CELL INTERACTIONS IN THE IMMUNE RESPONSE IN VITRO

V. Specific Collaboration via Complexes of Antigen and Thymus-Derived Cell Immunoglobulin*

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Cell interaction between thymus-derived (T)[1] and nonthymus-derived (B) lymphocytes occurs in the antibody response to many antigens, such as heterologous erythrocytes (1–3) and hapten-protein conjugates (4). Recently, it was demonstrated that cell collaboration in the response to erythrocyte and hapten-protein antigens occurs efficiently with T and B lymphocytes cultured in a double-chamber system, separated by a cell impermeable nucleopore membrane (5–7). Thus direct contact of T and B lymphocytes was not essential, and cooperation was mediated by a subcellular component or components. This form of cooperation was markedly antigen specific. Only T cells which had been specifically activated to the carrier antigen used in vitro augmented antibody production (5, 6). This degree of antigen specificity of cooperation in vitro was the same as found in previous in vivo studies, such as those of Mitchison (4) and others (8, 9). The experiments to be described below provide some clues as to the nature of the specific cooperative factor and its mode of action.

Materials and Methods

Animals.—Inbred CBA/H/Wehi mice were used for most experiments. For some studies congenitally athymic "nude" (nu/nu) mice provided a source of lymphoid cells uncontami-

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; ATC, activated thymus cells; B lymphocytes, nonthymus-derived lymphocytes; CFA, Freund's complete adjuvant; DNP, dinitrophenyl; DRC donkey red cells; FCS, fetal calf serum; FγG, fowl gamma globulin; Fla, flagella; KLH, keyhole limpet hemocyanin; MIF, migration-inhibitory factor; PEC, peritoneal exudate cells; POL, polymeric flagellin; SF, antigen-specific factor; SRC, sheep red cells; T-TDL, thoracic duct lymphocytes for irradiated mice injected with thymocytes.

nated by T cells (10). These were kindly provided by Dr. M. C. Holmes, and their breeding and maintenance was described previously (11).

Antigens.—Heterologous erythrocytes, sheep red cells (SRC) or donkey red cells (DRC), were collected and stored as detailed elsewhere (12). The dinitrophenyl (DNP) determinant was conjugated to flagella (Fla), polymeric flagellin (POL), keyhole limpet hemocyanin (KLH), or fowl gamma globulin (FγG), as described elsewhere (12). Conjugation ratios used were $DNP_{1.3}POL$, $DNP_{1.7}Fla$, $DNP_{800}KLH$ (per 8×10^6 mol wt), and $DNP_{8-12}F\gamma G$. Unless otherwise specified, POL or Fla refers to antigens derived from the *Salmonella adelaide* strain (1338). POL_{871} is derived from *Salmonella typhimurium*.

Immunization.—Some mice were primed with 25 μg of dinitrophenylated flagella of *Salmonella adelaide* 1-3 months before use in culture.

Activation of Thymus Cells.—This was performed as described previously (13). Basically, lethally irradiated mice were injected with thymocytes and with antigen. 6-7 days later their spleens were used as a source of activated (or educated) thymus cells (ATC).

Tissue Culture.—Mouse spleen cells were cultured in a modified Marbrook-Diener culture system, as described recently (13). Eagle's minimal essential medium with supplementary nonessential amino acids was obtained from Grand Island Biological Company, Grand Island, N. Y. This was supplemented with 5% fetal calf serum (FCS; BioCult Ltd., Sydney, Australia), 100 μg/ml streptomycin, and 100 units/ml penicillin G, and was buffered with sodium bicarbonate. Cultures were placed in a humidified incubator at 37°C in an atmosphere of 10% CO_2 in air. Culture chambers consisting of two distinct compartments were constructed using a pair of concentric glass tubes, one of which fitted inside the other, the whole system being inserted into a 125 ml Erlenmeyer flask, as described elsewhere (6). The bottom end of the inner cylinder was usually sealed off by a nucleopore membrane of 0.2 μ pore size (General Electric Company, Schenectady, N. Y.), thereby forming the upper compartment. The lower compartment was always sealed off with a dialysis membrane. ATC were placed in the upper compartment and various cell populations in the lower chamber. Control cultures were set up using dialysis membrane closed tubes of the same diameter as the outer tube of the double-chamber cultures. Unless otherwise specified 1 μg/ml DNP KLH was used as antigen.

Treatment with Isoantisera.—AKR anti-θC3H serum was prepared by the method of Reif and Allen (14). It was assayed and used as described previously (13).

Enumeration of Antibody-Forming Cells (AFC).—AFC were detected by Cunningham and Szenberg's modification (15) of the hemolytic plaque assay as outlined previously (13). AFC to POL strain 871 (POL_{871}) were detected by coating cells with POL_{871} using $CrCl_3$ as a coupling agent, as described by R. Langman (personal communication).

Macrophage Depletion.—The active adherence column technique of Shortman et al. (16) was used to obtain macrophage-depleted suspensions of DNP Fla-primed spleen cells, as described in detail in reference 16.

Macrophages.—Peritoneal exudate cells were obtained from 6-8-month old CBA mice injected 4 days previously with 1 ml of proteose peptone broth (Difco Laboratories, Inc., Detroit, Mich.). In some instances these peritoneal exudate suspensions (containing about 60% macrophages) were depleted of their T cell content by treatment with anti-θ serum and complement, as described previously (13). Peritoneal exudate cells were sometimes also depleted of their content of nonadherent cells by passage through a glass bead column. The adherent cells were eluted with Rabinowitz' solution containing ethylenediaminetetraacetate and by mechanical agitation of the beads (16).

Trypsinization of Macrophages.—Macrophages were washed three times in serum-free HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid; Calbiochem, Sydney, Australia)-buffered Eagle's medium and trypsinized in that medium (protein free) at concentrations of 100 μg of crystalline trypsin (Calbiochem) per ml, 10^6 macrophages/ml for 10 min

at 37°C. The reaction was stopped by the addition of 2 ml of FCS, and the cells were sedimented twice through FCS.

Antisera.—An outbred Hall Institute mouse antiserum to DNP FγG was produced by injecting adult animals three times with 100 μg of DNP FγG emulsified in Freund's complete adjuvant (CFA; Difco Laboratories, Inc.). This had an agglutination titer of 5120, using DRC coated with DNP human gamma globulin. A rabbit anti-KLH antiserum was raised by two injections of 1 mg of KLH emulsified in CFA. This had a titer of 6400, using SRC coated with KLH using chromic chloride.

A polyvalent rabbit anti-mouse immunoglobulin antiserum was generously donated by Dr. P. J. Russell. Rabbit anti-mouse κ-chain antiserum (R84) and specific rabbit anti-mouse μ-chain antiserum (R19AC) were generously prepared and donated by Dr. N. L. Warner. The anti-κ-chain antiserum was made specific by absorption with myeloma proteins bound to polyaminostyrene beads. All three anti-immunoglobulin sera were absorbed twice with mouse thymocytes at a cell to serum ratio of 1 to 10.

Purification of IgM.—NZB serum was fractionated by salt precipitation (45% saturated ammonium sulfate) and Sephadex G-200 chromatography (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) to field IgM. Its purity was checked by immunoelectrophoresis.

