INTERACTION OF HUMAN THYMUS-DERIVED AND NON-THYMUS-DERIVED LYMPHOCYTES IN VITRO

Induction of Proliferation and Antibody Synthesis in B Lymphocytes by a Soluble Factor Released from Antigen-Stimulated T Lymphocytes*

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It is established that cell interactions between thymus-derived (T) and non-thymus-derived (B) lymphocytes are required for antibody responses to many antigens, such as heterologous erythrocytes and protein antigens (1–4). Various hypotheses have been proposed to explain the mechanism of T- and B-cell interactions (5) that affect a precursor cell, derived from the bone marrow, to differentiate into an antibody-producing cell (6, 7). In the mouse, T cells activated by antigen secrete a soluble factor that is capable of replacing the helper effect of intact T cells in the induction of antibody production by B cells (8–10). Less is known about interactions between human T and B lymphocytes. Successful separation of human blood lymphocytes into T cell-rich and B cell-rich populations has recently been achieved using centrifugation in discontinuous density gradients of 17–35% bovine serum albumin (BSA)[1] (11). Cells sedimenting in the middle portion of a BSA gradient (23–27% BSA) are enriched in T lymphocytes, while cells sedimenting at the bottom of the gradient (27–35% BSA) are almost exclusively B lymphocytes.

Previous work has demonstrated that, upon encounter with antigen, purified human T cells from immune donors release a lymphocyte mitogenic factor (LMF) into culture fluid supernatants (12). Under the influence of LMF, B cells proliferate and undergo morphologic and functional changes. These changes include appearance of rough endoplasmic reticulum (RER), loss of

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; E, sheep red blood cell; EAC3, EAC1423; LMF, lymphocyte mitogenic factor; MI, mitogenic index; P supernatant, supernatant containing mitogenic factor; PBS, phosphate-buffered saline; PFC, plaque-forming cells; R supernatant, control supernatant containing TT antigen but no mitogenic activity; RER, rough endoplasmic reticulum; RIEP, radioimmunoelectrophoresis; SDS, sodium dodecyl sulfate; SRBC, sheep red blood cells; TT, tetanus toxoid antigen.

surface C3 receptors, and synthesis of antibody. The antibodies synthesized include specificity for the antigen present in the cell culture fluids containing the T-cell mitogen.

Materials and Methods

Human Lymphocytes.—Suspensions of human blood and tonsil lymphocytes were prepared as described elsewhere (11). Cell suspensions were passed through a 50 × 3 cm column of glass beads at 37°C to remove adherent cells. Final suspensions always contained more than 90% viable lymphocytes. All donors were immunized to tetanus toxoid (TT).

Fractionation of Lymphocytes on Gradients of BSA.—Albumin gradients were constructed in 16 × 125 mm plastic tubes by layering 1 ml of the albumin solutions in 2% decrements, starting with 35% and ending with 19% BSA solutions (13). 5×10^8 cells were suspended in 1 ml of 17% BSA and layered on top of the gradient. Tubes were centrifuged at 10°C and 900 *g* for 45 min. Nine fractions were obtained: fraction 1 represents cells at the interface between 17 and 19% albumin; fraction 9, those between 33 and 35% albumin. Lymphocytes from each of the nine fractions were washed three times in Medium 199 (Microbiological Associates, Inc., Bethesda, Md.) before use.

Tissue Culture.—Lymphocytes were cultured in Medium RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 15% heat-inactivated human serum obtained from donors with type AB, Rh+ erythrocytes. Cultures of blood lymphocytes were supplemented with penicillin G (50 U/ml) and streptomycin (50 μg/ml). Cultures of tonsil lymphocytes received, in addition, kanamycin (50 μg/ml) and mycostatin (50 μg/ml). Cultures were placed in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in air.

Antigens.—A single preparation of tetanus toxoid (lot no. LP-346) was used throughout all the experiments. It contained 2.3 mg of protein per ml. Half of this protein was precipitable by human hyperimmune antitetanus antibodies. The tetanus toxoid was always dialyzed against culture medium before use in lymphocyte cultures. TT was coupled to agarose beads (Sepharose 2B; Pharmacia Fine Chemicals, Piscataway, N.J.) using the cyanogen bromide activation method of Axen and Ernback (14). The efficiency of coupling was 78%.

Preparation of Lymphocyte Mitogenic Factor (LMF) from T Lymphocytes.—Blood lymphocytes sedimenting in fraction 4 of the BSA gradient (interface of 23–25% BSA) served as a source of T cells. These lymphocytes respond vigorously to both antigen and allogeneic stimulation, react with sheep red blood cells (E [45–50% form E rosettes containing four or more red cells per rosette]), and react very poorly (less than 2% reactive cells) with either EAC1423 (EAC3) or fluorescent antisera to human immunoglobulins.

LMF was prepared from BSA fraction 4 lymphocytes as described previously (12). In brief, cells were cultured at a concentration of 10^7 cells/ml and incubated for 3 days at 37°C in a humidified atmosphere of 5% CO_2 in air (stage I cultures, Fig. 1). Cultures for mitogen production received TT (10 μg/ml) at the onset of the incubation period and were designated P cultures (preincubated with antigen). Control cultures received TT (10 μg/ml) at the end of the incubation period and were designated R cultures (reconstituted with antigen). Incubation was stopped by pelleting the cells for 15 min at 200 *g*. The supernatants, P and R, were pipetted off, filtered through a 0.45 μm filter (Millipore Corp., Bedford, Mass.), and stored at −20°C until tested.

Assay of the Mitogenic Activity of Supernatants on B Lymphocytes.—Almost pure populations of B cells were provided by tonsil and blood lymphocytes that sediment in fraction 7 of the BSA gradient (interface of 29 and 31% BSA). Cells in this fraction do not transform in response to antigen, react very readily (more than 95%) with both EAC3 and fluorescent antisera to human immunoglobulins, and react very poorly with E (less than 5%). Furthermore, no cells are present in fractions 6–9 of the gradient from the blood of boys with X-linked agammaglobulinemia who lack circulating B cells (11).

