CELLULAR RECOGNITION BY MOUSE LYMPHOCYTES IN VITRO

I. Definition of a New Technique and Results of Stimulation by Phytohemagglutinin and Specific Antigens*

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(Received for publication 3 December 1969)

The lymphoid cells of man, rat, guinea pig, rabbit, and other species respond in vitro to a variety of specific and nonspecific stimuli. Metabolic and morphologic changes in a stimulated cell population lead to cell division and elaboration of several biologic potent effector substances into the medium (1, 2). Despite the obvious genetic and operational advantages of the laboratory mouse for in vitro studies of cellular recognition, use of this species has been limited by the short life span and lack of sustained stimulated cell division permitted by tissue culture conditions.

Dutton (3) and Heumer and colleagues (4) have devised the most successful in vitro culture methods for mouse spleen cells; the former is now being widely used for the investigation of primary antibody synthesis in mouse spleen cell populations, and the latter for the study of cell to cell interaction. In other studies, cell division was shown to follow phytohemagglutinin (PHA)[1] stimulation of mouse lymphoid cells (5–7) and production of various effector substances followed alloantigen and PHA stimulation. (8–11) On the other hand, no culture medium capable of supporting extended viability and stimulation of mouse lymphoid cells has been described (12).

This series of papers describes and characterizes in detail a technically simple and dependable method of cultivating mouse lymphoid cells in vitro, and describes the results of studies of stimulation and other events of cellular recognition which the technique permits. This paper reports methods and requirements

* This research was supported in part by the National Institutes of Health, Grant No. HD-00384; the American Cancer Society, Grant No. ACS-T-463; an American Cancer Society Institutional Grant IN-62-G; and National Institute of General Medical Science Training Grant GM-01996.

‡ Work done as National Institute of Allergy and Infectious Diseases trainee Grant No. 5 TI AI 0218.

[1] *Abbreviations used in this paper:* AMLG, anti-mouse lymphocyte globulin; MRBC, mouse erythrocytes; a-MRBC, anti-mouse erythrocyte agglutinins; NCS, nuclear Chicago solubilizer; PHA, phytohemagglutinin; PHA-P, phytohemagglutinin P; PPD, purified protein derivative; RPMI, Roswell Park Memorial Institute medium; SRBC, sheep erythrocytes; TCA, trichloroacetic acid.

for mitogenic stimulation of mouse lymphoid cells with phytohemagglutinin and certain specific antigens. Subsequent papers in this series concern the results of mixed cell reactions involving a variety of strong and weak histocompatibility antigens (13), characterization of mitogenic factors elaborated in mixed cell reactions, and stimulation by tumor specific transplantation antigens.[2] A preliminary report of some early results of this investigation has been made elsewhere (14, 15).

Materials and Methods

Tissue Culture Medium.—Throughout the experiments reported, the tissue culture medium employed was a complete medium designated Roswell Park Memorial Institute (RPMI) 1640, obtained from Grand Island Biological Co., Grand Island, N. Y. For certain experiments, the medium was supplied deficient in arginine and asparagine and these amino acids were added as required by the experiments. All media employed contained 100 units of penicillin and 100 μg streptomycin per ml. 5% fresh human serum which had been heated at 56°C for 30 min was added before use. Lots of complete medium, thus constituted, were always used for experiments within 24 hr of preparation. It was soon established that human serum varies considerably in its capacity to support lymphocyte stimulation; thus, the serum from a single donor was used for each complete experiment. Under identical conditions of cell origin and number, and of stimulus, medium containing serum from a single donor gave test results having a high order of day to day and week to week reproducibility (see text).

Culture Conditions.—Each tube always contained 3.0 ml of medium, and, except where indicated, 15×10^6 mouse spleen leukocytes. The tubes (13 × 100, Falcon plastic, No. 2037, disposable screw-capped type) were incubated at 37°C, loosely capped, for varying periods of time, in a 5% CO_2 in air atmosphere and 80% humidity, inclined at a 10° angle.

Animals and Cell Preparation.—Mice used for these studies were either purchased from Jackson Laboratories, Bar Harbor, Maine, or derived from our own inbred lines, originally obtained as pedigreed stock, and kindly supplied by Jackson Laboratories. Strains employed A/J; C57Bl/6; C57Bl/10, and its congenic sublines B10-M, B10-LP, and B10-D2; BALB/C; CBA; and DBA-2. F_1 hybrids were all obtained from Jackson Laboratories, together with the appropriate parental strains to be included in a single experiment.

Cells were obtained by removing the spleen, thymus, or lymph nodes under sterile conditions. Each organ was gently minced with scissors and the tissue fragments screened through 60-mesh stainless steel gauze into the complete tissue culture medium. The cells were drawn through a 25 gauge needle to produce a suspension of single cells. The cells were then counted and distributed to the culture tubes. The spleen cell suspension usually consisted of about 95% mononuclear cells.

Assay of Anti-Mouse Antibody.—Anti-mouse erythrocyte agglutinins (a-MRBC) were assayed by adding 0.25 ml of 10% washed A/J erythrocytes to 0.25 ml of serial two-fold dilutions of serum in phosphate-buffered saline, pH 7.2; the mixture was incubated 120 min at 37°C and the hemagglutinin titer was read visually after 18 hr at 4°C. For studies in which the a-MRBC was eluted from mouse erythrocytes (MRBC) for further investigation, serum from a donor having a 1:20 titer of a-MRBC was incubated at 37°C for 30 min and at 4°C for 18 hr, with an equal volume of thrice washed A/J erythrocytes. The agglutinin was eluted from these erythrocytes after washing twice with buffered saline, either by adding one volume of ether at 37°C according to the method of Rubin (16) or by heating the cells in 7% bovine albumin at 56°C for 15 min by the method of Landsteiner and Miller (17). The supernatants

[2] Adler, W. H., T. Takiguchi, and R. T. Smith. Manuscript in preparation.

from these procedures were collected by centrifugation, concentrated, and used to determine cytotoxicity, hemolysis, and attachment to mouse lymphocyte membranes by the fluorescent antibody technique. Cytotoxicity was assayed by the method of Gorer and O'Gorman (18) and membrane immunofluorescence by the method of Möller (19). The eluted antibody was identified by the Ouchterlony technique employing various reagent anti-human immunoglobulins prepared in this laboratory.

Thymectomy.—Thymectomy was performed in newborn mice by the method described by Sjodin, et al (20) and verified as complete by gross examination when the animals were sacrificed for the experiments reported.

PHA Stimulation.—Phytohemagglutinin P (PHA-P, Difco) was employed as supplied, suspended in sterile water, at 10 microliters per tube, except as indicated in the text. Marked leukoagglutination was always evident in cultures stimulated with this reagent.

BCG Immunization Procedures and PPD Stimulation.—In experiments in which BCG sensitization was attempted, 0.05 ml of a 12-day old culture of bacillus Calmette-Guérin, obtained from Dr. Joseph Shands, Department of Microbiology, University of Florida, was given subcutaneously; spleen or other cells were later harvested at varying intervals. The in vitro test for stimulation of these cells was identical to that for PHA, except that the cultures were terminated at 72 hr; 24 hr after adding ^{3}H-thymidine. Purified protein derivative (PPD) was obtained from Merck, Sharp & Dohme (Lot No. 4492), West Point, Pa.

Human dialysate transfer factor was prepared by the method of Lawrence (21) from the peripheral leukocytes of an individual known to be strongly tuberculin-positive from skin testing. The dialysate obtained was lyophilized and reconstituted in tissue culture medium for the experiments described. Its effectiveness was verified by its capacity to render human peripheral lymphocytes from a tuberculin-negative donor reactive to PPD in vitro by the technique of Fireman, et al. (22). A single amount of the dialysate, 0.1 ml containing transfer factor derived from 6×10^6 lymphocytes was employed. It was incubated with 15×10^6 mouse spleen cells for 30 min at 37°C, followed by washing, and addition of medium and PPD as indicated.

Sheep Erythrocyte Immunization Procedures and SRBC Stroma Stimulation.—Mice were immunized by a single intravenous injection of 0.1 ml of a 50% suspension of sheep erythrocytes (SRBC) and spleen cells were taken at varying intervals for in vitro stimulation with SRBC stroma. Animals designated hyperimmune had received three injections of SRBC over a 3-wk interval before sacrifice. The SRBC stroma were prepared by hypotonic lysis and repeated washing of fresh SRBC, then lyophillized and used for in vitro stimulation after reconstitution in tissue culture medium.