RESULTS

Locus of Action of the Collaborative Factor from Activated Thymus Cells.—The use of cultures with two chambers separated by a cell impermeable nucleopore membrane has permitted the detection of a subcellular mediatior of cell cooperation. Thymus cells activated by KLH (ATC_{KLH}) produced a factor which enhanced the anti-DNP response to DNP KLH of spleen cells from DNP Fla-primed mice in vitro (6). The augmentation of the response did not depend on the presence of T cells in the lower culture chamber (6). Thus the factor acted on B cells either directly, or indirectly through the agency of other cells, such as macrophages. The capacity of the subcellular factor from ATC_{KLH} to induce a response to DNP KLH in vitro in lymphoid cells depleted of adherent cells was thus investigated. The effectiveness of macrophage depletion was monitored by measuring the response of the lower compartment to a macrophage-dependent antigen, DRC. The results shown in Table I demonstrate that the collaborative anti-DNP response to DNP KLH was virtually abolished by adherent cell depletion but was restored by the addition of small numbers of peritoneal exudate cells, suggesting that the collaborating factor acted through the agency of macrophages.

Capacity of Peritoneal Exudate Cells to Transfer the Factor Capable of Immunizing B Cells.—Because the collaborative factor acted through the agency of adherent cells (Table I), it was possible to perform the double-chamber culture experiments in an indirect manner, as described in Fig. 1. Double-chamber cultures were set up with ATC in the upper chamber separated from peritoneal exudate cells (PEC) by a nucleopore membrane. These were incubated with antigen for 1–3 days before the PEC were harvested, washed, and added in graded numbers to lymphoid cell populations containing B cells. The effect of adding such pretreated peritoneal exudate cells to cultures of DNP Fla-primed spleen cells is shown in Fig. 2. A good anti-DNP response occurs when

the optimal number, about 10^5 peritoneal cells, is used. No source of DNP antigen apart from the pretreated PEC was added to the cultures during the second period of culture. The specificity of the immunization by T cell and

TABLE I

Effect of Macrophage Depletion on the Capacity of ATC to Collaborate across a Nucleopore Membrane

Cells cultured		Antibody response (AFC/culture ± SE)	
Upper	Lower	DNP	DRC
NIL	DNP Fla-primed spleen	0	1290 ± 140
ATC$_{KLH}$	" " "	1260 ± 310	1450 ± 210
NIL	DNP Fla-primed LC*	0	35 ± 30
ATC$_{KLH}$	" " "	30 ± 30	25 ± 20
"	" " " ‡	650 ± 110	710 ± 180
	Peritoneal cells		

2×10^6 ATC$_{KLH}$ were cultured in the presence of 1 μg/ml DNP KLH in the upper compartment of double-chamber flasks which contained either:

* 40×10^6 DNP Fla-primed spleen cells or adherence column-purified spleen cells (LC)
‡ 4×10^5 peritoneal cells were added as a source of macrophages.
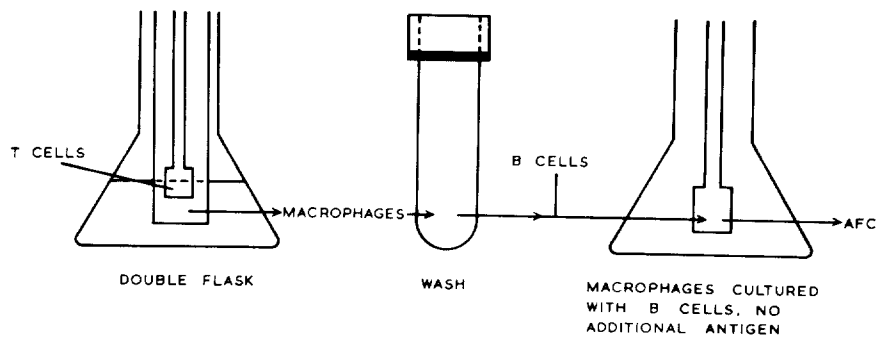
DOUBLE TRANSFER

FIG. 1. Macrophage transfer protocol for the generation of AFC in B cells using macrophages which had been cultured with ATC and antigen as the sole source of DNP antigen.

antigen-pretreated PEC was demonstrated by the following controls: (a) The response to another antigen (DRC) in the same flasks was not altered by the presence of cultured peritoneal cells. (b) Peritoneal cells cultured in the presence of DNP KLH and thymus cells activated to another antigen (fowl gamma globulin) or cultured in the absence of ATC did not induce a response to DNP. (c) Peritoneal cells cultured with ATC$_{KLH}$, but with unconjugated KLH, did

not induce a response to DNP. Some cultures of DNP Fla-primed spleen cells were immunized with 0.1 μg/ml DNP POL. The anti-DNP response generated by the optimal numbers of PEC was about 40% of that induced by the optimal concentration of DNP POL. Depending on the time of assay and the range of peritoneal macrophage numbers tested, PEC-induced responses ranged from 30 to 120% of that generated by DNP POL, with a mode of 40–50%.
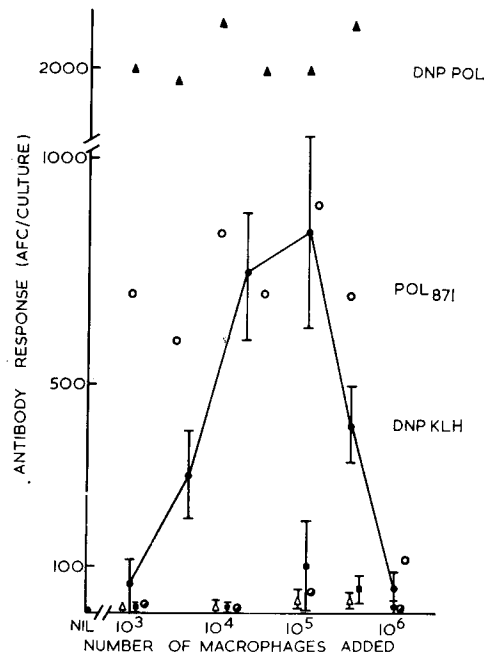

FIG. 2. Capacity of macrophages cultured with ATC_{KLH} and DNP KLH to induce anti-DNP responses in DNP Fla-primed spleen. ▲, DNP response to DNP POL in the presence of macrophages; ●, DNP response to macrophages cultured with ATC_{KLH} + DNP KLH; ■, DNP response to macrophages cultured with ATC_{KLH} + KLH; ◉, DNP response to macrophages cultured with $ATC_{F\gamma G}$ + DNP KLH; △, DNP response to macrophages cultured with DNP KLH alone; ○, POL anti-flagellin response to POL_{871}. Period of culture was 4 days after macrophage transfer.

Cell Type in Peritoneal Exudate Capable of Transferring the Mediator of Cell Collaboration.—Peritoneal exudate cell populations contain chiefly macrophages but also some T and B lymphocytes. To ensure that the essential cell involved in transferring the capacity to immunize B cells was a macrophage-like cell, peritoneal exudate cells initially containing an average of 60% macrophages, as ascertained by morphological criteria, were subjected to adherence column fractionation by the method of Shortman et al. (16). By this means a population of 90–95% macrophage-like cells was obtained. To ensure that the T cells

in the peritoneal exudate were not the active component, the effect of treatment with AKR anti-θC3H serum and complement was also investigated. Table II indicates that lymphocyte depletion either by column adherence or by treatment with anti-θ serum did not abrogate the capacity of peritoneal exudate cells preincubated with ATC and antigen to specifically augment immune responses. Thus adherent cells, presumably macrophages, were the cells involved in mediation of cell collaboration in vitro. The term "macrophage" will henceforth be used to describe the peritoneal exudate-derived cell active in cell collaboration.