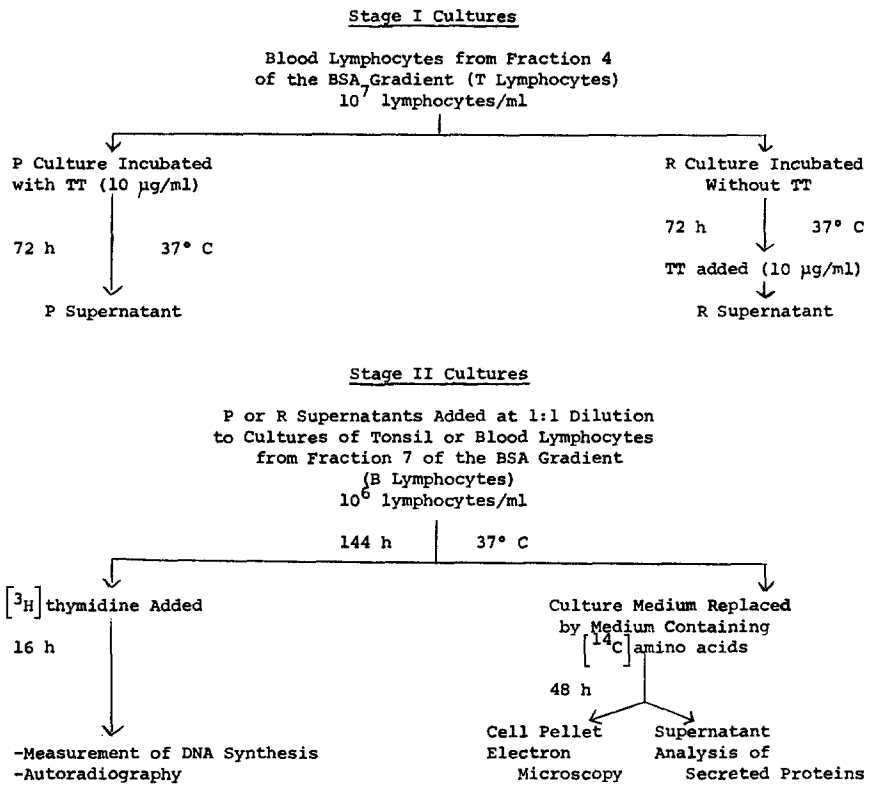


FIG. 1. Experimental design utilized for the preparation of human T cell-derived LMF and for the assay of its activities on human B lymphocytes.

The ability of paired P and R supernatants to stimulate DNA synthesis in B lymphocytes was assayed as follows: Lymphocytes were suspended at a concentration of 1×10^6 cells/ml, and four sets of 1 ml cultures were made in duplicate (stage II cultures, Fig. 1). In the first two sets, cells were incubated in the presence of P and R supernatants that were added at a 1:1 dilution to fresh culture medium. The third set was incubated in the presence of TT (5 μg/ml); and the fourth set, a control, received no additions. After an incubation period of 6 days, the degree of incorporation of [^{3}H]thymidine into DNA in each culture was determined. This was done as detailed in reference 11. Results were expressed as the number of counts per minute (cpm) of [^{3}H]thymidine incorporated by each culture. Mitogenic activity was considered present when the number of counts per minute incorporated by cultures stimulated with P supernatants exceeded the number of counts per minute incorporated by cultures stimulated with R supernatants. A measure of the degree of mitogenic activity, mitogenic index (MI), was defined as the following ratio:

$$\text{MI} = \frac{\text{cpm incorporated by stage II cultures stimulated by P supernatants}}{\text{cpm incorporated by stage II cultures stimulated by R supernatants.}}$$

Autoradiography.—Cultures of B lymphocytes were pulsed with 5 μCi/ml of [^{3}H]thymidine (20 mCi/mM, New England Nuclear, Boston, Mass.). 16 h later, the cells were washed and

smears were made in triplicate on glass slides. Smears were fixed in methanol and covered with Kodak AR-10 stripping film (Eastman Kodak Company, Rochester, N.Y.). 6–8 wk later, slides were developed in Kodak D-19 developer, stained with Giemsa stain, and examined microscopically. Cells with more than eight nuclear grains were considered to have divided and taken up the label specifically. A minimum of 100 cells was counted per smear.

Rosette Formation with E and EAC3.—EAC3 was prepared according to the method of Borsos and Rapp (15) using components of human complement. Rosette formation between lymphocytes and sheep red cell intermediates E and EAC3 was performed as described in references 16 and 17, except that with E the initial stage of the reaction was carried out at room temperature. Separation of rosette-forming cells from non-rosette-forming cells was performed on Ficoll-Hypaque gradients (18).

Electron Microscopy.—Cell pellets of 1×10^6 lymphocytes were fixed in 2.5% glutaraldehyde in 0.08 M cacodylate buffer, pH 7.4, containing 0.1 M sucrose and 4.5×10^{-3} M $CaCl_2$ for 1 h at 4°C. They were rinsed three times in 0.08 M cacodylate buffer, pH 7.4, containing 0.18 M sucrose and postfixed in 1.3% OsO_4 in s-collidine, pH 7.4, for 1 h at 4°C (19). The cells were stained en bloc with 1.5% uranyl acetate (20) in 0.05 M maleate buffer, pH 6.2, and dehydrated in graded ethanol and embedded in Epon. Thin sections were cut with a diamond knife (E. I. DuPont de Nemours and Co., Wilmington, Del.) on an LKB Ultratome III (LKB Instruments Inc., Rockville, Md.) and picked up on carbon-coated grids. They were counterstained with lead citrate (21) and examined in a Philips 300 electron microscope. In order to assess the number of cells that showed enlargement after incubation with and without LMF, 500 and 200 cells in each sample, respectively, were counted. Their size was roughly estimated at a primary magnification of 6,800 by observing whether their borders fell within the perimeter of a 4 cm diameter circle inscribed on the fluorescent screen of the electron microscope. At the above magnification, 4 cm represent an actual length of 6 μm.

