CELL-TO-CELL INTERACTION IN THE IMMUNE RESPONSE

VII. Requirement for Differentiation of Thymus-Derived Cells*, ‡

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Cooperation between thymus-derived (T)[1] and nonthymus-derived (B) cells occurs in primary and secondary humoral antibody responses of mice to heterologous erythrocytes and serum proteins (1–3). Recent experiments have demonstrated that T as well as B cells can react to antigen specifically and therefore dictate the specificity of the response (1, 4). Furthermore, the requirement for T cells in antibody production appears to depend on the way in which antigen is presented rather than on the specificity of the antigenic determinant concerned (5). T cells thus act as helper cells in facilitating the response of B cells to antigen. As recirculating small lymphocytes (6), T cells would be particularly well suited to the task of picking up antigen and concentrating it in regions in lymphoid tissues where B cells reside. Concentration may be an entirely passive process in which antigen is focused directly onto B cells, antigen bridges linking T and B cells (7). On the other hand, antigen concentration may occur only after differentiation of T cells: in other words, these may first have to interact with some determinants on the antigen, differentiate, and divide to elaborate some factor that mediates the induction process. This factor may be highly specific,

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[1] *Abbreviations used in this paper:* B, nonthymus-derived; FALG, fowl anti-mouse lymphocyte globulin; FALS, fowl anti-mouse lymphocyte serum; FγG, fowl immunoglobulin G; FγG AP, alum-precipitated FγG given with 2×10^9 killed pertusis organisms; HGG, human gamma globulin; HRBC, horse erythrocytes; NIP, 4-hydroxy-3-iodo-5-nitrophenylacetic acid; PFC, plaque-forming cells; RALG, rabbit anti-mouse lymphocyte globulin; SRBC, sheep erythrocytes; T, thymus-derived; TDL, thoracic duct lymphocytes; TxBM, adult thymectomized, irradiated, and marrow protected.

a type of immunoglobulin molecule (IgX), serving to concentrate other determinants of the antigen onto B cells (8); or it may be a nonimmunoglobulin, nonantigen, specific factor carrying information in the form of the tertiary structure of the antigenic determinant and capable of activating B cells (9). Alternatively, the factor may be nonspecific, acting at short range to lower the threshold of stimulation of B cells, a type of pharmacological mediator analogous to those produced in delayed hypersensitivity reactions (10).

If T cells act simply as passive carriers of antigen, their function should be mimicked by inert antigen-coated nonreactive cells, provided these are distributed in vivo in the same way as T cells. The experiments reported here were designed to test this possibility. It will be shown that triggering of either primed or unprimed B cells, residing in the spleen, cannot be achieved by delivering into the spleen inert or nonreactive T or B cells artificially coated with the antigen in question, and that differentiation of specific T cells appears to be mandatory in a collaborative response.

Materials and Methods

Animals.—Male and female mice of the highly inbred CBA and C57BL strains and F_1 hybrids from crosses between these two strains were used. The pedigree and maintenance of these mice have been given previously (11). White Leghorn fowls, 6 months old, were obtained from a commercial breeder and kept in suitable poultry cages.

Cell Suspensions.—Suspensions of cells from thymus, marrow, spleen, and thoracic duct lymphocytes (TDL) were obtained as described before (11, 12).

Antigens.—Fowl immunoglobulin G (FγG), sheep erythrocytes (SRBC), and horse erythrocytes (HRBC) were obtained as described elsewhere (11–13). Human gamma globulin (HGG) was supplied from the Commonwealth Serum Laboratories, Melbourne, Australia, in 10-ml vials containing 160 mg protein/ml. 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) was prepared according to the method of Brownstone et al. (14). NIP azide was conjugated onto the proteins, FγG or HGG, according to the technique given in Brownstone et al. (14). The substitution ratio (groups of hapten per mole of protein) was determined spectrophotometrically at 430 mμ and the values for the extinction coefficient, k, was that given in Brownstone et al. (14). Conjugates with 10 hapten groups/molecule FγG or HGG were generally used.

Injections.—Cells and antigens were injected intravenously or intraperitoneally as stated below.

Operative Procedures.—The techniques of thymectomy (15) and of thoracic duct cannulation (11) have been given elsewhere.

Irradiation.—Details of the irradiation technique were given in the previous paper in this series (3). When thymectomized mice were irradiated, they received within 1–3 hr an intravenous injection of 5×10^6 syngeneic marrow cells. All irradiated mice were given polymyxin B (100,000 IU/liter) and neomycin (10 mg/liter) in the drinking water.

Immunization.—An intravenous injection of 4×10^8 washed SRBC or HRBC was used to immunize mice against these heterologous erythrocytes. Immunization to protein antigens and to hapten-protein conjugates was performed by giving 500 μg of alum-precipitated antigen and 2×10^9 killed pertussis organisms[2] intraperitoneally. The technique of alum precipitation was that of Proom (16). 100 μg of fluid protein antigen was given intraperitoneally as a booster in some experiments.

[2] Pertussis vaccine (4×10^{10} killed organisms/ml), Commonwealth Serum Laboratories Melbourne, Australia.