Capacity of Macrophages Preincubated with Antigen and ATC to Immunize Purified Lymphocyte Suspensions or Spleen Cells from Nude Mice.—Spleen cell populations deprived of their macrophage content by either adherence to glass

TABLE II

Comparison of the Capacity of Treated Peritoneal Exudate Populations to Immunize DNP-Primed Spleen in a Transfer Culture System

Cells initially cultured		Anti-DNP response (AFC/culture ± SE)
Upper	Lower	
NIL	Peritoneal exudate cells	0
ATC$_{KLH}$	Peritoneal exudate cells	680 ± 110
ATC$_{KLH}$	Column-purified macrophages	760 ± 80
ATC$_{KLH}$	Anti-θ-treated peritoneal exudate	510 ± 105

As a first step 2×10^6 ATC$_{KLH}$ were cultured for 2 days in the upper chamber with 1 μg/ml DNP KLH and 2×10^6 of peritoneal exudate or derivatives in the lower chamber. The peritoneal cells were washed, and 10^5 cells cultured with 1.5×10^7 DNP Fla-primed spleen cells for 3 days in the absence of further antigen. Responses to DRC in the same cultures were normal.

beads at 37°C or specific anti-macrophage serum treatment, or thoracic duct lymphocytes do not respond in vitro to thymus-dependent antigens such as DNP FγG, but respond normally to thymus-independent antigens such as DNP POL (17). These observations implied the participation of macrophages in the process of T-B lymphocyte collaboration but did not indicate their exact role. The experiments described in the preceding sections suggest that macrophages bind the collaborative factor derived from T cells. If this is their major function in the response to DNP KLH, then macrophages after exposure to ATC$_{KLH}$ and the antigen, DNP KLH, should immunize purified lymphocytes (macrophage depleted) in vitro. The results in Fig. 3 indicate that primed column-purified lymphocytes may indeed be immunized by these macrophages as effectively as the spleen cell population from which they were obtained. It was of interest that the DRC response of the lymphocyte cultures was not augmented by macrophages which had been cultured with antigen and ATC.

In a similar manner, spleen cell populations, uncontaminated by T cells, obtained from unprimed or DNP Fla-primed nude mice were cultured with various

numbers of CBA peritoneal macrophages (anti-θ-treated peritoneal exudate cells). Because the antibody response of allogeneic cell mixtures cultured in vitro is sometimes enhanced (18, 19), equivalent numbers of CBA peritoneal macrophages, exposed to DNP KLH in the absence of ATC_{KLH} , were used as controls. Fig. 4 indicates that T-deprived nu/nu spleen cell populations from both primed and unprimed mice may be immunized by macrophages which had been incubated with ATC and antigen. It is of interest that there was no non-specific enhancement of the antibody response in cultures of T-depleted
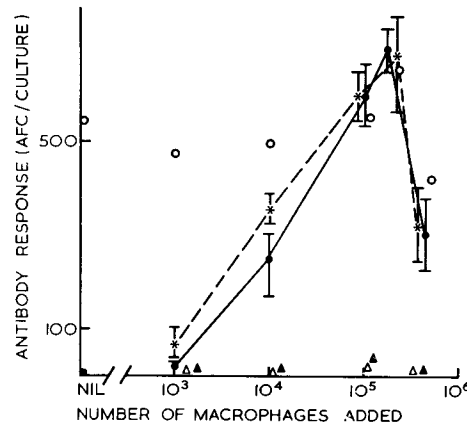

Fig. 3. Capacity of macrophages which had been cultured with ATC_{KLH} and DNP KLH to induce antibody responses in purified lymphocytes in a two-stage culture system. *, Response to DNP in cultures of spleen cells containing macrophages precultured with ATC_{KLH} and DNP KLH; ●, response to DNP in cultures of nonadherent lymphocytes containing macrophages precultured with ATC_{KLH} and DNP KLH; △, response to DNP in cultures of lymphocytes and untreated macrophages; ○, response to POL_{871} in cultures of lympho-cytes containing macrophages precultured with ATC_{KLH} and DNP KLH; ▲, response to DRC in cultures of lymphocytes with macrophages cultured with ATC_{KLH} and DNP KLH. Period of culture was 4 days after the macrophage transfer.

nu/nu spleen and T-depleted CBA macrophages. This was expected from previous studies which have demonstrated that augmentation of the response in allogeneic situations depends on the presence of T cells (7).

Kinetics of the Anti-DNP Response Induced by Various Immunogens.—The capacity of macrophages to immunize B cells in the absence of T cells suggested that these macrophages represent the final step of T cell interaction and that B cells are immunized at their surface. If this was indeed the case, the kinetics of the anti-DNP response of DNP Fla-primed spleen cells induced by macro-phages which had been cultured with DNP KLH and ATC_{KLH} should be different from the response induced by ATC_{KLH} and DNP KLH. For com-parison, the kinetics of the response to the thymus-independent antigen, DNP Fla, and the thymus-dependent DRC were compared. Markedly dif-

ferent kinetics were found (Fig. 5). The anti-DNP response to thymus-independent DNP Fla or macrophages as immunogens began a day earlier than the response to thymus-dependent DNP KLH or DRC. It was of interest that while the response in the presence of ATC_{KLH} mixed with the B cells began later, it eventually reached a level of twice the magnitude as that induced by the preincubated macrophages (Fig. 5).

Specificity of the Augmentation of the Response by Macrophages Exposed to Activated Thymus Cells and Antigen.—Column-purified macrophages were
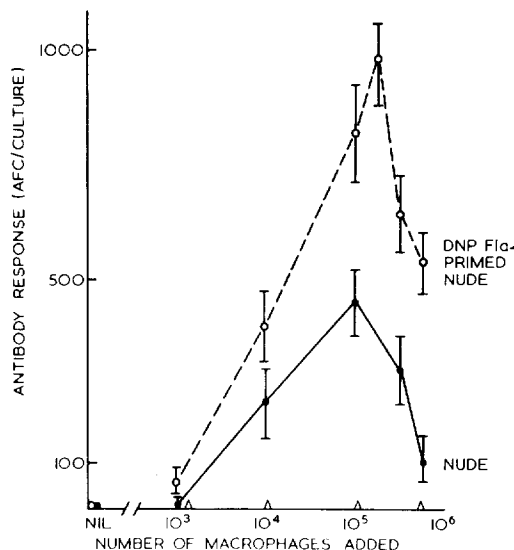

Fig. 4. Capacity of macrophages precultured with ATC_{KLH} and DNP KLH to immunize T-depleted (nu/nu) spleen cells. O, Anti-DNP response of DNP-primed spleen cells; ●, anti-DNP response of unprimed spleen cells; △, anti-DNP response of primed spleen cells with normal (untreated) macrophages. Period of culture was 4 days after the macrophage transfer.

cultured in the bottom compartment of double chambers together with ATC_{KLH} in the upper chamber and various combinations of KLH, DNP KLH, FγG, and DNP FγG for 2 days. Macrophages harvested from these cultures were washed and then cultured with DNP Fla-primed spleen cells for a further 3 days (Table III). An anti-DNP response occurred in the presence of DNP linked to KLH, but not with these determinants on separate molecules as KLH (1 μg/ml) and 1–100 μg/ml of DNP FγG. Unconjugated KLH inhibited the response to DNP KLH confirming results obtained by Mitchison in cell transfer experiments (20). It was of interest that in this particular competitive system small concentrations of the unconjugated carrier protein (KLH) inhibited the response, whereas large amounts of unconjugated protein were needed if carrier-primed spleen cells were used either in vitro (21) or in vivo (20).