Determination of Protein Synthesis.—After a 6-day incubation period in the presence of T-cell supernatants, cell pellets were washed and then suspended at 2×10^6 cells/ml in Medium 199 deficient in L-leucine, L-valine, and L-isoleucine. This medium was supplemented with 15% dialyzed serum. In early experiments, human serum was used; but in later experiments, use was made of fetal calf serum. 1 μCi of L-[^{14}C]leucine (260 Ci/mM), 0.5 μCi of L-[^{14}C]valine (220 Ci/mM), and 0.5 μCi of L-[^{14}C]isoleucine (250 Ci/mM) were added per 1 ml culture. 48 h later, the supernatants were collected, centrifuged for 20 min at 1,000 *g*, and filtered through a 0.45 μm filter. Part of the supernatant was precipitated with 10% trichloroacetic acid (TCA). The precipitate was washed three times in 10% TCA, solubilized with Soluene, and transferred to scintillation vials (22). 10 ml of a toluene scintillation fluid (20.3 g of 2,5-diphenyloxazole and 1.45 g of 1,4-bis [2-(5-phenyloxazolyl)]-benzene in 3.8 liters of toluene) was added to each vial, and the samples were counted in a liquid scintillation spectrometer (Tri-Carb; Packard Instrument Co., Inc., Downers Grove, Ill.). Another part of the supernatant was applied to a Sephadex G-200 column (Pharmacia Fine Chemicals). The sample was eluted with 0.15 M NaCl, and the protein concentration in the effluent was determined by extinction measurements at 280 nm. The effluent was collected in 1.8-ml fractions. 100 μl of each fraction was mixed with 10 ml of Aquasol (New England Nuclear), and its radioactivity was counted by scintillation spectrometry.

Analysis of Secreted Proteins.—Supernatants and supernatant fractions obtained after Sephadex G-200 fractionation were analyzed for the presence of secreted antibodies by the following methods.

Radioimmunoelectrophoresis (RIEP): The antisera used were commercial antisera to whole human serum and to human immunoglobulins (Behring Diagnostics, Somerville, N. J.), rabbit antihuman gamma G globulin antiserum, and goat antisera to human gamma M and gamma A. Antisera to individual immunoglobulins were rendered specific by passage through appropriate immunoabsorbent columns.

Immunoprecipitation with antisera to human immunoglobulins: The following antigen-

antibody systems were added at equivalence to the samples tested: rabbit antihuman gamma G globulin–goat antirabbit gamma globulin; rabbit antihuman mu chain–goat antirabbit gamma globulin; normal rabbit serum–goat antirabbit gamma globulin. Samples were incubated for 1 h at 37°C and overnight at 4°C. The precipitates that formed were washed three times at 4°C in phosphate-buffered saline (PBS), solubilized in Soluene, and their radioactivity was determined. Radioactivity specifically precipitated with each system was taken as the total amount of radioactivity precipitated with this system *minus* the amount of radioactivity precipitated in the presence of normal rabbit serum–goat antirabbit gamma globulin. Antisera were purchased from Miles Laboratories, Inc., Kankakee, Ill., and from Microbiological Associates, Inc.

Specific immunoadsorption to cross-linked TT: Aliquots from each sample were incubated for 1 h at 37°C with an excess of TT cross-linked to agarose. After standing in the cold (4°C) for 3 h, the agarose beads were pelleted at 1,000 *g*, washed in PBS, until no radioactivity was detectable in the washings, dissolved in Soluene, and counted for radioactivity. Parallel aliquots were incubated with an equal volume of agarose beads or with an equal volume of TT cross-linked to agarose in the presence of an excess of human anti-TT antibody.

Local Hemolysis in Gel Assay.—1 ml of cell suspension containing 2×10^6 cells/ml was added to 2.5-cm. tissue culture dishes. Dishes were incubated on a rocker platform (8 oscillations/min) in an atmosphere of 83% N_2, 10% CO_2, and 7% O_2. Cultures were fed daily with 50 μl of "nutritional cocktail" and with 30 μl of human or fetal calf serum. Nutritional cocktail was prepared as described in reference 23.

The number of plaque-forming cells (PFC) against sheep red blood cells (SRBC) coated with TT was assayed after an incubation period of 7 days by the method of Jerne and Nordin (24). TT was coupled to SRBC by the bis-diazobenzidine procedure (25). Normal rabbit serum absorbed with TT-coated SRBC was used as a source of complement. Similarly absorbed rabbit antihuman gamma G globulin was used to assay for indirect PFC.

RESULTS

Effect of Lymphocyte Mitogenic Factor (LMF) on DNA Synthesis by B Lymphocytes.—Tonsil and blood B lymphocytes sedimenting in fraction 7 of the BSA gradient showed a marked acceleration of their DNA synthesis in the presence of P supernatants containing LMF (Table I). These cells did not transform in the presence of either antigen (TT) alone or control (R) supernatants. 93–98% of cells in BSA fraction 7 were EAC3-reactive cells, and more than 90% of them stained with immunofluorescent antisera to human

TABLE I

Stimulation of DNA Synthesis in Blood and Tonsillar B Lymphocytes by LMF

Source of responding lymphocytes	BSA fraction number	EAC3-reactive cells	Immunofluorescent staining cells	cpm of [^{3}H]thymidine incorporated per culture				P-R cpm	Mitogenic index P/R
				Control	TT	Stimulated with P supernatant	Stimulated with R supernatant		
		%	%						
Tonsil	7	93	96	683±231	739±171	15,330±4,058	811±216	+14,519±3,872	18.8±5.10
Blood	7	88	90	780±251	835±187	14,053±2,263	927±246	+13,126±3,033	15.1±3.30

Cultures consisted of 10^6 cells. Values represent the arithmetic mean ± SD derived from experiments on five tonsils and six bloods.

immunoglobulins (Table I). LMF caused transformation of both autologous and allogeneic fraction-7 lymphocytes. Maximal stimulation of DNA synthesis occurred after an incubation period of 6 days (Fig. 2). At this time, MI ranged from 7.5 to 32.6. Autoradiographic studies revealed that by day 6 of culture, as many as 60–70% of the cells were dividing (Fig. 2).

