BIOLOGICAL EXPRESSIONS OF LYMPHOCYTE ACTIVATION

I. Effects of Phytomitogens on Antibody Synthesis In Vitro*

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(Received for publication 22 September 1972)

In recent years the nonspecific plant mitogens phytohemagglutinin (PHA),[1] concanavalin A (Con A), and pokeweed mitogen (PWM) have been widely used to investigate fundamental problems in lymphocyte biology. The sequence of events after activation of lymphocytes by these substances is similar to the sequence which follows an encounter with specific antigen. The activation by mitogens of a majority of lymphocytes of a particular type, contrasted with the very limited number of cells specifically stimulated by antigen, provides a powerful tool for study of the events and consequences of lymphocyte activation. The heterogeneity of lymphoid cells as reflected by their proliferative responses to mitogens has recently been an object of considerable interest in several laboratories. It is now generally agreed that soluble Con A and PHA exclusively stimulate thymus-derived lymphocytes (T cells) (1–4). Conversely, when these T cell mitogens are covalently bound to certain particulate carriers such as sepharose beads (5) or plastic Petri dishes (6), bone marrow-derived lymphocytes (B cells) are activated. Con A is also reported to activate B cells in the presence of supernates from Con A-stimulated T cells (4). PWM is apparently less specific and stimulates both T and B lymphocytes (2).

The consequences of T cell activation by mitogen have been the more intensively investigated. The morphological events of blast transformation and mitosis were recognized first (7). Recently attention has focused on the differentiation of mitogen-stimulated T cells for performance of the diverse functions of fully differentiated antigen-stimulated T lymphocytes. In this regard it has been reported that Con A and PHA stimulate production of mediators of delayed hypersensitivity such as

* This investigation was supported by U.S. Public Health Service Grant AI-09897 from the National Institute of Allergy and Infectious Diseases and by a grant from The Anna Fuller Fund.

‡ Recipient of U.S. Public Health Service Special Fellowship 1-F03-AI-50,602 from the National Institute of Allergy and Infectious Diseases.

§ Recipient of U.S. Public Health Service Research Career Development Award 1K4-AI-70,173 from the National Institute of Allergy and Infectious Diseases.

[1] *Abbreviations used in this paper:* B cells, bone marrow-derived cells; Con A, concanavalin A; HBSS, Hanks' balanced salt solution lacking sodium bicarbonate; HRBC, horse erythrocytes; [^{3}H]TdR, tritiated thymidine; PFC, plaque-forming cells; PHA, phytohemagglutinin; PHA-P, phytohemagglutinin-purified; PWM, pokeweed mitogen; T-cells, thymus-derived cells.

skin-reactive factor (8, 9), migration inhibitory factor (10), and lymphotoxin (11, 12). Moreover, it has been shown that these substances can induce development of a population of cytotoxic lymphocytes, or "killer cells" (13–15).

It is now apparent that development of an antibody response to complex, multideterminant antigens requires a cooperative interaction between lymphocytes of thymus and bone marrow derivation (16). Antibody synthesis per se is an exclusive property of B lymphocytes, whereas T cells function in a permissive or "helper" capacity which is as yet poorly understood. Under physiological conditions the activities of both cell types are antigen dependent and antigen specific (17). Although it is clear that mitogens can nonspecifically replace antigen stimulation for many cellular immune functions, it is not clear whether they can similarly activate or modulate helper T cell activity for humoral immunity. The studies to be reported were undertaken to examine the effects of nonspecific mitogen-induced activation of lymphocytes on antibody synthesis in vitro.

Materials and Methods

Mice.—Male C57BL/6J and BALB/cJ mice were obtained from Jackson Laboratory, Bar Harbor, Maine, and maintained on laboratory chow and acidified-chlorinated water ad lib. Experiments were performed with 8- to 16-wk old animals.

Mitogens.—Twice recrystallized concanavalin A (Con A) (Nutritional Biochemicals Corporation, Cleveland, Ohio) was stored at room temperature in saturated NaCl solution and sterilized by filtration through a 0.45 μm Millipore membrane (Millipore Corporation, Bedford, Mass.) saturated with Con A. Sterile phytohemagglutinin-P (PHA-P) (Wellcome Reagents, Ltd., Beckenham, England) and pokeweed mitogen (PWM) (Grand Island Biological Co., Grand Island, N. Y.) were stored in lyophilized form at -20°C. All mitogens were diluted with Hanks' balanced salt solution (HBSS) before use and were subsequently stored at 4°C and used within 1 wk of dilution.

Erythrocyte Antigens.—Sheep (SRBC) and horse (HRBC) erythrocytes (Grand Island Biological Co.) were obtained in Alsever's solution and washed three times in HBSS by centrifugation before use.

Spleen Cell Cultures.—Preparation of spleen cell suspensions and techniques of cell culture have been described in detail previously (18–20). Briefly, single cell suspensions of spleens were prepared by gentle teasing and sedimentation of tissue fragments and debris. Cells cultured for antibody synthesis were suspended in Eagle's minimal essential medium, supplemented with L-glutamine, nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum (Reheis Co., Inc., Kankakee, Ill.; lot E21806) and containing penicillin, streptomycin, and nystatin. Cultures were established at 10^7 spleen cells per ml with 10^7 heterologous erythrocytes. 1-ml cultures were maintained in 35-mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) at 37°C on a continuously rocking platform in a humidified atmosphere of 10% CO_2, 7% O_2, and 83% N_2 (19). Experimental groups contained three cultures which were pooled for assay of hemolytic plaque-forming cells. Cells cultured for DNA synthetic responses to mitogens were maintained in triplicate as 1-ml cultures in 12 $\times$ 75 mm plastic tubes (Falcon Plastics) containing 10^6 cells in RPMI-1640 medium supplemented with L-glutamine and 5% fetal bovine serum and containing penicillin, streptomycin, and nystatin at 37°C in a humidified atmosphere of 5% CO_2 in air (20).

Hemolytic Plaque Assay.—The plaque-forming cell (PFC) response of cultured cells was assayed by enumerating IgM or IgG antibody-producing cells by the slide modification of the localized hemolysis-in-gel technique of Jerne previously described (19). Three slides were prepared from pooled cultures in experimental groups and PFC responses are expressed as the mean PFC per culture. To permit pooling of data from replicate experiments with differing control PFC responses, control data were normalized to responses of 1,000 IgM or 200 IgG PFC per culture and experimental data were derived by multiplying observed responses by the normalization factor.