Induction of Tolerance to FγG.—Adult mice were rendered tolerant to FγG by the cyclophosphamide technique described in a previous paper in this series (17). An intraperitoneal injection of 5 mg alum-precipitated FγG and 2×10^9 killed pertussis organisms (FγG AP) was followed the next day by a subcutaneous injection of cyclophosphamide[3] using a dose of 1 mg/10 g body weight. The mice were used 2 wk later. Assays for tolerance were performed in some mice after challenging with 500 μg of FγG AP either by measuring the serum-binding capacity using radioiodinated FγG (18) or by determining splenic anti-FγG plaque-forming cells (PFC) (13).

Preparation of Antisera.—The following antisera were used: (*a*) mouse anti-H2 sera (CBA anti-C57BL and C57BL anti-CBA) prepared as described before (11); (*b*) mouse anti-FγG serum obtained by immunizing CBA mice with one intraperitoneal injection of 500 μg of FγG AP followed 6 wk later by one intraperitoneal injection of 100 μg of fluid FγG, the mice being bled 7–10 days later; (*c*) anti-SRBC immunoglobulin G (13); (*d*) fowl anti-mouse lymphocyte serum (FALS) prepared by giving fowls two intravenous injections of 10^9 CBA or (CBA × C57BL)F_1 thymus cells 3 wk apart followed 1 wk later by bleeding. The immunoglobulin G-containing fraction was separated from the serum by the technique described previously (13) and the fowl anti-mouse lymphocyte globulin (FALG) fraction titrated for its lymphoagglutinating activity and stored in small aliquots at −20°C; (*e*) rabbit anti-mouse lymphocyte globulin (RALG) and normal rabbit globulin were prepared as described previously (19); (*f*) anti-θ C3H serum (20).

Measurement of Antibody Activity.—The anti-SRBC titer of the serum of mice immunized to SRBC was determined by hemagglutination assay (21). The titer of mouse anti-FγG serum was measured either by its hemagglutination activity against SRBC coated with FγG in the form of fowl anti-SRBC immunoglobulin G, as described elsewhere (13) or by microprecipitation assay with FγG-^{125}I, in a manner analogous to the previously described method of testing for anti-immunoglobulin sera (18). The antibody activity of the FALG and RALG preparations were titrated by the techniques of lymphocytotoxicity and lymphoagglutination (22).

Detection of Antibody-Forming Cells.—PFC in spleen cell suspensions were detected according to the method of Cunningham and Szenberg (23). The following target erythrocytes were used: (*a*) HRBC or SRBC to detect anti-HRBC and anti-SRBC PFC, respectively; (*b*) FγG-coated SRBC to detect anti-FγG PFC according to the technique of Miller and Warner (13); (*c*) NIP azide-coated SRBC according to the technique of Pasanen and Mäkelä (24). Guinea pig serum served as a source of complement and a rabbit anti-mouse immunoglobulin serum diluted appropriately was added to detect indirect PFC as described elsewhere (25, 26).

Preparation of Antigen-Coated Cells.—Lymphoid cell suspensions from the spleen or thoracic duct lymph were coated with antigen according to one of two methods. (*a*) FALG-coated cells were prepared as follows: 8×10^7 TDL in 3.75 ml Eisen's balanced salt solution (27) was added 0.25 ml of FALG (4 mg IgG/ml) of known lymphoagglutinating titer. The suspension was incubated for 1 hr at 37°C and washed twice before injection, 2×10^7 cells being given intravenously to each recipient. (*b*) A second method utilized the ability of B cells to bind antibody-antigen complexes (28). Anti-FγG–FγG.NIP-coated cells were obtained as follows: 5×10^7 spleen cells/ml were incubated for 30 min at 37°C with 1:5 dilution of mouse anti-FγG serum (hemagglutinating titer 1:256). The cells were then spun once, the supernatant removed, and the pellet resuspended (without washing) in Dulbecco's balanced salt solution (29) containing 100 μg FγG.NIP/ml, 5×10^7 cells being suspended in 2 ml. After incubation for a further 30 min at 37°C, the cells were washed twice and 2×10^7 injected intravenously into each recipient.

Radioisotopes.—^{51}Cr as Na_2CrO_4 in isotonic saline and ^{125}I were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. FALG was trace labeled with ^{125}I

[3] Cyclophosphamide (endoxan, asta), Charles McDonald, Caringbah, Australia.

by the method of Byrt and Ada (30), using a low substitution rate (1 iodine atom/molecule) so as not to interfere with antibody activity.

Labeling Cells Radioactively and Tracing Their Distribution.—In some experiments, TDL were labeled with ^{51}Cr and their localization in the liver and spleen, 4 hr after intravenous injection into mice, was determined according to the technique described by Martin (31). In other experiments, TDL were incubated in vitro with trace labeled FALG-^{125}I (1 μg FALG/10^7 cells) for 1 hr at 37°C. Excess radioactivity was removed by centrifugation through fetal calf serum[4] gradients. Approximately 4×10^7 FALG-^{125}I–coated TDL were injected intravenously into mice as described below. Radioautographs of spleen sections and TDL smears were prepared, at appropriate times, the slides being dipped in Eastman Kodak NTB-2 emulsion, developed, and stained.

