten-specific lymphocytes from unimmunized mice. In the present paper we present evidence for the functional integrity of the purified cell populations. We demonstrate the capacity of enriched DNP- and NIP-specific lymphocytes to respond in vitro to DNP or NIP coupled to a T-independent carrier, polymerized flagellin.

Materials and Methods

Mice. Specific pathogen-free (6–8-wk old), CBA mice were used in all experiments.

Antigens. Dinitrophenylated polymerized flagellin was prepared as described previously: $DNP_{0.1}$-POL (17). 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) was coupled to POL according to the method of Brownstone et al. (18): $NIP_{0.2}$-POL. The indices represent the conjugation ratio per residue of monomeric flagellin.

Cell Fractionation. Red cell and damaged cell-free spleen cell suspensions were prepared as described previously (19). Unless otherwise stated, 10^8 spleen cells suspended in Hepes buffered Eagle's minimal medium (HEM) were rocked for 60 min in dishes coated with a thin layer of DNP-gelatin or NIP-gelatin at 4°C as described previously (19). Unbound cells were washed off and 10 ml HEM (37°C) was added to each dish in order to melt the gelatin layer. The suspended binding cells were harvested from the dishes and centrifuged through underlayers of fetal calf serum (FCS). Cells harvested from 5 to 20 dishes were pooled in 10 ml HEM, centrifuged again, and resuspended in a small volume of HEM for treatment with collagenase. In some experiments dishes were used which were coated with cross-linked DNP-gelatin layers. DNP-gelatin-coated dishes were prepared as usual, rinsed with phosphate-buffered saline (PBS, pH 7.3), then treated with glutaraldehyde (3 ml of a 2.5% solution of glutaraldehyde in PBS) for 1 h at room temperature and rinsed again with PBS. Spleen cells were fractionated in these dishes as usual and the bound cells counted under an inverted microscope. 10 to 20 areas were counted at 80-fold magnification (a total of at least 500 cells). The correlation between the area count and the total cell count was 1 to 14,600 as determined by area counts in unfixed dishes and total cell counts in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.) after resuspension of the binding cells by melting the gel at 37°C.

Collagenase Treatment. Hapten-gelatin treated and fractionated spleen cells were treated with collagenase in order to remove hapten-gelatin which remained bound to the cells (19). 10^7 or less spleen cells were suspended in 0.9 ml HEM and 0.1 ml collagenase (1 mg/ml; A grade, Calbiochem, San Diego, Calif.) was added. The cells were kept at 37°C for 10 min or at 4°C for 20 min and then washed twice with HEM (5% FCS). In the course of this procedure a substantial number of cells was lost (up to 50%) particularly when very small cell numbers were treated with collagenase (19).

Tissue Culture. Up to 10^7 spleen cells were cultured in 2 ml medium in plastic trays containing 25 compartments, 20 x 20 mm^2 (Filtrona, Melbourne). Alternatively, up to 2×10^6 spleen cells were cultured in 0.2 ml medium in flat based Microtest II tissue culture plates (System Cooke, Greiner, Germany). In the following the latter is referred to as "microculture system". The culture medium used was Eagle's minimum essential medium buffered with bicarbonate and for microcultures in addition with 10 mM Hepes. It contained mercaptoethanol (10^{-4} molar), FCS (5%), streptomycin

(100 U/ml) and penicillin (100 U/ml). DNP-POL or NIP-POL was added to the cultures to a final concentration of 100 ng/ml. A detailed description of the microculture system will be published elsewhere (B. L. Pike, manuscript in preparation).

Feeder Cell Populations. Spleen cells from irradiated mice or mitomycin-treated spleen cells were used as feeder cells in the microculture system (10^6 cells per culture). Irradiated spleen cells were obtained from mice immediately after lethal irradiation (850 R). Spleen cells were treated with mitomycin (50 μg/ml) for 30 min at 37°C and washed twice with HEM (5% FCS) before suspension in culture medium. Alternatively, low numbers of normal and fractionated spleen cells were cultured in the presence of 10^6 spleen cells depleted in DNP- or NIP-specific cells. Depletion of spleen cells in hapten-specific lymphocytes was performed in dishes coated with hapten-gelatin layers which were in some experiments fixed with glutaraldehyde.

Plaque-Forming Cell (PFC) Assay. The spleen cell cultures were harvested on day 3, washed once with cold HEM and resuspended in 0.5 to 2 ml HEM (5% FCS). Direct anti-DNP, anti-NIP, and anti-SRBC-PFC were assayed according to the method of Cunningham and Szenberg (20). DNP- and NIP-coupled SRBC were prepared as described previously (19).

