CELL INTERACTIONS IN THE IMMUNE RESPONSE IN VITRO

III. Specific Collaboration across a Cell Impermeable Membrane*

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Interaction between thymus-derived (T) lymphocytes[1] and nonthymus-derived (B) lymphocytes is essential for optimal antibody production to many antigens, including heterologous erythrocytes (1–3), serum proteins (4, 5), and hapten-protein conjugates (6, 7). In contrast, other antigens such as polymeric flagellin (POL) (8), polyvinylpyrrolidone (9), and pneumococcal polysaccharide Type III (10), which display a large number of repeating determinants, can apparently immunize B cells directly. The requirement for T cells in the immune response would thus appear to be related to the physical structure of the antigen, and not to the specificity of its antigenic determinants. Confirmation of this concept was obtained in an in vitro system by coupling the hapten, the dinitrophenyl (DNP) determinant, to different carriers, one of which, donkey red cells (DRC), elicits a thymus-dependent response and the other, POL, a thymus-independent response. When DNP antigens in these two physical forms were cultured with B cells obtained from adult thymectomized, heavily irradiated and bone marrow–protected (ATXBM) mice, an anti-hapten response occurred with DNP POL but not with DNP DRC (8).

Direct triggering of B cells by polyvalent antigens is thought to be related to their capacity to form multiple bonds with receptor sites on the B cells' surfaces. Multiple bond formation greatly prolongs the duration of the antigen-

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[1] *Abbreviations used in this paper:* AFC, antibody-forming cells; ATC, activated thymus cells; ATC_{DRC}, ATC_{SRC}, $ATC_{F\gamma G}$, ATC_{KLH}, activated thymus cells to DRC, SRC, FγG, and KLH; ATXBM, adult thymectomized, irradiated and bone marrow–protected mice; B cells, thymus-independent lymphocytes; DNP, 2,4 dinitrophenyl determinant; DNP FγG, DNP KLH, DNP POL, etc., dinitrophenylated protein; DRC, donkey red cells; FCS, fetal calf serum; Fla, flagella of *Salmonella adelaide*; FγG, fowl immunoglobulin G; KLH, keyhole limpet hemocyanin; POL, polymeric flagellin; SRC, sheep red cells; T cells, thymus-dependent lymphocytes.

receptor interaction (11), despite the rapid turnover of B cell receptors (12). The physiological function of cell collaboration as suggested by Mitchison (13) could well be to construct a similar matrix of determinants for immunogens lacking a repeating epitope pattern. The precise mechanism of antigen concentration by T cells in cell collaboration is unknown. The simplest model, proposed by Mitchison (13) and Rajewsky et al. (14) on the basis of their studies of hapten carrier systems, involves the binding of antigen via its carrier determinants to T cells which then "focus" the inducing determinants, also present on the same molecule, on to the corresponding receptors of B cells. This process is passive, with antigen bridges linking T and B cells of appropriate specificities. Other work, however, both in vitro (15) and in vivo (16) implies that cell collaboration is an active process requiring the differentiation and division of T cells with elaboration of a factor capable of activating B cells. These two contrasting hypotheses are readily amenable to test in vitro, by determining whether a collaborative response can occur when T and B cells are physically separated by a cell impermeable membrane. In preliminary experiments, some evidence for cooperation across a filter was indeed found (17). The purpose of this communication is to establish the veracity of the phenomenon in a variety of cooperative systems and to examine critically its parameters and the specificity of the factor(s) released.

Materials and Methods

Animals.—Mice of the highly inbred CBA/H/Wehi or (CBA × C57BL)F_1 hybrid strains were used in most experiments. For some studies nude (nu/nu) mice provided a source of lymphoid cells uncontaminated by T cells (18, 19).

Thymectomy.—Thymectomy was performed in adult CBA mice at 5–7 wk of age and in neonatal CBA mice within 24 hr of birth, according to the methods of Miller (20).

Irradiation.—Intact or thymectomized animals were exposed to total body irradiation in a Philips (RT 250) 250 kev machine (Philips Electronic Instruments, Mount Vernon, N. Y.) under conditions described previously (8). Adult thymectomized mice were given 750 rads 2–4 wk postoperatively and were reconstituted with 3–5 × 10^6 syngeneic bone marrow cells and were used as outlined in an earlier paper (8). Neonatally thymectomized mice received 300 rads at 8 days of age and then were injected four times at weekly intervals with 10^8 (CBA × C57BL)F_1 thymocytes (21). The chimeras produced possessed T cells derived from the thymus cell inoculum and B cells from the CBA host which has very few T cells of its own (22). Their spleen cells were used at 3–4 months of age.

Immunization of Mice.—CBA mice were injected with either 5 × 10^8 sheep red cells (SRC), 5 × 10^8 DRC, or 25 μg dinitrophenylated flagella of *Salmonella adelaide* (DNP Fla). Spleen cells were harvested 1–3 months postpriming and washed twice before use.