Antimetabolite Treatment of Lymphocytes.—5×10^7 TDL/ml Dulbecco's solution were incubated in vitro at 37°C for ½ hr with 25 μg mitomycin C[5]/ml, and then washed twice. The viability of the mitomycin-treated TDL was 98% as determined by the trypan blue exclusion technique (32).

Statistical Analysis.—Calculations of the geometric means, upper and lower limits of the SE and P values according to the nonparametric rank test, were performed using an IBM 7044 computer. In comparison of the means of any two groups of observations, a significance level of 0.05 was chosen.

EXPERIMENTAL PROCEDURE

The aim of these experiments was to study the response of thymus-deprived mice given lymphocytes which lacked specific T cells capable of reacting with a particular antigen but which could be artificially coated with that antigen. One further requirement was that the cells should home to the spleen after injection. In one set of experiments TDL from mice rendered specifically tolerant to FγG were incubated with FALG which coated the cells because of its antibody activity. FALG was found not to be immunosuppressive in mice and had neither cytotoxic nor opsonizing effects. The FALG-coated cells migrated as normal lymphocytes after intravenous injection into mice. A second set of experiments utilized the recent finding that B cells can be coated with antibody-antigen complexes (28). Spleen from thymectomized, irradiated, and marrow protected (TxBM) mice served as a source of B cells which were incubated with anti-FγG–FγG.NIP and injected together with NIP-sensitive cells into irradiated mice. The capacity of the TxBM mice in the first set of experiments and of the irradiated mice in the second set to produce an antibody response to the antigen in question was then tested with or without added T cells.

RESULTS

Effect of Incubating Mouse TDL with FALG.—The lymphoagglutinating and lymphocytotoxic titers of two batches of FALG against mouse TDL are shown in Table I. Even normal FγG had some agglutinating activity. This was increased markedly by immunizing fowls with mouse thymus lymphocytes. The FALG preparations were not cytotoxic for mouse TDL in the presence of guinea pig complement. This was expected since avian immunoglobulins do not bind mammalian complement (33).

[4] Fetal calf serum was obtained from Commonwealth Serum Laboratories.

[5] Mitomycin C (Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan).

Distribution of FALG-Coated TDL.—The localization of FALG-incubated ^{51}Cr-labeled TDL was studied after intravenous injection into normal mice. Radioactivity was determined in the liver and spleen 4 hr after injection. No significant "opsonization" of mouse TDL resulted from incubation with FALG (Table II). By contrast, a high proportion of TDL were opsonized by the reference RALG preparation, as had been found in previous work (22, 31).

TABLE I

Effects of FALG on Mouse TDL In Vitro

Preparation	Lymphoagglutinating titer	Lymphocytotoxic index*
Dulbecco's medium	0	0
FγG	1:16	0
$FALG_1$	1:128	0
$FALG_2$	1:64	0

* Trypan blue exclusion technique (32).

TABLE II

Diversion of Mouse TDL from Spleen to Liver after Incubation with Various Antisera

Serum used	TDL diverted to liver (in excess of normal percentage)*, ‡
	%
NFS§	1.40
$FALS_1$	1.39
$FALS_2$	1.41
FγG	1.09
$FALG_1$	1.07
$FALG_2$	0.99
RALG	79.00

* For calculations see reference 31.

‡ Average of eight test mice used for each serum.

§ NFS, normal fowl serum.

Radioautographs of smears of TDL incubated with FALG-^{125}I were prepared. More than 95% of these cells showed binding of the radioiodinated material. 4×10^7-labeled TDL were injected intravenously either into intact mice, which were killed 4, 12, and 24 hr later to provide spleen sections, or into mice in which a thoracic duct fistula had been established. Among the TDL emerging from these mice in the ensuing 12–36 hr, no cell carrying labeled material was detected. Examination of the spleen sections revealed that even as early as 4 hr after injection, only the occasional cell still carried the FALG, the majority of the label being scattered throughout the white pulp.

TABLE III

Serum Anti-FγG Antibody in (CBA × C57BL)F1 Mice Given FγG AP and Cyclophosphamide 2 Wk before

Group*	Pretreatment	Antigen challenge 2 wk later	Reciprocal serum antibody titers 15 days postchallenge	
			50% precipitation of FγG-^{125}I by serum	Hemagglutinin titer (SRBC)
1	Day 1: saline Day 2: saline	500 μg FγG AP and 4×10^8 SRBC	120 160 160 240	128 256 512 256
2	Day 1: saline Day 2: cyclophosphamide	500 μg FγG AP and 4×10^8 SRBC	160 240 240 320	256 64 512 1024
3	Day 1: 5 mg FγG AP Day 2: cyclophosphamide	500 μg FγG AP and 4×10^8 SRBC	<1 <1 <1 <1	64 256 256 512

* Four mice per group.