Results

Binding of Normal Spleen Cells to Dishes Coated with DNP- or NIP-Gelatin. In the experiments described in the following section, fractionated spleen cells were pooled from 5 to 20 dishes, treated with collagenase, and then counted in a hemocytometer. The proportion of binding cells could not be determined by this cell count because of a very inconsistent cell loss during the course of treatment with collagenase. However, the variation in the number of binding cells was very low from experiment to experiment. For convenience the exact proportion of binding cells was therefore determined in separate experiments.

Normal spleen cells were suspended in HEM after removal of damaged cells and red cells and rocked in DNP-gelatin or NIP-gelatin coated dishes at 4°C (10^8 spleen cells per dish). After 60 min the unbound cells were washed off with Eisen's Medium. The bound cells were suspended in 10 ml HEM (37°C) by melting the gels and counted in a Coulter Counter. In two experiments the spleen cells were fractionated in dishes coated with a DNP-gelatin layer which had been fixed by treatment with glutaraldehyde. The number of bound cells were counted in situ using an inverted microscope and the total number of cells bound per dish calculated as described in Materials and Methods.

The number of spleen cells binding to DNP-gelatin dishes increased with the conjugation ratio of DNP-gelatin (Table I). Mixed DNP-gelatin-gelatin layers bound somewhat less cells than pure DNP-gelatin layers. Fixation of the gel with glutaraldehyde did not affect the number of binding cells.

DNP-Specific Unresponsiveness of Spleen Cells Treated with DNP-Gelatin. In previous experiments we were unable to demonstrate an anti-DNP response to DNP-POL of normal spleen cells which were bound to DNP-gelatin-coated dishes and recovered by melting the DNP-gelatin. A possible reason for this failure was the presence of DNP-gelatin on the surface of cells recovered by melting the adsorbent (19). We therefore tested the antihapten response of unfractionated normal spleen cells after pretreatment with hapten-coupled gelatin. Normal spleen cells were incubated at 37°C in HEM containing no inhibitors or various concentrations of DNP_4-gelatin or NIP_3-gelatin. After 30 min the cells were washed twice with cold HEM through underlayers of FCS and

TABLE I

Binding of Normal Spleen Cells to Dishes Coated with Different Hapten-Gelatin Conjugates

Immunoadsorbents	No. of binding cells ($\times 10^{-3}$) per dish	
DNP_2-gelatin	367 ± 17	265 ± 48*
DNP_4-gelatin	463 ± 52	302 ± 41*
DNP_9-gelatin	812 ± 107	
DNP_{16}-gelatin	2,214 ± 223	2,063 ± 117‡
NIP_3-gelatin	83 ± 3	

Normal spleen cells were rocked in dishes coated with thin layers of DNP-gelatin or NIP-gelatin (10^6 cells per dish) for 60 min at 4°C. Unbound cells were washed off and bound cells were suspended in 10 ml HEM (37°C) by melting the gel and counted in a Coulter Counter. The figures show the number of binding cells per dish (mean of three or more replicates ± SE). The data are pooled from five different experiments.
* DNP-gelatin was mixed with an equal volume of unconjugated gelatin.
‡ DNP-gelatin layers were cross-linked by glutaraldehyde. In these dishes, cells were counted in situ under an inverted microscope.

resuspended in microculture medium. DNP-POL and/or NIP-POL was added as indicated in Table II.

DNP-POL stimulated only the generation of anti-DNP PFC and NIP-POL of anti-NIP PFC. Both a normal anti-DNP and a normal anti-NIP response were obtained in cultures containing both antigens. Pretreatment with DNP-gelatin affected the anti-DNP response but not the anti-NIP response. However, pretreatment with NIP-gelatin reduced only marginally the anti-NIP response in cultures containing both DNP-POL and NIP-POL. DNP-sepcific unresponsiveness was also induced by DNP-gelatin treatment of normal spleen cells at 4°C for 20 min (Table III and IV).

Reversibility of DNP-gelatin-Induced Unresponsiveness to DNP-POL by Treatment with Collagenase. Pretreatment of normal spleen cells with different DNP-gelatin conjugates (1 mg/ml) for only 20 min at 4°C abolished almost completely the subsequent in vitro response to DNP-POL (Table IV). DNP-specific cells bound presumably highly aggregated DNP-gelatin which could not be washed off. However, these DNP-gelatin aggregates could be removed from the cell surface by treatment with collagenase (19). Treatment with collagenase (100 μg/ml) for 20 min at 4°C restored the anti-DNP response of spleen cells pretreated with DNP_{16}-gelatin or lower substituted DNP-gelatin. The response of spleen cells treated with highly substituted DNP-gelatin was only restored completely if the cells were treated with collagenase for 15 min at 37°C (Table IV). Unresponsiveness induced by preincubation of spleen cells for 2 h at 37°C could still be reversed by treatment with collagenase (Table III). The anti-DNP response of untreated or gelatin-treated spleen cells was not altered by collagenase treatment.