Antigens.—Heterologous erythrocytes (SRC and DRC) were collected and stored as described previously (8). The DNP determinant was coupled to flagella, POL, fowl immunoglobulin G (FγG), or keyhole limpet hemocyanin (KLH) as described elsewhere (22–24). Conjugation ratios used were $DNP_{1.5-2.3}$ Fla, DNP_1POL, $DNP_{8-12}F\gamma G$, and $DNP_{800}KLH$ (per 8 × 10^6 molecular weight).

Cell Suspensions.—Thymocytes and spleen cells were prepared as described elsewhere (15).

Activation of Thymus Cells.—Activated thymus cells (ATC) were prepared by the method

of Miller and Mitchell (25). 10^8 thymocytes were injected intravenously into irradiated (800 rads) syngeneic recipients together with various antigens. SRC or DRC (5×10^8 per recipient) were given intravenously, and KLH or FγG emulsified in Freund's complete adjuvant (100 μg per recipient) was injected intraperitoneally. 6–8 days later the spleens were removed and single cell suspensions were made and washed thoroughly by centrifugation through fetal calf serum (FCS).

Tissue Culture.—Mouse spleen cells were grown in a modified Marbrook-Diener culture system as described recently (26). 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% 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 with two distinct departments were designed using a pair of concentric

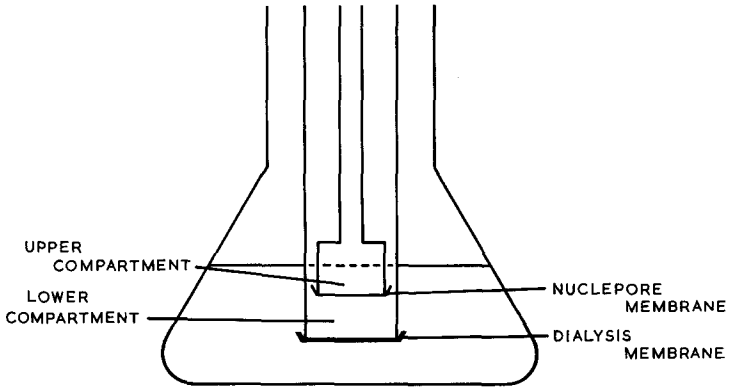

FIG. 1. Double chamber cultures, formed by concentric glass tubes, suspended in a reservoir of medium. T cells were placed in the upper compartment, and B cell–containing populations in the lower compartment.

glass tubes, one of which fitted inside the other, the whole system being inserted into a 125 ml Erlenmeyer flask. The bottom end of the inner cylinder was sealed off by a nuclepore membrane, usually of 1 μ size (General Electric Company, Schenectady, N. Y.) thereby forming the upper compartment. In some experiments nuclepore membranes of 0.1, 0.2, and 0.4 μ pore size were employed, or this type of membrane was replaced by a dialysis membrane (Union Carbide Corp., New York). The lower compartment was always sealed off with a dialysis membrane. ATC were placed in the upper compartment and various cell populations containing B cells in the lower chamber. Control cultures were set up using inserts of the same diameter as the double chamber cultures. A diagrammatic representation of a double culture is shown in Fig. 1.

Treatment with Isoantisera.—AKR anti-Θ C3H serum was prepared by the method of Reif and Allen (27). It was assayed and used as described previously (8). CBA anti-C57BL antiserum was raised by five to six biweekly intraperitoneal injections of 10^7 C57BL spleen cells and was tested for cytotoxicity in the same manner as for anti-Θ sera. For experimental purposes 3×10^8 chimeric spleen cells were incubated in 4 ml of CBA anti-C57BL serum diluted 1 in 4 for 30 min at 37°C, washed twice, and agarose-absorbed guinea pig serum (28) was added as a source of complement for a further 37° incubation period of 45 min.

Treatment with Inhibitory Agents.—Antimycin A and actinomycin D were obtained from

Sigma Chemical Co., St. Louis, Mo. ATC were treated with either 10^{-5} M antimycin A or 0.1 μg/ml actinomycin D, as described previously (15). These concentrations were selected because of their known capacity to exert a selective inhibitory effect on the in vitro activity of ATC without interfering nonspecifically with the integrity of B cells (15).

Enumeration of Antibody-Forming Cells (AFC).—AFC were detected by Cunningham and Szenberg's modification (29) of the hemolytic plaque assay as outlined elsewhere (22).

Preparation of Rabbit Anti-Mouse IgG-^{125}I.—The IgG fraction of a polyvalent rabbit anti-mouse immunoglobulin serum was obtained by Sephadex (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) chromatography. Iodination with iodide-^{125}I (IMS-3), Radiochemical Centre, Amersham, England) was performed according to the method of Byrt and Ada (30). The iodinated immunoglobulin had a substitution ratio of approximately 1 iodine atom per molecule of IgG and was stable for at least a week after preparation.

Labeling of Lymphoid Cells.—Spleen cells from CBA or C57BL mice or mixtures of the two cell types were incubated for 30 min at 37°C with normal mouse serum or CBA anti-C57BL serum at a concentration of 10^7 cells/ml of serum diluted 1 in 10. After washing twice, the cell pellets were resuspended in rabbit anti-mouse IgG-^{125}I for 30 min at 4°C. Noncell-bound radioactivity was removed by centrifugation through FCS gradients (30) Smears for radioautography on gelatin-coated slides were dipped in NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.), developed approximately 1 wk later, and stained with Giemsa solution. 1000 viable mononuclear cells with the features of small lymphocytes were counted per slide. The conditions of radioautography were adjusted so that B cells were not labeled by the anti-IgG–^{125}I.