TABLE IV

Anti-FγG PFC Response in Spleens of Nontolerant or FγG-Tolerant (CBA × C57BL)F1 Mice or TxBM CBA Mice

Group	Pretreatment of mice	Antigen challenge	No. of mice in group*	Anti-FγG PFC per spleen	
				19S PFC‡ (4–5 days)	7S PFC‡ (6–7 days)
1	Day 1: saline Day 2: saline	500 μg FγG AP 2 wk later	12	20,530 (23,240–18,140)§	58,010 (76,400–44,050)
2	Day 1: saline Day 2: cyclophosphamide	500 μg FγG AP 2 wk later	12	16,850 (17,960–15,800) ‖	33,060 (40,080–27,260) ‖
3	Day 1: 5 mg FγG AP Day 2: cyclo.	500 μg FγG AP 2 wk later	12	70 (180–30) ‖	5 (17–2) ‖
4	TxBM	500 μg FγG AP 2 wk postirradiation	16	510 (700–370)	2 (4–1)

* Half the mice were killed at 4–5 days to determine 19S PFC, the other half at 6–7 days to assay 7S PFC.
‡ 19S PFC, direct plaque-forming cells; 7S PFC, indirect or developed plaque-forming cells.
§ Geometric means, upper and lower limits of SE.
‖ *P* values <0.005.

Induction of Tolerance to FγG in Adult Mice.—The cyclophosphamide method was highly successful in inducing specific immunological tolerance to FγG in mice as indicated by the low serum antibody titers and splenic anti-FγG PFC (Tables III and IV). The state of unresponsiveness was specific since the mice could respond normally to SRBC (Table III). The peak 19S and 7S anti-FγG PFC response in FγG-tolerant mice was of the same order of magnitude as that occurring after challenging (TxBM) mice with FγG AP (Table IV). The low response of these thymectomized animals is in accordance with previous studies indicating thymus-dependency of the humoral antibody response of mice to FγG (13).

TABLE V

Serum Anti-FγG Antibody in TxBM CBA Mice Receiving TDL from Nontolerant or FγG-Tolerant (C57BL × CBA)F1 Mice

TDL given	Antigen challenge in vivo	Anti-FγG antibody (%FγG-^{125}I bound/10 μl antiserum)			Range of peak reciprocal anti-SRBC hemagglutinin titer (15 or 22 days)
		15 days	22 days	42 days	
None	500 μg FγG AP and 4×10^8 SRBC	12.1 (7)*	2.8 (6)	26.0 (3)	<2–4 (7)
2×10^7 cells from FγG-tolerant mice	500 μg FγG AP and 4×10^8 SRBC	2.6 (11)	0.5 (11)	2.2 (6)	32–1024 (11)
2×10^7 cells from cyclophosphamide-pretreated mice	500 μg FγG AP and 4×10^8 SRBC	11.5 (12)	50.4 (10)	61.8 (5)	32–1024 (12)

* No. of mice is shown in parentheses.

Adoptive Transfer of Antibody Response to FγG.—Since TxBM CBA mice failed to respond to FγG, they were used as recipients of (CBA × C57BL)F1 TDL to study the capacity of normal and tolerant TDL populations to transfer adoptive primary antibody responses to FγG. When 500 μg of FγG AP was given in vivo to TxBM mice, only recipients of nontolerant TDL could respond to FγG (Table V). TxBM mice receiving TDL from FγG-tolerant donors had very little detectable serum antibody to FγG, although they could now produce antibody to SRBC. When instead of giving FγG in vivo, the TDL were incubated in vitro with FALG, washed, and injected intravenously into TxBM mice, again only recipients of nontolerant TDL responded to FγG whereas mice given FγG-tolerant TDL failed to do so, although a good response to SRBC was obtained (Table VI). It is clear from these results that FALG is not immunosuppressive in mice and that FALG-coated TDL can adoptively transfer antibody responsiveness to FγG provided they are obtained from nontolerant donors.

Similar results were obtained in separate experiments when the response was measured in terms of PFC per spleen. Here the control antigen could not be SRBC (since FγG-sensitized SRBC were the target erythrocytes used in the

TABLE VI

Serum Anti-FγG Antibody in TxBM CBA Mice Receiving FALG Incubated TDL from Nontolerant or FγG-Tolerant (C57BL × CBA)F₁ Mice

TDL given	Antigen challenge in vivo	Anti-FγG antibody (% FγG-^{125}I bound/10 μl antiserum)			Range of peak reciprocal anti-SRBC hemagglutinin titer (15 or 22 days)
		15 days	22 days	42 days	
None	4×10^8 SRBC	0.5 (8)*	0.2 (8)	1.0 (6)	<2–16 (8)
2×10^7 cells from FγG-tolerant mice	4×10^8 SRBC	1.2 (19)	1.4 (18)	12.1 (10)	32 –128 (13)
2×10^7 cells from cyclophosphamide-pretreated mice	4×10^8 SRBC	22.6 (11)	17.1 (11)	42.0 (8)	32–512 (8)

* No. of mice is shown in parentheses.