Locus of Binding of Collaborative Factor to Macrophages.—Because macrophages could take up the mediatior of cell cooperation (Fig. 2), it was of interest to determine whether this material was membrane bound or entered these cells. Trypsinization of macrophages which had been cultured with ATC_{KLH} and DNP KLH was performed as described in Materials and Methods. These tryp-
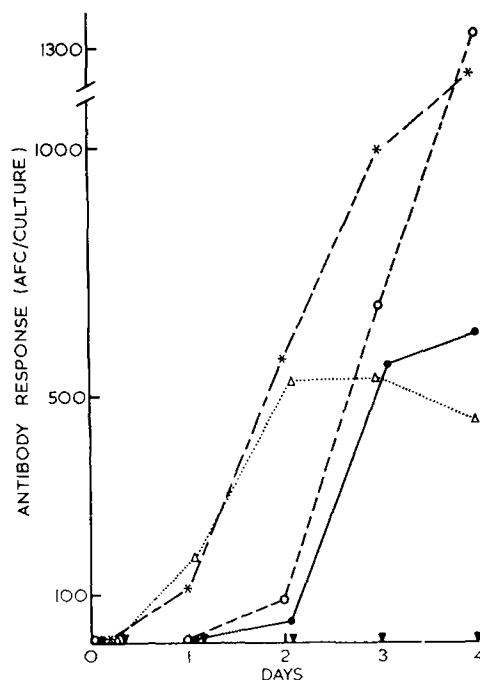

FIG. 5. Kinetics of the antibody response in vitro of DNP Fla-primed spleen cells. *, Anti-DNP response to DNP Fla; △, anti-DNP response to DNP KLH and ATC_{KLH}-cultured macrophages; ○, anti-DNP response to ATC_{KLH} and DNP KLH in the same culture as DNP Fla-primed spleen cells; ▼, anti-DNP response to DNP KLH; ●, anti-POL response to POL_{871}. The kinetics of the DRC response were virtually the same as those of ATC_{KLH} and DNP KLH and for simplicity were omitted.

sinized macrophages were still functional since they could restore responses of purified lymphocytes to erythrocyte antigens (unpublished data). The capacity of a pool of macrophages, which had been exposed to ATC_{KLH} and DNP KLH, to immunize DNP Fla-primed spleen cells before and after trypsinization is compared in Fig. 6. Trypsinization virtually abolished the capacity of these cells to induce anti-DNP responses. Thus a factor mediating immunization was present on the surface of macrophages.

Presence of Antigen on the Surface of "Collaborating" Macrophages.—Because the immunizing material was present in the surface of macrophages, its nature

could be investigated by the use of antisera. The presence of DNP deter-
minants on the surface of macrophage was ascertained by using a mouse
antiserum to DNP FγG. Various amounts of this antiserum were added to
cultures of 15 × 10⁶ DNP Fla-primed spleen cells and 10⁵ macrophages (anti-
θ-treated peritoneal exudate cells) which had been cultured in double-chamber
flasks with DNP KLH and ATC$_{KLH}$. Small concentrations of this anti-DNP
antiserum inhibited the anti-DNP response to either macrophages or to DNP
POL in parallel cultures. The specificity of the antibody-induced suppression
was indicated by the normal response of the same cultures to DRC (Fig. 7).

TABLE III

Immunogenicity of Macrophages Cultured with ATC_{KLH} and Various Antigens

Cells initially cultured		Anti-DNP response (AFC/culture ± SE)
Upper	Lower	
ATC$_{KLH}$ + KLH	Macrophages	90 ± 40
ATC$_{KLH}$ + DNP KLH	Macrophages	810 ± 160
ATC$_{KLH}$ + DNP KLH + 1 μg/ml KLH	Macrophages	150 ± 40
ATC$_{KLH}$ + DNP KLH + 10 μg/ml KLH	Macrophages	83 ± 29
ATC$_{KLH}$ + DNP KLH + 100 μg/ml KLH	Macrophages	0
ATC$_{KLH}$ + KLH + 1 μg/ml DNP FγG	Macrophages	47 ± 25
ATC$_{KLH}$ + KLH + 10 μg/ml DNP FγG	Macrophages	90 ± 40
ATC$_{KLH}$ + KLH + 100 μg/ml DNP FγG	Macrophages	55 ± 20
ATC$_{FγG}$ + 1 μg/ml DNP FγG	Macrophages	365 ± 120

10⁵ column-purified peritoneal macrophages harvested from the above cultures were
again cultured for 4 days together with 1.5 × 10⁷ DNP Fla-primed spleen cells. Only macro-
phages which had been exposed to ATC$_{KLH}$ and DNP physically linked to KLH (on the
same molecule) or ATC$_{FγG}$ and DNP FγG were immunogenic. Responses to DRC in the
same cultures were always normal. 1 μg/ml of KLH or DNP KLH were always used. 3 × 10⁶
ATC were used per culture.

Experiments of a similar design were performed using a rabbit anti-KLH
antiserum. This antiserum, even in low concentrations, inhibited the anti-DNP
response to macrophages which had been cultured with ATC and antigen. In
contrast, the anti-KLH antiserum did not suppress the response to either DRC
present in the same cultures or to DNP Fla present in parallel cultures of DNP-
primed spleen cells (Fig. 8). The latter observations indicated that the rabbit
anti-KLH antiserum did not have suppressive natural anti-DNP activity (22),
and established that KLH determinants were present on the surface of the
macrophages which had been cultured with DNP KLH and ATC$_{KLH}$. The
simplest explanation for the presence of both KLH and DNP determinants
on the surface of these macrophages was that they were coated with the whole
antigen molecule, DNP KLH.