Depletion of Hapten-Specific Antibody-Forming Cell Precursors. Normal spleen cells were rocked in gelatin or DNP-gelatin-coated dishes for 60 min at 4°C. Unbound cells were washed off, pooled from 5 to 10 dishes, and aliquots were

TABLE II

Spleen Cell Response to DNP-POL and NIP-POL: Effect of Pretreatment with DNP-Gelatin and NIP-Gelatin

Pretreatment		Antigen	PFC per culture	
Inhibitor	mg/ml		Anti-DNP	Anti-NIP
—	—	DNP-POL	330 ± 26	30 ± 9
—	—	NIP-POL	30 ± 3	298 ± 31
—	—	DNP-POL + NIP-POL	375 ± 45	288 ± 26
DNP_4-gelatin	5	"	47 ± 8	317 ± 32
DNP_4-gelatin	0.5	"	150 ± 30	319 ± 30
NIP_3-gelatin	5	"	312 ± 41	186 ± 28
NIP_3-gelatin	0.5	"	296 ± 67	282 ± 32

Spleen cells were suspended in HEM or HEM containing different concentrations of DNP-gelatin or NIP-gelatin and kept at 37°C for 30 min. The cells were then washed twice with HEM through underlayers of FCS resuspended in culture medium and aliquots cultured in the microculture system (10^6 cells per culture) with DNP-POL and/or NIP-POL as indicated. The figures represent the number of DNP-PFC or NIP-PFC per culture (mean of five replicates ± SE).

TABLE III

Reversibility of Unresponsiveness to DNP-POL Induced by Various DNP-Gelatin Conjugates

Pretreatment	DNP-PFC per culture	
	Collagenase treatment:	
	−	+
—	1,906 ± 132	1,918 ± 86
Gelatin	2,020 ± 118	1,934 ± 117
DNP_4-gelatin	116 ± 26	2,018 ± 143
DNP_{16}-gelatin	70 ± 12	1,934 ± 56
DNP_{24}-gelatin	42 ± 14	1,150 ± 107
DNP_{24}-gelatin	—	1,720 ± 86*

Normal spleen cells were kept in HEM or HEM containing gelatin or DNP-gelatin (1 mg/ml) for 20 min at 4°C. The cells were then washed twice and treated with collagenase (+) or not (−). For treatment with collagenase cells were incubated with 100 μg/ml collagenase for 20 min at 4°C or in one case (*) for 15 min at 37°C. The cells were then cultured (10^7 cells per culture) with DNP-POL. The figures represent the number of DNP-PFC per culture (mean of four replicates ± SE).

treated or not treated with collagenase and then cultured with DNP-POL. The anti-DNP response of cells depleted in DNP_4-gelatin dishes and DNP_{16}-gelatin dishes was about 25% and 10% of the control response (Table V). However, collagenase treatment recovered the response of the depleted cell populations partially, particularly in the case of cells fractionated in DNP_{16}-gelatin-coated dishes. Collagenase treatment had no effect on the response of spleen cells

TABLE IV

Reversibility of Unresponsiveness to DNP-POL Induced by DNP-Gelatin at 4°C and 37°C

Pretreatment			DNP-PFC per culture	
			Collagenase treatment:	
			−	+
Gelatin	4°C,	20 min	2,340 ± 212	2,130 ± 178
Gelatin	37°C,	120 min	1,882 ± 144	1,762 ± 83
DNP_8-gelatin	4°C,	20 min	120 ± 18	2,000 ± 107
DNP_8-gelatin	37°C,	120 min	82 ± 22	1,655 ± 142

Normal spleen cells were kept in HEM containing gelatin or DNP_8-gelatin (1 mg/ml) and then washed twice and treated with collagenase (15 min, 37°C) or not as indicated. The cells were then cultured (10^7 cells per culture) with DNP-POL. The figures represent the number of DNP-PFC per culture. (mean of five replicates ± SE).

TABLE V

Depletion of DNP-POL Responding Cells

Immunoadsorbents	Spleen cells cultured		DNP-PFC per culture	
			Collagenase treatment:	
			−	+
—	Unfractionated	10^7	2,115 ± 153	2,085 ± 141
Gelatin	Unbound	10^7	2,055 ± 231	2,150 ± 144
DNP_4-gelatin	Unbound	10^7	335 ± 52	695 ± 181
DNP_{16}-gelatin	Unbound	10^7	175 ± 29	890 ± 123
DNP_4-gelatin fixed	Unbound	10^7	635 ± 90	735 ± 74
DNP_{16}-gelatin fixed	Unbound	10^7	855 ± 65	800 ± 180

Spleen cells were rocked in dishes coated with thin layers of gelatin, DNP-gelatin, or DNP-gelatin which have been fixed by glutaraldehyde treatment. After 60 min unbound cells were washed off, treated with collagenase (15 min, 37°C) or not and aliquots were cultured with DNP-POL. The figures represent the number of DNP-PFC per culture (mean of five replicates ± SE).

depleted in dishes coated with DNP-gelatin which was cross-linked by treatment with glutaraldehyde. Depletion in these dishes was still incomplete and equally effective using DNP_4-gelatin and DNP_{16}-gelatin, although five times as many cells were bound to the latter (Table I). The response of gelatin-dish "absorbed" cells was normal. Furthermore, fractionation in DNP-gelatin dishes depleted only anti-DNP but not anti-NIP AFCP and fractionation in NIP-gelatin dishes depleted only anti-NIP but not anti-DNP AFCP (Table VI).