TABLE VII

Anti-FγG PFC Response in Spleens of TxBM CBA Mice Receiving TDL from Nontolerant or FγG-tolerant (CBA × C57BL) F₁ Mice

TDL given	Antigen challenge in vivo	No. of mice in group*	Peak anti-FγG PFC per spleen		Per cent reduction of PFC with	
			19S PFC	7S PFC	CBA anti-C57BL serum	C57BL anti-CBA serum
None	500 μg FγG AP 2 wk post-irradiation	16	510 (700–370)‡, §	2 (4–1)§	—	—
2×10^7 cells from FγG-tolerant mice	500 μg FγG AP	12	230 (760–70) ‖	210 (300–140) ‖	—	—
2×10^7 cells from cyclophosphamide-pretreated mice	500 μg FγG AP	12	2620 (3270–2090) ‖	16,600 (20,350–13,530) ‖	25% (19S) 10% (7S)	83% (19S) 90% (7S)

* Half the mice were killed at 5–6 days to determine 19S PFC, the other half at 7 days to assay 7S PFC.
‡ Geometric means, upper and lower limits of SE.
§ Data from Table IV, group 4.
‖ P Values < 0.005.

anti-FγG PFC assay). HRBC were used instead. When FγG was given in vivo, only TxBM recipients of nontolerant TDL could produce a good anti-FγG PFC response. Treatment of these PFC with anti-H2 serum identified them as derived from the cells of the TxBM hosts and not from the TDL (Table VII).

When the TDL were incubated in vitro with FALG, only nontolerant TDL enabled their hosts to respond by producing anti-FγG PFC and these were derived, not from the TDL, but from the hosts. The responsiveness to HRBC in TxBM recipients of both nontolerant and FγG-tolerant TDL, incubated in vitro with FALG, established the specificity of tolerance in the TDL population and the failure of FALG to act as an immunosuppressant in mice (Table VIII). The inability of tolerant TDL, artificially coated with FγG (in the form of FALG), to induce a response in TxBM hosts, which do not lack B cells, suggested that normal T cells with receptors specific for FγG were required to facilitate antibody formation. Whether differentiation of specific T cells was essential was examined with the aid of antimetabolites.

Effect of Mitomycin C on the Ability of TDL to Transfer Antibody Responsiveness.—The ability of normal TDL to transfer anti-FγG activity to TxBM mice is evident from the above results. This occurred both when the recipients were challenged in vivo with FγG or when they were not challenged but received, instead, FALG-coated cells. By contrast, TDL from FγG-tolerant mice failed to transfer responsiveness to FγG under identical experimental situations. As shown in Table IX, this failure (group 1) could readily be reversed by supplementing with normal TDL (group 2). If, however, the normal TDL had previously been incubated in vitro with mitomycin C and then washed, they were unable either to transfer, on their own, a primary adoptive response to FγG or HRBC (group 4), or to reverse the failure of tolerant TDL to allow TxBM hosts to respond to FγG (group 5). The fact that a response to HRBC was obtained in TxBM recipients of a mixed population of FγG-tolerant TDL and mitomycin-treated normal TDL excluded the possibility of carry over of antimetabolite from one population to the other. As seen in Table X, the ability of mitomycin-treated TDL from FγG-primed mice to interact with the B cells (obtained by anti-θ treatment of FγG-primed spleen cells) was likewise impaired. The degree of impairment (10–100-fold) was measured by comparing the collaborative response of 10^7 mitomycin-treated primed TDL (group 7) with that of graded numbers of untreated primed TDL (groups 4 and 6). Reversal of mitomycin-induced suppression with normal TDL (group 9) established that carry over to B cells did not occur. The viability of TDL exposed to the antimetabolite was determined by using the dye exclusion technique: over 98% of cells were viable according to this parameter. The localization of ^{51}Cr-labeled mitomycin-treated TDL after injection into mice was found to be normal (Table XI). It would seem, therefore, that mitomycin impaired the ability of specific T cells to collaborate with B cells in both primary and secondary responses. Presumably this occurred as a result of interference with differentiation and/or division.

Adoptive Transfer of Antibody Response to NIP.—In the above experiments, a tolerant TDL population (composed mostly of T cells [6]) was artificially coated with the antigen and used essentially as antigen-coated "particles" able

TABLE VIII

Anti-FγG PFC Response in Spleens of TxBM CBA Mice Receiving FALG- incubated TDL from Nontolerant or FγG-tolerant (CBA × C57BL)F_1 Mice

TDL given	Antigen challenge in vivo	No. of mice in group*	Peak anti-FγG PFC per spleen		% reduction of anti-FγG PFC with		Peak 19S anti-HRBC PFC
			19S PFC	7S PFC	CBA anti-C57BL serum	C57BL anti-CBA serum	
None	500 μg FγG AP + 4×10^8 HRBC 2 wk post-irradiation	16	510 (700–370)‡, §	2 (4–1)§	—	—	390 (690–230)
2×10^7 FALG-coated cells from FγG-tolerant mice	4×10^8 HRBC	16	140 (160–120) ‖	2 (4–1) ‖	—	—	32,230 (40,640–25,560)
2×10^7 FALG-coated cells from cyclophosphamide-pretreated mice	4×10^8 HRBC	16	7690 (8890–6640) ‖	14,730 (16,640–13,040) ‖	20% 19S 0% 7S	99% 19S 99% 7S	29,210 (38,680–22,070)

* Half the mice were killed at 5–6 days to determine 19S anti-FγG PFC, the other half at 7 days to assay 7S anti-FγG PFC and 19S anti-HRBC PFC.

‡ Geometric means, upper and lower limits of SE.