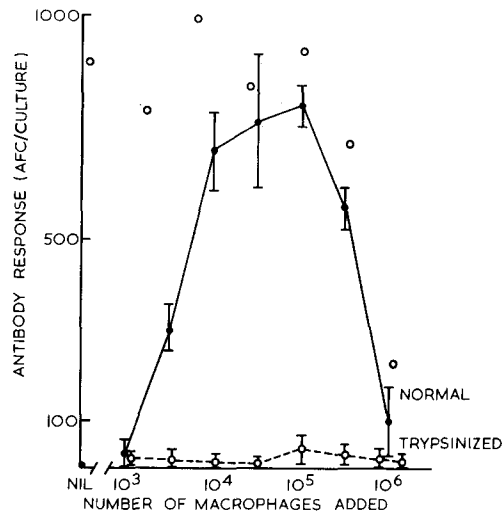*Presence of Immunoglobulin Determinants in the Collaborating Factor.*—Be-

Fig. 6. Abolition by trypsinization of the response to DNP induced by macrophages which had been cultured with ATC_{KLH} and DNP KLH. ●, DNP response to macrophages before trypsinization; ○, DNP response to macrophages after trypsinization. Open circles not joined by line represent the response to POL_{871} in cultures containing trypsinized macrophages. Period of culture was 4 days after the macrophage transfer.
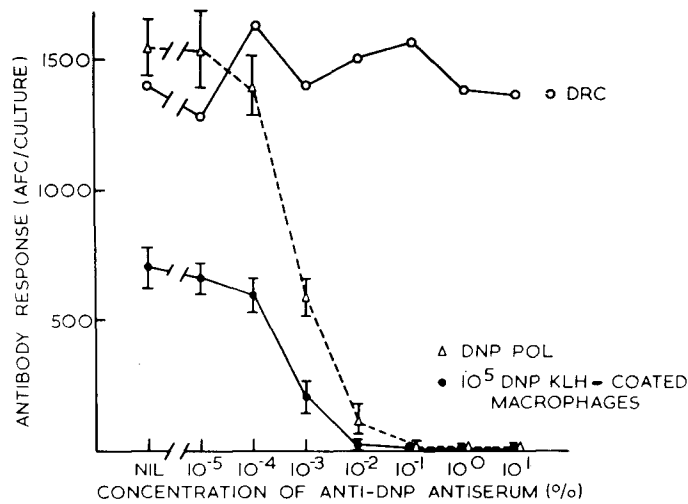

Fig. 7. Suppression by anti-DNP serum of the anti-DNP response to macrophages pretreated with ATC_{KLH} and DNP KLH. ●, Anti-DNP response to pretreated macrophages in presence of antiserum; ○, anti-DRC response to pretreated macrophages in presence of antiserum; △, anti-DNP response to DNP POL in presence of antiserum. Period of culture was 4 days after the macrophage transfer.

cause macrophages cultured with DNP KLH at either high or low concentrations, but without T cells (Fig. 2; and unpublished data), cannot immunize DNP Fla-primed spleen cells against DNP in vitro, ATC must contribute a component to the mediator of cell collaboration which binds to macrophages. The antigen specificity of the collaborating factor suggested that it might be immunoglobulin in nature. To investigate this possibility ATC_{KLH} were incubated at 4°C with 1 μg/ml DNP KLH for 1–2 hr, washed, and then cultured in the upper chamber of double flasks with macrophages (column-
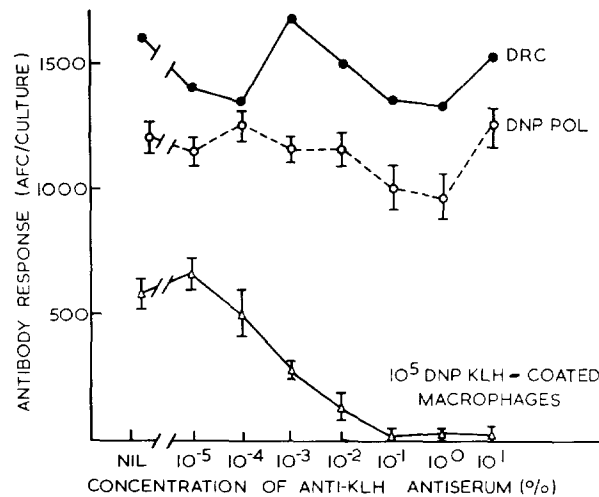

Fig. 8. Suppression by anti-KLH serum of the anti-DNP response to macrophages pretreated with ATC_{KLH} and DNP KLH. △, Anti-DNP response to pretreated macrophages in presence of antiserum; ●, anti-DRC response to macrophages in presence of antiserum; ○, anti-DNP response to DNP POL in presence of antiserum. Period of culture was 4 days after the macrophage transfer.

purified or anti-θ-treated peritoneal exudate cells) in the lower chamber. Preliminary experiments had indicated that the eventual anti-DNP responses induced on DNP Fla-primed spleen cells by macrophages were similar in magnitude with preincubated T cells as when 1 μg/ml DNP KLH was present for the entire period of culture in double flasks. This modification of the experimental design with preincubation of ATC with antigen ensured that the antigen, DNP KLH, could bind to ATC receptors. Thus a rabbit anti-mouse immunoglobulin serum (anti-Ig) added to macrophages in double-chamber cultures did not inhibit the generation of the anti-DNP response by merely blocking the binding of DNP KLH to T cell receptors. Shown in Fig. 9 is the effect of a polyvalent anti-Ig serum on the capacity of the collaborating factor to express itself on macrophages. Even very small concentrations of the anti-Ig antiserum inhibited completely the anti-DNP response which could be generated by 10^5 of these macrophages, as compared with
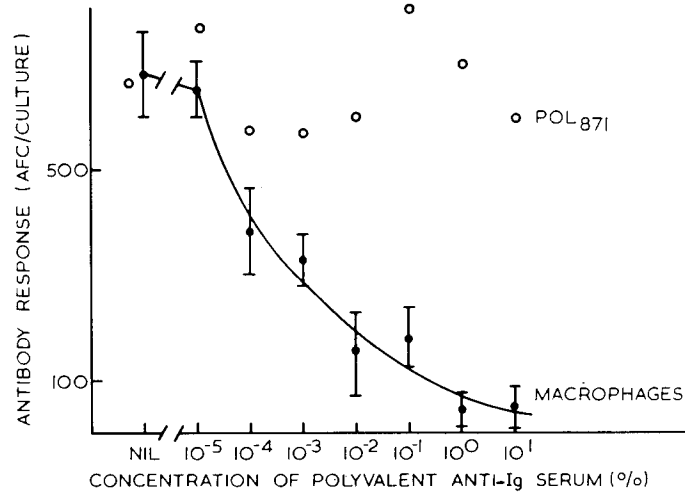
FIG. 9. Capacity of polyvalent anti-Ig serum to inhibit the response to macrophages which had been cultured with ATC_{KLH} and DNP KLH. ●, Anti-DNP response to 10^5 macrophages exposed to various concentrations of anti-Ig serum; O, anti-POL response to POL_{871} in the same cultures. Period of culture was 4 days after the macrophage transfer.
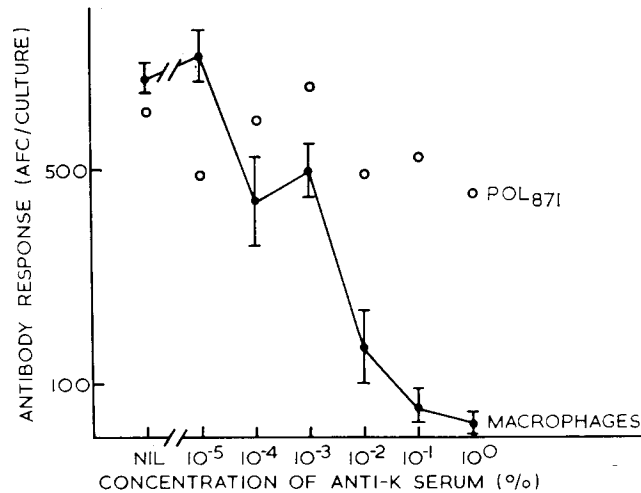