Tritiated Thymidine Incorporation.—The incorporation of tritiated thymidine ($[^3H]TdR$) (Schwarz/Mann, Orangeburg, N. Y.) into DNA in mitogen-stimulated cultures was assayed by adding 1.0 μCi of the isotope to cultures for the final 18 h of a 72-h culture period. Cells from individual cultures were harvested on 0.45-μm Millipore membranes and washed once with cold phosphate-buffered saline containing 1 mM unlabeled thymidine (Schwarz/Mann), twice with cold 5% trichloroacetic acid containing 1 mM unlabeled thymidine, and once with cold 95% ethanol. Dry filter membranes were placed in Aquasol (New England Nuclear Corp., Boston, Mass.) and radioactivity was assayed in a Beckman liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) Data are expressed as mean counts per minute (cpm) of triplicate cultures.

Cell Enumeration and Viability Determination.—Nucleated cells were enumerated with a Model B Coulter Counter (Coulter Electronics, Hialeah, Fla.). Cell viability was determined by trypan blue exclusion; cells were stained for 30 s in 0.04% trypan blue, fixed with an equal volume of 4% acetic acid, and counted in a hemacytometer.

Iodination of Con A.—Con A was iodinated by a modification of the method of Greenwood, Hunter, and Glover (21). 5 mg Con A in 200 μl saturated NaCl were added to 1 mCi carrier-free $[^{125}I]Na$ (New England Nuclear Corp.) at 4°C. 20 μl chloramine-T (5 mg/ml) (Eastman Kodak Co., Rochester, N. Y.) and 20 μl 0.5 M phosphate buffer pH 7.5 were added to the $[^{125}I]$Con A mixture and the iodination reaction was allowed to proceed with occasional mixing for 10 min. The reaction was halted by adding 40 μl $Na_2S_2O_5$ (5 mg/ml) and 30 μl KI (10 mg/ml). Unbound ^{125}I was removed by exhaustive dialysis at room temperature against 1 M NaCl; 1 mM KI was present in the initial dialysate. Radioactivity of the $[^{125}I]$Con A and of spleen cells after injection of $[^{125}I]$Con A into mice was measured in a Packard gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Con A content was quantified from a dilution curve of trichloroacetic acid-precipitable cpm per ng of $[^{125}I]$Con A.

Treatment of Spleen Cells with Anti-θ Serum and Complement.—AKR anti-θC3H/HeJ serum was prepared by the method of Raff (22) and stored at -20°C until use. The anti-θ serum used had 90% cytotoxicity titer of 1:250 against C57BL/6 thymus cells by ^{51}Cr release assay. Cytotoxic activity against C57BL/6 thymus and spleen cells was completely absorbed from the serum by reacting 3 ml serum with brain homogenate from three C3H/HeJ mice at 4°C for 1 h three times. Spleen cells were treated with neat anti-θ serum for 30 min at 4°C (10^8 cells per ml neat anti-θ serum), washed once with HBSS, and resuspended in 4 ml of a 1:4 dilution of noncytotoxic guinea pig complement (Baltimore Biological Laboratories, Baltimore, Md.) containing 10 μg/ml deoxyribonuclease (Worthington Biochemical Co., St. Louis, Mo.) per 10^8 cells. Cells were incubated with complement for 30 min at 37°C, washed twice in HBSS, and resuspended in medium. Control cells were treated with brain-absorbed anti-θ serum and complement. PFC responses in anti-θ-treated C57BL/6 spleen cell cultures were restored by addition of 3×10^6 normal C57BL/6 lymph node cells (20).

Cell Irradiation.—Spleen cells were irradiated in plastic Petri dishes with a General Electric Maximar 250 Type III X-ray Therapy Unit operating at 250 kVp, 15 mA. Irradiation was delivered at 140 R/min through 0.5 mm Cu, 1.0 mm Al filters. After irradiation, cells were washed in HBSS and resuspended in fresh medium.

RESULTS

Effects of Phytomitogens on Primary Immune Responses In Vitro.—In an initial series of experiments Con A or PHA-P at varying concentrations was added at culture initiation to dissociated spleen cell cultures stimulated with 10^7 SRBC. Cultures were harvested at 5 days and assayed for IgM PFC. Similar results were obtained with both mitogens (Figs. 1 and 2). At submitogenic doses

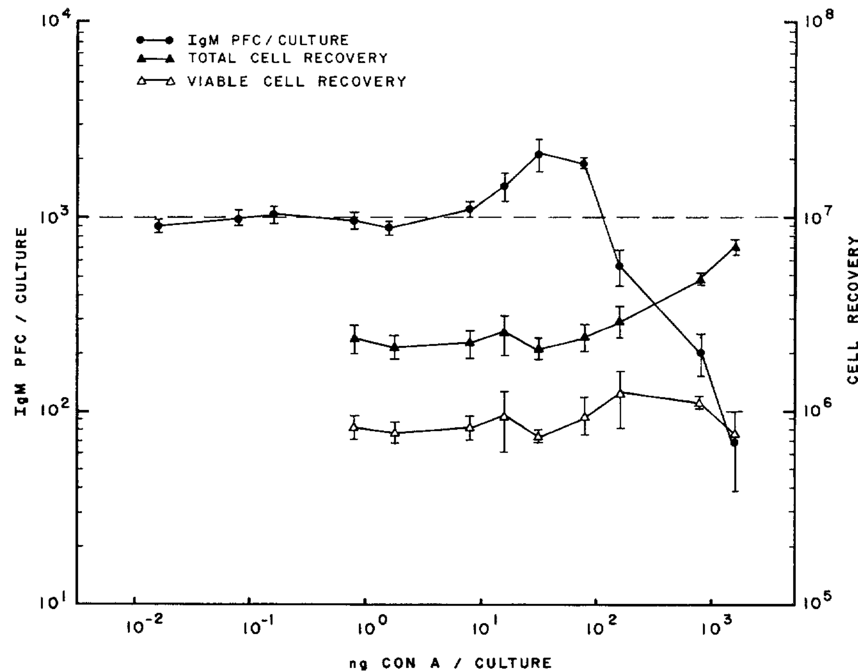

FIG. 1. Effects of graded doses of Con A added to spleen cell cultures at initiation on the 5-day primary IgM PFC response to SRBC. Data represent means and standard errors of means of four separate experiments normalized to a control response of 1,000 PFC per culture.

of 15–75 ng Con A or PHA-P per culture, enhancement of PFC responses of about twofold was observed. Conversely, after addition of mitogenic concentrations of Con A or PHA-P, 150–2,500 ng/culture, PFC responses were profoundly suppressed. Submitogenic doses of Con A and PHA-P had no effect on either total or viable cell recovery at 5 days, whereas mitogenic doses substantially increased total cell recovery but had little or no effect on the absolute number of viable cells recovered. Similar data were obtained when assays were performed at 3 days, at the peak of mitogen-stimulated cell division, rather than at 5 days, when there was little continuing effect of Con A or PHA-P on [^{3}H]TdR incorporation. Addition of mitogen to cultures immediately before assay had no effect on PFC recovery.