Response of EAC3-Reactive vs. EAC3-Nonreactive Cells in Fraction 7 of the BSA Gradient to Stimulation by LMF.—To confirm that the mitogen-responsive cells in fraction 7 of the BSA gradient were B lymphocytes, fraction-7 cells were separated on the basis of their reactivity with the sheep cell intermediates E and EAC3. As shown in Table II, the E-nonreactive but EAC3-reactive cells, though incapable of responding to antigen alone, showed a vigorous proliferative response in the presence of mitogenic (P) supernatants. The minor

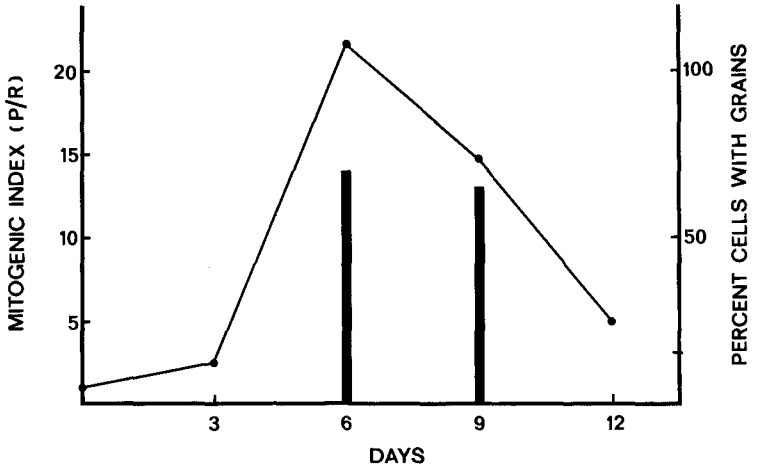


FIG. 2. Time-course of the proliferative response of tonsil and blood lymphocytes sedimenting in fraction 7 of the BSA gradient in response to LMF. Vertical bars indicate the percentage of cells having more than eight nuclear grains on autoradiography.

TABLE II

Response to LMF and Reactivity With E and EAC3 of Cells in BSA Fraction 7

Reactivity with SRBC intermediates	Total cell number	cpm of [^{3}H]thymidine incorporated per culture				P-R cpm	Mitogenic index P/R
		Control	TT	Stimulated with			
				P supernatant	R supernatant		
	%						
EAC3 reactive	96	650±137	617±188	11,750±2,176	840±226	+10,910±1,820	14.50±3.80
EAC3 nonreactive	4	1,080±219	11,360±3,410	24,220±4,835	13,680±2,108	+10,540±3,227	1.78±0.51
E reactive	7	1,033±287	10,205±3,015	20,765±5,115	9,633±2,337	+11,132±3,516	2.16±0.43
E nonreactive	93	788±251	821±240	12,175±2,612	771±212	+11,404±1,738	15.80±4.20

Cultures consisted of 10^5 cells each. Values represent the arithmetic mean ± SD of five experiments with EAC3 and three experiments with E.

population of E-reactive EAC3-nonreactive cells (4–7% of the total cell number) found in fraction 7 behaved differently. When isolated, these cells proliferated in the presence of antigen (TT) alone and showed a low MI (2–3) in the presence of mitogenic (P) supernatants. This behavior is characteristic of circulating T lymphocytes (12).

Loss of the EAC3 Activity after Stimulation by LMF.—Tonsil B lymphocytes from BSA fraction 7 were cultured for 10 days in the presence of P and R supernatants. At different time intervals, the percentage of EAC3-reactive cells present in the cultures was determined. The results, shown in Fig. 3 *a*, indicated that in the cultures incubated with P supernatants there was a progressive loss in the percentage of EAC3-reactive cells. At the onset of

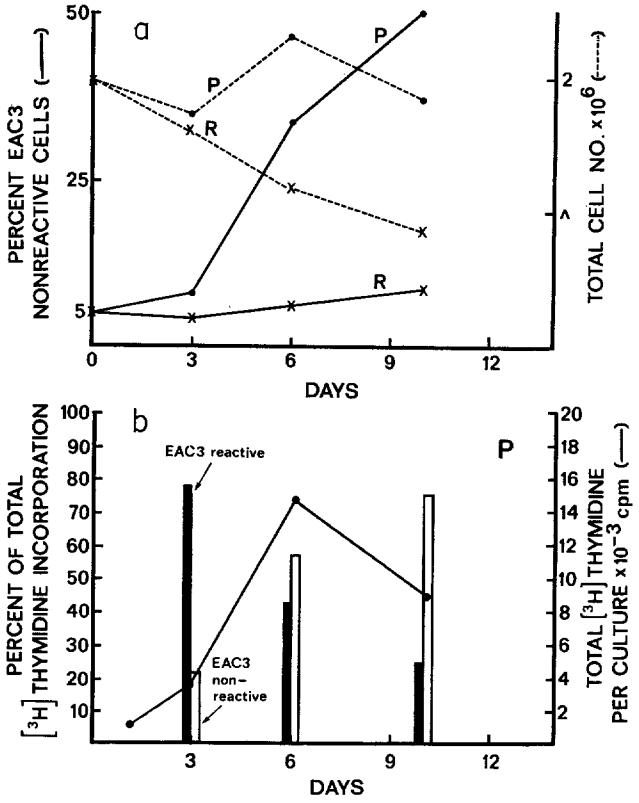


FIG. 3. Interrelation of EAC3 reactivity and response to LMF in tonsil lymphocytes sedimenting in fraction 7 of the BSA gradient. (*a*) Percent EAC3-nonreactive cells vs. length of incubation time with P (mitogenic) or R (control) supernatants. The solid lines (—) indicate the percent of EAC3-nonreactive cells. The interrupted lines (-------) indicate the total number of cells present in culture. (*b*) Relative contribution of EAC3-reactive (full bars) vs. EAC3-nonreactive (open bars) cells to the total [^{3}H]thymidine incorporation (solid lines) in cultures incubated with LMF-containing (P) supernatants.

incubation, 95% of the cells were EAC3 reactive; by day 10, only 50% of the cells in the mitogen (P)-stimulated cultures were EAC3 reactive. In the cultures incubated with control (R) supernatants, the percentage of EAC3-reactive cells remained constant, 92–95%, throughout the period of incubation. This finding, taken together with the absolute cell counts (Fig. 3 *a*), indicated that lymphocytes from fraction 7 of the BSA gradient lost their EAC3 receptors after stimulation with mitogen. This was not due to the release of tryptic activity into the medium with subsequent destruction of the trypsin-sensitive EAC3 receptor. Medium collected from cell cultures incubated for 9 days in the presence of P supernatants did not decrease the EAC3 reactivity of freshly isolated fraction-7 tonsil lymphocytes.