Method of Assaying Cell Stimulation

Euchrysine staining: Cells from experimental and control tubes were suspended by vigorous mechanical agituation; one drop of the suspension was taken and added to one drop of 1:100,000 final dilution of euchrysine (K & K Laboratories, Inc., Plainview, N. Y.) in buffered saline, according to the method of Allison and Mallucci (23). The resultant preparation was examined immediately by fluorescent microscopy at a magnification of 400 times.

Acid phosphatase staining: Acid phosphatase staining was performed on acetone:fixed smears of stimulated and unstimulated cells at varying intervals by the method of Burstone (24). The cells were scored by counts of 100 cells at 1000 times magnification, as having zero or one (negative), or more than one (positive) phosphatase positive granules in the cytoplasm.

Blast transformation: The occurrence of blast transformation was determined by light microscopy of Wright-Giemsa stained cell preparations, at intervals in culture using the morphologic criteria described by Chessin, et al (25) for human cell populations. The results are given as per cent of cells in the population scored as lymphoblasts.

^{3}H-thymidine incorporation assay: In all tests, 1.0 microcurie (μCi) of tritiated thymidine (^{3}H-thymidine; Schwarz Biochemical, specific activity 1.9 curie/mmole; 1 μCi per 2 μl) was added to each tube for the final 24 hr of culture, after which the cultures were terminated. At termination, the tubes were centrifuged at 1,500 rpm, the resultant cell button washed twice with cold 0.15 M NaCl and twice with 5% trichloroacetic acid (TCA) at 4°C. After this, the precipitates were washed once with absolute methanol. After TCA precipitation and washing, the precipitates were transferred to glass tubes and allowed to dissolve in 0.25 ml of nuclear Chicago solubilizer (NCS) for 2 hr at 45° C. 15 ml of scintillation fluid (toluene, containing *p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP) 100 mg/liter and 2,5-diphenyloxazole (PPO) 5 g/liter) was then added to the solubilized precipitate. The solution was transferred to screwtop vials and counted in a Beckman Model CPM-100 liquid scintillation counter after 24 hr accommodation. The counting efficiency under the conditions described is 80%.

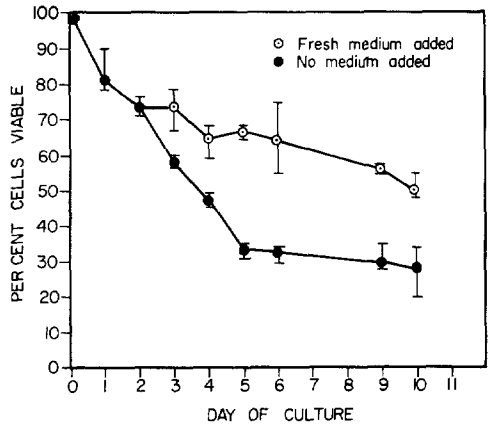

FIG. 1. Viability of C57Bl/6 spleen cells in RPMI 1640 culture medium plus 5% human serum. In these experiments, 15 × 10^6 C57Bl/6 spleen cells per tube were incubated for the indicated period of time and viability determined by trypan blue exclusion. The points shown represent: cultures of 15 × 10^6 cells with no added medium (●); cultures of 15 × 10^6 cells fed by adding one-half volume of medium every other day, beginning on day 2 (⊙). The values shown are means and ranges of 5–10 tubes.

This technique gave replicate values consistently within 5 to 10%. The data obtained are presented as average values if duplicates only were performed; as mean value and range of values if 3 to 5 replicate tubes were included; or as a mean value plus or minus (±) the standard error (SE) of the mean when 6 to 12 tubes were included.

RESULTS

Characterization of the Culture Conditions.—The only medium of many tried which provided conditions for in vitro stimulation consisted of an amino acid rich tissue culture fluid, RPMI 1640, to which was added 5% fresh, heated human serum. The survival of mouse spleen cells from all strains cultured in this medium had a characteristic pattern exemplified by that shown in Fig. 1. Roughly linear decrease in viability occurred during the first 5 to 7 days; at that time the cultures contained about 30% viable cells. After that, a slower

rate of decline in viability was observed; 20–30% of cells survived 10–12 days. After 10 days, the proportion of cells surviving often increased and some relatively long-term cultures have been established.

The proportion of surviving cells in the first 10 days of culture could be increased (Fig. 1) by adding increments of fresh medium at 48 hr intervals. The actual number of cells surviving under the conditions of this experiment are more difficult to determine because of the variable proportion which adhere to the vessel during the culture period. In general, however, the decline of cell numbers under the culture conditions illustrated in Fig. 1, roughly paralleled

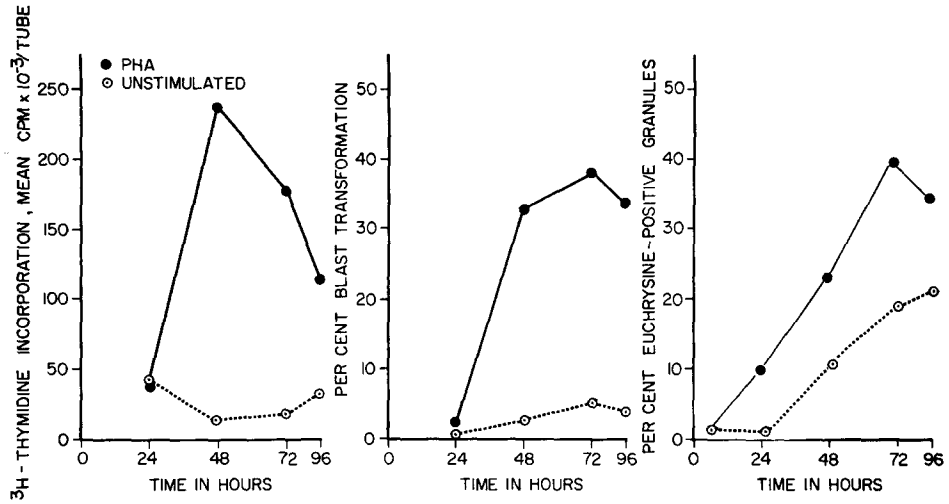

FIG. 2. Time-course of PHA stimulation of A/J spleen cells. 15 × 10^6 cells per tube in 3 ml were incubated with or without PHA for the indicated periods of time. ^{3}H-thymidine incorporation was determined for the last 24 hr of each period. Per cent blast transformation and euchrysine staining were determined on the indicated day. The values given represent the means of 3–5 replicate tubes.

that of viability. The culture conditions adopted for subsequent studies consisted of 15 × 10^6 cells in 3 ml of RPMI 1640 with 5% fresh, heat inactivated human serum added.

When PHA was added to replicate tubes of mouse spleen cells in this medium, the same constellation of effects was observed which has been reported for lymphoid cells of other species. The time course of several of these effects is illustrated by the experiments shown in Fig. 2. By 24 hr an increase in the proportion of cells containing either euchrysine-positive supravitally stained lysosomes or acid phosphatase-containing granules appeared in the PHA-stimulated cells. Positive cells increased over the 72 hr period of culture to a maximum of 30–40% in the case of euchrysine granules and nearly 100% in the case of

acid phosphatase granules. During this interval, however, the discrimination between the control and the stimulated cultures by these assays decreased, since numbers of granules rapidly increased in the unstimulated cells also. This proved to limit the practical values of both the euchrysine and phosphatase granule assay for discrimination quantitatively between the stimulatory capability of different mitogenic substances.