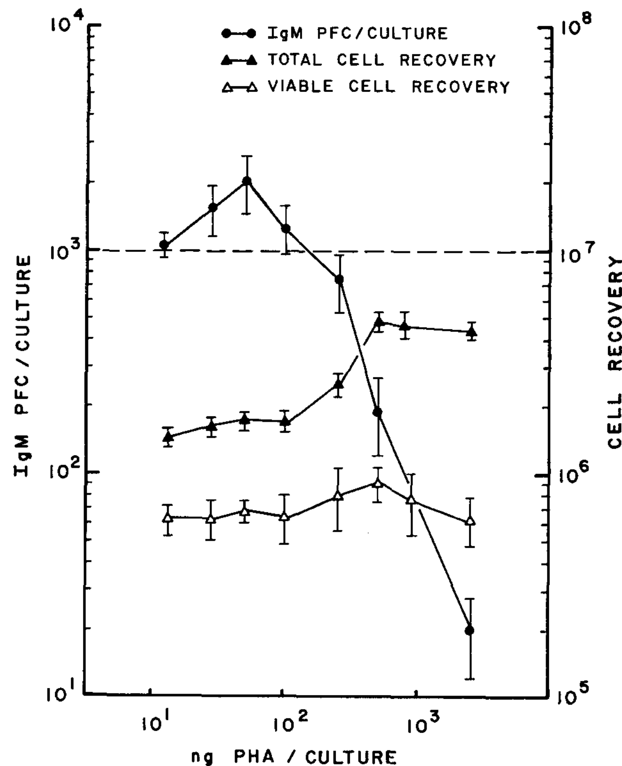

FIG. 2. Effects of graded doses of PHA-P added to spleen cell cultures at initiation on the 5-day primary IgM PFC response to SRBC. Data represent means and standard errors of means of five separate expeiiments normalized to a control response of 1,000 PFC per culture.

Diverse results were obtained by varying the time of mitogen addition to cultures. Treatment of cultures with submitogenic doses of Con A or PHA-P 24 h or more after culture initiation had no significant effect on PFC responses. However, in contrast with their suppressive effect when added at time 0, mitogenic doses of both Con A and PHA-P, as well as PWM, augmented PFC responses when added at 48 h (Fig. 3). Neither enhancement nor suppression of PFC responses was noted after treatment in vitro between 72 and 120 h. The enhancement of PFC responses induced by Con A added to cultures at 48 h was consistently greater than enhancement observed when either PHA-P or PWM was added at this time; mean augmentation after Con A addition was 4.5-fold with a range of 2- to 10-fold increase in the PFC response.

Study of the kinetics of PFC enhancement after Con A addition at 48 h revealed that this effect was manifest remarkably quickly (Fig. 4). PFC responses in cultures to which Con A was added immediately before harvesting were not different from responses in control cultures. When cultures were har-

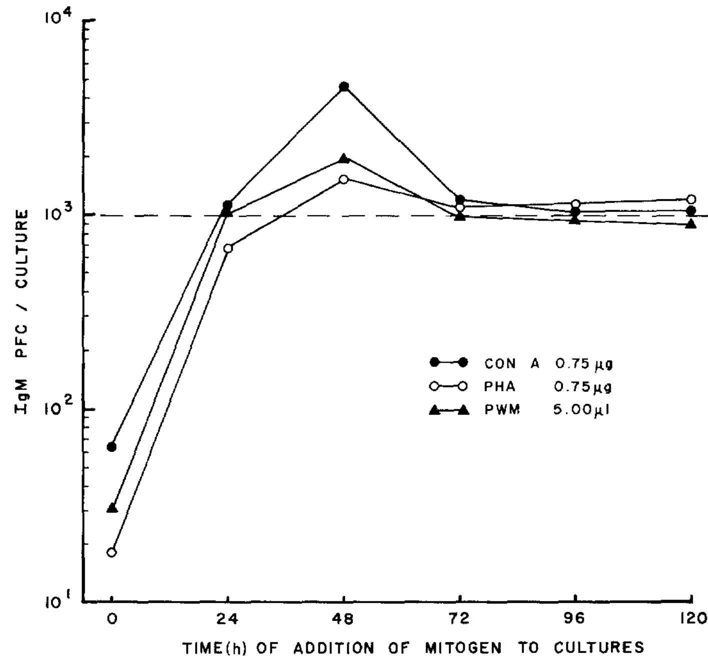

FIG. 3. Effects of adding mitogens 48 h after culture initiation on 5-day primary IgM PFC responses in vitro. Data represent mean PFC responses from three experiments normalized to a control response of 1,000 PFC per culture.

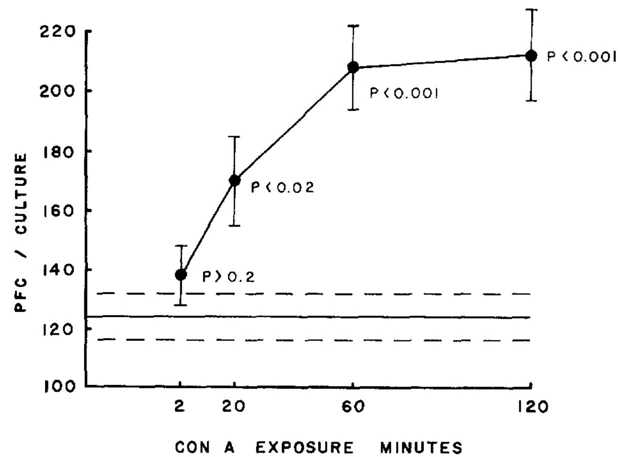

FIG. 4. Enhancement of primary IgM PFC response at 48 h after brief incubation with Con A, 0.75 μg/culture, immediately before assay. Data represent means and standard errors of means of absolute PFC/culture recovered in four experiments with eight individually assayed cultures per data point per experiment.

vested 1 h after addition of Con A, however, the response was enhanced to 170% of the control value, a difference which was statistically significant with $P < 0.001$ by two-tailed Student's t test.

The suppressive and enhancing effects of Con A were not unique to spleen cells of the C57BL/6 strain which mount vigorous anti-SRBC responses in vitro but are relatively poor responders to mitogens as determined by [^{3}H]TdR incorporation (23). PFC responses of BALB/c spleen cells, which respond less vigorously to SRBC (19) but more actively to Con A and PHA-P, were either suppressed (at 0 time) or enhanced (at 48 h) by 0.75 μg/culture Con A, effects analogous to those on C57BL/6 cells (Fig. 5).