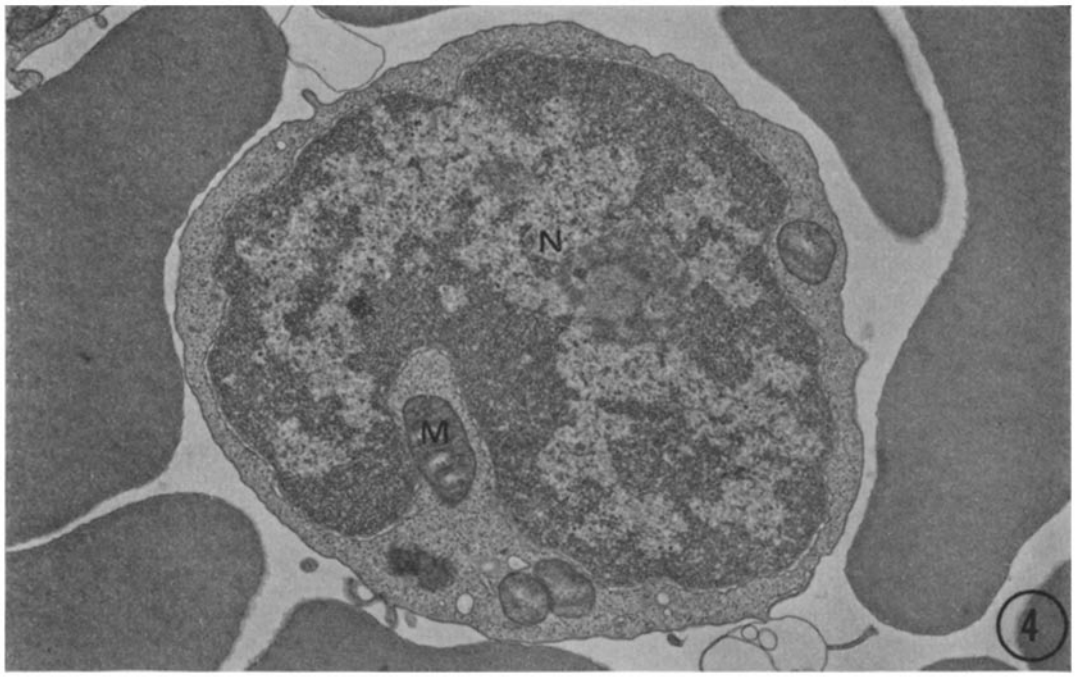
The relative contribution of EAC3-reactive and EAC3-nonreactive lymphocytes to the total amount of [^{3}H]thymidine incorporated into DNA in cultures stimulated with P supernatants is shown in Fig. 3 *b*. Early in culture (day 3) the majority of cells incorporating [^{3}H]thymidine were EAC3-reactive cells, while late in culture (day 10) most of them were EAC3-nonreactive cells. These results were confirmed by autoradiography.

Appearance of RER in B Lymphocytes Stimulated with LMF.—Fig. 4 depicts the typical electron microscopic appearance of a tonsil lymphocyte from fraction 7 incubated for 10 days in the presence of control (R) supernatants. There was scanty cytoplasm, very few organelles, and little smooth endoplasmic reticulum. Only 0.5% of these cells have a diameter greater than 6 μm. The morphology of these lymphocytes is similar to that of lymphocytes freshly isolated from fraction 7.

After a 10-day incubation period in the presence of P supernatants, 50–60% of the lymphocytes present in culture have become larger cells measuring more than 6 μm in diameter and had a greater amount of cytoplasm. Their morphologic features are seen in Fig. 5. There was a well-developed Golgi apparatus; RER was present, together with numerous, non-membrane-bound ribosomes.

Effect of LMF on Protein Synthesis by Lymphocytes in BSA Fraction 7.—Supernatants of mitogen-treated B lymphocytes contained three- to fivefold more radioactive TCA-precipitable material than supernatants of control cultures treated with R supernatants. When supernatants from stage II cultures were separated on Sephadex G-200, this increase in TCA-precipitable, labeled protein was distributed in all three major protein fractions (Fig. 6). Calculations based on the integration of the area under the curve showed that the increase was greatest in the 7S fraction. The increase in radioactive proteins secreted by the P supernatant-treated cultures was distributed as follows: three- to fivefold in the 19S fraction; five- to ninefold in the 7S fraction, and three- to fivefold in the albumin-region fraction.

Radioimmunoelectrophoresis (RIEP) of Secreted Proteins.—On RIEP, supernatants from mitogen-treated cultures incubated in the presence of normal human serum gave three labeled precipitin lines corresponding to alpha-2