§ Data from Table IV, group 4.

‖ *P* values <0.005.

to deliver the antigen into the spleen where B cells capable of producing antibody reside. No response was obtained in the absence of specific T cells which could differentiate and/or divide. In the following experiments, B cells, not T cells, were used as antigen-bearing particles. The technique utilized the ability of B cells to bind antibody-antigen complexes (28). Cells from the spleen of TxBM mice (which contain B cells relatively uncontaminated by T cells) were

TABLE IX

Anti-FγG PFC Response in Spleens of TxBM CBA Mice Receiving FALG-Incubated TDL from Nontolerant or FγG-tolerant CBA Mice: Effect of Mitomycin Treatment of Normal TDL as a Source of Unprimed T Cells

Group	TDL given	Antigen challenge in vivo	No. of mice in group	7S PFC per spleen at 7 days	
				Anti-FγG*	Anti-HRBC‡
1	2×10^7 FALG-incubated cells from FγG-tolerant CBA mice	4×10^8 HRBC	6	4 (8–2)§	33,480 (36,000–31,150)
2	2×10^7 FALG-incubated cells from FγG-tolerant CBA mice + 2×10^7 cells from normal CBA mice	4×10^8 HRBC	6	7100 (7790–6470)	43,980 (58,920–32,830)
3	2×10^7 FALG-incubated cells from normal CBA mice	4×10^8 HRBC	4	8380 (11,330–6200)	25,640 (28,180–23,320)
4	2×10^7 mitomycin-treated and FALG-incubated cells from normal CBA mice	4×10^8 HRBC	4	10 (5–2)	250 (1630–40)
5	2×10^7 FALG-incubated cells from FγG-tolerant CBA mice + 2×10^7 mitomycin-treated cells from normal CBA mice	4×10^8 HRBC	6	400 (590–270)	58,350 (73,280–46,470)

* The differences between anti-FγG PFC in groups 1 and 2, 3 and 4, and 3 and 5 were significant ($P < 0.01$) Anti-FγG PFC in groups 2 and 3 did not differ significantly.

‡ The differences between anti-HRBC PFC in groups 3 and 4 were significant ($P < 0.01$).

§ Geometric means, upper and lower limits of SE.

preincubated in vitro with anti-FγG–FγG·NIP and injected with or without normal TDL (a source of T cells) into hosts that had received 800 R total body irradiation. Only mice given both cell types could produce an anti-NIP antibody response (Table XII). Thus, when B cells were used as antigen-bearing particles a primary response did not take place, unless T cells were present. Radioautographic observations, to be given in detail elsewhere,[6] have shown that B cells

[6] A. Basten, J. F. A. P. Miller, J. Sprent, and J. Pye. Receptors for antibody-antigen complexes on B lymphocytes. Manuscript in preparation.

TABLE X

Anti-FγG PFC Response in Spleens of Irradiated Mice Receiving T and B Cells: Effect of Mitomycin C Treatment of Primed TDL as a Source of Primed T Cells

Group	Cells given	No. of irradiated recipients*	7S anti-FγG PFC per spleen at 7 days‡
1	5×10^6 NMS-treated§ spleen cells from FγG-primed mice	5	38,170 (47,860–30,020)
2	5×10^6 anti-θ-treated spleen cells‖ from FγG-primed mice (primed B cells)	5	130 (180–90)
3	10^5 TDL from FγG-primed mice + primed B cells	5	5600 (8730–3620)
4	10^5 TDL from FγG-primed mice	6	150 (410–50)
5	10^6 TDL from FγG-primed mice + primed B cells	6	46,370 (69,000–31,170)
6	10^6 TDL from FγG-primed mice	5	5710 (8520–3820)
7	10^7 TDL from FγG-primed mice¶	5	224,000 (400,000–145,000)
8	10^7 mitomycin-treated TDL** from FγG-primed mice + primed B cells	6	9320 (13,400–6480)
9	10^7 mitomycin-treated TDL** from FγG-primed mice	5	290 (1270–70)
10	10^7 mitomycin-treated TDL** from FγG-primed mice + primed B cells and 5×10^6 normal TDL	5	60,440 (81,060–45,000)
11	10^7 normal TDL	5	1460 (1830–1170)

* 100 μg fluid FγG/recipient.

‡ The differences in PFC responses between groups 1 and 2, 2 and 3, 2 and 5, 5 and 8, 8 and 10 were significant (P values <0.05).

§ NMS, normal mouse serum.

‖ Cells incubated with anti-θ serum (1:4) at concentration 15×10^7 cells/ml plus guinea pig complement (1:6) at concentration of 5×10^7 cells/ml.

¶ The addition of primed B cells did not significantly enhance the PFC response in this instance and the data have therefore been excluded.

** TDL incubated with 25 μg/ml mitomycin C at a concentration of 5×10^7 cells/ml.

coated in this way homed to the spleen with labeled antigen which eluted during the next 24 hr.