It has been reported that although soluble Con A activates only T cells, it binds equally to T and B cells (3, 24). Before addition to cultures, Con A solutions (30 μg/ml) were absorbed three times at 4°C for 30 min with bone marrow or thymus cells at a final concentration of 20% cells (Table I). Both the suppressive activity of a mitogenic dose (0.75 μg/culture) and the enhancing activity of

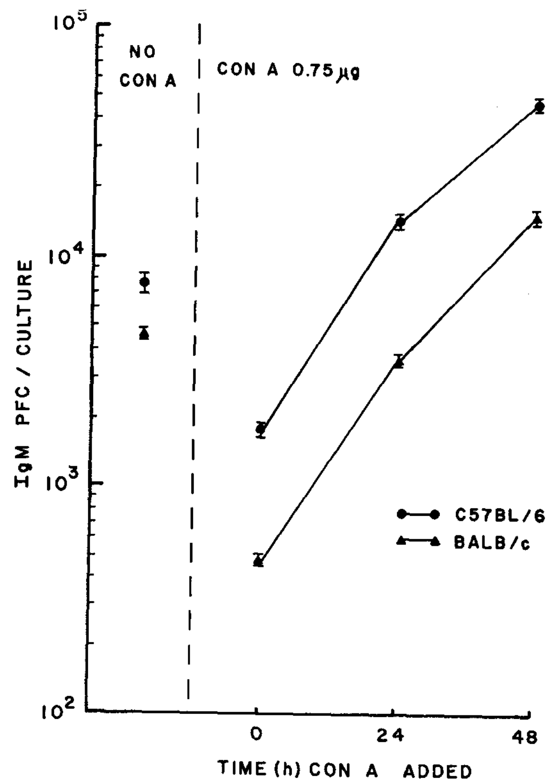

FIG. 5. Comparison of effects of Con A on 5-day primary IgM PFC responses to SRBC in C57BL/6 and BALB/c spleen cell cultures. Con A, 0.75 μg/culture, was added at 0, 24, or 48 h incubation. Data represent absolute IgM PFC per culture.

TABLE I

Effect of Absorption of Con A with Bone Marrow and Thymus Cells on Con A-Induced Modulation of PFC Responses

Unabsorbed Con A dose μg/culture	IgM PFC per culture		
	Unabsorbed Con A	BM-absorbed Con A*	Thymus-absorbed Con A*
None	1,850	—	—
0.75	500	2,140	2,024
0.15	1,640	2,844	3,300
0.075	9,324	2,725	2,100
0.03	2,824	2,850	2,600

* Con A solution absorbed three times at 4°C for 30 min with bone marrow (BM) or thymus cells at a final concentration of 20% cells.

a submitogenic dose (0.075 μg/culture) of Con A were absorbed with either cell type. As a protein which possesses sites for covalent binding to various carbohydrates including cross-linked dextran, Con A can also be absorbed from solution in this manner (25). A Con A solution, 40 ml at 300 μg/ml, was absorbed once with 1 g of Sephadex G-75. Absorbed or unabsorbed solutions were then added to SRBC-stimulated cultures at 0 or 48 h and to 1-ml cultures in vertical stationary tubes to assay [^{3}H]TdR incorporation. Sephadex absorbed both the mitogenic activity and the enhancing activity for PFC responses of mitogenic doses of Con A added to cultures at 48 h (Table II). The suppressive activity on PFC responses of the same doses added at culture initiation was also markedly reduced although not completely abrogated by a single Sephadex absorption.

Effects of Antigen Exposure on Con A-Induced Enhancement of PFC Responses. —Experiments were designed to ascertain whether previous activation of spleen cells by specific antigen was required for manifestation of enhancement of PFC responses by Con A added at 48 h, and whether prior activation by one antigen altered the effect of Con A on responses to a second antigen (Table III). Data presented are IgM PFC responses to SRBC and HRBC assayed after 5 days in culture. The PFC responses in cultures assayed after 7 days were consistently lower than 5-day responses whether antigen was added at 0 time or 48 h or both, but the Con A-induced alteration of responses was qualitatively the same at both 5 and 7 days. First, Table III notes the effect of Con A, 0.75 μg/culture, added at 48 h on background PFC recovery (group A). In the absence of added antigen, background responses to both SRBC and HRBC were increased. Second, if SRBC was added 48 h after culture initiation, simultaneous with addition of Con A, 0.75 μg/culture, enhancement of the anti-SRBC PFC response was observed (group B). Finally, enhancement of responses to both SRBC and HRBC was also observed when either antigen was added at 0 time and the second antigen was added with 0.75 μg Con A 48 h later (group C).

TABLE II

Effect of Absorption of Con A with Sephadex G-75 on Con A-Induced Modulation of PFC Responses

Unabsorbed Con A dose, μg/culture	Absorption*	[³H]TdR	IgM PFC per culture	
			Con A 0 h‡	Con A 48 h‡
		cpm		
None		4,480	4,953	4,953
1.5	−	34,773	83	19,920
0.75	−	33,596	391	17,440
0.38	−	6,675	1,227	10,213
1.5	+	5,075	1,340	5,147
0.75	+	4,440	2,680	5,267
0.38	+	6,904	4,693	5,307

* The Con A solution, 40 ml at 300 μg/ml, was absorbed once at room temperature for 1 h with 1 g Sephadex G-75.

‡ Absorbed or unabsorbed Con A solution added to cultures at culture initiation or 48 h later.

TABLE III

Role of Antigen Exposure on Enhancement of PFC Responses by Con A In Vitro

Experimental group	Time SRBC added	Time HRBC added	Con A* (0.75 μg/culture)	Anti-SRBC‡ PFC per culture	Anti-HRBC‡ PFC per culture
	h	*h*			
A	None	None	−	55	14
	None	None	+	295	46
B	48	None	−	583	—
	48	None	+	1,320	—
C	0	None	−	3,600	5
	0	48	−	3,735	212
	0	48	+	9,750	1,248
	None	0	−	142	705
	48	0	−	668	1,980
	48	0	+	1,350	5,160

* Con A, 0.75 μg/culture, added at 48 h.

‡ Data represent IgM PFC response to both sheep erythrocytes (SRBC) and horse erythrocytes (HRBC) at 5 days. Erythrocyte antigen dose was 10^7 erythrocytes per culture.

These experiments indicated that Con A expanded the background pool of PFC to both SRBC and HRBC and that prior activation of cultures by specific antigen was not required for manifestation of Con A-induced enhancement of PFC responses. Moreover, the time of Con A addition to cultures rather than time of antigen exposure determined the effect of Con A on antibody synthesis.