Enrichment of Hapten-Specific Antibody-Forming Cell Precursors

DNP-POL RESPONSE OF NORMAL AND PURIFIED DNP-SPECIFIC SPLEEN CELLS IN THE ABSENCE OR PRESENCE OF MITOMYCIN-TREATED OR IRRADIATED "FEEDER" CELLS. Dif-

TABLE VI

Specific Depletion and Enrichment of Anti-DNP and Anti-NIP AFCP

Immunoadsorbents	Spleen cells cultured		PFC per culture	
			Anti-DNP	Anti-NIP
DNP_4-gelatin (*)	Unfractionated	10^6	247 ± 36	157 ± 27
	Unbound	10^6	133 ± 22	157 ± 23
	Unbound	10^6		
	+ unfractionated	2×10^5	201 ± 25	205 ± 23
	Unbound	10^6		
	+ bound	2×10^4	816 ± 79	148 ± 21
	Unbound	10^6		
	+ bound	2×10^5	1,898 ± 65	243 ± 25
NIP_3-gelatin	Unfractionated	10^6	201 ± 37	209 ± 47
	Unbound	10^6	241 ± 38	85 ± 25
	Unbound	10^6		
	+ unfractionated	2×10^5	348 ± 40	190 ± 32
	Unbound	10^6		
	+ bound	2×10^4	321 ± 25	1,831 ± 175

Normal spleen cells were fractionated in dishes coated with DNP_4-gelatin (* mixed with an equal volume of unconjugated gelatin) or NIP_3-gelatin as described in Materials and Methods. Bound cells were recovered by melting the gel and pooled from 15 dishes (input: 10^8 spleen cells per dish) and unbound cells were pooled from five dishes (input: 3×10^7 spleen cells per dish). Unfractionated and fractionated spleen cells were treated with collagenase (15 min at 37°C) and cultured in the microculture system as indicated. Both DNP-POL and NIP-POL were added to each culture. The figures represent the PFC per culture (mean of five replicates ± SE).

ferent numbers of unfractionated and purified DNP-specific spleen cells were cultured in the microculture system and stimulated with DNP-POL. The cell dose response relationship in cultures of normal spleen cells was not a straight line (Fig. 1 A), the optimal cell dose being 8×10^5 cells per culture. More than 2×10^5 unfractionated spleen cells were required to obtain a significant anti-DNP AFC response. However, some anti-DNP AFC were generated in cultures containing only 10^4 purified DNP-specific spleen cells. Although the culture conditions in cultures with low cell density were suboptimal, AFC were generated by small cell numbers containing a sufficient proportion of responding cells.

The numbers of purified hapten-specific spleen cells obtained after fractionation and collagenase-treatment were very small because of the small proportion of binding cells and a substantial cell loss in the course of the treatment with collagenase (19). Therefore, we had to establish optimal culture conditions for the generation of AFC by low cell numbers. We approached this problem by using mitomycin-treated or lethally irradiated spleen cells as "feeder" cells. Different numbers of normal spleen cells were cultured in the presence of 10^6 feeder cells