RESULTS

Cell Collaboration across a Nuclepore Membrane In Vitro.—Interaction between T and B cells in vitro has been reported by many workers (7, 31–35). In a preliminary communication we reported that CBA thymus cells activated to KLH can induce an anti-DNP response to DNP KLH in syngeneic DNP Fla–primed spleen cells immunized with DNP KLH, regardless of whether the ATC_{KLH} and hapten-primed cell populations were mixed or were separated from each other by a nuclepore membrane of 1 μ pore size (17). The data in Table I confirm this observation and demonstrate a similar effect with several other antigenic systems (SRC, DRC, DNP FγG). Furthermore, the efficiency of cell interaction was not significantly impaired by insertion of a nuclepore barrier. This is well illustrated by the experiments depicted in Fig. 2, in which limiting dilutions of ATC_{KLH} were used to detect any subtle differences in efficiency. Approximately 10^5 ATC_{KLH} proved to be the lowest effective dose of helper cells in both the double as well as the single chamber cultures.

Cell Impermeability of a Nuclepore Culture System.—The experiments reported above imply the existence of a soluble factor capable of stimulating antibody production by B cells. The validity of this conclusion, however, depended on excluding the leakage of significant numbers of T cells into the compartment containing B cells. Several different approaches were used for this purpose. First, double chambers with 10^7 T cells in the upper compartment, but no cells at all in the lower chamber, were set up. After culture periods of 1 or 2 days, the fluid from the lower compartment was centrifuged and subjected to micro-

scope examination. Although cell debris were found in all cases, only 1 out of 36 cultures examined contained intact cells (approximately 10^4). The second approach to exclude leakage utilized a radioautographic technique. 10^7 C57BL spleen cells were placed in the upper compartment of a double flask and 10^7 CBA spleen cells in the lower, the two populations being separated by 1 μ pore size membrane. The flasks were cultured for 24 hr, after which cells from the

TABLE I
Collaboration across a Nuclepore Membrane

Culture flasks	Cells		Antibody response (AFC/culture ± SE)
	T	B*	
Single	—	Anti-Θ-treated SRC-primed spleen cells	520 ± 160
"	ATC_{SRC}	" " " "	32,080 ± 1470
Double	"	" " " "	12,060 ± 2680‡
Single	—	Anti-Θ-treated DRC-primed spleen cells	210 ± 110
"	ATC_{DRC}	" " " "	18,640 ± 1680
Double	"	" " " "	12,420 ± 3100
Single	—	DNP Fla-primed spleen cells	0
"	$ATC_{F\gamma G}$	" " "	2840 ± 260
Double	"	" " "	2890 ± 480
Single	—	DNP Fla-primed spleen cells	0
"	ATC_{KLH}	" " "	3420 ± 180
Double	"	" " "	2950 ± 320

* The B cell populations used also contained macrophages. Each value represents the arithmetic mean of four to eight cultures ± the standard error of the mean. 3×10^7 cells were cultured in the B cell compartment. Antigen was present in both compartments of double chambers. DNP FγG and DNP KLH were used in the latter two experiments, respectively. Antigen doses used were 3×10^6 red cells/culture, 1 μg/ml of DNP FγG or DNP KLH.

‡ The response in double chambers in this experiment was lower than in single chambers ($P < 0.05$), whereas in others the difference was not significant. The magnitude of anti-erythrocyte responses in these cultures was always greater than the anti-hapten responses.

lower chamber were harvested and treated with CBA anti-C57BL serum followed by radioiodinated rabbit anti-mouse IgG. The sensitivity of the method was calibrated by mixing known and varying numbers of C57BL spleen cells from the same donors used above to samples of a standard suspension of CBA cells. As shown in Table II, the technique permitted detection of less than 1% of contaminating cells. 12 cultures comprising two experiments were analyzed in this way (Table III). In only one of these was there detectable leakage, with 1–3% of cells in the lower chamber carrying H-2^b (C57BL) isoantigens on their surface.

The effect of omitting antigen from the T cell compartment provided a third independent way of excluding significant T cell leakage. In this case, a system involving a particulate antigen, SRC, was employed. ATC_{SRC} pretreated with ammonium chloride to lyse residual erythrocytes (36) and then washed was used as a source of T cells relatively uncontaminated by antigen, and spleen