Effects of Administration of Con A In Vivo on Primary Immune Responses In Vitro.—In order to ascertain whether enhancement or suppression of primary immune responses in vitro could also be induced by administration of Con A in vivo, mice were injected with Con A intravenously 24 h before culture initiation. When 10^7 spleen cells from mice so treated were cultured, effects were similar to those obtained by adding Con A directly to cultures. A small dose of Con A (6 μg intravenously, T_{-1}) augmented the PFC response about fourfold, whereas a large dose (150 μg intravenously, T_{-1}) caused suppression of the response of similar magnitude (Fig. 6). No effect was observed when cultures were established 30 min after Con A injection. Dose-dependent suppression and enhancement of PFC responses were both maximal 24 h after injection of Con A; the

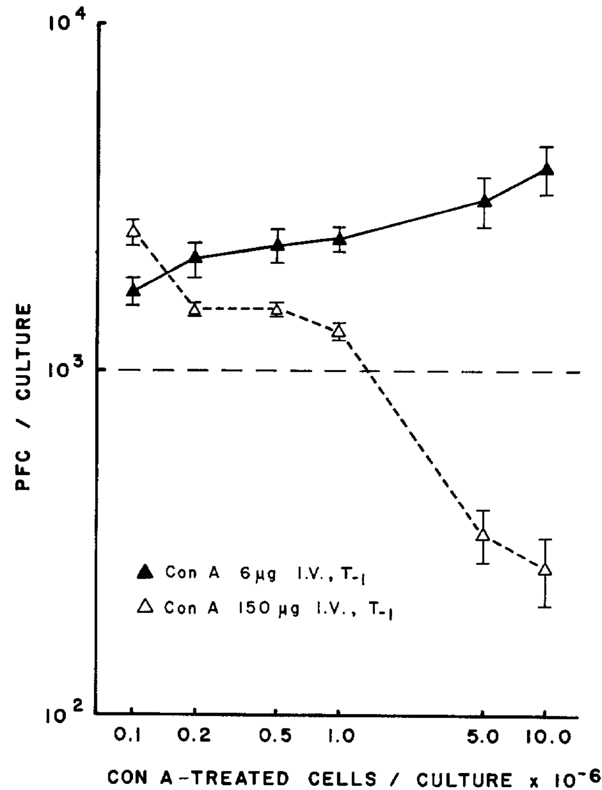

FIG. 6. Suppression or enhancement of 5-day primary IgM PFC responses to SRBC in cultures of graded numbers of spleen cells from Con A-injected mice and normal spleen cells. All cultures contained a total of 10^7 cells. Mice were injected intravenously with 6 μg or 150 μg Con A 24 h before preparation of spleen cell suspensions. Data represent means and standard errors of means of PFC per culture from four experiments normalized to a control response of 1,000 PFC per culture.

effects gradually decayed as the interval between Con A administration and culture initiation was increased to 5 days.

Fig. 6 also illustrates the result of mixing normal spleen cells with cells from mice injected with Con A 24 h previously. All cultures contained a total of 10^7 cells with graded increments from 1 to 100% of the cells being derived from Con A-treated mice. Synergistic enhancement of PFC responses was observed when small numbers of cells from mice injected with 6 μg Con A were mixed with larger numbers of normal cells. As few as 10^5 spleen cells from mice injected with 6 μg of Con A approximately doubled PFC responses when mixed with 9.9×10^6 normal spleen cells. When 1×10^6 cells from Con A-treated mice were mixed with 9×10^6 normal cells, the response was enhanced about 2.5-fold. As previously noted, a pure inoculum of Con A-treated cells had a PFC response approximately four times greater than that of untreated control cells. Interestingly, despite the substantial suppression of responses in cultures of 10^7 spleen cells from recipients of 150 μg of Con A, enhancement of about 2.5-fold, rather than suppression, was observed when 10^5 of these cells were mixed with 9.9×10^6 normal spleen cells.

It was thought that the enhancement of PFC responses observed when small numbers of cells from Con A-treated mice were mixed with much larger numbers of normal cells could have been due to transfer of Con A to the cultures with the spleen cells, rather than the consequence of activation of the small number of Con A-treated cells themselves. This problem was approached by the direct measurement of the amount of [^{125}I]Con A present in 10^5 spleen cells from mice injected 24 h before with various doses of [^{125}I]Con A (Table IV). When these data are compared with the Con A dose-response curve (Fig. 1), it is apparent that the amount of [^{125}I]Con A transferred with 10^5 Con A-treated cells 24 h after injection of 150 μg was at least one order of magnitude less than that required for enhancement of PFC responses when Con A was added directly to cultures. Moreover, after injection of 6 μg, insufficient Con A was transferred 24 h later to affect PFC responses at any cell dose up to 10^7/culture.

TABLE IV

Amount of [^{125}I]Con A in 10^5 Spleen Cells 24 h After Intravenous Injection

Con A dose injected	[^{125}I] Con A per 10^5 spleen cells*
μg	*ng*
1	0.0024 ± 0.0004
6	0.0276 ± 0.0016
30	0.2259 ± 0.0149
150	1.5756 ± 0.1292

* Data represent the means and standard errors of the means of ng [^{125}I]Con A in 10^5 spleen cells from four mice per dose injected with [^{125}I]Con A 24 h previously. Trichloroacetic acid-precipitable cpm in 10^5 spleen cells were converted to nanograms Con A from a standard curve of trichloroacetic acid-precipitable cpm in known [^{125}I]Con A solutions.

Further characterization of the cell population mediating enhancement solidified the argument that transferred Con A did not account for the synergistic enhancement of PFC responses observed when mixing normal and Con A-treated spleen cells. Spleen cells from mice injected with 6 μg Con A were treated with anti-θC3H serum and complement before mixing with normal cells. Control Con A-treated cells were exposed to brain-absorbed anti-θ serum and complement. Treatment with unabsorbed anti-θ serum and complement completely abolished the 2- to 2½-fold enhancement of responses mediated by control Con A-treated cells (Table V). The effectiveness of treatment with anti-θ serum and complement was demonstrated by the profound suppression of PFC responses in cultures of 10^7 anti-θ-treated cells from Con A-injected mice.

Characteristics of Con A-activated cells with enhancing activity were also delineated by exposure of these cells to 2000 R X-irradiation 24 h after injection of 6 μg Con A in vivo and before mixing with normal cells (Fig. 7). Irradiation completely abolished the capacity of these cells cultured alone to mount an immune response. However, the magnitude of enhancement of PFC responses of normal spleen cells induced by 10^5 to 10^6 spleen cells from Con A-treated animals was not altered by 2,000 R irradiation.