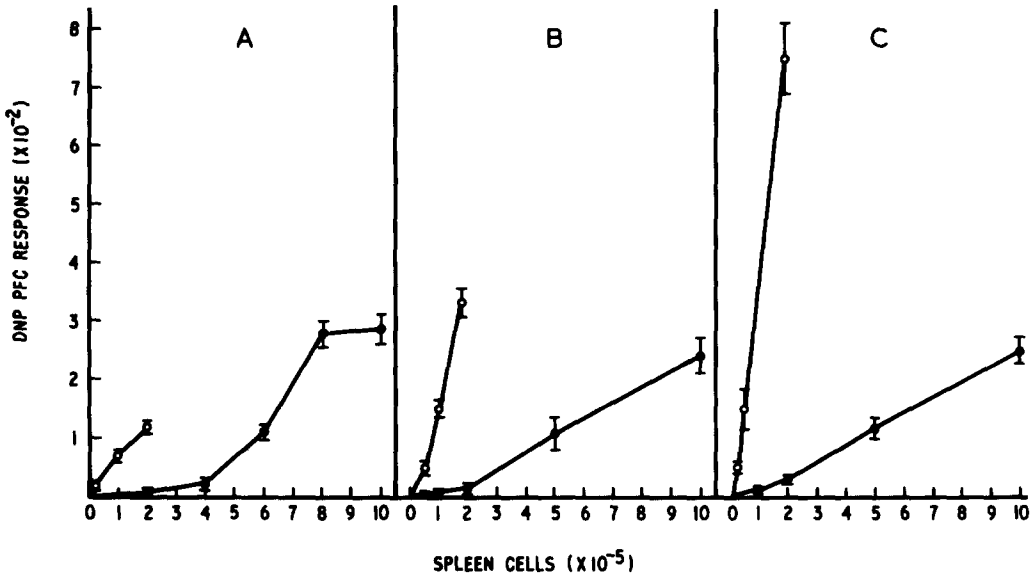

FIG. 1. DNP-POL response of unfractionated and purified DNP-specific spleen cells in the absence or presence of feeder cells. Various numbers (abscissa) of unfractionated (●) or purified DNP_4-gelatin binding cells (○) were cultured with DNP-POL either alone (A) or in the presence of 10^6 mitomycin-treated (B) or irradiated (C) feeder cells. No anti-DNP-AFC were generated in cultures containing 10^6 feeder cells only. The figures show the number of anti-DNP-AFC per culture obtained on day 3 (mean of five replicates ± SE).

which gave no anti-DNP AFC response when cultured alone (Fig. 1 B and C). The limiting number of normal spleen cells giving a significant anti-DNP response was somewhat lower in the presence of feeder cells. However, the cell dose response relationship was still not a straight line. Again the response of small numbers of purified DNP-specific cells obtained from DNP_4-gelatin coated dishes was enriched compared with the response of unfractionated cells. The response of only 1.8×10^5 DNP-gelatin-binding cells was three times greater than the response of 10^6 unfractionated cells in the presence of unresponsive irradiated feeder cells (Fig. 1 C). Mitomycin-treated feeder cells were less effective in enhancing the response of normal and purified spleen cells (Fig. 1 B).

DNP-POL RESPONSE OF NORMAL AND PURIFIED DNP-SPECIFIC CELLS IN THE PRESENCE OF SPLEEN CELLS DEPLETED IN DNP-SPECIFIC CELLS. We tested the capacity of DNP-unresponsive but otherwise normal spleen cells, to support the response of small numbers of competent spleen cells in culture. A source for the former cell population were spleen cells depleted in DNP-gelatin-coated dishes. Fig. 2 shows the anti-DNP response of different numbers of unfractionated and purified DNP-specific spleen cells in the presence of 10^6 depleted cells in three different experiments. The diminished response of the depleted cell populations was reconstituted by addition of normal spleen cells. In this system optimal numbers of anti-DNP PFC were obtained with low cell numbers in contrast to the response of normal spleen cells obtained in the presence of mitomycin-treated or irradiated feeder cells.