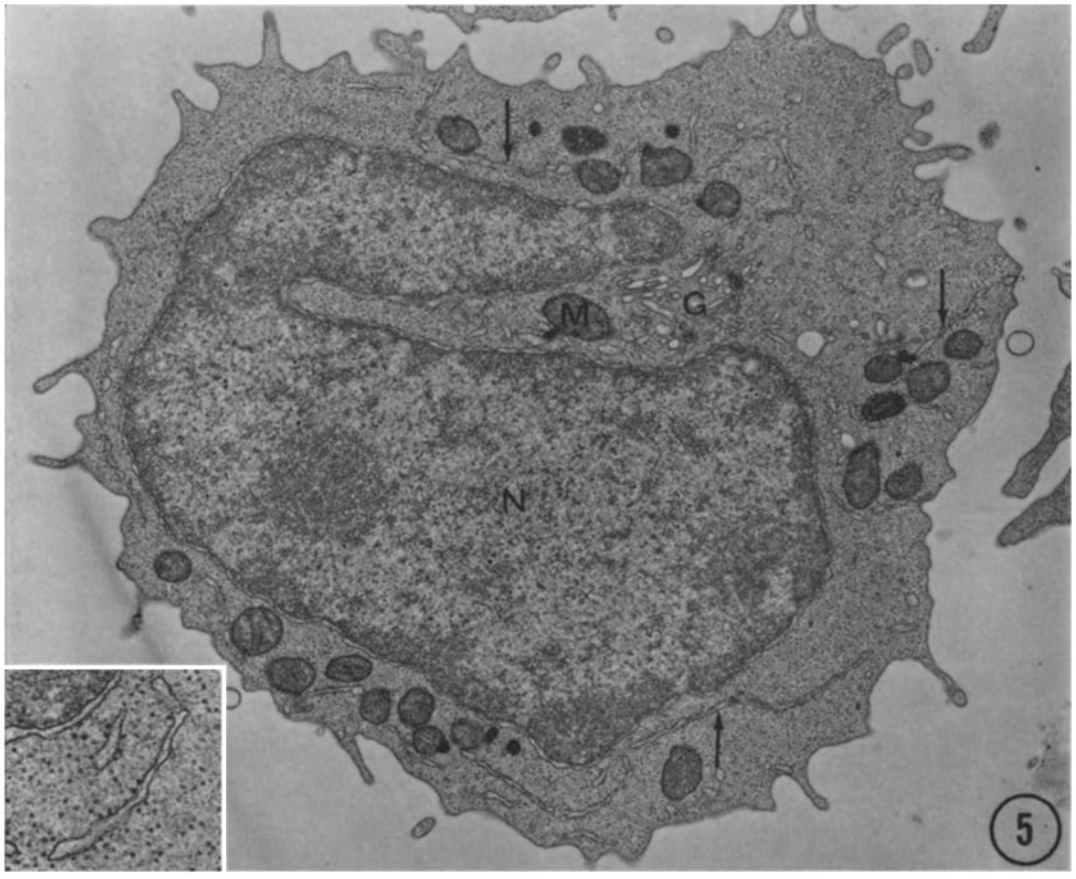


FIG. 4. Tonsil B lymphocyte incubated in the presence of R (control) supernatant for 10 days. The cell contains a large central nucleus (*N*) surrounded by a thin rim of cytoplasm in which there are a few mitochondria (*M*) and numerous free ribosomes. × 14,800.

FIG. 5. Lymphocyte obtained from the same tonsil as the cell in Fig. 4 and incubated in the presence of P (mitogenic) supernatant for 10 days. The nucleus (*N*) is surrounded by abundant cytoplasm in which many mitochondria (*M*) and the Golgi apparatus (*G*) are seen. In addition to free ribosomes, the cytoplasm contains several profiles of RER (arrows). × 14,800. Insert shows two profiles of RER on which membrane-bound ribosomes are clearly visible. × 37,000.

macroglobulin, gamma G globulin, and albumin (Fig. 7 *a*). The labeling of albumin was later shown to be an artifact and not to represent *de novo* synthesis, as supernatants from cultures incubated in the presence of fetal calf serum exhibited only two labeled precipitin lines corresponding to alpha-2 macroglobulin and to gamma G globulin.

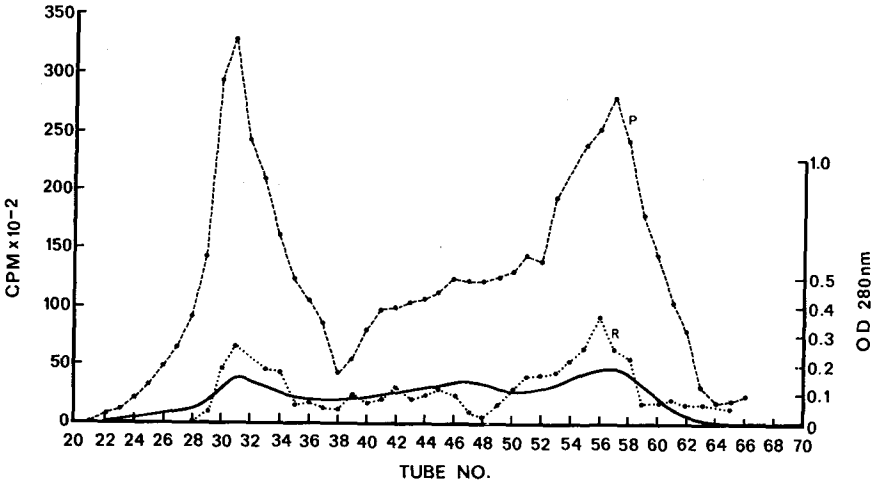


FIG. 6. Sephadex G-200 separation of supernatants from mitogen (P)-treated and control (R)-treated cultures of tonsil cells of BSA fraction 7. Cultures were pulsed for 48 h with [^{14}C]-amino acids (2.0 μCi/ml) before harvesting. The interrupted lines represent the radioactivity in the effluent. The optical density readings of the effluent are represented by the solid line and are due to the presence of 15% normal human serum in the culture medium.

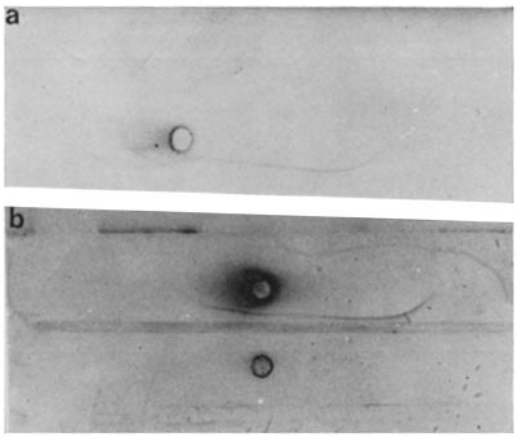


FIG. 7. RIEP of supernatants from tonsil B lymphocytes incubated in the presence of LMF. (*a*) Antiwhole human serum in the trough; unfractionated supernatant in the well. (*b*) Antihuman gamma G globulin in the trough; Sephadex G-200 fraction II (7S) of supernatants in the upper well; Sephadex G-200 fraction I (19S) of supernatants in the lower well.

Simultaneously processed supernatants from paired control-treated cultures gave precipitin lines corresponding to alpha-2 macroglobulin but no or a very faint line corresponding to gamma G globulin. When Sephadex G-200 fractions of supernatants from mitogen-treated cultures were treated, only the 7S fraction gave a precipitin line with antihuman gamma G globulin (Fig. 7 *b*). None of the fractions formed precipitin lines with antisera to human alpha and mu chains of IgA and IgM immunoglobulins, respectively.

Immunoprecipitation of Secreted Proteins with the Double-Antibody Technique.—15% of the total TCA-precipitable radioactivity present in supernatants of mitogen-treated cultures was specifically precipitated with a rabbit antiserum to human gamma G globulin. The corresponding figure for control-treated cultures was 4.8% (Table III). Assuming that all precipitated counts were incorporated into *de novo* secreted proteins and considering the absolute number of precipitable counts in each supernatant, the data obtained indicated that there was an eightfold increase in gamma G globulin secretion after treatment of B lymphocytes with the T-cell mitogen. Analysis of the Sephadex G-200 fractions of the supernatants revealed that most of the precipitated radioactive gamma G globulin was found in the 7S fraction (Table III). In supernatants of both mitogen- and control-treated cultures, insignificant amounts of radioactivity were precipitated with the goat antiserum to human mu chain.