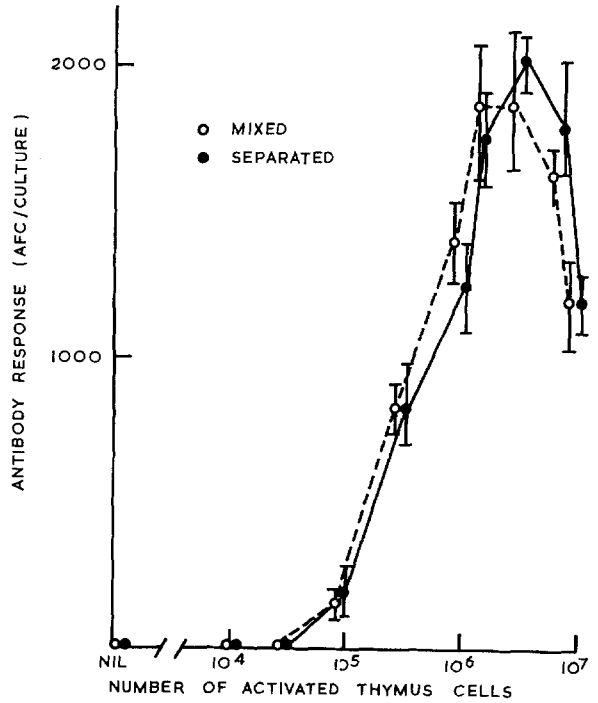

FIG. 2. Cooperation between T and B cells was as efficient when they were mixed or separated by a 1 μ pore size nuclepore membrane. ATC_{KLH} were cultured with DNP Fla-primed spleen cells and 1 μg/ml DNP KLH. The anti-DNP response was assessed 4 days later. Each value represents the arithmetic mean of 3 cultures $\pm$ the standard error of the mean. Comparable results were obtained in 31 similar experiments.

cells from SRC-primed mice treated with AKR anti-Θ serum served as a source of B cells. The data shown in Table IV indicate that a collaborative response across a nuclepore membrane did not occur unless additional SRC were added to the upper compartment. If ATC_{SRC} had leaked into the lower chamber, which contained antigen as well as B cells, a response would have been detected in the presence of as few as 10^4 ATC. Furthermore, the use of nuclepore membranes of even smaller pore size, namely, 0.1, 0.2, and 0.4 μ (*vide infra* Table VIII), still permitted effective collaboration to occur.

Specificity of Cell Collaboration across a Nuclepore Membrane.—The helper activity of T cells in vivo is usually highly specific with respect to the antigen

used (e.g. 13, 21, 25). It was therefore essential to ascertain whether the soluble factor elaborated by T cells (Table I) displayed specificity. Two aspects were studied, namely, the antigenic specificity of the ATC capable of producing significant amounts of the factor, and, in addition, the factor's specificity for B cells. Several populations of ATC were prepared and tested for their capacity to enhance the response of DNP Fla–primed spleen cells to DNP KLH. Fig. 3 indicates that activation of T cells to the specific antigen was mandatory for the production of effective quantities of collaborative factor in the syngeneic system under study. Furthermore, the response to another antigen, DRC,

TABLE II

*Detection of Contaminating Lymphocytes in a Mixed Lymphoid Cell Population. Sensitivity of the Technique**

C57BL cells present‡	Pretreatment	Cells labeled with anti-mouse IgG-^{125}I/10^3 cells	
		Exp. 1	Exp. 2
(%)			
0	CBA anti-C57BL serum	4	6
0.3	" " "	12	14
1	" " "	20	22
3	" " "	51	58
10	" " "	114	123
30	" " "	344	324
50	" " "	473	560
50	Normal mouse serum	4	2

The conditions of labeling and radioautography were such that B cells were not labeled (e.g. lines 1 and 8). With longer exposures anti-Ig-^{125}I-labeled B cells directly (52, 53).

* The number of cells bearing *H-2*^{b} (C57BL) isoantigens was detected by a sandwich technique in which lymphocytes were treated with appropriate antiserum followed by a polyvalent rabbit anti-mouse IgG-^{125}I (Materials and Methods).

‡ Obtained by adding varying numbers of C57BL spleen cells to samples of a standard suspension of CBA cells.

placed in the B cell compartment was not significantly enhanced by the factor. Its specificity for B cells was subsequently confirmed in five other experiments.

Capacity of ATC to Collaborate with Unprimed B Cells across a Nuclepore Membrane.—The experiments described above involved the use of various sources of primed B cells. It was thus of interest to determine whether unprimed B cells could respond in the double culture system. For this purpose three populations of lymphocytes containing unprimed B cells were used, namely, spleen cells from normal or ATXBM CBA mice and from nude mice. Their capacity to yield an anti-DNP response to DNP KLH in the presence of ATC_{KLH} was compared with that of DNP-primed cells (DNP Fla–primed spleen) (Table V). Appreciable specific antibody production was obtained whether T and B cells were mixed together or separated by a cell impermeable

barrier. Not surprisingly, the magnitude of these responses was smaller than those obtained with primed B cells.

Site of Action of the Collaborative Factor.—The ability of thymus cells activated to SRC to collaborate with primed B cells of the same specificity was

TABLE III

Detection of Contaminating Lymphocytes in a Mixed Lymphoid Cell Population. Degree of Leakage across a Nuclepore Membrane

Cultures*	Cells labeled with anti-mouse IgG-^{125}I after pretreatment with	
	Normal mouse serum	CBA anti-C57BL serum
	per 10^3	
Experiment 1‡		
1	4	2
2	4	5
3	0	2
4	2	3
5	3	3
6	2	2
Experiment 2‡		
1	0	4
2	4	34§
3	4	2
4	3	5
5	2	4
6	4	6

* Each number represents an individual culture flask. Two separate experiments are shown.