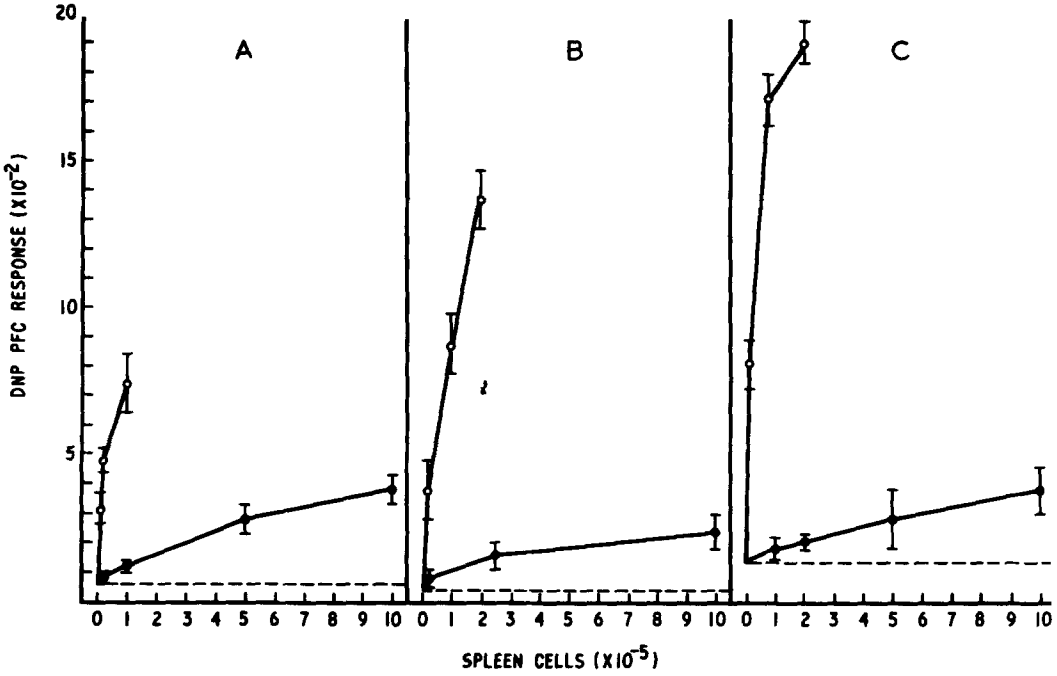

FIG. 2. DNP-POL response of unfractionated and purified DNP-specific spleen cells in the presence of spleen cells depleted in DNP-specific spleen cells. Fractionation of spleen cells was performed in dishes coated with DNP_4-gelatin (A) a mixture of equal volume of DNP_4-gelatin and gelatin (B) or DNP_2-gelatin and gelatin (C). For depletion dishes with glutaraldehyde-fixed gels were used. The figures represent the anti-DNP-AFC response to DNP-POL of 10^6 depleted spleen cells alone (horizontal, broken line) or with various numbers (abscissa) of unfractionated (●) or purified DNP-specific ○) spleen cells (mean of five replicates ± S.E.).

The purified DNP-specific cells were enriched in DNP-POL responding cells. The response of only 2×10^4 purified cells was greater than the response of 10^6 unfractionated cells. Very similar results were obtained using DNP-tolerant "feeder" cells (J. W. Stocker, manuscript in preparation). The degree of enrichment was dependent on the DNP-gelatin conjugation ratio used for the fractionation. There was no significant difference between the response of spleen cells binding to DNP_4-gelatin layers (Fig. 2 A) and cells binding to layers of a mixture of equal volumes of DNP_4-gelatin and gelatin (Fig. 2 B). The degree of depletion in dishes which were coated with these gels was also of the same order of magnitude. However, fractionation in dishes coated with a mixture of DNP_2-gelatin and gelatin resulted in greater enrichment but less depletion (Fig. 2 C).

SPECIFICITY OF ENRICHMENT. Spleen cells were fractionated in dishes coated with a mixture of DNP_2-gelatin and gelatin or with NIP_3-gelatin. 3×10^6 spleen cells were fractionated per dish to obtain cell populations depleted in hapten-specific cells and 10^8 spleen cells per dish to obtain purified hapten-specific cells. Unfractionated and hapten-gelatin binding cells were cultured in the presence of

depleted cells after treatment with collagenase (15 min, 37°C) with both NIP-POL and DNP-POL (Table II). The cultures were harvested after 3 days and the cells from each culture assayed for anti-SRBC, anti-DNP, and anti-NIP-PFC. The SRBC-PFC background was the same in all cultures (10 to 20 PFC per culture). The antihapten response of the unbound cell populations was specifically depleted. Cultures containing small numbers of purified DNP-specific cells gave enriched anti-DNP but normal anti-NIP responses whereas NIP-specific cells gave highly enriched anti-NIP responses and normal anti-DNP responses.

The specific enrichment of anti-DNP AFCP was approximately 100-fold and of NIP-POL AFCP 300-fold assuming that the culture conditions for stimulation of AFCP and for clonal expansion were similar in cultures containing 2×10^4 purified cells and 2×10^5 unfractionated cells.

Discussion

We describe in the preceding paper a new method for isolation of hapten-specific lymphocytes. Spleen cells were fractionated in dishes coated with a thin layer of insoluble hapten-gelatin at 4°C. The great majority of the binding cells recovered by melting the gel were Ig-positive B lymphocytes (19). The experiments described in this article are concerned with the question of whether these cells have any functional significance in a humoral immune response to the specific hapten. The in vitro IgM response to a hatpen coupled to polymerize flagellin provides a simple model to study the immunocompetence of hapten-specific B lymphocytes which is independent of thymus-dependent (T) cells and macrophages (21).

A common disadvantage of methods for separation of antigen-specific cells is that antigen is present on the surface of the isolated cells (19). DNP-gelatin has been detected on the surface of cells recovered from DNP-gelatin-coated dishes by melting (19). In the present paper we demonstrate that DNP-gelatin bound to DNP-specific cells induced unresponsiveness to DNP-POL. Previously we failed to demonstrate any reduction in a primary adoptive anti-DNP response to DNP-POL of spleen cells which had been pretreated with DNP-gelatin (30). However, the anti-DNP response of DNP-gelatin-treated cells was almost completely abolished earlier in the adoptive immune response (unpublished data). It is probable that DNP-gelatin is removed rapidly from the surface of immunocompetent cells since it is very susceptible to digestion by proteases (31).

