CELL INTERACTIONS IN THE IMMUNE RESPONSE IN VITRO

II. The Requirement for Macrophages in Lymphoid Cell Collaboration*

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Collaboration between thymus-derived (T)[1] and nonthymus-derived (B) lymphocytes occurs in the induction of antibody formation to many antigens, including heterologous erythrocytes (1, 2), serum proteins (3), and hapten-protein conjugates (4). Macrophages are also involved in the induction of the antibody response to many antigens both in vivo (5, 6) and in vitro (7). Ultrastructural observations have suggested a close relationship between macrophages and immunocompetent cells (8), and clusters of macrophages and lymphocytes have been observed in antigen-stimulated cultures (9, 10). Using enzymes or radioiodinated antigens it was shown that there was a structural relationship between antigen-bearing macrophages and antibody-forming cells (10, 11). Furthermore, antigen-bearing macrophages augment immune responses (5, 6). These observations suggested that macrophages are involved in immunization, but did not clarify their exact role. Experiments performed in tissue culture, using sheep erythrocytes (SRC) and polymerized flagellin of *Salmonella adelaide* (POL) suggested that macrophages break down antigens of large size into smaller immunogenic particles (7). While studying antibody responses to dinitrophenylated (DNP) proteins in vitro, it was noted that macrophages were required for anti-DNP responses to some, but not all, DNP hapten-protein conjugates (12). The experiments reported here establish that antibody responses to protein antigens involving T and B lymphocyte collaboration also require the presence of macrophages. In other words, macrophages participate in the process of T-B lymphocyte cooperation.

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[1] *Abbreviations used in this paper*: AFC, antibody-forming cells; AMS, antimouse macrophage serum; B, nonthymus-derived; DNP, dinitrophenyl determinant; DRC, donkey red cells; FγG, fowl gamma globulin; Fla, flagella; KLH, keyhole limpet hemocyanin; MON, monomeric flagellin; POL, polymeric flagellin; SRC, sheep erythrocytes; T, thymus-derived; TDC, thoracic duct cells.

Materials and Methods

Antigens.—Flagella (Fla), polymerized flagellin (POL) of *Salmonella adelaide*, were prepared by the method of Ada et al. (13) and oxidized monomeric flagellin (MON) by the method of Parish (14). DNP Fla, DNP POL, DNP MON, DNP fowl gamma globulin (DNP FγG), and DNP keyhole limpet hemocyanin (DNPKLH) were prepared as described (15). A single batch of each of these conjugates was used. Donkey red cells (DRC) and SRC were aged for 1–3 wk before use.

Animals.—CBA/H/Wehi mice of either sex were used throughout.

Priming of Mice.—Mice were injected one to three times intraperitoneally at 6–10 wk of age with 25–100 μg of antigen. Except for DNP Fla or DNP POL, these antigens were emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). Spleen cells for culture were obtained 4–16 wk after the last priming dose.

Tissue Culture.—Mouse spleen cells were cultured in Diener (16) culture flasks in Eagle's minimal essential medium containing 5% fetal calf serum (17). Spleen cells from primed mice were always washed before culture.

Removal of Macrophages.—The column purification method of Shortman et al. (18) was used. Briefly, spleen cell suspensions, in a culture medium containing 50% mouse serum, were passed through a column of large siliconized glass beads at 37°C. The effluent is a purified population of lymphocytes (>99%), extensively depleted of phagocytes as judged by morphological, functional, or immunological criteria (18).

Peritoneal Exudate.—CBA mice aged 6–8 months were injected intraperitoneally with 1 ml of proteose peptone broth (Difco Laboratories). Exudate cells were harvested 4 or 5 days later.

Thoracic Duct Cannulation.—DNP-primed mice were cannulated by Dr. John Sprent, using the technique of Miller and Mitchell (19).

Antimacrophage Serum.—Specific rabbit antimouse macrophage serum (AMS) was prepared and made macrophage specific as described by Feldmann and Palmer (20) and Shortman and Palmer (21).

AKR Anti-θ-C3H Serum.—Peritoneal exudate cells were treated with AKR anti-θ-C3H serum and complement under conditions optimal for killing T cells (17).