‡ Controls are shown in Table II. Experiments with the same number, i.e. 1 and 2, correspond.

§ Significant leakage of cells. Between 1 and 3% of cells in lower chamber were contaminants. Since there were 10^7 cells in the lower chamber, this represents a leak of 10^5–3×10^5 cells.

TABLE IV

Effect of Omitting Antigen from the T Cell Compartment

Cells					Antigen with T cells	Antibody response (AFC/culture ± SE)
T		B*				
—		Anti-Θ-treated SRC-primed spleen			+	340 ± 120
10^3	ATC_{SRC}	"	"	"	+	360 ± 40
10^4	"	"	"	"	+	1420 ± 180
10^5	"	"	"	"	+	3860 ± 240
10^6	"	"	"	"	+	6280 ± 1100
3×10^6	"	"	"	"	+	12,640 ± 840
3×10^6	"	"	"	"	−	290 ± 110

Each value is the arithmetic mean of six cultures ± the standard error of the mean.

* The B cell population used contained macrophages. 3×10^7 cells were in each culture. SRC were present in the B cell compartment.

unaffected by treatment of the B cell source with anti-θ serum and complement (Table VI). In other words, the factor released by ATC in the upper

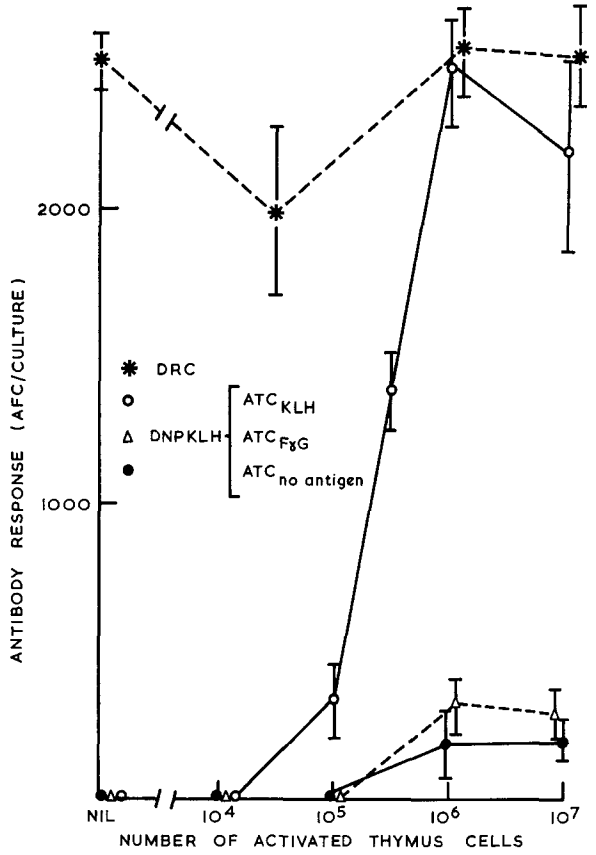

FIG. 3. Specificity of cell collaboration across a nuclepore membrane. (✳—✳) DRC response of B cell compartment (40 × 10^6 DNP Fla–primed spleen cells) of cultures containing ATC_{KLH}. DRC responses of cultures containing other ATC were comparable. Other lines represent anti-DNP response of B cell compartment to 1 μg/ml DNP KLH in the presence of ATC_{KLH}, $ATC_{F\gamma G}$, or thymocytes injected into an irradiated host without additional antigen. No marked increase in the anti-DNP response was found unless thymus cells were activated to the carrier protein used in vitro.

compartment appeared to act on the B cell itself or macrophages, and not on residual T cells in the lower compartment. It was also necessary to ensure that the same was true in carrier hapten systems, where two different determinants are involved. T-depleted populations of hapten (DNP)–primed B cells were obtained by treating either CBA spleen cells with AKR anti-θ C3H serum and

complement, or spleen cells from chimeric mice[2] (*vide supra*) with CBA anti-C57BL serum and complement. These were then placed in the lower compartment of a double chamber and cultured together with, or separated from, ATC_{KLH} in the presence of DNP KLH as described previously. Completeness of T depletion was verified by the lack of DRC response in these cultures (Table VI). Despite this, the magnitude of anti-DNP antibody production by the cells in the lower chamber was as great in the absence as in the presence of T cells primed to the haptenic determinant in the same culture compartment.

TABLE V

Collaboration of ATC_{KLH} and Normal B Cells across a Nuclepore Membrane

Culture flasks	Cells		Antibody response (AFC/culture ± SE)
	T	B*	
Single	—	DNP Fla-primed‡ spleen cells	120 ± 80
"	3×10^6 ATC_{KLH}	" " "	2840 ± 180
Double	" × " "	" " "	2420 ± 320
Single	—	Normal CBA spleen cells	0
"	3×10^6 ATC_{KLH}	" " " "	375 ± 180
Double	" × " "	" " " "	754 ± 320
Single	—	Nude spleen cells	0
Double	3×10^6 ATC_{KLH}	" " "	620 ± 126
Single	—	ATXBM spleen cells	0
"	4×10^6 ATC_{KLH}	" " "	620 ± 30
Double	" × " "	" " "	495 ± 20

Each value represents the arithmetic mean of four cultures ± the standard error of the mean. Anti-DNP AFC in response to 1 μg/ml DNP KLH are shown.