In addition to the expected increase in number and size of lysosomal granules in stimulated mouse cells, blast transformation, as judged by the morphologic

TABLE I

*Time-Course of Stimulation of A/J Spleen Cells by PHA, in Relationship to Lysosome Appearance, Blast Transformation, and ^{3}H-Thymidine Incorporation**

Time intervals and conditions of the experiment	Euchrysine-positive granules (mean of cells with one or more)		Acid phosphatase-positive granules (mean of cells with more than one)		Blast transformation (mean of positive cells)		^{3}H-thymidine incorporation (mean cpm per tube)‡		Ratio: PHA/control
	Control	PHA	Control	PHA	Control	PHA	Control	PHA	
	%	%	%	%	%	%	*cpm*	*cpm*	
24 hr; ^{3}H-thymidine added at beginning	3.0	7.7	13	88	0.6	4.3	44,661	44,522	1.0
48 hr; ^{3}H-thymidine added at 24 hr	4.6	26.0	48	98	2.7	33.2	12,911	237,015	18.3
72 hr; ^{3}H-thymidine added at 48 hr.	4.7	26.2	—	—	5.3	38.9	15,761	178,969	11.4
96 hr; ^{3}H-thymidine added at 72 hr.	5.8	25.7	—	—	3.6	34.4	34,228	119,003	3.5
72 hr; ^{3}H-thymidine added and medium changed at 48 hr	5.5	30.0	—	—	2.9	36.7	18,916	333,887	17.6
96 hr; ^{3}H-thymidine added at 72 hr; medium changed at 48 hr	5.5	24.0	—	—	3.5	34.2	41,633	245,638	5.9

* In this experiment, 15×10^6 A/J spleen cells per tube were examined at intervals for the four parameters indicated. See Materials and Methods for procedure. The medium change was accomplished by replacing one-half of the culture fluid with fresh medium.

‡ Mean values are of duplicate tubes.

criteria described for stimulated human cells (25) occurred in 30–40% of the cell populations by the end of the 2nd day of culture. This proportion rose only slightly thereafter. In contrast, unstimulated cells showed less than 5% blast transformation during 10 days of culture.

The time course of blast transformation was paralleled roughly by incorporation of ^{3}H-thymidine as determined at intervals in both stimulated and unstimulated cells. Maximum incorporation after PHA stimulation occurred between 24 and 48 hr and declined thereafter. Unstimulated cells have a high degree of incorporation of ^{3}H-thymidine in the first hours of culture but this declines to reach a steady state later in cultivation.

Experiments of similar type, but illustrating the effect of adding fresh tissue culture medium to the system at 48 hr, are shown in Table I. Note that additional ^{3}H-thymidine incorporation followed the addition of fresh medium in PHA-stimulated cultures, but not in control cultures. However, no significant increase in the proportion of lymphoblasts in the culture or of granules stained by euchrysine was seen. This table also shows that the ratio of incorporation of ^{3}H-thymidine in stimulated cell cultures divided by that of unstimulated cell cultures in the PHA system is maximal at 48 hr and declined after that time.

These time course studies of PHA stimulation of mouse lymphoid cells give data comparable to those reported for human and rat systems (26, 27). The interval between establishment of the culture and the beginning of DNA synthesis was somewhat shorter, however, and an earlier maximum rate of synthesis of DNA was achieved in comparison to these other systems.

TABLE II

*Dose Response Relationship between PHA Concentration and ^{3}H-Thymidine Incorporation by A/J Spleen Cells**

PHA-P added per tube	^{3}H-thymidine incorporation (mean and range cpm per tube)		Ratio of incorporation: PHA/control
microliters	*cpm*	*cpm*	
0	2,216	(1,989–2,436)	
1	79,685	(71,534–85,798)	35.9
5	94,992	(91,498–100,303)	42.9
10	122,952	(119,091–129,806)	55.5
25	50,587	(48,561–52,142)	22.8
50	16,197	(14,173–18,967)	7.3
100	4,241	(4,074–4,408)	1.9

* In this experiment, varying amounts of PHA, as indicated, were added to 15×10^6 spleen cells, incubated for 24 hr, ^{3}H-thymidine was then added and the experiment terminated at 48 hr. The data shown represent mean and range for 3–5 replicate tubes.

These experiments provided the basis for establishing a standard culture period of 48 hr for PHA stimulation; assaying stimulation based upon tritiated thymidine incorporation during the final 24 hr of this period. The agreement between replicate tubes in such experiments was high; usually on the order of 5%.

The relationship between PHA concentration and thymidine incorporation in several different strains of mouse spleen cells was examined across a 100-fold range of PHA concentrations. Results of a typical experiment are shown in Table II. Optimal stimulation, both in terms of total thymidine incorporation and discrimination as expressed by the ratio of incorporation was greatest at 10 microliters per tube. Amounts of PHA greater than this gave decreasing degrees of stimulation.

Another significant variable in this system proved to be the number of cells per culture. Experiments were devised in which this number was varied between 1×10^6 and 50×10^6 cells with a constant stimulus of 10 microliters PHA. A typical dose response curve of this type is shown in Fig. 3, expressed as ^{3}H-thymidine incorporation and as ratio of incorporation. For cell numbers below 3×10^6 per tube, tritiated thymidine incorporation was low both in stimulated and unstimulated cells. Above 5×10^6 per tube, the increments of PHA stimu-

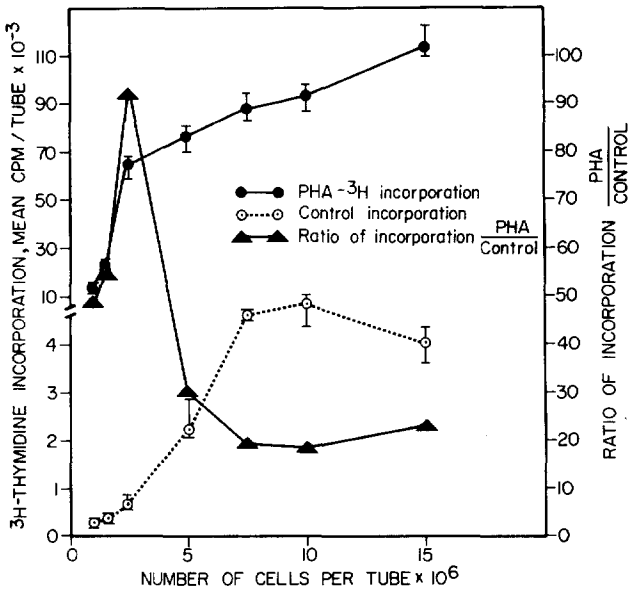

FIG. 3. Relation between cell numbers and PHA stimulation. In this experiment, C57B1/6 spleen cells in the indicated numbers per tube were incubated 48 hr alone, or with 10 microliters PHA-P for 48 hr; at 24 hr, ^{3}H-thymidine was added. The points represent mean values for 3–5 replicate tubes and the range for PHA-stimulated (●), and control tubes (⊙); the ratio of incorporation (▲) was calculated from the mean values.

lated incorporation were smaller for the stimulated than the unstimulated cell populations, so that a maximum ratio of incorporation with this amount of PHA was attained at 3 to 5 million cells per tube. Because constant values for incorporation in unstimulated cells are found over a wide range of cell numbers around 15×10^6 cells per tube, this value was chosen for most subsequent experiments. In experiments for which high discrimination is desirable, or in which higher cell numbers represented an operational problem, the optimal ratio of cells to stimulant should be determined.

Variation was found in the degree of stimulation by PHA in cultures of cells from various strains of mice (Table III). Variability was evident both in total

incorporation values and the ratio of incorporation under identical cultural conditions. The greatest total incorporation values were obtained in cultures of BALB/C cells; the greatest discrimination observed was by those of C57Bl/6. DBA-2, by contrast, was minimally stimulated by PHA despite a close genetic relationship to BALB/C. B10-LP and B10-M, both congenic sublines of C57Bl/10, showed uptake and ratios of incorporation comparable to their stem line.