Gelatin is a T-dependent antigen capable of stimulating helper cells for antibody production to its own antigenic determinants (22) or to haptens coupled to it (23). However, DNP-gelatin is a weak immunogen in vivo (23) and nonimmunogenic in vitro in the absence of activated T-helper cells (unpublished data). Hapten-specific B-cell unresponsiveness after brief treatment of lymphocytes with relatively high doses of polyvalent nonimmunogenic hapten-conjugates has been described by several authors (for review see 24, 35, 36). It has been suggested that the interaction of B cells with haptens coupled to T-dependent carrier molecules in the absence of T-helper cells induced tolerance (24). However, we do not know the mechanism by which specific unresponsiveness was induced by DNP-gelatin in our experiments. Pretreatment of spleen cells with

NIP-gelatin, even at high concentrations, reduced the anti-NIP response marginally. Early in the culture period DNP-gelatin and NIP-gelatin may be shed from the binding cells. Subsequently, the DNP-gelatin concentrations in the cultures containing DNP-gelatin pretreated cells was sufficiently high to inhibit the stimulation by DNP-POL. In the case of NIP-gelatin-pretreated cells only very few cells had NIP-gelatin attached to their surface. Thus the stimulation of NIP-specific cells by NIP-POL was not markedly inhibited by NIP-gelatin which was released from the NIP-gelatin binding cells in the culture fluid. Experiments are in progress to clarify the differential effects of DNP-gelatin and NIP-gelatin. For the experiments presented in this article, it was only important that DNP-gelatin-induced unresponsiveness could be reversed by treatment with collagenase. Similarly, DNP-specific tolerance induced by DNP-ovalbumin or DNP-L-GL could be broken easily by treatment with trypsin (24, 35).

Although gelatin is easily digested by trypsin, we used collagenase to avoid digestion of cell-surface proteins and nonspecific stimulatory effects of trypsin treatment (35). Collagenase treatment had no effect on the immune response of normal spleen cells to DNP-POL or NIP-POL. Spleen cells binding to DNP-gelatin-coated dishes which were recovered by melting the gel did not respond to hapten-conjugated POL unless treated with collagenase. Furthermore, the decreased anti-DNP response of cells depleted in DNP-gelatin-coated dishes was partially due to unresponsiveness induced by DNP-gelatin, particularly when using highly conjugated DNP-gelatin which is known to be less stable than more lightly substituted DNP-gelatin conjugates (25). It is likely that this effect was due to release of cells from the immunoadsorbents after binding since treatment of normal spleen cells with supernatants of DNP-gelatin dishes did not affect their responses to DNP-POL. Similarly, specific lymphocytes escaped from antigen-coated glass bead columns with antigen bound to their surface (26), although free antigen has not been eluted from such columns in detectable amounts (27).

There was no recovery of the anti-DNP response from cells depleted in dishes coated with DNP-gelatin cross-linked by glutaraldehyde. The failure to deplete normal spleen cells completely in anti-DNP AFCP even using highly substituted DNP-gelatin, raises the question of whether some anti-DNP AFC are derived from B cells with no receptors or from B cells with no DNP-specific receptors. However, the aim of our experiments was to enrich rather than deplete immunocompetent cells.

The number of purified cells available for tissue culture was very low although up to 10^9 spleen cells were fractionated in one particular experiment and the binding cells were pooled from 10 to 20 dishes. This was due to a substantial cell loss during the treatment of small numbers of purified spleen cells with collagenase, although collagenase did not affect the viability of the cells (19). A problem encountered in culturing low cell numbers is the lower than expected PFC response by low numbers of cells to antigenic stimulation as depicted by the shoulder in limiting dilution curves (for review see 28).

In our microculture system, there was also no linear cell dose response relationship. More than 2×10^5 normal spleen cells were required to obtain a

significant anti-DNP response. However, anti-DNP PFC were detected in cultures containing only 2×10^4 purified DNP-specific cells. Thus, differentiation into PFC was possible in low density cultures providing a sufficient number of responding cells.

The limiting number of unfractionated spleen cells which gave a significant response was slightly lower in the presence of nonresponding feeder cells, although there was still no linear cell dose response relationship. The in vitro AFC response of low numbers of spleen cells to SRBC can be enhanced by adding lethally irradiated spleen cells to the cultures as feeder cells which presumably supply radioresistant adherent or thymus-derived cells (29). In our experiments the presence of feeder cells had a more striking effect on the response of small numbers of purified cells. It is possible that the more pronounced effect of feeder cells on the response of purified DNP-specific cells was due to supplementation of a cell type not present in the purified cell population.

Mitomycin-treated spleen cells appeared to be less effective as feeder cells than irradiated spleen cells. Low numbers of normal spleen cells responded optimally to DNP-POL in the presence of spleen cells partially depleted in DNP-responding cells. In this system the response of low numbers of purified cells was highly enriched and exceeded by almost 10-fold the response obtained from optimal numbers of unfractionated cells. Very similar results were obtained using spleen cells from DNP-tolerant mice as "feeder" cells (J. W. Stocker, unpublished data).