* The B cell populations also contain macrophages. 4×10^7 cells were cultured in the B cell compartment.

‡ The figures for collaboration with primed B cells are included for comparison.

Thus in a syngeneic system, the factor released from ATC can trigger B cells independently of other T cells. Similar results were described in Table V using unprimed B cells.

Effect of Pretreatment of ATC by Metabolic Inhibitors on their Capacity to Collaborate with B Cells across a Nuclepore Membrane.—Feldmann and Basten (15) have recently demonstrated that antimycin A and actinomycin D can specifically inhibit the capacity of ATC to collaborate with either primed or unprimed B cells. In other words, interaction between T and B cells in im-

[2] Neonatally thymectomized CBA mice, irradiated and injected with (CBA × C57BL)F_1 T cells. These have CBA B cells, but F_1 T cells (Materials and Methods).

munization is an active metabolic process probably requiring protein synthesis on the part of T cells. One would therefore expect these agents to interfere with a transfilter collaborative response. As shown in Table VII this indeed proved to be the case, with both actinomycin D– and antimycin A–treated T cells incapable of effectively collaborating. It was proven that B cell function in the cultures was normal because of the normal response to DRC in the same cultures. Thus the requirement for T cell participation in cell collaboration

TABLE VI

Collaboration of ATC_{KLH} and DNP-Primed B Cell Populations across a Nuclepore Membrane

Cells		Antibody response (AFC/culture ± SE)	
T	B*	DNP	DRC
—‡	DNP-primed spleen cells	0	1860 ± 210
10^6 ATC_{KLH}	" " "	960 ± 140	1940 ± 380
—	Anti-Θ-treated DNP Fla–primed spleen cells	0	25 ± 20
10^6 ATC_{KLH}	" " " "	940 ± 190	75 ± 40
—	DNP Fla–primed chimeric mouse spleen cells	0	1960 ± 420
2×10^6 ATC_{KLH}	" " " "	790 ± 192	2770 ± 430
—	DNP Fla–primed chimeric mouse spleen cells treated with anti-C57BL serum	0	205 ± 60
2×10^6 ATC_{KLH}	DNP Fla–primed chimeric mouse spleen cells treated with anti-C57BL serum	1140 ± 170	300 ± 179

Each value represents the arithmetic mean of six cultures ± the standard error of the mean pooled from two experiments. Antigen concentrations used were 3×10^6 DRC and 1 μg/ml DNP KLH.

* The B cell populations also contain macrophages. 4×10^7 cells were cultured in the B cell compartment.

‡ Cultures performed in single chamber flasks of same diameter as the doubles. Other cultures performed in double chambers.

seemed to be due to the need to elaborate a subcellular antigen-specific product.

Pore Size of Membranes Permitting T-B Cell Interaction.—The capacity of dialysis membrane and nuclepore membranes of different pore size (0.1, 0.2, 0.4, and 1.0 μ) to permit cell interaction was compared (Table VIII). Only a marginal effect (increase) on responsiveness was noticed with dialysis membranes. This was the same degree of augmentation as when ATC to a nonrelated antigen were employed, i.e., it was not antigen specific (Fig. 3). In contrast, all the nuclepore membranes proved to be equally effective.

TABLE VII

Inhibiting Effect of Pretreatment of ATC with Actinomycin D (act. D) or Antimycin A (anti. A) on Cell Collaboration across a Nuclepore Membrane

Cells		Antibody response (AFC/culture ± SE)	
T	B*	DNP	DRC
—	DNP Fla-primed spleen cells	0	2880 ± 240
5×10^5 ATC_{KLH}	" " "	370 ± 8	3240 ± 620
5×10^5 ATC_{KLH}	" " "	1240 ± 190	3330 ± 180
2×10^6 "	" " "	2330 ± 600	1970 ± 290
10^5 ATC_{KLH} (act. D)	" " "	30 ± 40‡	2420 ± 310
5×10^5 " "	" " "	40 ± 30‡	1990 ± 620
2×10^6 " "	" " "	7 ± 5‡	2845 ± 720
10^5 ATC_{KLH} (anti. A)	" " "	0‡	3110 ± 170
5×10^5 " "	" " "	37 ± 30‡	2970 ± 640
2×10^6 " "	" " "	0‡	2360 ± 475

Each value represents the arithmetic mean of six cultures ± the standard error of the mean. DRC and DNP KLH were used as antigen throughout.

* The B cell population also contains macrophages. 3×10^7 cells were cultured in this compartment.

‡ $P < 0.005$ compared with untreated controls (Student's t test).