FIG. 10. Capacity of anti-κ chain antiserum to inhibit the response to macrophages which had been cultured with ATC_{KLH} and DNP KLH. ●, Anti-DNP response to 10^5 macrophages exposed to various concentrations of anti-κ chain serum; O, anti-POL response to POL_{871} in the same culture. Period of culture was 4 days after the macrophage transfer.

the response induced by 10^5 macrophages cultured in the presence of 1 % pooled normal rabbit serum, DNP KLH and ATC_{KLH}. The inhibitory effect of the anti-Ig serum was due to its action in the initial culture period, i.e. on macrophage binding and not directly on B cells, because the response to a control

antigen (POL₈₇₁) of DNP Fla-primed spleen cell cultures containing macrophages which had been exposed to anti-Ig serum was normal (Fig. 9).

Experiments of a similar design were performed using a rabbit anti-mouse κ-chain antiserum (anti-κ) during the initial culture period. Fig. 10 illustrates
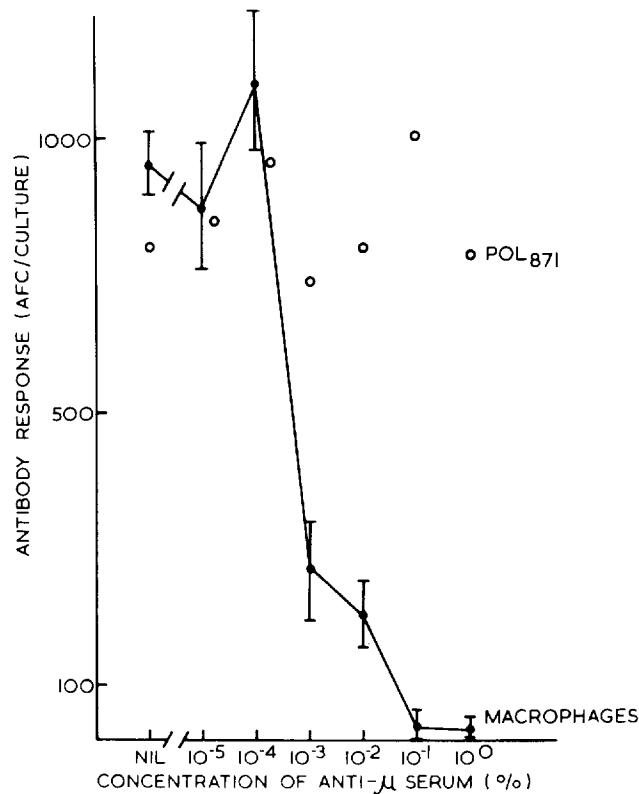

Fig. 11. Capacity of specific anti-μ chain antiserum to inhibit the response to macrophages which had been cultured with ATC_{KLH} and DNP KLH. ●, Anti-DNP response to 10⁵ macrophages exposed to various concentrations of anti-μ chain antiserum; O, anti-POL response to POL₈₇₁ in the same cultures. Period of culture was 4 days after the macrophage transfer.

the capacity of this antiserum to block the binding of the collaborative factor to macrophages as evidenced by the failure of 10⁵ macrophages which had been cultured with ATC and antigen in the presence of anti-κ serum to induce anti-DNP responses in DNP Fla-primed spleen cells. Using a rabbit anti-mouse μ-chain antiserum (anti-μ), made specific for μ-chains by absorption, results of a similar nature were obtained (Fig. 11). Anti-μ serum did not block the binding of the collaborative factor in the presence of 100 μg/ml of a purified NZB mouse IgM preparation (Table IV).

DISCUSSION

Experiments using double-chamber cultures with the two compartments separated by a cell impermeable nucleopore membrane have demonstrated that contact of T and B lymphocytes is not essential for efficient cell interaction in in vitro hapten carrier and anti-erythrocyte culture systems (5–7). Thus collaboration was mediated by a subcellular component or a "factor." Because both the induction of this factor, and once induced its immunological effects, were markedly antigen specific, this factor will be termed "antigen-specific factor" or abbreviated as SF. The double-culture system was used here to

TABLE IV

Capacity of Anti-μ Serum to Inhibit the Binding of the Collaborative Factor to Macrophages

Cells cultured initially		Serum	Anti-DNP response (AFC/culture ± SE)
Upper	Lower		
NIL	Macrophages	NIL	0
NIL	Macrophages	NRS	0
NIL	Macrophages	anti-μ	0
ATC$_{KLH}$	Macrophages	NIL	710 ± 90
ATC$_{KLH}$	Macrophages	NRS	605 ± 180
ATC$_{KLH}$	Macrophages	anti-μ	105 ± 60
ATC$_{KLH}$	Macrophages	anti-μ + IgM	695 ± 210

3×10^6 ATC$_{KLH}$ were cultured with 2×10^6 anti-θ-treated peritoneal exudate cells as a source of macrophages for 2 days. The macrophages were harvested and washed, and 10^5 cells were cultured for 3 days with 1.5×10^7 DNP Fla-primed spleen cells and no other source of antigen. Responses to DRC were normal throughout. 0.1% normal rabbit serum or anti-μ chain serum were used, and 100 μg/ml of mouse IgM. In other experiments abrogation of the suppression by anti-μ antiserum has also obtained using 10 or 1 μg/ml of mouse IgM.

delineate the locus of action of this mediator of collaboration and to define its nature more precisely.

The initial double-chamber experiments were performed using whole spleen cell suspensions as a source of antibody-forming cell precursors (6). A combination of methods (6) was used to exclude the possibility that the antigen-specific factor merely acted as a T cell adjuvant, like complexes of polyadenylic and polyuridylic acid (23). Thus the SF must act directly on B cells or indirectly via macrophages. By using purified (macrophage-depleted) lymphocyte suspensions, the latter possibility was found to be correct, which opened up many experimental avenues and greatly simplified the identification of the SF. As a source of macrophages, peritoneal exudate cells from unprimed mice were either used as such or were further purified by column adherence or by the use of anti-θ serum and complement. Macrophages which had been incubated in the bottom compartment of double-chamber cultures together with specially activated thymus cells in the upper compartment and antigen were subsequently

capable of immunizing DNP Fla-primed spleen cells (Fig. 2), unprimed spleen cells (unpublished data), primed lymphocytes (macrophage-depleted spleen) as shown in Fig. 3, and DNP-primed or unprimed T-depleted (nu/nu) spleen cells (Fig. 4). In all these experiments only the response to antigens with which macrophage and activated thymus cells had been initially incubated in double flasks was augmented. The specificity of the effect of the collaborating factor was investigated by using a combination of both conjugated and unconjugated proteins. In the presence of ATC_{KLH} and DNP KLH, macrophages were able to subsequently induce anti-DNP response in B cells. This reaction was blocked by the presence of unconjugated KLH. ATC_{KLH} cultured with both KLH and DNP FγG did not yield macrophages capable of inducing anti-DNP responses (Table III), demonstrating that the T cell component of the cooperative factor did not cause just any antigen present in the culture to bind in an immunogenic manner to macrophages, but only those antigens recognized by T cells.