Antibody-Forming Cell Assays.—Antibody-forming cells (AFC) were enumerated using the technique of Cunningham and Szenberg (22). To detect anti-DNP AFC, SRC were coated with dinitrophenylated rabbit anti-SRC Fab' (DNP-SRC). Specific anti-DNP AFC were obtained by subtracting counts of anti-SRC AFC. IgG responses were detected in vitro, but since their magnitude was only 10–60% of the IgM response, and always varied in parallel with it, only IgM results are presented here.

RESULTS

Effect of Removal of Macrophages on the Anti-DNP Response In Vitro.—In both the primary and secondary responses, spleen cell populations depleted of their content of phagocytes respond normally in vitro to POL, but not to SRC (7, 20). Spleen cell suspensions from mice primed to DNP FγG, DNP KLH, or DNP MON can be immunized with the corresponding antigen in vitro (15, 23). These responses, unlike that to DNP POL, require the participation of carrier-reactive T cells in vitro (15, 17). Removal of macrophages suppressed responses to DNP MON and DNP FγG in vitro but not to DNP Fla (Table I). Effective depletion of macrophages from spleen cell populations was monitored by the inhibition of the response of purified lymphocytes to DRC in vitro. Since DNP

Fla immunized purified lymphocyte suspensions normally (Table I), DNP-re-active B cells were present in the purified lymphocyte suspensions. The poor anti-DNP responses of purified lymphocytes from DNP FγG–primed spleen after 4 days in culture could be due to a delay in the generation of antibody responses. Thus assays were performed 2–6 days after initiation of culture. At all these times the lymphocytes were incapable of responding to DNP FγG in the absence of additional macrophages (Fig. 1).

Effect of Specific Antimacrophage Serum on the Anti-DNP Response In Vitro.–

TABLE I

Suppression of Collaborative Anti-DNP Responses by Removal of Macrophages

Cells	Antigen*	Immune response (AFC/culture ± SE)	
		DNP	DRC
DNP Fla–primed spleen cells	DNP Fla	1200 ± 106	—
" " " " "	DNP MON	865 ± 112	—
DNP Fla–primed lymphocytes‡	DNP Fla	1080 ± 96	—
" " " "	DNP MON	48 ± 40	—
DNP FγG–primed spleen cells	DNP Fla	2820 ± 146	1430 ± 290
" " " " "	DNP FγG	2520 ± 502	1140 ± 312
DNP FγG–primed lymphocytes‡	DNP Fla	1620 ± 187	30 ± 20
" " " "	DNP FγG	8 ± 5	25 ± 18
DNP MON–primed spleen cells	DNP Fla	1197 ± 285	3400 ± 180
" " " " "	DNP MON	592 ± 227	2725 ± 680
DNP MON–primed lymphocytes‡	DNP Fla	1050 ± 283	24 ± 18
" " " "	DNP MON	0	16 ± 12

* Antigen concentrations used 0.1 μg/ml of $DNP_{1.5}Fla$, 1 μg/ml of $DNP_{1.2}MON$ or $DNP_{12}F\gamma G$. These were the optimal concentrations. Each value is the arithmetic mean of four cultures ± the standard error of the mean. Similar results were obtained in 12 other, experiments of this type.

‡ Lymphocytes obtained by the active adherence method of Shortman et al. (18).

It was recently demonstrated that specific antimouse macrophage serum, which reacted with macrophages but not with lymphocytes, inhibited antibody production to SRC, but not to POL in vitro (20, 21). It was thus of interest to determine the effect of AMS on the anti-DNP response in vitro. Two different batches of AMS were used. Both suppressed the response to DRC and to DNP KLH, but not that to DNP Fla (Table II).