TABLE VIII

Collaboration across Membranes of Different Pore Size

Membrane	Cells		Antibody response (AFC/culture ± SE)	
	T	B*	Expt. 1	Expt. 2
—	—	DNP Fla-primed spleen cells	0	0
—	2×10^6 ATC_{KLH}	" "	1640 ± 120	2210 ± 600
Dialysis	" × " "	" "	180 ± 40	210 ± 60
Nuclepore				
0.1 μ	" × " "	" "	2246 ± 140	1536 ± 303
0.2 μ	" × " "	" "	1566 ± 168	1866 ± 140
0.4 μ	" × " "	" "	2250 ± 160	1350 ± 69
1.0 μ	" × " "	" "	1580 ± 656	1640 ± 460

Each value represents the arithmetic mean of four cultures ± the standard error of the mean. DNP KLH was used throughout.

* The B cell populations also contain macrophages. 4×10^7 DNP Fla-primed spleen cells were cultured.

DISCUSSION

The requirement for helper T cells in antibody production to many antigens is now well established (1–7), but the mechanism of their interaction with antibody-forming cell precursors (B cells) is still controversial. Two main

theories exist to explain cell collaboration: the first, the antigen-focusing hypothesis, postulates the formation of an antigen bridge between T and B cells of appropriate specificities (13, 14), whereas in the second, it is suggested that T cells need not come into intimate relationship with B cells but upon activation by antigen may elaborate a factor capable of triggering the latter (15, 37).

The development of a double chamber culture system in which T and B cells are separated by a cell impermeable barrier has permitted us to differentiate between these two hypotheses. In most experiments T cells and antigen were placed in the upper compartment and syngeneic B cells and antigen in the lower, with mixing of the two cell populations being prevented by a nuclepore membrane. Collaborative responses to both heterologous erythrocytes (SRC and DRC) and hapten-protein conjugates (DNP FγG and DNP KLH) were achieved in this culture system (Table I). Since the hapten carrier system was effectively background-free, i.e. generated minimal anti-DNP AFC in the absence of activated or primed carrier reactive helper cells, this response despite the lower total number of AFC generated was used in the majority of experiments to be discussed.

The sensitivity of the technique permitted an accurate comparison of the efficiency of interaction when ATC were mixed or separated from hapten-sensitive cells (Fig. 2). The minimal differences obtained strongly implied an important role for a soluble mediator in the usual mode of T-B cell collaboration. The validity of this assumption, however, depended on excluding the leakage of significant numbers of T cells through the nuclepore membrane. Tears in the membrane were easy to detect, and were usually caused by rough handling during preparation of the culture flasks. They were detectable during harvesting of the cells as a gentle squirt with a Pasteur pipette produced an obvious leakage. All cultures were so tested, and those with leaks excluded from the experimental results. With practice, only 1–3% of culture vessels suffered this fate. Several other more sensitive methods were employed to detect subtler forms of T cell leakage, for example through the larger pores of the nuclepore membranes. All of them, including a radioautographic technique, which could detect the presence of less than 1% of contaminating cells, failed to reveal significant escape of T cells from the upper to the lower compartment (Tables II–IV and VIII).

Cell collaboration in vivo is a markedly antigen-specific process in which T cells, as well as B cells, can dictate the specificity of the response (13, 21, 25. 38). It was therefore mandatory to establish the specificity both of elicitation of the T cell–derived factor and of its effect on B cells reactive to different antigenic determinants. The specific nature of thymus cell activation was demonstrated by the failure of even large numbers of ATC_{SRC} or $ATC_{F\gamma G}$ with, as well as without, antigen to produce a significant anti-DNP response to DNP KLH (Fig. 3). The specificity of the mediator for B cells was tested in a different way, by comparing the anti-DNP and anti-DRC responses in cultures

of DNP Fla–primed spleen cells immunized with both DNP KLH and DRC. Since only the anti-DNP response was enhanced (17, Table VI, Fig. 3), the soluble factor appeared to be antigen specific, both in its induction and its effector stages.

The demonstration that the same factor stimulated unprimed, as well as primed B cells (Table V), added further credence to its role as a physiological mediator of collaborative responses, especially since in vitro T cells are usually rate limiting (23).

Neither group of experiments formally defined the precise site of action of the factor in the lower compartment. Although the most likely final target was presumably the B cell, it was necessary to exclude an effect on the nonactivated T cells present. This could take the form either of a mitogenic reaction or of an adjuvant-like effect analogous to that of complexes of polyadenylic and polyuridylic acid (39). The development of normal antibody responses in the presence of the T cell factor by unprimed B cells, from either ATXBM spleen or nude spleen (Table V), coupled with the failure of T cell depletion of two populations of hapten-primed B cells (Table VI) to impair the capacity to respond to the factor, excluded a significant contribution by other T cells. In other words, the mediator of collaboration in a syngeneic system in vitro can trigger B cells independently of T cells other than those from which it was derived.

