CELL INTERACTIONS IN THE IMMUNE RESPONSE IN VITRO

II. THE REQUIREMENT FOR MACROPHAGES IN LYMPHOCYTE COLLABORATION*

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Collaboration between thymus-derived (T) 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 radiiodinated 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 adelaidae (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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Abbreviations used in this paper: AFC, antibody-forming cells; AMS, antimouse macrophage serum; B, nonthymus-derived; DNP, dinitrophenyl determinant; DRC, donkey red cells; FyG, fowl gamma globulin; Flk, 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 adelaidae*, 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 low gamma globulin (DNP F\(\gamma\)G), and DNP keyhole limpet hemocyanin (DNP KLH) 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 \(\mu\)g 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).

**Anti-macrophage 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-\(\theta\)-C3H Serum.**—Peritoneal exudate cells were treated with AKR anti-\(\theta\)-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 F\(\gamma\)G, 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 F\(\gamma\)G 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-reactive 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).

Table I

| Cells                      | Antigen* | Immune response (AFC/culture ± SE) |
|----------------------------|----------|-------------------------------------|
| 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                          |
|                            | DNP FγG  | 2520 ± 502                          |
| DNP FγG-primed lymphocytes‡ | DNP Fla  | 1620 ± 187                          |
|                            | DNP FγG  | 8 ± 5                               |
| DNP MON-primed spleen cells | DNP Fla  | 1197 ± 285                          |
|                            | DNP MON  | 592 ± 227                           |
| DNP MON-primed lymphocytes‡ | DNP Fla  | 1050 ± 283                          |
|                            | DNP MON  | 0 ± 16                              |

* Antigen concentrations used 0.1 μg/ml of DNP₁₂Fla, 1 μg/ml of DNP₁₂MON or DNP₁₂FγG. 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

![Figure 1](image_url)

**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
(O), spleen

DNP FγG

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
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.

| Serum          | Antigen | Immune response (AFC/culture ± SE) |
|----------------|---------|-----------------------------------|
|                |         | DNP DRC                           |
|                | DNP Fla | 2640 ± 445                        |
| 10% NRS        | DNP KLH | 1600 ± 380                        |
| 10% NRS        | DNP Fla | 2040 ± 425                        |
| 10% AMS(A)     | DNP KLH | 1040 ± 310                        |
| 10% AMS (A)    | DNP Fla | 120 ± 22                          |
| 10% AMS (B)    | DNP KLH | 135 ± 45                          |
| 10% AMS (B)    | DNP Fla | 1010 ± 210                        |

Two different batches of AMS were used, A or B. 1 μg/ml of DNP FLA or 0.1 μg/ml of DNP 1,4-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.

| Cells*            | Antigen | Immune response (AFC/culture ± SE) |
|-------------------|---------|-----------------------------------|
|                   |         | DNP DRC                           |
| TDC               | DNP Fla | 860 ± 140‡                        |
| TDC               | DNP Fla | 525 ± 113                         |
| TDC               | DNP Flg | 0                                 |
| TDC + 5 × 10⁴ macrophages§ | DNP Flg | 355 ± 40                         |
| TDC + 1.5 × 10⁵ macrophages | DNP Flg | 1230 ± 420                       |
| TDC + 5 × 10⁵ macrophages | DNP Flg | 885 ± 253                        |

* TDC were from mice that had been primed in vivo with two injections of DNP Flg.
‡ 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 φ-C3H and complement-treated peritoneal exudate cells. 0.1 μg of DNP 1,4-Fla or 1 μg/ml of DNP 12-Fyg was used.

(15, 17), but not to thymus-dependent DNP Flg 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 Flg-primed spleen cells or purified lympho-
cytes was compared. Graded numbers of these cells were added to $10^7$ DNP Flα-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 cells* | DNP-primed spleen | Immune response (AFC/culture ± SE) |
|---------------|-------------------|------------------------------------|
|               |                   | DNP                                |
| —             | ±                 | 1210 ± 280                         |
| $2 \times 10^7$ FγG spleen | — | 20 ± 10 |
| $2 \times 10^7$ FγG lymphocytes | — | 0 |
| $10^7$ FγG spleen | ± | 1620 ± 160 |
| $3 \times 10^6$ “ | ± | 1210 ± 145 |
| $10^6$ “ | ± | 410 ± 180 |
| $3 \times 10^5$ “ | ± | 40 ± 25 |
| $10^7$ FγG lymphocytes | ± | 1380 ± 260 |
| $3 \times 10^6$ “ | ± | 1040 ± 170 |
| $10^6$ “ | ± | 490 ± 165 |
| $3 \times 10^4$ “ | ± | 0 |

* 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 DNPαFlα. Each value represents the arithmetic mean of four cultures ± the standard error of the mean. Cultures were immunized with 1 μg of DNPαFγG and $3 \times 10^6$ 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 FyG-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 FyG 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 FyG) 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 FyG-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 FyG, 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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