Response of Thoracic Duct Lymphocytes In Vitro.—Thoracic duct cells (TDC), especially those obtained in the first 12 hr, are a population devoid of mature phagocytes (24) which have been extensively used in adoptive immunological studies (25, 26). TDC thus provided another means of testing whether macrophages were required for collaborative anti-DNP responses in vitro. TDC of

mice primed twice with DNP FγG were cultured in vitro with optimal concentrations of DNP Fla, DNP FγG, and DRC for 3 or 4 days. There was absolutely no response to DRC or to DNP FγG, but the same TDC responded well to DNP Fla (Table III). The addition of a small number of an enriched population of macrophages, depleted of T cells by treatment with AKR anti-θ-C3H
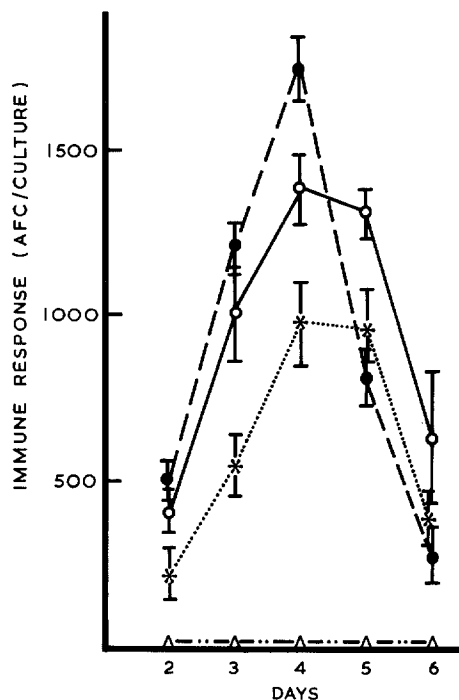

FIG. 1. Kinetics of the anti-DNP response of DNP FγG–primed lymphocytes or spleen cells to 1 μg/ml of DNP FγG or 0.1 μg/ml of DNP POL in vitro. 10⁵ macrophages were used to restore some lymphocyte cultures.

(●), lymphocyte (LC)–DNP POL
(✳), LC and macrophages ⎫
(△), LC   ⎬ DNP FγG
(○), spleen   ⎭

serum and complement, restored the capacity of TDC to respond to both DRC and DNP FγG in vitro (Table III).

Restoration of Collaborative Anti-DNP Responses of Lymphocytes with Peritoneal Exudate Cells.—To check that macrophages were the missing component anti-θ-treated peritoneal exudate cells (>85% macrophages) were cultured with column filtrate cells and DNP FγG. They restored the anti-DNP (and anti-DRC) response of purified lymphocytes from DNP FγG–primed spleen

(Fig 2). This experiment demonstrates that the incapacity of purified lymphocytes to respond is due to a lack of macrophages.

Helper Activity of Column-Purified Lymphocytes.—Column-purified lymphocytes respond well to DNP Fla, which can immunize in the absence of T cells

TABLE II

Suppression of Collaborative Anti-DNP Response by Anti-Macrophage Serum

Serum	Antigen	Immune response (AFC/culture ± SE)	
		DNP	DRC
—	DNP KLH	2640 ± 445	840 ± 0
—	DNP Fla	1600 ± 380	1420 ± 240
10% NRS	DNP KLH	3040 ± 425	1080 ± 155
10% NRS	DNP Fla	1660 ± 310	1040 ± 80
10% AMS(A)	DNP KLH	120 ± 22	180 ± 85
10% AMS (A)	DNP Fla	1230 ± 120	160 ± 45
10% AMS (B)	DNP KLH	135 ± 45	105 ± 75
10% AMS (B)	DNP Fla	1010 ± 210	165 ± 58

Two different batches of AMS were used, A or B. 1 μg/ml of $DNP_{800}KLH$ or 0.1 μg/ml of $DNP_{1.5}Fla$ were used. Each value represents the arithmetic mean of four cultures ± the standard error of the mean. Similar results were obtained in two other experiments. In the absence of added antigen, there was no background response to DNP, but 80 ± 40 AFC/culture to DRC.

TABLE III

Immune Response of Thoracic Duct Cells (TDC) In Vitro

Cells*	Antigen	Immune response (AFC/culture ± SE)	
		DNP	DRC
TDC	DNP Fla	860 ± 140‡	0
TDC	DNP Fla	525 ± 113	0
TDC	DNP FγG	0	0
TDC + 5 × 10^4 macrophages§	DNP FγG	355 ± 40	85 ± 30
TDC + 1.5 × 10^5 macrophages	DNP FγG	1230 ± 420	257 ± 32
TDC + 5 × 10^5 macrophages	DNP FγG	885 ± 253	115 ± 24

* TDC were from mice that had been primed in vivo with two injections of DNP FγG.