The reasons for strain variations in responsiveness to PHA are not understood. It could be related to the number of PHA receptors on the cell surfaces involved, differing sensitivity to, or requirements for, certain elements of the

TABLE III

*Comparison of Spleen Cells from Various Mouse Strains in Their Capacity for ^{3}H-Thymidine Incorporation With and Without PHA Stimulation**

Strain of origin	^{3}H-thymidine incorporation (mean and range cpm per tube)				Ratio of incorporation: PHA/control
	Control		PHA		
	cpm	*cpm*	*cpm*	*cpm*	
A/J	6,407	(5,782–6,831)	168,029	(150,741–175,048)	26.2
C57Bl/6	956	(872–1,095)	119,678	(110,746–127,828)	125.2
DBA-2	9,238	(8,709–10,009)	56,109	(50,548–62,060)	6.1
BALB/C	5,695	(4,924–6,193)	318,357	(305,650–345,683)	56.0
CBA	5,135	(4,687–5,442)	176,836	(169,542–193,090)	34.4
B10-LP	5,593	(5,028–6,316)	84,205	(73,822–89,969)	15.1
(A/J × C57Bl/6 F-1	4,556	(4,193–4,975)	179,782	(173,833–186,176)	39.5
B10-M	4,147	(3,778–5,160)	95,566	(84,150–98,895)	23.0

*In this experiment, a single serum donor was employed for preparation of the medium in order to compare cells from various mouse strains with respect to the background and PHA-stimulated incorporation of ^{3}H-thymidine under identical cultural conditions. 15×10^6 cells in 3 ml per tube were incubated with or without PHA for 24 hr; ^{3}H-thymidine was added, and the experiment was terminated at 48 hr. The values given represent means of 6–8 replicate tubes.

medium in which the stimulation takes place, or differing proportions of PHA-sensitive cells in the spleen cell suspension.

The earliest experiments with this system suggested that the human serum component was an important variable which determined the degree and discrimination of stimulation. Three elements appear to contribute to this source of variability. First, heat inactivation of the serum at 56°C for 30 min is required to negate a cytotoxic effect of human serum upon mouse spleen cells. Second, the heated serum must be used fresh to achieve consistent and predictable results over a period of time. Storage at 4°C or −20°C for any significant period of time resulted in a medium which gave undependable and variable degrees of stimulation. The character of lability to low temperature is at present unknown. As shown in Table IV, however, and exemplified by experience throughout this period of investigation, fresh, heat inactivated serum from the same

donor provides a highly reproducible degree of stimulation to spleen cells of the same strain, day to day and week to week.

The third major component of human serum proved to constitute an absolute requirement for mouse cell stimulation under these conditions. This is a naturally occurring anti-mouse erythrocyte agglutinin found in the serum of nearly every adult human being. The presence and titer of this agglutinin was positively

TABLE IV

*Stimulation of Varying Numbers of A/J or C57Bl/6 Spleen Cells by PHA; Day to Day Reproducibility of Experimental Results using Same Serum Donor**

No. of reacting cells per tube × 10^6	Day	^{3}H-thymidine incorporation (mean and range cpm per tube)				Ratio of incorporation: PHA/control
		Control		PHA		
		cpm	*cpm*	*cpm*	*cpm*	
C57Bl/6 cells	0	242	(227–260)	13,025	(12,563–13,483)	54.0
1 × 10^6/tube	5	282	(253–325)	11,799	(10,841–13,536)	62.6
	19	238	(228–246)	13,261	(12,889–13,002)	54.5
C57Bl/6 cells	0	2,393	(2,342-2,454)	51,821	(46,626–56,640)	21.7
5 × 10^6/tube	5	2,679	(2,501–2,891)	72,600	(68,190–76,425)	27.0
	19	2,865	(2,659–2,985)	73,207	(70,764–75,357)	25.3
C57Bl/6 cells	0	4,856	(4,808–4,914)	88,335	(86,416–91,918)	18.2
10 × 10^6/tube	5	4,257	(4,148–4,367)	94,213	(92,695–95,731)	22.2
	19	4,638	(4,569–4,708)	91,817	(92,791–96,844)	19.8
C57Bl/6 cells	0	4,020	(3,787–4,363)	116,248	(110,180–116,433)	29.2
15 × 10^6	5	4,406	(3,998–4,680)	124,843	(118,403–130,723)	28.3
	19	3,830	(3,791–3,892)	—		—

* In each experiment shown, fresh serum from a single donor was used to repeat the same experiment on the days as indicated. The number of cells indicated were incubated with or without PHA-P, 10 microliters per tube for 24 hr; ^{3}H-thymidine was then added and the experiment terminated at 48 hr. The data given are the mean values and ranges for 3–4 replicate tubes done on the indicated day.

correlated with ^{3}H-thymidine incorporation of paired control and PHA-stimulated tubes over the range of titers of 1–5 to 1–40. A summary of data demonstrating this relationship is shown in Table V. Increasing titers of this agglutinin were correlated directly with the level of thymidine incorporation in PHA-stimulated cultures. However, incorporation by unstimulated cells increased 10-fold over the same range of titers. Consequently, the ratio of incorporation was highest in medium containing low-titered sera, and thus, whenever possible, individuals with the lowest titers were employed for all experiments. It was also established within the limited number of individuals

tested, that there is little day to day or week to week variation in the titer in any given individual over a period of time.

Absorption of the human anti-MRBC agglutinin with MRBC, as shown in Table V, removed almost all capacity of the medium to support stimulation by PHA. The characteristics of the element removed were determined on the eluate

TABLE V

*Effect of Human Anti-Mouse Erythrocyte Agglutinin on ^{3}H-Thymidine Incorporation by Normal and PHA-Stimulated A/J Spleen Cells**

Anti-mouse RBC agglutinin titer of serum donors	No. of paired determinations	^{3}H-thymidine incorporation (average cpm per tube and range)		Ratio of incorporation: PHA/control
		Control	PHA	
		cpm	*cpm*	
1–5	15	2,113 (1,364–2,945)	141,089 (118,027–182,602)	68
1–10	20	8,348 (4,313–13,244)	165,232 (127,694–191,962)	20
1–20	16	17,789 (12,279–31,216)	176,328 (115,776–277,967)	10
1–40	16	28,506 (18,400–47,234)	245,835 (187,391–288,443)	8.2
1–10 (Absorbed with mouse RBC‡)	10	101 (45–160)	182 (51–500)	1.8

* Data from 67 experiments in which 15 × 10^6 A/J spleen cells in 3 ml medium were stimulated, or not, with 10 microliters PHA-P. The media employed for individual experiments contained human serum from various donors. The data are arrayed on the basis of the anti-mouse erythrocyte agglutinin titer of the donor serum. Each experiment consisted of 3–5 control tubes paired with an equal number of PHA tubes. The data given here represent the average of the mean values for comparable tubes in each experiment, and the range of values observed.

‡ For these experiments, the medium was made from the serum of a single donor, having a 1–10 titer. The serum was absorbed with an equal volume of washed A/J erythrocytes and the resultant serum used to prepare the medium.

from MRBC which had been incubated in human serum. Elution by either of two techniques (see Materials and Methods) designed to dissociated antigen-antibody complexes yielded into the supernate proteins identified as gamma G and gamma M immunoglobulins. Moreover, this eluate had the property of combining with and coating mouse lymphoid cells at 37°C after 30 min, as revealed by ring fluorescence when fluorescein-labeled anti-human fragment III

was added. These eluates, moreover, had the property of mediating cytotoxicity of mouse spleen cells in titers over 1–500, when guinea pig complement absorbed with MRBC was added to the system. Therefore, the major factor in human serum necessary to the medium was identified as an anti-mouse lymphocyte globulin (AMLG). This requirement may be related to the observation that guinea pig lymphoid cells are stimulated to mitosis by anti-lymphocyte sera and this effect is dose-dependent (28).

The stimulatory capacity of the anti-lymphocyte globulin alone in this system is shown by the results of experiments in which PHA was added at intervals after establishment of cultures, then incorporation assayed after an additional

TABLE VI

*Loss of Responsiveness to PHA Stimulation in Long-Term Cultures of C57Bl/6 Spleen Cells**

Age of culture in days	^{3}H-thymidine incorporation (mean and range cpm per tube)		
	Control	PHA	Ratio of incorporation: PHA/control
	cpm	*cpm*	
3	11,757 (9,435–12,967)	42,686 (41,030–43,737)	3.6
6	18,094 (16,813–19,782)	38,305 (36,182–43,233)	2.1
10	53,400 (50,600–56,271)	56,731 (55,100–58,801)	1.06

* C57Bl/6 spleen cells, 15×10^6 per tube were incubated without PHA until 48 hr before the indicated age. At that time (1, 4, and 8 days, respectively), PHA, 10 microliters per tube, was added to the cell with fresh medium. 24 hr later, ^{3}H-thymidine was added and the cultures were terminated on the indicated day. The values shown represent mean and range of 3–5 replicate tubes.