The B cells in the above experiment were derived from nonprimed mice. It remained possible that primed B cells could be induced to respond in the absence

TABLE XI

Effect of Mitomycin on Localization of Mouse TDL in Spleen and Liver

Medium used to incubate TDL*	Spleen radioactivity: liver radioactivity
Dulbecco's solution	1.63
Dulbecco's solution containing 25 μg mitomycin/ml	1.38

* ^{51}Cr-labeled TDL incubated for 30 min at 37°C; 5×10^6 TDL injected into each of four recipients; livers and spleens removed 4 hr later.

TABLE XII

Adoptive Transfer of Primary Anti-NIP PFC Response by Antigen-Coated B Cells

No. and source of B cells	No. and source of T cells	No. of irradiated recipients	19S anti-NIP PFC per spleen at 7 days
2×10^7 spleen cells from TxBM mice preincubated with anti-FγG antibody and FγG·NIP	—	10	40 (60–20)*· ‡
2×10^7 spleen cells from TxBM mice preincubated with anti-FγG antibody and FγG·NIP	5×10^6 normal TDL	10	710 (800–640)‡
—	5×10^6 normal TDL	10§	70 (80–65)

* Geometric means, upper and lower limits of SE.

‡ Addition of spleen cells from TxBM mice, not incubated with antibody and antigen, made no difference to these results.

§ These 10 mice received 500 μg of FγG AP in vivo.

of T cells. Since TxBM mice could not be primed to NIP by immunizing with FγG·NIP, the following two experiments were performed. Mice were immunized with HGG·NIP and used 1–2 months later as donors of spleen cells containing B cells primed to NIP. These cells were injected into irradiated mice

which were challenged with either HGG·NIP or FγG·NIP. The former produced a good response, the latter, a poor one (Table XIII). The response of mice challenged with FγG·NIP was considerably enhanced by adding spleen cells from other mice primed only to the heterologous carrier protein, FγG. These results essentially confirm previous work with hapten-protein conjugates, in which the necessity for carrier-primed T cells was established (7). In a second experiment, irradiated mice received a mixed population of two types of spleen cells: one was obtained from TxBM mice and incubated in vitro with anti-FγG–FγG·NIP; the other from HGG·NIP-primed mice. The irradiated mice were not challenged with antigen other than that carried by the cells from TxBM mice. As shown in Table XIV, a good anti-NIP PFC response was obtained. This might at first sight imply that antigen-coated B cells could substitute for carrier-primed T cells. If, however, the spleen cells from HGG·NIP-primed mice

TABLE XIII

Adoptive Transfer of Anti-NIP PFC Response in CBA Mice

Donor of spleen cells*	Antigen challenge in vivo‡	No. of irradiated recipients	7S anti-NIP PFC per spleen at 7 days
HGG·NIP-primed mice	HGG·NIP	6	452,000 (598,180–341,490)§
HGG·NIP-primed mice	FγG·NIP	8	4210 (8740–2020)
HGG·NIP-primed mice and FγG-primed mice	FγG·NIP	5	118,170 (126,900–110,050)

* 2×10^7 spleen cells from each donor given to each recipient.

‡ 100 μg of fluid antigen given intraperitoneally.

§ Geometric means, upper and lower limits of SE.

were pretreated in vitro with anti-θ serum and complement, to eliminate the majority of T cells from this population, the NIP response was abolished (Table XIV). It was restored by supplementing with normal spleen cells (which contain T cells). The results therefore indicate that antigen-coated B cells cannot substitute for T cells even when primed B cells are present. T cells are thus required for both normal and primed B cells to respond to the antigen.

DISCUSSION

Two points clearly emerge from the experiments described. First, T cells are specifically involved in inducing unprimed or primed B cells to produce antibody. Second, T cells must, themselves, react with the antigen and undergo differentiation before they can influence B cells.

The requirement for T cells was examined by attempting to induce a response in unprimed or primed B cells to antigens carried on the surface of cells which were essentially inert or nonreactive. Antigen-coated cells were prepared in two different ways. In the first, TDL from FγG-tolerant mice were incubated in

vitro with FALG (which was shown not to be immunosuppressive in mice) to provide a source of FγG-coated cells. These had normal migratory characteristics and therefore homed to the spleen in the same way as uncoated TDL. On injection into thymectomized irradiated mice, a response to HRBC but not to FγG was obtained even though the hosts were shown to contain B cells capable of responding to FγG (Table VIII). The second method of coating cells with antigen was based on the fact that B cells possess a receptor for antibody-antigen complexes (28). Cells from the spleens of TxBM mice were incubated in

TABLE XIV

Adoptive Transfer of Secondary Anti-NIP PFC Response by Antigen-Coated B Cells and NIP-Primed Cells

Group	Source of B cells*	Source of NIP-primed cells*	Supplementary cells	No. of irradiated recipients	7S anti-NIP PFC per spleen at 7 days‡
1	Spleen cells from TxBM mice preincubated with anti-FγG antibody and FγG·NIP	Spleen cells from HGG·NIP-primed mice preincubated with NMS	—	10	18,495 (20,545–16,645)§
2	Spleen cells from TxBM mice preincubated with anti-FγG antibody and FγG·NIP	Spleen cells from HGG·NIP-primed mice preincubated with anti-θ‖ serum	—	9	70 (170–25)
3	Spleen cells from TxBM mice preincubated with anti-FγG antibody and FγG·NIP	Spleen cells from HGG·NIP-primed mice preincubated with anti-θ‖ serum	5×10^5 normal spleen cells	4	80 (360–20)
4	Spleen cells from TxBM mice preincubated with anti-FγG antibody and FγG·NIP	Spleen cells from HGG·NIP-primed mice preincubated with anti-θ‖ serum	10^7 normal spleen cells	4	2640 (3340–2090)
5	Spleen cells from normal mice preincubated with NMS and FγG·NIP	Spleen cells from HGG·NIP-primed mice preincubated with NMS	—	6	15 (40–5)

* Number of cells was 2×10^7/recipient.