‡ Response at 3 days. All other responses were measured at 4 days. Each value represents the arithmetic mean of four cultures ± the standard error of the mean. Similar results were obtained in another experiment.

§ AKR anti-θ-C3H and complement-treated peritoneal exudate cells. 0.1 μg of $DNP_{1.5}$-Fla or 1 μg/ml of $DNP_{12}F\gamma$G was used.

(15, 17), but not to thymus-dependent DNP FγG or DNP MON (Table I). It was thus conceivable that helper cells may have been selectively retained by the column purification procedure, which traps about 50% of lymphocytes (18). Thus the helper cell content of FγG-primed spleen cells or purified lympho-

cytes was compared. Graded numbers of these cells were added to 10^7 DNP Fla–primed spleen cells, and the response to DNP FγG was determined after 4 days in vitro. Table IV indicates that the FγG-primed purified lymphocytes enhanced the anti-DNP response to DNP FγG. Titrating the number of FγG-re-
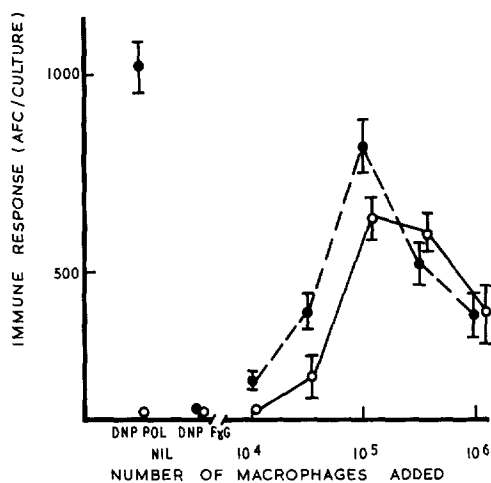

FIG. 2. Restoration of the collaborative responses of DNP FγG–primed lymphocytes by macrophages. The period of culture was 4 days. (●), DNP; (○), DRC.

TABLE IV

Helper Activity of Column-Purified Lymphocytes

Helper cells*	DNP-primed spleen‡	Immune response (AFC/culture ± SE)	
		DNP	DRC
—	+	20 ± 10	1210 ± 280
2 × 10^7 FγG spleen	—	480 ± 20	1620 ± 10
2 × 10^7 FγG lymphocytes	—	0	0
10^7 FγG spleen	+	1620 ± 160	990 ± 210
3 × 10^6 "	+	1210 ± 145	1080 ± 145
10^6 "	+	410 ± 180	1170 ± 190
3 × 10^5 "	+	40 ± 25	970 ± 80
10^7 FγG lymphocytes	+	1380 ± 260	1620 ± 245
3 × 10^6 "	+	1040 ± 170	1090 ± 170
10^6 "	+	490 ± 165	1210 ± 240
3 × 10^5 "	+	0	970 ± 155

* Helper cells were obtained from the spleen of mice injected twice with 100 μg of FγG emulsified in Freund's complete adjuvant.

‡ 10^7 DNP-primed cells, from the spleen of mice injected with 25 μg of DNP_2Fla. Each value represents the arithmetic mean of four cultures ± the standard error of the mean. Cultures were immunized with 1 μg of $DNP_{12}FγG$ and 3 × 10^6 DRC.

Similar results were obtained in two other experiments.

active cells indicated that the degree of helper activity of lymphocytes was not less than that of spleen cells (Table IV). Since FγG-reactive helper cells, and DNP-reactive B cells, were present in functionally normal numbers in purified lymphocyte suspensions, the essential cellular component lacking for the anti-DNP responses to DNP FγG was macrophages.

DISCUSSION

Since the antibody response to SRC requires the participation of both T cells and macrophages, the concept of a three-cell interaction has arisen (27, 28). However, because the response of primed or normal mouse spleen cells in vitro to POL or fragmented SRC (7, 20, 21) did not require the participation of macrophages, it was suggested that one function of these cells is to reduce antigen particles to a more immunogenic size. This investigation was aimed at more precisely defining the role of macrophages in immune induction. It was found that the generation of antibody responses involving T and B cell interaction also requires the participation of macrophages. This observation restricts the possible mechanisms of T-B lymphocyte cooperation.