48 hr in culture. In such experiments, illustrated by that shown in Table VI, PHA was added after 1, 4, or 8 days, to cultures of C57Bl/6 spleen cells. These results indicate that, during the course of cultivation in human serum containing medium, the cell population becomes progressively refractory. This suggests that AMLG either has preempted sensitivity to the superimposed specific stimulus, or that the vigorous stimulation of a non–PHA-sensitive component of the cell population has masked that of a PHA-sensitive component. It may also be that the PHA-sensitive cell population has not survived in the culture.

Demonstration of an Arginine Requirement for Stimulation.—Several recent studies have indicated unique requirements for certain nonessential amino acids by lymphoid cells, either for growth or for response to mitogenic stimulation. Certain lines of mouse lymphoma cells have a growth-limiting requirement for asparagine (29); these cells either die or fail to divide in vitro in its

absence, or they disappear when asparagine deficiency is artificially induced in vivo (30). It has recently been reported (31, 32) that lymphoid cells are inhibited from responding to several mitogenic stimuli by a factor extracted from *Mycoplasma* and identified as consisting, in part, of arginase. It was of interest,

TABLE VII

*Inhibition of PHA Stimulation of A/J Spleen Cells in Arginine-Deficient Medium; Restoration of Reactivity with Arginine**

Medium employed	^{3}H-thymidine uptake of culture‡ (Mean per tube and range)		
	Control	PHA	Ratio of incorporation: PHA/control
	cpm	*cpm*	
Serum A:			
RPMI 1640	15,821 (15,770–16,001)	151,806 (150,556–153,419)	9.5
RPMI 1640 plus 200 mg/liter arginine	12,897 (12,317–13,217)	156,575 (155,585–157,481)	12.2
RPMI 1640, arginine-deficient	7,555 (6,897–8,421)	27,956 (23,982–30,172)	3.7
RPMI 1640 arginine-deficient plus 200 mg/liter arginine	11,456 (10,632–11,972)	145,064 (138,783–151,772)	12.7
Serum B:			
Dialyzed with RPMI 1640	13,410 (12,854–13,665)	112,827 (99,677–119,577)	8.4
Dialyzed with arginine-deficient RPMI 1640; arginine-deficient RPMI 1640	6,323 (5,370–6,957)	19,769 (19,454–20,165)	3.1
Dialyzed with arginine-deficient RPMI 1640; arginine deficient RPMI 1640 plus 200 mg/liter arginine	9,573 (9,015–9,770)	95,899 (90,193–101,833)	10.1

* Two experiments in which tubes containing 15×10^6 A/J spleen cells in 3 ml of the various media, composed as indicated, were incubated with or without 10 microliters PHA for 48 hr. ^{3}H-thymidine was added at 24 hr, and the cultures terminated at 48 hr. The sera in the second experiment shown were dialyzed against two changes of the indicated medium before use. The sera for the two experiments shown were taken from different individuals.

‡ The values given represent means and range of 3–6 replicate tubes.

therefore, to determine if the apparent superiority of RPMI 1640, as compared to other standard media tried might be associated with its very high arginine content. Experiments were designed to detect a requirement for either arginine or asparagine in the stimulation by PHA of ^{3}H-thymidine incorporation by mouse spleen cells. Table VII illustrates two experiments in which the require-

ment of arginine for stimulation by PHA was demonstrated under different circumstances. In one experiment, arginine-deficient medium was compared with regular medium and with that in which arginine-deficient media had been replenished to the original level. It was clearly shown that incorporation of tritiated thymidine by PHA-stimulated cells was markedly reduced in the arginine-deficient medium. Moreover, if the arginine content of the medium was reduced further by dialysis of the serum against arginine-deficient medium as shown in the second illustrative experiment, PHA-stimulated incorporation was reduced somewhat more. It was concluded that arginine is a requirement for optimal stimulation of mouse lymphoid cells by PHA, but not an absolute

TABLE VIII

*Comparison of PHA Stimulation of Spleen, Lymph Node, and Thymus Cells from Mature C57Bl/6 Mice**

Source of cells	^{3}H-thymidine incorporation (mean and range cpm per tube)		Ratio of incorporation: PHA/control
	Control	PHA	
	cpm	*cpm*	
Spleen	8,639 8,569–8,777	108,321 95,731–117,592	12.5
Lymph node	306 283–315	4,980 4,508–5,419	16.6
Thymus	3,334 3,209–3,411	12,515 12,096–12,982	3.8

* In this experiment, 15×10^6 cells from the organ indicated in 3 ml of medium per tube, were incubated 24 hr. ^{3}H-thymidine was then added and the experiment was terminated at 48 hr. Serum from a single donor was employed for the medium. The data represent mean and range values of 3–4 replicate tubes in each category.

requirement. Even in the absence of any known source of arginine, some stimulation occurred in all experiments. No requirements for asparagine could be detected. The role of arginine appears to be limited to the first 24 hr of the culture period; for feeding the cultures additional arginine alone after this period failed to support additional incorporation which occurs when the cultures are fed whole medium, as described earlier.

PHA-Stimulation of Cells from Animals of Different Age and from Different Sources of Lymphoid Tissue.—It has already been established in other species that the stimulatory effect of mitogenic substances is related to the proportion of small lymphocytes present in the cell preparation involved. Proportions of these cells vary in the spleen, lymph nodes, thymus, thoracic duct contents, and the peripheral blood (33). Accordingly, the relative susceptibility to stimulation of mouse spleen, lymph node, and thymus cells was examined, since these vary

considerably in their contents of mature lymphocytes. Results shown in Table VIII illustrate the findings. Cells from the spleen gave the highest incorporation of thymidine per 15×10^6 cells of the organs studied, but its ratio of incorporation was not as high as those from lymph nodes harvested from the axillary and neck areas. Thymus cell preparations were stimulated, but minimally, and with a low ratio of incorporation. It seems probable that these latter results can be explained by the presence in the preparations of lymphoid cells derived from thymic stroma or aggregations of lymphoid cells attached to the thymus. No

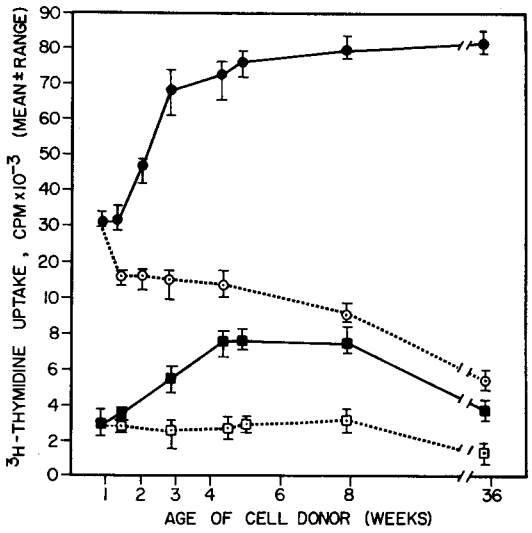

FIG. 4. Age variations in PHA stimulation of C57Bl/6 spleen and thymus cells. Spleen or thymus cells were taken from groups of C57Bl/6 mice of the indicated ages; 15×10^6 cells were cultured per tube together with 10 λ PHA-P for a total period of 48 hr. ^{3}H-thymidine was added at 24 hr. The mean value and range of ^{3}H-thymidine incorporation for cells from animals of each age group are shown for both stimulated (●, spleen; ■, thymus) and unstimulated (⊙, spleen; ⊡, thymus) cultures. Serum from the same donor was used in the medium throughout this experiment.

attempt was made to relate the relative numbers of small lymphocytes and other cell types in these preparations to the degree of stimulation.

The proportion of lymphocytes to other cell types, as well as the absolute numbers in the thymus, lymph node, and spleen vary with age (34); therefore, age was examined as a variable in susceptibility to PHA stimulation. For these studies, experiments were performed by taking spleen and thymus cells from C57Bl/6 mice of differing age and comparing the effects of PHA stimulation. Results of this type experiment are shown in Fig. 4. In contrast to the case of the human lymphocyte, where PHA stimulation can be demonstrated early in

fetal life (35), relatively little stimulation of the mouse lymphocyte was evident at the time of birth. Only during the 2nd wk of age did susceptibility to PHA stimulation become significant. This was accompanied by decreasing values for incorporation in the control cultures. As the susceptibility to PHA stimulation appeared with age in thymus cells, the ratio of incorporation increased to a maximum at 4 wk and thereafter fell toward the newborn level by 36 wk of age. Little change in values of incorporation by unstimulated cells occurred throughout the period.