Effects of Con A on IgG PFC Responses.—The foregoing data represent observations of the effects of phytomitogens on IgM PFC responses. It clearly was

TABLE V

Effect of Anti-θC3H and Complement Treatment of Spleen Cells from Mice Injected with Con A 24 h Previously on Their Ability To Enhance PFC Responses by Normal Spleen Cells

Normal cells per culture × 10^{-6}	Con A spleen cells per culture × 10^{-6}	Treatment of Con A cells*	IgM PFC per culture‡
10 Spleen	0	—	1,000
9.9 Spleen	0.1	Brain-absorbed anti-θ + C	1,570
9.8 "	0.2	" " " + "	1,603
9.5 "	0.5	" " " + "	2,066
9 "	1.0	" " " + "	1,907
0 "	10.0	" " " + "	2,623
9.9 Spleen	0.1	Anti-θ + C	980
9.8 "	0.2	" + "	986
9.5 "	0.5	" + "	1,120
9 "	1.0	" + "	1,140
0 "	10.0	" + "	58

* Spleen cells from mice injected intravenously 24 h before with 6 μg Con A were treated with neat AKR anti-θC3H or brain-absorbed anti-θC3H for 30 min at 37°C, 10^8 cells/ml antiserum. Cells were then washed and incubated for 30 min at 37°C with 1:4 guinea pig complement containing 10 μg/ml deoxyribonuclease.

‡ Data represent means of IgM PFC per culture from three separate experiments. Data have been normalized to a control response of 1,000 PFC per culture.

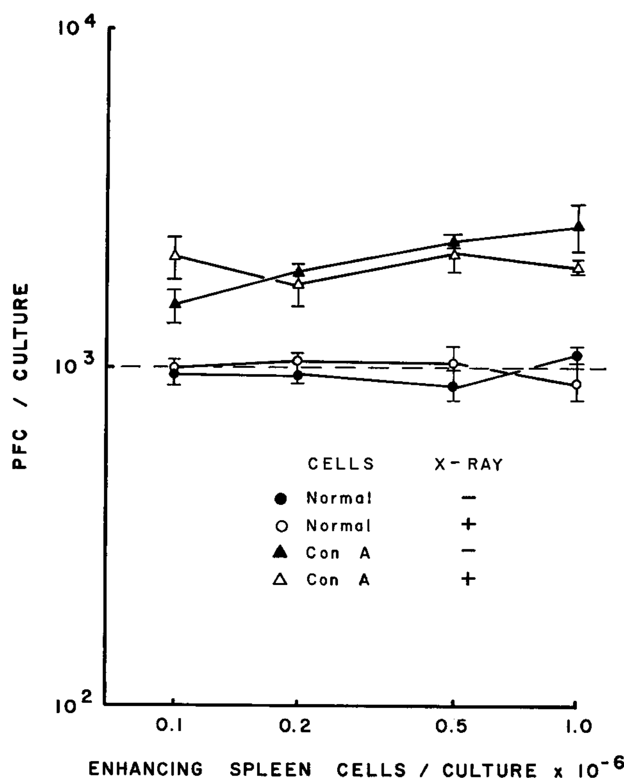

FIG. 7. Radiation resistance of the enhancing effect of small numbers of spleen cells from Con A-injected mice on IgM PFC responses of normal spleen cells. All cultures contained a total of 10^7 cells. Mice were injected intravenously with 6 μg Con A 24 h before preparation of spleen cell suspensions. Cells were exposed to 2,000 R X-irradiation immediately before mixing with normal spleen cells in culture. Data represent means and standard errors of means from three separate experiments normalized to a control response of 1,000 PFC per culture.

important to establish whether these effects were confined to the IgM response or whether synthesis of antibodies in other immunoglobulin classes was also affected. Spleen cells were consequently treated either in vitro or in vivo with Con A doses and regimens previously shown to enhance or suppress IgM synthesis, and assays for IgM and IgG PFC were performed in parallel (Table VI). In all experiments the effects on IgM and IgG antibody responses were concordant. Both suppression and enhancement of IgG PFC development were observed and the direction of change was consistently the same as the effect on IgM responses.

DISCUSSION

These experiments demonstrate that the nonspecific phytomitogens can substantially enhance or suppress primary PFC responses to heterologous erythrocytes in vitro. The effects can be obtained by administration of mitogen either

TABLE VI

Effect of Con A on IgG PFC Responses In Vitro

Treatment mode	Con A		PFC response*	
	Dose	Time of Con A treatment	IgM	IgG
	μg	h		
—	None	—	1,000	200
In vitro	0.75	0	27	8
In vitro	0.75	48	2,365	906
In vivo	6.0	−24	2,213	632
In vivo	150.0	−24	84	43

* Data represent means of IgM and IgG PFC per culture from three separate experiments. Data have been normalized to control responses of 1,000 IgM PFC and 200 IgG PFC per culture.

in vivo or in vitro and is critically dependent upon both dose and time of mitogen treatment. A small number of mitogen-activated radioresistant T cells derived from mice injected with Con A enhance the PFC responses of much larger numbers of normal spleen cells. The mechanisms of the modulation of PFC responses by mitogen-activated T cells are not as yet clear. Our observations, however, are consistent with presently emerging concepts of T cell-mediated control of antibody synthesis by B cells (17).

Suppression of PFC responses generally followed treatment of spleen cells with mitogenic doses of Con A or PHA before or concomitant with culture initiation. Suppression was not due simply to elaboration of toxic substances into the culture medium since 10-fold suppression of PFC responses was not associated with reduction in viable cell recovery. Moreover, suppression was manifest both during and after the period of intense mitogenic activity in the cultures. Studies with [^{125}I]Con A established that suppression of antibody synthesis was a secondary effect mediated by activated T lymphocytes rather than a direct effect of mitogen-lymphocyte interaction. This was demonstrated by the substantial inhibition of PFC responses in cultures where equal numbers of normal spleen cells and spleen cells from mice injected with 150 μg Con A 24 h previously were mixed. These cultures contained approximately 80 ng of Con A, which was transferred with the spleen cells from Con A-injected mice and was a dose of Con A which enhanced rather than suppressed PFC responses when added directly to cultures. These data support recent observations from several laboratories that a population of thymus or thymus-derived lymphocytes stimulated with soluble antigens (26–31) or allogeneic cells (32) can suppress B cell responses to antigen. Although this "suppressor" T cell function remains, as yet, poorly understood, concordance of the suppression and enhancement of both IgM and IgG PFC responses in the present experiments is important since it suggests that the regulatory function of T cells involves direct quantitative

control of antibody production rather than a permissive effect on synthesis of one class of antibody and consequent negative feedback on another class of antibody.

The cell population mediating enhancement of PFC responses when large numbers of normal spleen cells were mixed with much smaller numbers of cells from Con A-injected mice was more fully characterized. The data clearly indicate that this enhancing effect was mediated by activated T cells since it was completely and specifically abrogated by treatment of the cells from Con A-injected animals with anti-θ serum and complement before mixing with normal cells. Although susceptible to treatment with anti-θ serum, the capacity of these cells to enhance PFC responses of normal cells was resistant to 2,000 R X-irradiation. In both respects, therefore, these cells are entirely similar to antigen-induced T helper cells described by others (17, 33–36).