‡ The differences in PFC responses between groups 1 and 2, 4 and 2, 4 and 3 were significant ($P < 0.002$, $P < 0.01$ $P < 0.05$).

§ Geometric means, upper and lower limits of SE.

‖ Treatment with anti-θ serum as in Table X.

vitro with anti-FγG–FγG·NIP, washed, and injected into irradiated mice. A 19S anti-NIP antibody response was obtained, but only if normal T cells, in the form of TDL, were also given (Table XII). A 7S anti-NIP antibody response occurred in irradiated mice given both NIP-coated B cells, prepared as above, and NIP-primed cells obtained from the spleens of HGG·NIP-primed mice (Table XIV). This response was, however, abolished by eliminating the T cells present in the spleens of the primed mice by treatment with anti-θ serum and complement (Table XIV). Some reconstitution of the NIP response was achieved by supplementing with normal spleen cells. T cells must thus be specifically involved in inducing unprimed or primed B cells to produce antibody.

The antigen-coated nonreactive T or B cells evidently acted as a means of concentrating antigen into the spleen where it eluted and presumably was made available to specific T cells. Only then could collaboration take place.

The ability of irradiated recipients of spleen cells from HGG·NIP-primed mice and of FγG·NIP-coated B cells to produce a substantial anti-NIP response (Table XIV) provides another example of overriding of the carrier effect (34). In the present model, overriding occurred only in the presence of T cells since anti-θ serum treatment of spleen cells from HGG·NIP-primed mice abrogated the response. The data are therefore consistent with the previous observation that, for an adoptive memory response, T cells are essential but need not be derived from mice primed to the antigen in question: in other words, normal T cells can substitute for specifically primed T cells (3).

Treatment of TDL (a source of T cells) from both unprimed and primed mice with mitomycin C impaired their ability to collaborate with B cells (Tables IX and X). In contrast, Katz et al. found little impairment in function of carrier specific cells (known to be T cells [35, 36]) after irradiation in vitro (37). The effectiveness of mitomycin C in abrogating T cell function may therefore depend not so much on its antimitotic activity as on its capacity to interfere with cell differentiation at the dose level used (38). The failure of mitomycin[7] as well as irradiation (39) to prevent collaboration of activated thymus cells (which have already undergone some degree of differentiation) with B cells in vitro is consistent with this interpretation. Taken together these findings suggest that the helper function of T cells may be dependent on cell differentiation, not on cell division. Thus before collaboration can occur, T cells need to be activated by antigen to differentiate to produce some factor essential for triggering B cells. As mentioned in the introduction, this factor may be specific or nonspecific with respect to the antigenic determinants concerned.

SUMMARY

Experiments were designed to test the possibility that thymus-derived (T) cells cooperate with nonthymus derived (B) cells in antibody responses by acting as passive carriers of antigen. Thoracic duct lymphocytes (TDL) from fowl γG-tolerant mice were incubated in vitro with fowl anti-mouse lymphocyte globulin (FALG), which was shown not to be immunosuppressive in mice. On transfer into adult thymectomized, irradiated, and marrow protected (TxBM) hosts together with a control antigen, horse RBC, a response to horse RBC but not to fowl γG was obtained. By contrast, TxBM recipients of nontolerant, FALG-coated TDL responded to both antigens and the antibody-forming cells were shown to be derived from the host, not from the injected TDL. These findings suggested that, under the conditions of the experiment, triggering of unprimed

[7] Feldmann, M., and A. Basten. Unpublished data.

B cells in the spleens of TxBM hosts was not achieved with antigen-coated tolerant lymphocytes.

Another model utilized the ability of B cells to bind antibody-antigen complexes. Spleen cells from TxBM mice, incubated in vitro with anti-fowl γG–fowl γG·NIP, were injected with or without normal TDL (a source of T cells) into irradiated hosts. Only mice given both cell types could produce an anti-NIP antibody response. In a further experiment, spleen cells from HGG·NIP-primed mice were injected together with NIP-coated B cells (prepared as above) into irradiated hosts. A substantial anti-NIP antibody response occurred. If, however, the T cells in the spleens of HGG·NIP-primed mice were eliminated by treatment with anti-θ serum and complement, the NIP response was abolished. It was concluded that antigen-coated B cells could not substitute for T cells either in the primary or secondary response.

Treatment of T cells from unprimed or primed mice with mitomycin C impaired their capacity to collaborate with B cells on transfer into irradiated hosts. Taken together these findings suggest that before collaboration can take place T cells must be activated by antigen to differentiate and in so doing may produce some factor essential for triggering of B cells.

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