Immunoadsorption to Cross-Linked Tetanus Toxoid (TT).—Table IV shows the amount of radioactivity that specifically adsorbed to insolubilized TT from

TABLE III

Immunoprecipitation of Supernatants from Cultures of Tonsil B Lymphocytes

Source of supernatants	Sephadex G-200 fraction	Total TCA-precipitable radioactivity	Radioactivity specifically precipitated with			
			Antihuman γ-chain		Antihuman μ-chain	
		cpm	*cpm*	%	*cpm*	%
P (mitogen)-stimulated culture	U	6,648	965	14.9	15	0.23
	I	1,962	60	3.1	11	0.57
	II	1,875	748	40.1	4	0.21
	III	3,112	71	2.0	6	0.19
R-stimulated culture (control)	U	2,503	120	4.8	40	1.60
	I	638	20	3.2	22	3.40
	II	601	66	11.1	6	1.00
	III	1,213	25	2.1	7	0.46

U represents unfractionated supernatants; I, 19S; II, 7S fraction; III, albumin region fraction. After Sephadex G-200 fractionation, each fraction was concentrated to a volume equal to the volume of the supernatant applied to the column. Experiments were carried on 0.2-ml aliquots from each sample. Values represent the arithmetic mean of duplicate determinations on a pair of supernatants from P- and R-stimulated cultures. Similar results were obtained in two different experiments.

paired supernatants of mitogen- and control-treated cultures. There was a ninefold increase of specifically adsorbable radioactivity in supernatants of mitogen-treated cultures. Analysis of the Sephadex G-200 fractions of the supernatants revealed that almost all this increase was found in the 7S fraction (Table IV).

Plaque-Forming Cells (PFC).—After 7 days of culture in the presence of P and R supernatants, tonsil and blood lymphocytes from BSA fraction 7 were

TABLE IV

Immunoadsorption of Supernatants from Cultures of Tonsil B Lymphocytes

Source of supernatants	Sephadex G-200 fraction	Total TCA-precipitable radioactivity	Radioactivity (cpm) adsorbed to			Radioactivity specifically adsorbed to Sepharose-TT	
			Sepharose	Sepharose-TT	Sepharose-TT in the presence of anti-TT		
		cpm		*cpm*		*cpm*	%
P (mitogen)-stimulated culture	U	4,313	41	261	37	224	5.2
	I	1,270	16	46	13	33	2.6
	II	1,152	15	181	15	166	14.4
	III	2,192	12	78	10	68	3.1
R-stimulated culture (control)	U	1,162	23	23	58	27	2.7
	I	347	13	13	29	18	2.9
	II	222	18	18	24	16	3.5
	III	619	12	12	31	14	2.7

U represents unfractionated supernatants; I, 19S fraction; II, 7S fraction; III, albumin region fraction; sepharose-TT, tetanus toxoid cross-linked to agarose; anti-TT, human anti-tetanus toxoid antibodies. After Sephadex G-200 fractionation, each fraction was concentrated to a volume equal to the volume of supernatant applied to the column. Experiments were carried on 0.2-ml aliquots from each sample. Values represent the arithmetic mean of duplicate determinations on a pair of supernatants from P- and R-stimulated cultures. Similar results were obtained in two different experiments.

assayed for both direct (IgM) and indirect (IgG) PFC against SRBC coated with TT. It was found in three different experiments that tonsil B lymphocytes responded to the T cell-derived mitogen by a 23- to 42-fold increase in the number of indirect PFC to the TT antigen present in the T cell supernatants (Table V). There was no increase over the low baseline value of indirect PFC to the same antigen.

DISCUSSION

Relatively pure populations of human B and T lymphocytes were obtained by the method of density gradient centrifugation in bovine serum albumin (BSA). Cells in fraction 4 of the BSA gradient contained less than 5% B lymphocytes and exhibited T cell behavior in vitro, while cells in fraction 7

TABLE V

Effect of Mitogenic Factor on the Antibody Response of Tonsil B Lymphocyte to TT

Type of PFC	PFC/culture			
	Control	Stimulated with		
		TT	P supernatant	R supernatant
Direct (IgM)	2 ± 1	3 ± 2	3 ± 2	2 ± 2
Indirect (IgG)	0	2 ± 2	88 ± 17	3 ± 3

Cultures of 2×10^6 cells/ml were assayed for PFC against SRBC coated with TT. Each value represents the arithmetic mean ± SD of five experiments.

of the gradient contained around 95% B lymphocytes exhibiting EAC3 reactivity and surface staining with fluorescent antisera to human immunoglobulins (11). Human B lymphocytes neither proliferated nor engaged in antibody synthesis in response to antigen alone. However, culture fluid supernatants from antigen-stimulated T lymphocytes contained an activity that caused blast transformation of B lymphocytes. The lymphocyte mitogenic factor (LMF) could not be related to transplantation antigens for the following reasons: supernatants were active on autologous B lymphocytes; no mitogenic activity was present in control (R) supernatants that were not stimulated with antigen; B lymphocytes responded poorly if at all to allogeneic stimulation in one-way mixed lymphocyte cultures (11). LMF release was immunologically specific, as it occurred only in response to antigens to which the cell donors were immune. Lymphocytes from newborns failed to release LMF in response to stimulation with TT. Vaccinia virus antigen induced release of LMF only by cells of individuals immunized with vaccinia. LMF was a product of T lymphocytes exclusively; it was shown to be released normally by lymphocytes from individuals with X-linked agammaglobulinemia who lacked B cells. Furthermore, B lymphocytes of both peripheral blood and tonsils failed completely to release LMF. These B cells, nonetheless, responded vigorously to LMF. It was shown that 60% of them underwent blast transformation in response to the T-cell mitogen (12).

Together with its proliferative response to LMF, B lymphocytes lost their C3 receptor. By day 9 of culture, 50% of B cells remaining in culture were EAC3 nonreactive vs. 5% of them at day 0. This loss was not due to the release of tryptic activity in the culture medium with subsequent destruction of the trypsin-sensitive C3 receptors, since medium from these cultures did not affect the EAC3 reactivity of freshly isolated lymphocytes from fraction 7 of the BSA gradient. Loss of C3 receptors could not be attributed to selective lysis or loss of EAC3-reactive cells for the following reasons. Cultures of lymphocytes from fraction 7 after elimination of those cells that formed E rosettes contained 98–100% EAC3-reactive cells at day 0 and exhibited similar loss of EAC3 reactivity. By day 9 of culture, despite the loss of EAC3 reactivity,

a number (30–50%) of EAC3-nonreactive cells continued to stain for surface or intracellular immunoglobulins. No decrease in the number of EAC3-reactive cells was seen in cultures incubated with control (R) supernatants.