Trypsinization of macrophages established that the immunogenic SF resides on the surface of these cells (Fig. 6). This permitted the use of various antisera to investigate the nature of the SF. Because macrophages which has been incubated with ATC_{KLH} and DNP KLH and induced anti-DNP responses in vitro possessed both DNP and KLH determinants on their surface (Figs. 7 and 8), the native antigen, DNP KLH, must be one component of the SF. However because macrophages incubated with DNP KLH but without activated thymus cells, or with thymocytes activated to the wrong antigen, were not immunogenic in vitro (Fig. 2), there was a specific T cell contribution to the SF. By using polyvalent anti-Ig, anti-κ chain, and specific anti-μ chain antisera, the immunoglobulin nature and the class specificity of the T cell contribution was ascertained (Figs. 9–11). Thus the antigen-specific factor, which passes through nucleopore but not dialysis membranes, is a complex of T cell IgM and antigen. Recently it was found[2] that the activated thymus cell populations as used in the above experiments release molecules of mol wt 180,000 with antigenic specificity, containing both light and μ-like peptide chains as judged by electrophoresis, and which bind to peritoneal macrophages.[3]

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[2] Feldmann, M., R. E. Cone, and J. J. Marchalonis. 1972. Cell interactions in the immune response in vitro. VI. Mediation by monomeric T cell surface IgM. Manuscript in preparation.

[3] Since populations of ATC from the spleen of heavily irradiated mice injected with thymocytes may contain a few residual B lymphocytes, it was essential to establish that the specific cooperative factor identified as T cell IgM was indeed of T cell origin. Several lines of experimental evidence, to be detailed elsewhere, suggest that the cooperative IgM is indeed of T cell origin.

First, ATC treated with anti-θC3H antiserum and complement no longer produce the SF. Secondly, treatment of ATC with anti-Ig or anti-κ serum and complement, a regime which kills B cells, does not diminish the production of SF (M. Feldmann, unpublished data). Thirdly, thoracic duct lymphocytes for irradiated mice injected with thymocytes (T-TDL),

The above studies have delineated a mechanism of specific T-B lymphocyte cooperation in the antibody response which is diagrammatically represented in Fig. 12. Activated thymus cells release their monomeric IgM receptors complexed with antigen. This complex binds to the surface of macrophages, where it forms a lattice of antigenic determinants. B cells are immunized upon interacting with this matrix of determinants. One of the attractive features of this model of cell interaction is that the final step of cooperation, namely the
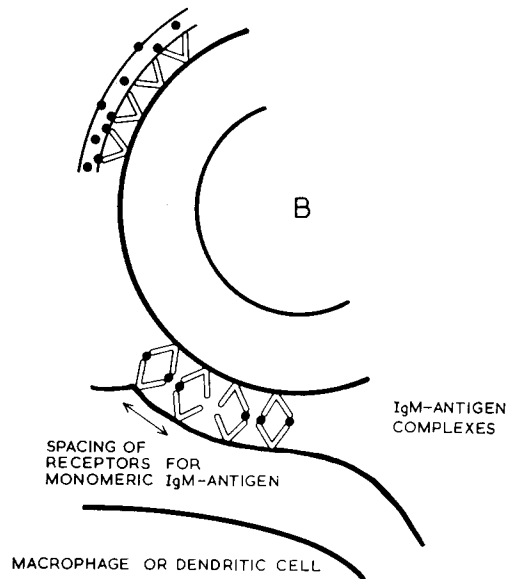

FIG. 12. Theoretical model of cell interaction fitting best with the available data. Immunization of B lymphocytes occurs due to a matrix of antigenic determinants bound by the T cell IgM to the surface of a macrophage or possibly a dendritic-type cell. The spacing of the receptors for T cell IgM on the surface of macrophages seems to only permit the formation of an immunogenic lattice of antigen.

immunization of B cells, could involve the same interactions between antigenic determinants and receptor molecules as that which occurs in the process of immunization with thymus-independent antigens such as DNP POL or DNP Fla (12, 13, 42).

This mechanism of cell cooperation probably represents the events at the initiation of the antibody response (Fig. 5) and would have the rigid antigenic

a population which contains less than 0.3% B cells (50), nevertheless cooperate just as efficiently as ATC obtained from the spleen of these mice (M. Feldmann and J. Sprent, manuscript in preparation). Furthermore, studies by R. E. Cone, M. Feldmann, and J. J. Marchalonis (unpublished data) have indicated that monomeric IgM from the surface of B cells (nu/nu spleen), unlike monomeric IgM from ATC, does not bind to macrophages. These considerations taken together indicate that the SF originates from T cells.

specificity known to usually occur in both primary and secondary responses
(7, 20, 24). It seems possible that there may be another component of the
cooperative interaction, which is also produced by T cells, but is not antigen
specific and augments the response of already triggered B cells to both thymus-
dependent and thymus-independent antigens (6, 7). This may be the same
factor which is produced in allogeneic mixtures of lymphoid cells in vitro
(7, 18, 19) and acts optimally at a later stage in the antibody response than
the antigen-specific factor. Evidence for the existence of such a nonspecific
factor in syngeneic systems has also been obtained by the author using ATC
of different specificities (reference 6; Fig. 5; P. Adams and K. Shortman,
personal communication; reference 51). Fig. 5 demonstrates that the antibody
response to macrophages which had been coated with SF, while starting much
earlier than the response initiated by activated thymocytes present in the
same cultures, only reached about half the magnitude of the response of
the latter. In the latter instance both SF and the nonspecific factor would be
present, and the presence of the nonspecific factor, which augments B cell
responses, may explain the twofold greater response occurring by the 4th day
of the response.

The mechanism of cell cooperation favored by the above experiments and
described in Fig. 12 resembles in certain features models previously suggested
by other workers in this field. The specific T cell component of the SF certainly
fits in well with the "carrier antibody" concept theoretically postulated by
Bretscher and Cohn (25) and is indeed "antibody" by both electrophoretic[2]
and serological criteria (Figs. 9–11). Furthermore it possesses carrier specificity.[2]
However carrier antibody acts via the intermediary action of macrophages
(e. g. Fig. 2) and not by directly transmitting an immunogenic signal to B cells,
as these authors have suggested (25). In a recent review, Miller et al. (24) pro-
posed several models of T-B cell cooperation. In one of these, T cell immuno-
globulin, "IgX" and antigen, was envisaged as being the mediator of cell co-
operation. Similarly, Mitchison, Taylor, and Rajewsky (26) discussed several
possible models of cell interaction. One of these involved a T cell immunoglobu-
lin which bound to macrophages. On the available evidence in 1970, they con-
sidered other mechanisms more likely. Lachmann (27) has suggested that
"antigen-specific migration-inhibitory factor (MIF)" may be the mediator of
cell cooperation. This model is conceptually the same as shown in Fig. 12, with
the exception of the details of the entity involved in the binding of antigen to
macrophages. It is not inconceivable that in in vivo MIF may also be involved
in conjunction with complexes of T cell receptor and antigen. Guttman and
Weissman (28) have proposed that opsonic antibody from T cells binds to
dendritic cell processes, and that antigen B cell interaction occurs at this site.
Basically this is the mechanism described in Fig. 12. It is not known, but it is
possible that cooperation in vivo usually acts through the mediation of a cell
which resembles a dendritic cell, rather than the usual phagocytic macrophage

(see also reference 28). It is possible that the peritoneal macrophage containing cell populations used as a source of accessory cells in vitro is functionally heterogenous and that the cell involved in cooperation in vitro could resemble a dendritic cell.