Purified populations of primed lymphocytes, obtained by three different techniques, namely adherence to glass beads, antimacrophage serum, or by thoracic duct cannulation, did not respond to the priming antigen (e.g. DNP FγG) after 2–6 days in vitro (Tables I, II, III; Fig. 1). In contrast, spleen cells from these primed mice responded well in culture. Since the major difference in the cellular composition of responding and nonresponding cell populations was the presence of macrophages in the former, it was likely that these cells were essential for the generation of antibody responses to thymus-dependent antigens in vitro. This proposition was established since DNP-reactive B cells, as judged by the normal response of lymphocytes to DNP Fla, were present (Tables I, II, III); the helper cell content of FγG-primed lymphocytes was the same as that of spleen (Table IV). Furthermore, small numbers of a purified macrophage population (>85%) markedly enhanced the anti-DNP responses of lymphocytes to thymus-dependent antigens (Table III; Figs. 1 and 2). Since DNP Fla, which is macrophage independent (12), is a much larger antigenic molecule than DNP FγG, DNP MON, or DNP KLH (15), the role of macrophages in these responses cannot be simply to reduce the size of the antigen. Rather, macrophages must be involved in T-B cell collaboration.

The mechanism of interaction between T and B cells is not known. Mitchison et al. (29) proposed that T cells pick up antigen by its carrier determinants and "focus" the inducing determinant on the same molecule onto receptors on B cells. Antigen thus bridges receptors on T and B cells. This hypothesis is rendered unlikely as it does not involve macrophages. It has recently been demonstrated that B cells (but not T cells) bind to macrophages (30) and that division of lymphocytes and AFC are generated in proximity to macrophages both in vivo (8, 11) and in vitro (9, 10). These results, taken together with those here,

indicate that the effective site of T-B collaboration, i.e. of B cell immunization, is at the surface of phagocytes. Recently, Feldmann and Basten (31) found that T cell helper activity, even of activated thymus cells which need no further cell division, was abolished by inhibitors of RNA and protein synthesis. These findings suggested that T cells may elaborate a "factor" which immunizes B cells. Evidence for the existence of such a factor was found in vitro, since T and B cell populations separated from each other by a cell-impermeable nuclepore membrane cooperated as efficiently as when they were mixed in the same culture (32). The product which diffuses through the nuclepore membrane exhibited antigenic specificity since it was only formed in appreciable amounts by activated thymus cells of the correct antigenic specificity, and only enhanced the response of B cells reactive to determinants linked to the specificities recognized by T cells (32).

These results are thus consistent with the hypothesis that the specific component of cell cooperation is a soluble factor, possibly a T cell receptor ("IgX") complexed with antigen (33), a concept similar to that of "carrier antibody" (34). This complex of IgX and antigen could bind to the surface of macrophages to form a lattice of repeating antigenic determinants with a surface akin to that of DNP POL (or DNP Fla) which immunizes B cells without the participation of either T cells or macrophages. This concept explains the linked requirement of T cells and macrophages, the heightened immunogenicity of antigen on the surface of macrophages (35), and implies a common mechanism of B cell activation with thymus-dependent and thymus-independent antigens.

SUMMARY

The requirement for macrophages in thymus-dependent antibody responses was studied in vitro. Three different macrophage-deficient cell populations were studied: spleen cells passed through a glass bead column at 37°C, spleen cells cultured with specific antimacrophage serum, and thoracic duct lymphocytes. These cell populations from mice primed to dinitrophenylated (DNP) fowl gamma globulin were unable to respond to the homologous conjugate in vitro. DNP-reactive B cells were present in normal proportions, since all three macrophage-depleted populations responded normally to macrophage-independent and thymus-independent DNP flagella. Carrier-reactive T cells were present, as the helper capacity of carrier-primed spleen cells was the same as carrier-primed lymphocytes, and thoracic duct lymphocytes are a well-established source of helper cells. The inhibition of the cooperative response was thus due to removal of macrophages, and this was proven by restoration of thymus-dependent anti-DNP responses by small numbers of anti-θ-treated peritoneal exudate cells. These results suggest that macrophages are essential in cell collaboration. While their exact function in cell collaboration is not yet known, the above observation suggests that the mechanism of T-B collaboration involves the surface of macrophages.

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