The amounts of radioactive [^{3}H]thymidine incorporated at different time intervals by the EAC3-reactive and the EAC3-nonreactive cells of fraction 7 varied (Fig. 2 *a*). In early cultures (day 3), most of the label was in the EAC3-reactive population, while later (day 10), the situation was reversed and most of the label was in the EAC3-nonreactive population. This was not due to an excessive proliferation of the original E-reactive cells found at day 0 (5% of the total), as similar data were obtained after elimination of all E-reactive cells at day 0. It was concluded that in the presence of the T-cell mitogen, B cells start to divide, lose their C3 receptor, and presumably continue dividing. The number of divisions a B cell undergoes after the loss of its receptor cannot at the present time be determined. In the mouse, it is known that the antibody secreting plaque-forming cell (PFC) lacks the C3 receptor found on the B lymphocyte (26).

After stimulation with LMF, the cytoplasm of the B lymphocytes enlarged considerably, the Golgi apparatus became conspicuous, and rough endoplasmic reticulum (RER) was visible (Figs. 4 and 5). The presence of RER is considered evidence that the B cell has turned from a quiescent to a secretory state. This change was confirmed by the analysis of the B-cell supernatants for evidence of newly secreted proteins. There was a three- to fivefold increase in secreted protein after stimulation with LMF (Fig. 5). There was evidence for *de novo* synthesis of alpha-2 macroglobulin and gamma G globulin as shown by RIEP (Fig. 7 *b*). Labeling of human albumin occurred only when human serum was present in the culture medium and was shown to be an artifact. Analysis of *de novo* secreted proteins by disk gel electrophoresis (27, 28) confirmed this finding. Radioactivity in fraction I of Sephadex G-200 was found in a high mol wt protein, which reacted only with antisera to alpha-2 macroglobulin and did not split into subunits after treatment with sodium dodecyl sulfate (SDS) and mercaptoethanol. After similar treatment, radioactivity in fraction II of Sephadex G-200 distributed into heavy and light chains as determined by SDS-acrylamide gel electrophoresis. Six- to eightfold more IgG was secreted into the supernatant fluids of cultures incubated with LMF (Table III). No evidence of increased secretion of IgM immunoglobulin was found. 35% of the IgG secreted into the supernatant fluid of mitogen-stimulated cultures was specific antibody to tetanus toxoid as ascertained by precipitation with insoluble antigen. That indirect cell plaque formation increased after stimulation of B cells with LMF, supported the finding of 7S IgG antibody in the culture supernatants. The increase in PFC is sizable (23- to 42-fold) when it is expressed as a ratio of PFC found in stimulated vs. control cultures.

A number of T-cell factors have been recently implicated in the initiation of immune responses by B cells in the mouse. LMF is different from the allogeneic

factor of Dutton et al. (5), Katz et al. (29), and Schimpl and Wecker (8), both by the mode of its production and by its inability to pass through a dialysis membrane (12, 30, 31). It is also different from the antigen-specific factor described by Feldmann and Basten. This factor is believed to be a high mol wt molecule (more than 150,000) related to monomeric IgM (30, 31). LMF elutes after albumin on Sephadex G-200 columns and is probably less than 60,000 in mol wt (12). The LMF described in this report seems similar to the factor recently described in the mouse by Gorczynski et al. (33). These authors, as well as Andersson et al. (34), described a factor released by murine T cells in response to mitogenic, as well as antigenic, stimulation and capable of triggering antibody production in B cells. The activity of this factor was not antigen specific, and the T cell-releasing factor was found to be radiation sensitive, as is the case with the human T cell-releasing LMF (12). In view of the findings of Feldmann (32) and Schraeder (35), the question arises whether human LMF requires the mediation of macrophages. This is not definitely known. Despite passage of cells suspensions through prewarmed glass beads, there were small numbers of macrophages (about 1–2%) found in preparations of B cells derived from tonsils. When cells from BSA fraction 7 were rosetted with EAC3 in the absence of Mg^{++}, then separated on Ficoll-Hypaque gradients, the EAC3-reactive cells that sedimented to the bottom of the gradient were responsive to LMF. This finding militates against but does not rule out a possible requirement for a small number of macrophages to trigger the B cells.

The T cell-derived factor described in this communication accounts for only one facet of T- and B-cell interactions. It would be interesting to look in systems such as the one studied here for evidence of a regulatory feedback mechanism originating in the B cell and affecting T-cell function. There is already evidence for the presence of in vivo circulating immunoregulatory globulins (36). Preliminary evidence suggests that LMF is a protein that is active on thymocytes, as well as B lymphocytes (12). Pending the purification of LMF, it is difficult to know if one is dealing with a single molecular species or whether there are as many mitogenic factors as there are target cells and antigens.

SUMMARY

Relatively pure populations of human T and B lymphocytes were obtained from blood and tonsils using density gradient centrifugation in bovine serum albumin. Antigen alone was incapable of triggering the B lymphocyte into blast transformation or to secrete antibody. However, supernatants from tetanus toxoid-stimulated T cells obtained from immune donors contained a factor mitogenic for B lymphocytes. 50–60% of B cells responded to this lymphocyte mitogenic factor (LMF) by proliferation, loss of C3 reactivity, and change to a secretory state. LMF-stimulated B cells exhibited a three- to fivefold increase in protein secretion and a six- to eightfold increase in gamma G globulin secretion. *De novo* secreted IgG had specificity directed to the tetanus toxoid present

in the LMF containing T-cell supernatants. This was confirmed by an increase in the number of indirect plaque-forming cells to tetanus toxoid-coated sheep red blood cells after stimulation of B cells with LMF. It is proposed that in the course of the response to a previously encountered protein antigen, sensitized human T cells emit a signal in the form of a soluble product that, together with antigen, triggers B cells into division and antibody secretion. The experimental model utilized can be adapted to study human T-B cell cooperation under various conditions in normal individuals and in individuals with immunodeficiency diseases.

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