An essential feature of the above model of cell cooperation is the interaction of B lymphocytes with the surface of macrophage-like cells. Because the latter cells are not antigen specific and are relatively common, the interaction of specific B cells with macrophages is much more likely to occur than that of specific T with specific B cells. There is much evidence in the literature that the interaction of B cells and macrophages occurs. Schmidtke and Unanue (29) have demonstrated that B, but not T lymphocytes, adhere to macrophages in vitro. Clusters of lymphocytes with a central macrophage have been observed in spleen cell cultures generating both primary and secondary antibody responses in vitro (30–32). It has been shown that these clusters are essential for the generation of AFC and that antibody-forming cells usually arise in these clusters (30, 31). Furthermore, the cluster as a reflection of specific T-B cell cooperation explains the previously puzzling (in view of the usual nonspecificity of macrophages) antigen specificity of AFC produced in clusters of multiply immunized cultures (30).

The work of Fischer and his colleagues (e.g. reference 33) has amply documented the direct interaction of lymphocytes with dendritic macrophages bearing membrane-bound antigen. They noted that mitoses and AFC arose near macrophages. Miller and Avreamas (34) have observed the proximity of AFC to macrophages containing antigen in lymph nodes. Furthermore, Guttman and Weissman (28), Durkin et al. (35), Sprent (36), and Mitchell (37) have all noted that the principal class of lymphocytes in primary lymphoid follicles and at the peripheral region of germinal centers are B cells. This is the region of the lymphoid system through which are intertwined the processes of dendritic cells (38), which could thereby facilitate B cell antigen interaction.

The mechanism of cell cooperation shown in Fig. 12 also explains other facets of the antibody response. Firstly it explains the heightened immunogenicity of macrophage-bound antigen, found by Mitchison (39) and Unanue, Askonas, and Cerottini (40, 41). Macrophage-bound antigen, even in the absence of T cell component, may in some instances sufficiently resemble the T cell-mediated antigenic lattice that it efficiently immunizes B cells. Secondly, this mechanism of cell cooperation is fully compatible with the existing data concerning the mechanism of B cell signal discrimination between immunity and tolerance (12, 42). It was found that even large numbers of macrophages, even if they had been exposed to very large numbers of activated thymus cells and antigen, induced only immunity and never tolerance (Fig. 2). It should be noted that the lowered responses due to the presence of 10^6 macrophages were not antigen specific, confirming the prior results of Diener et al. (43). Thus the immunological properties of SF-coated macrophages were similar to DNP

POL conjugated with few DNP groups, which even at high antigen doses never induced tolerance (12). It is possible that the basis of this finding of "obligatorily immunogenic" DNP POL may be a reflection of the normal mechanism of cooperative immunization, via a lattice of determinants on the surface of a macrophage or dendritic cell. It is possible that receptors on macrophages for the SF may be spaced in such a way that the conditions for tolerance induction never occur (12, 42). The bivalency of the T cell component of the mediator of cell cooperation would bind antigen molecules in such a way as to favor the formation of bonds of antigen with both combining sites of a B cell receptor antibody molecule, which is important in immune induction (42).

Many features of the phenomenon of "antigenic competition" (reviewed in references 44–46) may be explained on the basis of the model of cell cooperation shown in Fig. 12, without postulating the existence of any T cell-derived inhibitor (44–46). If one assumes that during the generation of an antibody response the production of SF occupies many of the receptors for SF on macrophages, then the critical time relationship between antigen injections, which is often needed for efficient competition to occur, is readily explained, as there will be fewer sites available for the SF of the second antigen (44, 45). Similarly, competition would only occur in T cell-containing animals (46), and furthermore competition of the response to a thymus-independent antigen could not occur (45). Some evidence for this concept has already been obtained (Fig. 3); more details will be described elsewhere (J. W. Schrader and M. Feldmann, manuscript in preparation).[4]

It is possible that the participation of T cells in tolerance induction (47) may be due to the action of T cell IgM-antigen complexes directly on B cells or possibly on other T cells. Because the T cell monomeric IgM is presumably bivalent, it is possible that complexes of T cell carrier antibody and antigen are formed which, with the right ratio of antigen to antibody, would resemble the complexes of humoral antibody and antigen which can induce tolerance in vitro (48, 49). If binding sites for the T cell IgM on the surface of macrophages are saturated, such as at the height of an immune response, these complexes could interact directly with B cells and induce a degree of partial tolerance. This would be a self-regulatory homeostatic mechanism, preventing overimmunization, and which could also explain the so-called "infectious tolerance" (47).

<div align="center">SUMMARY</div>

The mechanism of interaction of T and B lymphocytes was investigated in an in vitro hapten carrier system using culture chambers with two compartments separated by a cell impermeable nucleopore membrane. Because specific cell interaction occurred efficiently across this membrane, contact of T and B

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[4] Note added in proof: Taussig and Lachmann (52) have independently proposed a similar, mechanism of antigen competition.

lymphocytes was not essential for cooperation which must have been mediated by a subcellular component or "factor." By using different lymphoid cell populations in the lower culture chamber and activated thymus cells in the upper chamber (with antigen present in both), it was found that the antigen-specific mediator acted indirectly on B cells, through the agency of macrophages. Macrophages which had been cultured in the presence of activated T cells and antigen acquired the capacity to specifically induce antibody responses in B cell-containing lymphoid populations. Trypsinization of these macrophages inhibited their capacity to induce immune responses, indicating that the mediator of cell cooperation is membrane bound. By using antisera to both the haptenic and carrier determinants of the antigen as blocking reagents, it was demonstrated that the whole antigen molecule was present on the surface of macrophages which had been exposed to activated T cells and antigen. Because specifically activated T cells were essential a component of the antigen-specific mediator must be derived from these cells. By using anti-immunoglobulin sera as inhibitors of the binding of the mediator to macrophages, the T cell component was indeed found to contain both κ- and μ-chains and was thus presumably a T cell-derived immunoglobulin.

It was proposed that cell cooperation is mediated by complexes of T cell IgM and antigen, bound to the surface of macrophage-like cells, forming a lattice of appropriately spaced antigenic determinants. B cells become immunized by interacting with this surface. With this mechanism of cell cooperation, the actual pattern of antigen-B cell receptor interactions in immunization would be the same with both thymus-dependent and independent antigens. An essential feature of the proposed mechanism of cell cooperation is that macrophage-B cell interaction must occur at an early stage of the antibody response, a concept which is supported by many lines of evidence. Furthermore this mechanism of cell interaction can be elaborated to explain certain phenomena such as the highly immunogenic macrophage-bound antigen, antigenic competition, the distinction between immunity and tolerance in B lymphocytes, and the possible mediation of tolerance by T lymphocytes.

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