TABLE IX

*Effect of Thymectomy on Stimulation of ^{3}H-Thymidine Incorporation by PHA into C57Bl/6 Spleen Cells**

Age of mice	^{3}H-thymidine incorporation into spleen cell cultures of thymectomized or sham operated littermates (cpm/tube)					
	Thymectomized			Sham operated		
	Control	PHA	Ratio: PHA/ control	Control	PHA	Ratio: PHA/ control
wk						
3	18,052 15,759–21,964	17,178 14,835–22,388	0.95	14,475 13,387–15,522	42,639 41,311–43,857	2.9
4	10,869 9,084–13,275	11,163 8,268–13,863	1.1	9,137 8,140–10,116	63,374 56,376–69,837	7.0
5	4,884 4,274–5,195	7,541 7,112–8,048	1.6	4,253 3,922–4,547	86,575 82,844–89,073	20.4

* In this experiment, C57B1/6 mice were thymectomized or sham operated at birth and at the indicated intervals, spleen cells were taken and cultured (15×10^6 cells per tube) with 10 microliters PHA for 48 hr. The data shown represent the mean values and range of 3–5 replicate tubes. The same serum donor was used for all media.

Effect of Thymectomy on PHA Stimulation in Spleen Cells.—Since PHA stimulation has been shown in other species to be dependent upon an intact thymus, the effects of thymectomy upon susceptibility of mouse spleen cells to stimulation was examined.

Animals were thymectomized or sham-operated at birth and their spleen cells examined in vitro for PHA stimulation by ^{3}H-thymidine incorporation at 3, 4, or 5 wk of age. The results are shown in Table IX.

Incorporation of thymidine as a result of PHA stimulation was markedly inhibited for the first 4 wk of age. At 5 wk, significant PHA stimulation of spleen cells from thymectomized animals was seen, but the response was much less than the sham-operated controls. It appears then that in mice, as in other species studied, the response to PHA is abolished by thymectomy and that this

is, therefore, a thymus-dependent function. It also seems probable that differing sensitivity to stimulation of populations of cells derived from the spleen, thymus, and lymph nodes reflects the proportion of thymus-dependent cells which are normally found in these organs.

Response of Mouse Spleen Cells to Antigen (Sheep Erythrocytes).—The studies described above led to the establishment of a standard model in which to examine the responsiveness to specific antigens. In vitro stimulation of spleen cell populations from immune and nonimmune mice has been examined for tubercu-

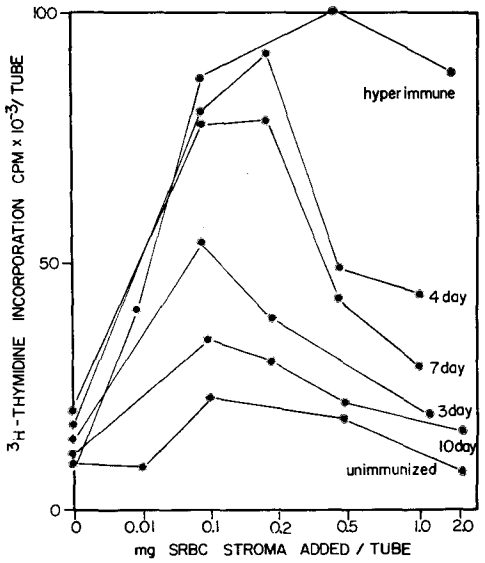

FIG. 5. CBA mouse spleen cells were taken at the indicated intervals after the donors received 0.1 ml of a 50% suspension of SRBC, intraperitoneally. Varying amounts of SRBC stroma were added to tubes containing 15 × 10^6 cells. ^{3}H-thymidine was added at 48 hr; and the cultures terminated at 72 hr. Serum from a single donor was used to constitute the medium. The points shown represent mean values of 3–5 replicate tubes.

lin and sheep erythrocyte antigens. Although sheep erythrocyte stroma represents a complex antigen, the system has been used extensively in mouse systems for study of population kinetics (36), and individual cells producing anti-SRBC (37) as well as providing a stimulus for eliciting a putative primary sponse in vitro (38).

It was found that normal spleen cell populations of CBA mice were stimulated significantly by SRBC as illustrated in Fig. 5. Moreover, after a single immunizing injection of sheep erythrocytes, enhanced thymidine incorporation was observed as early as the 2nd day and the maximum value was reached on the 4th day after antigen injection. Animals which had been hyperimmunized by re-

peated injections of sheep erythrocytes showed only slightly greater incorporation then those encountering the antigen for the first time, but had less tendency to be inhibited at high antigen doses than those cells involved in a first immune response.

Response of Mouse Spleen Cells to Specific Antigen (Tuberculin).—One of the earliest observations of cellular immunity in vitro was that of Pearmain (39) who showed that lymphocytes from tuberculin-positive human beings would transform into blasts if treated with tuberculin. This observation has been extensively confirmed in human and guinea pig systems (28). However, the lack

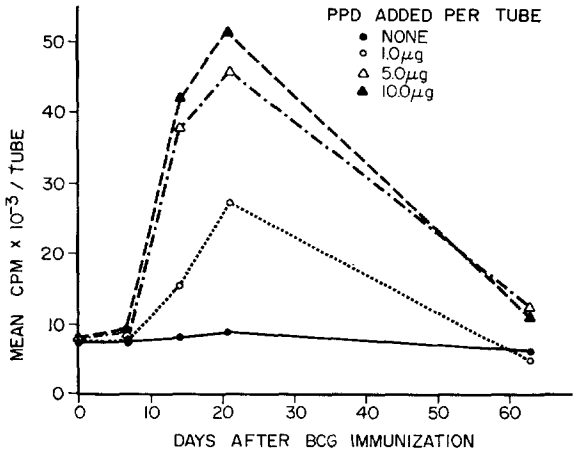

FIG. 6. PPD stimulation of BCG-immune spleen cells. Time-course of PPD stimulation of ^{3}H-thymidine incorporation by spleen cells taken from groups of C57Bl/6 mice which had received a single immunizing injection of living BCG organisms. Spleen cells were harvested at the indicated intervals, and 15×10^{6} cells per tube were cultured 72 hr with the amounts of PPD indicated. The data shown represent mean ^{3}H-thymidine incorporation of 3–5 tubes.

of an in vitro model has not permitted such studies in the mouse, despite extensive investigation of effects of immunization with BCG upon the laboratory mouse (40).

Extensive studies have been made of spleen cell reactivity to PPD in vitro at intervals after immunization of A/J or C57Bl/6 mice with BCG given by several different routes and by various immunizing schedules. In contrast to immunization by intraperitoneal or intravenous routes which induce little or no reactivity to PPD in spleen cell populations, subcutaneous administration of a relatively small number of living BCG organisms resulted in a specific susceptibility of spleen cells to PPD stimulation as indicated by ^{3}H-thymidine incorporation. Fig. 6 illustrates a typical experiment in which spleen cells from groups of BCG-immunized mice were taken at intervals after immunization and stimulated in vitro with varying amounts of PPD. It will be seen that no stimu-

lation occurred in cells taken 1 wk after BCG immunization, but the response was evident at 2 wk and maximal at 3 wk. This immune responsiveness rapidly diminished and was just detectable 10 wk later, as compared with nonimmune cell populations.