Quantitative considerations of the enrichment of AFCP in the purified cell populations are very limited, since the frequency of AFCP in our culture system is unknown. The number of PFC is an uncertain measure of the number of activated precursor cells, since clonal expansion takes place between antigenic stimulation and appearance of PFC. In fact, all AFC in our culture system were the result of cell proliferation since no PFC were obtained in cultures of mitomycin-treated or irradiated spleen cells. Both the precursor frequency and the burst size may be affected by cells and/or factors involved in the regulation of the immune response.

However, we can estimate the relative enrichment of precursor cells in the purified cell population by comparing the number of PFC generated by the optimal cell dose of different cell preparations. The enrichment of anti-DNP AFCP appeared to be higher in the cell population binding to more lightly substituted DNP-gelatin layers. Furthermore, heavily substituted DNP-gelatin was not more efficient than lightly substituted DNP-gelatin in depleting specific AFCP. The increased number of binding cells obtained by increasing the number of DNP groups of the immunoadsorbent represents presumably low affinity cells (19) which were not stimulated by DNP-POL to differentiate into AFC, or, if they were stimulated, their antibody products could not be detected in our plaque assay. A reverse correlation between the number of binding cells and the degree of enrichment of precursor cells was also found comparing NIP- and DNP-specific cells. The proportion of cells binding to lightly substituted DNP-gelatin was four to five times higher than the binding to NIP-gelatin with a comparable substitution ratio, but the enrichment of anti-NIP AFCP was two to three times higher than the enrichment of anti-DNP AFCP.

Although the purified hapten-specific cell populations gave much increased AFC responses compared with unfractionated cells, it is obvious that not all binding cells were stimulated by the relevant antigen to differentiate into AFC. Our data suggest that cells with low affinity receptors were not stimulated by antigen. However, this may reflect merely the limited sensitivity of the plaque assay. More low affinity cells are stimulated by T-independent antigens than is the case with T-dependent antigens (32, 33). Furthermore, many more anti-DNP AFC were demonstrated in DNP-POL-stimulated cultures using DNP-SRBC with a higher density (J. W. Stocker, personal communication). A specific immunological function of the binding cells which apparently failed to react to antigen cannot be ruled out. Some binding cells may be rendered tolerant by interaction with antigen, others may have differentiated into low affinity antibody-forming cells or memory cells. Furthermore, the binding cell population may contain the precursors of cells secreting Ig classes other than IgM (IgG or IgE), (34) and hapten-specific T cells.

One of the current tasks of immunology is to define specific immunogenic or tolerogenic signals and to correlate these with early changes in the metabolism of lymphocytes. The main reason for the lack of information in this field is the low frequency of antigen-binding cells in normal lymphoid cell populations. The method for purification of hapten-specific lymphocyte populations described in this and the preceding paper should be useful to study the immunological significance of antigen-binding and to characterize the immediate precursor cells of AFC, their interaction with other cell types and clonal expansion after antigenic stimulation.

Summary

Normal spleen cells were separated in dishes coated with thin layers of DNP-gelatin or NIP-gelatin into binding and nonbinding cells and stimulated in vitro with DNP- and/or NIP-conjugated polymerized flagellin (POL). Hapten-specific unresponsiveness was induced in the binding cell population by melting the gel at 37°C or in unfractionated cells by pretreatment with soluble hapten-gelatin and could be reversed by treatment with collagenase. A specific enrichment of anti-DNP and anti-NIP antibody-forming cell precursors (AFCP) could be demonstrated in the binding cell populations after treatment with collagenase in cultures with or without "feeder" cells. However, the response of small numbers of unfractionated and purified hapten-specific spleen cells was suboptimal even in the presence of mitomycin-treated or irradiated feeder cells.

Optimal numbers of anti-DNP (anti-NIP) antibody-forming cells were generated by small numbers of normal or purified spleen cells in the presence of spleen cells depleted of anti-DNP (anti-NIP) AFCP. In this system the response of only 2×10^4 purified hapten-specific cells was higher than the response of 10^6 unfractionated cells. Purified DNP-specific cells responded only to DNP-POL but not to NIP-POL and purified NIP-specific cells responded only to NIP-POL but not to DNP-POL. The degree of enrichment of anti-DNP AFCP decreased with increasing numbers of binding cells. NIP_3-gelatin layers bound four to five times less spleen cells than DNP_2-gelatin layers and the enrichment of anti-NIP AFCP (about 300-fold) was three times greater than the enrichment of anti-DNP

AFCP (about 100-fold). The immunological significance of hapten-gelatin binding cells which apparently failed to respond to antigen is discussed.

The author thanks Professor G. J. V. Nossal, Dr. A. Szenberg, and Dr. J. W. Schrader for their encouragement and advice, Mr. J. Pye for preparation of polymerized flagellin, and Ms. K. Fowler for excellent technical assistance.

Received for publication 29 October 1974.

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