The demonstration of a factor of this kind effectively excludes theories of collaboration which invoke physical contact between rare antigen-reactive T and B cells. Although the precise nature of the mediator remains to be elucidated, the experiments reported here shed some light on its properties. For example, ATC selectively treated with inhibitors of protein synthesis (Table VII) in nontoxic doses (15) fail to release the collaborative moiety, suggesting it to be a product of actively metabolizing, and not of dying, cells. Its marked antigenic specificity for both carrier-reactive cells and hapten-primed cells implied that its structure could comprise part of or all of the putative T cell receptor "IgX," and in addition antigen. In fact it could be a macromolecular "IgX-antigen" complex. Alternatively, the factor elaborated might be antigen induced, and carry specific information in the form of the tertiary structure of antigen, a factor analogous to that described by Valentine and Lawrence (40). However its failure to penetrate dialysis membranes (Table VIII), unlike transfer factor (41), argues against this interpretation. On the other hand, its relatively large molecular size might be consistent with an RNA antigen complex of the form envisaged by Fishman and Adler (42), although this possibility is difficult to reconcile with the nonspecificity of formation of RNA antigen complexes, as reviewed by Roelants (43). The use of ribonuclease in further experiments could well settle this problem. The data presented in this paper are also entirely consistent with the concept that an "antigen-specific migration-inhibition factor" may be the mediator of cell cooperation, which Lachmann has proposed (44).

These findings thus favor, but by no means prove, the release by activated T cells of receptor-antigen complex which acts as the mediator of T-B cell collaboration. Since recent experiments indicate that the receptor on T cells is an IgM molecule (45), probably of 7S size (46), this may be a 7S IgM-antigen complex. Furthermore, since it was found that macrophages are essential for cell collaboration to occur (47), and since B cells adhere to macrophages (48) and AFC arise in close proximity to macrophages in vivo (49, discussed in reference 17), it seems likely that the receptor-antigen complex may bind to the surface of macrophages, and that immunization may occur on the surface of macrophages or perhaps dendritic cells.[3] This mechanism of cell collaboration is similar to one of those proposed by Miller (5) and also to one of the possible modes of action of "carrier antibody," as proposed by Brestcher and Cohn (50). Thus a matrix of antigenic determinants could be formed mimicking the surface of a thymus-independent polymeric antigen such as POL. This hypothesis provides a mechanism by which the antigen determinant matrix-B cell receptor interactions may be the same with both thymus-dependent and thymus-independent antigens. In other words, it would permit a common final pathway for B cell immunization, regardless of the nature of the antigen used.

The majority of the studies discussed in this paper were performed in a syngeneic system in which the T cell contribution was largely antigen specific. It should however, be noted that some restoration of the transfilter response occurred with ATC of other specificities in the presence of their activating antigen (Fig. 3). Unlike the specific moiety, this "factor" can collaborate across dialysis, as well as nuclepore membranes (Table VII), and can be produced in larger quantities by interaction between allogeneic lymphocytes.[4] Its capacity to stimulate B cells regardless of their antigenic specificity clearly distinguishes it from the factor discussed here. Nonspecific enhancement of antibody production by graft-vs.-host reactions such as described by Dutton et al. (37) and by Katz et al. (51) could be due to excessive secretion of a factor of this kind. However it is difficult to envisage a major role for such a nonspecific factor in the physiological, syngeneic situations such as usually occurs in vivo, in which cell cooperation is markedly specific (1, 5, 6, 38). A comparison of the properties of the factor produced in allogeneic reactions with those of the specific collaborative factor is detailed elsewhere.[4]

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[3] Preliminary experiments (Feldmann, unpublished) support this concept. It has been shown that peritoneal macrophages cultured in the lower compartment of double chambers, with ATC_{KLH} and DNP KLH in the upper compartment, but not with DNP KLH alone, are capable of immunizing B cells. The capacity of these macrophages to immunize B cells can be inhibited by anti-KLH or anti-DNP antisera, indicating that antigen and a T cell product are indeed bound to macrophages.

[4] Feldmann, M., and A. Basten. 1972. Cell interactions in the immune response *in vitro*. IV. Comparison of the effects of antigen specific and allogeneic factors on the antibody response. Manuscript in preparation.

SUMMARY

Tissue cultures with two compartments, separated by a cell impermeable nuclepore membrane (1 μ pore size), were used to investigate the mechanism of T-B lymphocyte cooperation. It was found that collaboration was as effective when the T and B lymphocyte populations were separated by the membrane as when they were mixed together. Critical tests were performed to verify that the membranes used were in fact cell impermeable. The specificity of the augmentation of the B cell response by various T cell populations was investigated. Only the response of B cells reactive to determinants on the same molecule as recognized by the T cells was augmented markedly. Specific activation of thymocytes by antigen was necessary for efficient collaboration across the membrane. The response of both unprimed and hapten-primed spleen cells was augmented by the T cell "factor" although, as expected, hapten-primed cells yielded greater responses. The T cell factor acted as efficiently if T cells were present or absent in the lower chamber. Thus the site of action of the T cell factor was not on other T cells, but was either on macrophages or the B cells themselves.

The T cell–specific immunizing factor did not pass through dialysis membranes. The experiments reported here help rule out some of the possible theories of T-B cell collaboration. Clearly T-B cell contact was not necessary for successful cooperation to occur in this system. Possible theoretical interpretations of the results and their bearing on the detailed mechanism of T-B lymphocyte cooperation are discussed.

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