Table X illustrates the results of other experiments in which replicate cultures of spleen cells from BCG immune and normal A/J or C57Bl/6 mice were com-

TABLE X

*PPD Stimulation of ^{3}H-Thymidine Incorporation by Spleen Cells from A/J and C57Bl/6 Mice at Intervals after BCG Immunization**

Mouse strain and status	PPD added μg per tube	^{3}H-thymidine uptake of cultures at intervals after immunization (Mean cpm per tube ± SE)‡					
		preimmunization	1 wk	2 wk	3 wk	4 wk	9 wk
		cpm	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*
A/J; BCG immunized	0	8,067 ±97	11,929 ±251	10,651 ±475	—	13,626 ±273	—
	1	8,075 ±141	—	14,227 ±381	—	44,000 ±1,190	—
	5	—	17,804 ±757	36,772 ±1,505	—	77,359 ±571	—
	10	9,684 ±140	15,948 ±417	27,624 ±1,112	—	102,069 ±449	—
C57Bl/6; BCG immunized	0	7,661 ±64	7,691 ±71	7,930 ±96	8,643 ±415	—	5,743 ±97
	1	8,007 ±133	7,352 ±197	15,307 ±1,023	27,555 ±121	—	5,952 ±141
	5	—	8,282 ±255	37,992 ±542	46,197 ±893	—	12,094 ±240
	10	7,614 ±78	9,197 ±254	42,406 ±450	51,817 ±2,158	—	11,357 ±62
C57Bl/6§ not immunized	No transfer factor; no PPD	7,779 ±212	—	—	—	—	—
	Transfer factor; no PD	7,317 ±279	—	—	—	—	—
	Transfer factor; 1 μg PPD	11,793 ±378	—	—	—	—	—
	Transfer factor; 10 μg PPD	25,702 ±705	—	—	—	—	—

* A/J or C57Bl/6 mice were immunized with 0.05 ml of a 12-day culture of BCG subcutaneously, and spleen cells obtained from pools of animals at the indicated intervals. The indicated amounts of PPD were added to each tube. ^{3}H-thymidine was added to these cells at 48 hr and the experiment terminated at 72 hr.

§ To normal C57Bl/6 spleen cells, incubated at 37°C for 30 min with 0.1 ml dialysate transfer prepared from PPD reactive human peripheral leukocytes, were added either 1 μg or 10 μg PPD as indicated. ^{3}H-thymidine was added at 48 hr and the experiment terminated at 72 hr.

‡ The data represent the mean ±SE of 6 to 10 tubes in each group. Serum from a single donor was used in each of the experiments shown.

pared. These strains showed essentially the same dose response curve and time course of immunization. The time course of sensitization to PPD stimulation by BCG immunization as measured in these studies is almost precisely that observed by Blanden (40) in following the course of the PPD-induced footpad reaction in BCG-treated mice, suggesting a correlation between the in vitro events and the in vivo phenomenon.

Stimulation of Normal Mouse Spleen Cells by PPD after Adding Human Dialysate Transfer Factor.—A well-established property (21) of peripheral leukocytes from humans who react to PPD by skin test is the transfer of this reactivity to other PPD-negative individuals. This transfer does not require whole cells; in fact, it has been demonstrated that a low molecular weight material, extractable from these cells, has this transfer property. Fireman (22) has found that this dialysate transfer factor transmits PPD reactivity not only to intact individuals, but to the peripheral leukocytes of nonsensitive donors. It was of interest to determine whether transfer factor prepared from the peripheral leukocytes of a tuberculin-positive human donor would have sufficiently broad species specificity to endow unstimulated mouse spleen cells with a capacity to respond to PPD. Surprisingly, transfer factor derived from humans endowed mouse spleen cells with a capacity to respond specifically to PPD in vitro with a dose response relationship very much like that of mouse spleen cells derived from the BCG-immunized animals (Table X). The dialysate transfer factor used for these experiments had been established as effective by its capacity to endow nonsensitive human lymphocytes with susceptibility to in vitro stimulation by PPD. Preliminary studies show that the amount of transfer factor required to render a given population of mouse spleen cells susceptible has dose-response characteristics, suggesting that above a threshold amount, additional transfer factor gives no additional PPD responsiveness.

DISCUSSION

These studies show that under the cell culture conditions described, long term viability, transformation, and cell division of mouse lymphoid cells occurs in response to general mitogens and immunologically specific antigens. The numerous genetic and operational advantages of the mouse can be dependably exploited in an in vitro model for cellular recognition and in an analysis of the structural and functional consequences of stimulation. Three elements have been identified as necessary to mitogenic stimulation in this model: (*a*) a requirement in the medium for a naturally occurring antibody to a membrane component; (*b*) a relative requirement for arginine; and (*c*) an influence of age and neonatal thymectomy on susceptibility to stimulation by PHA. Through exploration of these variables which require control in order to yield reproducible data, some understanding of the mechanism can be obtained.

The natural anti-mouse erythrocyte agglutinin found in human serum has

broad specificity for mouse cells, agglutinating not only erythrocytes, but spleen and lymph node cells. When eluted from mouse erythrocytes, the agglutinin activity was contained in immunoglobulins of the gamma G and gamma M classes. These eluates were hemolytic and cytotoxic for spleen cells in the presence of complement. The eluate bound to lymphocyte membranes, as revealed by the indirect fluorescent technique. The interreaction, therefore, had the characteristics of specific antibody combining with a membrane antigen or receptor, and the antibody involved has been termed anti-mouse lymphocyte globulin (AMLG).

AMLG, thus characterized, has most of the attributes described by Landy and associates (41) to a ubiquitous natural antibody in human serum which was cytotoxic for sarcoma-37 cells of mouse origin. This antibody was found not only in human serum, but in serum of other primates, cows, some rabbits, dogs, birds and cats; but not in horse, sheep, guinea pig, mouse, rat, or fetal calf serum. This antibody was absorbed from serum by lymphoreticular cells and, to a much lesser extent, by muscle and kidney cells.

Nothing is currently known of the specific receptor or antigen on mouse lymphoreticular cells to which AMLG might be directed. By analogy to other natural antibodies showing broad specificity, such as the ABO and MN blood groups, Forssmann and other heterophile antibodies, membrane-bound mucopolysaccharide receptors seem highly probable.

When this receptor was engaged by AMLG in low concentration, a population of lymphoid cells derived from spleen or lymph node were made susceptible to PHA stimulation. The AMLG also permitted cell transformation and DNA synthesis in populations of immune cells when specific antigen was added, and it allowed detection of primary antigens and immune recognition of histocompatibility antigens (13).

Several mechanisms by which AMLG might exert these effects are conceivable. One is that it affects directly the initial recognition process, i.e., that AMLG functions by generating or unmasking recognition receptors on the cell membrane. A second possibility is that it functions only by supporting or enhancing the consequences of recognition in terms of the cell transformation and cell division assayed in these studies. A third possibility is that it acts to favor the operation of a two cell system.

The first proposed mechanism could be a two-step process in which sterically inaccessible receptors are unmasked by AMLG-produced conformational changes, permitting interaction of the antigen or PHA, and a sterically complementary membrane receptor. Evidence for masking of surface receptors by polysaccharides is available (42, 43) in the case of PHA on human lymphoblastoid cell lines (44) and immunoglobulin receptors on lymphoblastoid cell lines (45). A more complex mechanism of receptor generation might involve membrane turnover, conceivably stimulated by AMLG. A possibly related phenom-

enon is referred to as antigenic modulation (46). The rapid appearance of the earliest consequences of recognition place severe restrictions on the hypotheses involving receptor generation by any mechanism. Ramseier (9) and Granger (10) reported experiments, for example, showing evidence of release of chemotactic or cytotoxic factors into the medium, within 4 hr, as a consequence of specific in vitro recognition by mouse cells. The medium employed in both studies cited contained calf serum, which probably contained AMLG activity. Antigenic modulation in the thymic leukemia (TL) system occurs, however, with sufficient rapidity to be compatible with such early events. Arguing against this general type of hypothesis is the observation that receptors for PHA are already present on the cell membrane of rat thoracic duct lymphocytes (47), and therefore do not require generation. The evidence available does not rule out receptor generation or unmasking as the explanation for AMLG function in the mouse system, but it provides no direct support for this hypothesis.

The data reported here are also compatible with the second type of hypothesis; that AMLG has a function in supporting the consequences of recognition with no effect on actual receptors themselves. Evidence for this type mechanism includes the observation that when AMLG is present in high concentrations, spleen or lymph node populations were stimulated, *sui generis*, to undergo transformation and mitosis, as revealed by a greatly increased background incorporation of tritiated thymidine. PHA is also required in relatively high concentration for other in vitro models for cell-cell recognition in the mouse, which have been described by others (48, 49). In these experiments, the PHA effect has been ascribed to a capacity to agglutinate and approximate cell surfaces and thus facilitate recognition. In experiments reported here, mouse cells were not stimulated to divide by PHA in the absence of AMLG, despite the marked agglutination produced by PHA. These data suggest that the AMLG membrane receptor is not the same as that believed to interact with PHA, namely *N*-acetyl-galactosamine (50). The data do not exclude aggregation by AMLG as significant to its capacity to promote other specific recognition reactions.