Of particular interest are the paradoxical effects on PFC responses of adding to cultures small versus large numbers of spleen cells from mice injected 24 h previously with 150 μg Con A. Although PFC responses in cultures containing 50% or more of these cells were markedly suppressed, addition of 10^5 cells from these mice to cultures of 9.9×10^6 normal cells resulted in 2.5-fold enhancement of PFC responses. These observations suggest that the two effects, enhancement and suppression of PFC responses, may be mediated by a single population of regulatory T cells which promote antibody synthesis when present in small numbers and inhibit antibody synthesis when present in larger numbers. Alternatively, these same effects might be observed if two populations of cells with antagonistic functions were involved. If the frequency of helper cells were greater, but the potency per cell less than that of suppressor cells, at limiting dilutions the net effect of two antagonistic populations would be enhancement, whereas suppression would be the net result obtained with larger numbers of cells.

Andersson and coworkers (4) have observed that B lymphocytes proliferate when stimulated by Con A in the presence of humoral factors released by thymocytes. It is therefore conceivable that the enhancing activity of a mitogenic dose of Con A added after 48 h in culture reflected a direct effect of Con A on antibody-producing cells or their precursors. The progressive functional loss of the θ-alloantigen from cultured T cells[2] precluded analysis of derivation of cells activated by mitogens added to cultures at 48 h since T cells could not effectively be killed with anti-θ serum and complement at this time. It is apparent, however, that the enhancement of PFC responses did not depend entirely on mitogenesis in the responding clone of B cells since significant enhancement of PFC responses occurred within 1 h of addition of Con A to cultures at 48 h. This is many hours before DNA synthesis or cell replication can be detected in

[2] Pierce, C. W. 1972. Immune responses in vitro. VII. Loss of susceptibility of functional θ-bearing cells to cytotoxic action of anti-θ serum and complement in vitro. Submitted for publication.

mitogen-stimulated cultures (37) but is consistent with the observed early increases in protein and RNA synthesis (38–41). Moreover, enhancement of PFC responses after addition of submitogenic doses of Con A at culture initiation was also observed. These latter data emphasize that the nonspecific mitogens may have important effects on lymphocyte functions at doses insufficient to cause DNA replication, mitosis, or blast transformation.

The time-course of enhancement of PFC responses after addition of Con A to cultures at 48 h suggests possible mechanisms of this effect. In these circumstances Con A might stimulate active antibody synthesis earlier by committed precursors of PFC which, in the absence of Con A, would begin to secrete antibody somewhat later. Alternatively, Con A might provoke, either directly or via a T cell mediator, antibody synthesis by cells of marginal specificity for the stimulating antigen. Detailed studies, currently in progress, of the kinetics of PFC development should help delineate the correct mechanism. The increased recovery of background PFC to both SRBC and HRBC in vitro and the previously reported increase in background PFC after administration of PHA in vivo (42, 43) provide indirect support for the latter hypothesis.

It is apparent that further study of mitogen-induced effects on antibody synthesis will provide important information regarding regulatory mechanisms in immune responses. Experiments similar to those exploiting mitogen-induced lymphocyte activation for delineation of T cell functions in cellular immunity are in progress. In particular, the model should be useful in the investigation of soluble factors released from activated T cells which modulate and control antibody synthesis and in studies designed to establish the identity of or differences between cells or soluble cell products which mediate suppressor or helper T cell functions.

SUMMARY

The effects of nonspecific phytomitogens on primary plaque-forming cell (PFC) responses of mouse spleen cells to heterologous erythrocytes in vitro were studied. Spleen cell cultures treated with concanavalin A or phytohemagglutinin in vitro or established with spleen cells derived from mice injected with concanavalin A 24 h previously were similarly affected. In both cases, submitogenic doses resulted in substantial enhancement of PFC responses, whereas 10-fold larger doses were profoundly inhibitory. In contrast to the suppressive effects of mitogenic doses of phytomitogens added at culture initiation, addition of these same doses to cultures 48 h later resulted in increased PFC responses. This enhancement could be observed within 1 h after treatment and consequently could not be ascribed only to mitotic expansion of the antibody-synthesizing clone. Activation of spleen cells with specific antigen before mitogen treatment was not required for expression of the enhancing or suppressing effects on PFC responses. IgM and IgG PFC responses were similarly affected. Studies of cell interactions revealed that as few as 10^5 spleen cells obtained from mice treated

with concanavalin A in vivo synergistically enhanced the PFC responses of 10^7 normal spleen cells. This enhancement was mediated by mitogen-activated T lymphocytes which were resistant to 2000 R irradiation 24 h after activation. The relevance of these observations to emerging concepts of helper and suppressor T cell activity is discussed.

We are deeply grateful to Professor Baruj Benacerraf for his interest, encouragement, and advice during the course of these experiments.

REFERENCES

1. Janossy, G., and M. F. Greaves. 1971. Lymphocyte activation. I. Response of T and B lymphocytes to phytomitogens. *Clin. Exp. Immunol.* **9:**483.
2. Janossy, G., and M. F. Greaves. 1972. Lymphocyte activation. II. Discriminating stimulation of lymphocyte subpopulations by phytomitogens and heterologous antilymphocyte sera. *Clin. Exp. Immunol.* **10:**525.
3. Stobo, J. D., A. S. Rosenthal, and W. E. Paul. 1972. Functional heterogeneity of murine lymphoid cells. I. Responsiveness to and surface binding of concanavalin A and phytohemagglutinin. *J. Immunol.* **108:**1.
4. Andersson, J., G. Möller, and O. Sjöberg. 1972. B lymphocytes can be stimulated by concanavalin A in the presence of humoral factors released by T cells. *Eur. J. Immunol.* **2:**99.
5. Greaves, M. F., and S. Bauminger. 1972. Activation of T and B lymphocytes by insoluble phytomitogens. *Nat. New Biol.* **235:**67.
6. Andersson, J., G. M. Edelman, G. Möller, and O. Sjöberg. 1972. Activation of B lymphocytes by locally concentrated concanavalin A. *Eur. J. Immunol.* **2:**233.
7. Nowell, P. C. 1960. Phytohemagglutinin: an initiator of mitosis in cultures of normal human leukocytes. *Cancer Res.* **20:**462.
8. Pick, E., J. Krejci, and J. L. Turk. 1970. Release of skin reactive factor from guinea pig lymphocytes by mitogens. *Nature (Lond.).* **225:**236.
9. Schwartz, H. J., M. A. Leon, and R. P. Pelley. 1970. Concanavalin A-induced release of skin reactive factor from lymphoid cells. *J. Immunol.* **104:**265.
10. Pick, E., J. Brostoff, J. Krejci, and J. L. Turk. 1970. Interaction between "sensitized lymphocytes" and antigen *in vitro*. II. Mitogen-induced release of skin reactive and macrophage migration inhibitory factors. *Cell. Immunol.* **1:**92.
11. Granger, G. A., and W. P. Kolb. 1968. Lymphocyte *in vitro* cytotoxicity: mechanisms of immune and non-immune small lymphocyte mediated target L cell destruction. *J. Immunol.* **101:**111.
12. Haber, J., W. Rosenau, and M. Goldberg. 1972. Separate factors in phytohemagglutinin induce lymphotoxin, interferon, and nucleic acid synthesis. *Nat. New Biol.* **238:**60.
13. Perlmann, P., and G. Holm. 1969. Cytotoxic effects of lymphoid cells *in vitro*. *Adv. Immunol.* **11:**117.
14. Stavy, L., A. J. Treves, and M. Feldman. 1971. Effect of concanavalin A on lymphocyte-mediated cytotoxicity. *Nature (Lond.).* **232:**56.
15. Schwartz, H. J., and F. Wilson. 1971. Target cell destruction *in vitro* by concanavalin A-stimulated lymphoid cells. *Am. J. Pathol.* **64:**295.

16. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen-reactive cells. *Transplant. Rev.* **1**:3.
17. Katz, D. H., and B. Benacerraf. 1972. The regulatory influence of activated T cells on B cell responses to antigen. *Adv. Immunol.* **15**:1.
18. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
19. Pierce, C. W., B. M. Johnson, H. E. Gershon, and R. Asofsky. 1971. Immune responses in vitro. III. Development of primary γM, γG, and γA plaque-forming cell responses in mouse spleen cell cultures stimulated with heterologous erythrocytes. *J. Exp. Med.* **134**:395.
20. Mosier, D. E., and C. W. Pierce. 1972. Functional maturation of thymic lymphocyte populations in vitro. *J. Exp. Med.* **136**:1484.
21. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of I^{131} labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89**:114.
22. Raff, M. C. 1969. Theta isoantigen as a marker of thymus-derived lymphocytes in mice. *Nature (Lond.).* **224**:378.
23. Williams, R. M., and B. Benacerraf. 1972. Genetic control of thymus-derived cell function. I. In vitro DNA synthetic response of normal mouse spleen cells stimulated by the mitogens concanavalin A and phytohemagglutinin. *J. Exp. Med.* **135**:1279.
24. Greaves, M. F., S. Bauminger, and G. Janossy. 1972. Lymphocyte activation. III. Binding sites for phytomitogens on lymphocyte subpopulations. *Clin. Exp. Immunol.* **10**:537.
25. Agrawal, B. B. L., and I. J. Goldstein. 1965. Specific binding of concanavalin A to cross-linked dextran gels. *Biochem. J.* **96**:23c.
26. Baker, P. J., P. W. Stashak, D. F. Amsbaugh, B. Prescott, and R. F. Barth. 1970. Evidence for the existence of two functionally distinct types of cells which regulate the antibody response to Type III pneumococcal polysaccharide. *J. Immunol.* **105**:1581.
27. Möller, G., and G. Michael. 1971. Frequency of antigen-sensitive cells to thymus-independent antigens. *Cell. Immunol.* **2**:309.
28. Okumura, K., and T. Tada. 1971. Regulation of homocytotropic antibody in the rat. Inhibitory effect of thymocytes on the homocytotropic antibody response. *J. Immunol.* **107**:1682.
29. Kerbel, R. S., and D. Eidinger. 1972. Enhanced immune responsiveness to a thymus-independent antigen early after adult thymectomy: evidence for short-lived inhibitory thymus-derived cells. *Eur. J. Immunol.* **2**:114.
30. Jacobson, E. B., and L. A. Herzenberg. 1972. Active suppression of immunoglobulin allotype synthesis. I. Chronic suppression after perinatal exposure to maternal antibody to paternal allotype in (SJL $\times$ BALB/c)F_1 mice. *J. Exp. Med.* **135**:1163.
31. Eidinger, D., and H. Pross. 1972. Studies of antibody formation *in vitro* and in lethally irradiated reconstituted mice. Evidence for an inhibitory function of thymus-derived cell. *Scand. J. Immunol.* **1**:193.
32. Britton, S. 1972. When allogenic mouse spleen cells are mixed *in vitro* the T-cells

secrete a product which guides the maturation of B-cells. *Scand. J. Immunol.* **1:**89.

33. Chan, E., R. I. Mishell, and G. F. Mitchell. 1970. Cell interaction in an immune response *in vitro*: requirement for theta-carrying cells. *Science (Wash. D.C.).* **170:**1215.
34. Katz, D. H., W. E. Paul, E. A. Goidl, and B. Benacerraf. 1970. Radioresistance of cooperative function of carrier-specific lymphocytes in anti-hapten antibody responses. *Science (Wash. D.C.).* **170:**462.
35. Kettman, J., and R. W. Dutton. 1971. Radioresistance of the enhancing effect of cells from carrier immunized mice in an *in vitro* primary immune response. *Proc. Natl. Acad. Sci. U.S.A.* **68:**699.
36. Vann, D. C., and J. R. Kettmann. 1972. *In vitro* cooperation of cells of bone marrow and thymus origins in the generation of antibody-forming cells. *J. Immunol.* **108:**73.
37. Epstein, L. B., and F. Stohlman. 1964. RNA synthesis in cultures of normal human peripheral blood. *Blood.* **24:**69.
38. Rubin, A. D., and H. L. Cooper. 1965. Evolving patterns of RNA metabolism during transition from resting state to active growth in lymphocytes stimulated by phytohemagglutinin. *Proc. Natl. Acad. Sci. U.S.A.* **54:**469.
39. Kleinsmith, L. J., V. G. Allfrey, and A. E. Mirsky. 1966. Phosphorylation of nuclear protein early in the course of gene activation in lymphocytes. *Science (Wash. D.C.).* **154:**780.
40. Kay, J. E. 1968. Phytohemagglutinin: an early effect on lymphocyte lipid metabolism. *Nature (Lond.).* **219:**172.
41. Rosenberg, S. A., and R. Levy. 1972. Synthesis of nuclear-associated proteins by lymphocytes within minutes after contact with phytohemagglutinin. *J. Immunol.* **108:**1105.
42. Spreafico, F., and E. M. Lerner. 1967. Suppression of the primary and secondary immune response of the mouse by phytohemagglutinin. *J. Immunol.* **98:**407.
43. Hege, J. S., and L. J. Cole. 1967. Antibody plaque-forming cells in unsensitized mice. Specificity and response to neonatal thymectomy, X-irradiation and PHA. *J. Immunol.* **99:**61.