Experiments which bear upon the third mechanism which proposes that AMLG promotes interactions in a two cell system are those in which the donors of cells were thymectomized during the newborn period. Responsiveness to PHA stimulation was virtually abolished, but the "background" level of incorporation of ^{3}H-thymidine was not lower than in sham-operated controls. Stimulation of DNA synthesis by PHA in mouse spleen cell populations, therefore, appears to be a thymus-dependent function, presumably due to the presence of a population of thymus-derived cells. This conclusion is compatible with data derived from other animal cell populations studied in vitro (51, 52), and in vivo (53), and with data on congenital thymic deficiency of children (54, 55).

A second population of cells is present which is not thymus-dependent but which interacts with AMLG and initiates the DNA synthesis which constitutes the background incorporation levels. In experiments pertinent to this concept, to be reported elsewhere (56), two populations of mouse spleen cells were separated by albumin density gradient centrifugation. These populations differed markedly in their behavior in this in vitro system. High density small lymphocytes were stimulated by PHA and some specific antigens, but showed low background activity. Low density, large lymphoid cells were responsible for most of the background incorporation, but showed a lesser capacity to respond to PHA, and no response to PPD.

If it is accepted that two cell types are present in the complete in vitro system, the question is unresolved as to the role, if any, of the cell population which is non–thymus-dependent, and which is stimulated to initiate DNA synthesis by AMLG alone. If the two cell systems act together, the non–thymus-dependent cell population could conceivably be stimulated by AMLG to yield into its immediate environment factors which favor cell survival and cell division in the thymus-derived population. The latter cells would thereby be permitted to respond to specific antigens.

It is conceivable also that the thymus-derived and non–thymus-dependent population represents independent components of a complex mixture of cells in the spleen or lymph nodes. The response stimulated in the former would be irrelevant to the PHA or antigen-specific responses of the thymus-derived populations and represent simply the noise level of the technique. If this were the case, it would require that AMLG acts directly upon the cell membrane of the PHA- or antigen-responding cell as a costimulant to permit recognition or enhance viability and cell division.

Each of the hypotheses proposed to explain the function of AMLG in this system is compatible with existing data, and susceptible to further experimental testing. The concept of two independent cell systems with AMLG acting as a costimulant in the PHA or antigen-responsive population is favored by the most positive evidence available. However, no hypothesis in current form permits a full explanation of the nearly absolute requirement for AMLG for in vitro cell stimulation.

A second requirement of in vitro stimulation of mouse lymphoid cells was arginine. In an arginine-deficient medium, both background and PHA-initiated DNA synthesis were inhibited. Thus it seems probable that the arginine requirement is related to postrecognition events. Unlike the requirement for AMLG, the arginine effect was not absolute; deficiency did not abolish either type of stimulation. While this work was in progress, it was found (30, 31) that arginine was required for PHA-stimulated and specifically stimulated blast transformation of human and rabbit lymphocytes, and that the deficiency was also relative. Arginine was also required for DNA synthesis and growth of lymphoblastoid cell lines derived from normal individuals and patients having

infectious mononucleosis, or from biopsy specimens from patients with Burkitt's lymphoma (57).

No known biochemical pathway for arginine metabolism appears to explain these phenomena. An entirely different category of effects of arginine might actually be postulated—namely effects analogous to those of polylysine and other charged compounds in stimulation of micropinocytosis in macrophages (58). It is known that the arginine content of transformed lymphoid cells is higher than the unstimulated lymphocyte (59) and it is conceivable that this represents actively stimulated pinocytotic admission to the cytoplasm.

The age-related response of populations of lymphoid cells to PHA and other stimuli defines an operational variable which must be standardized for consistent results in this in vitro system. Beyond that, however, the data indicate that an in vitro equivalent exists for the well-established deficiency of newborn mice in responding to certain types of antigenic stimulation (60–62).

The general hyporeactivity of newborn mice to antigens has not been adequately explained. Argyris (63) has reported evidence that an element of immunologic hyporeactivity in the newborn mouse may be macrophage deficiency. The capacity to produce plaque-forming units in response to sheep erythrocyte immunization was augmented up to seven-fold by transferred syngeneic peritoneal exudate cells. She has suggested that the immaturity of the lymphoreticular system in newborn mice might be related to ineffective antigen processing or antigen localization.

In the system reported here, PHA-stimulated thymidine incorporation by populations of spleen cells was essentially nil, prior to 7 days of age, after which it rose rapidly to a maximal response by 40 days. The background level of incorporation, on the other hand, was relatively high at birth and thereafter fell progressively. These data might be interpreted as defining two populations of cells; one population which gives background thymidine incorporation, but which cannot itself respond to PHA, and a second which is PHA-sensitive, presumably thymus-derived, but absent from the spleen at birth. However, in newborn mice, as contrasted to thymectomized mice, background incorporation may represent ongoing cell division in a population of hematopoietic cells known to be present in the newborn spleen. The failure to demonstrate PHA-responsive cells at birth in the mouse is also inconsistent with reported susceptibility to PHA stimulation of newborn or fetal lymphoid cells of humans (34, 35, 64). The possibility should be considered, therefore, that lack of PHA responsiveness in newborn mouse cells is not necessarily related to the absence of responsive cells, but to a deficiency of the AMLG receptors. Such a mechanism has precedence in the observation that newborn mouse lymphoid cells lack, in their surface membrane, representative adult levels of histocompatibility antigens (13, 65).

The system reported here also permitted an analysis of the results of inter-

actions between specific antigens and populations of immune cells as compared with nonimmune cells. The two antigens examined thus far, SRBC stroma and PPD, have given results which are similar in many respects. The general dose-response relationship in terms of incorporation of ^{3}H-thymidine were similar in both systems; stimulation increased with increasing concentrations of antigen until a maximum value was reached. Addition of more antigen beyond this point gave progressive inhibition of incorporation. The concentration of antigen which gave maximal stimulation in both systems varied in cells obtained at various times during the course of immunization. For example, a concentration of SRBC stroma which was maximally stimulating to cells taken at 4 days post-immunization was inhibitory to cells taken at 3 or 10 days postimmunization.

Presumably, this dose-response relationship reflects the number of antigen-sensitive cells in the spleen population at the time assayed. It might be assumed that each of the cells requires an equivalent number of antigen hits to reach the threshold which stimulates DNA synthesis. In this case, the dose-response curve should reflect the amount of antigen required to trigger each member of the antigen-sensitive population present at any given time after immunization. This should give a plateau incorporation level, rather than inhibition in high doses. By analogy to antigen-antibody complexes, it might be postulated that antigen responsiveness in terms of DNA synthesis induced requires antigen bridges between antigen sensitive cells, a lattice effect. Excess antigen could combine with all sites on the reactive cells and block this effect, thus inhibiting DNA synthesis. It is also possible that excess antigen, beyond that required for maximum stimulation, is in some way cytotoxic or specifically inhibitory to the antigen-driven processes which usually lead to cell division.

The time-course of the specific response assayed in vitro differs in SRBC- and BCG-immunized mice. In the former, the earliest increase over background is at about 60 hr; it reaches a maximum level at 4–5 days and declines thereafter. This parallels very closely the timing of cellular reflections of antibody formation in the spleen after comparable immunization (66). BCG immunization initiates a slower tempo of cellular events, with earliest increase in incorporation occurring at the 2nd wk, the maximum achieved at 3–4 wk, and the response declining thereafter. This course follows the in vivo manifestations of delayed hypersensitivity in the mouse (40).

In both systems, however, and in the immune responses to alloantigens (13), the ultimate outcome is that susceptibility to specific stimulation in spleen cell populations returns to the preimmunization background level. This had occurred by 10–15 days after immunization in the case of SRBC stroma, by 10 wk after BCG, and by 2 wk after alloantigen injection (13). At face value, these data indicate that no greater number of antigen-sensitive cells exist after an antigenic experience, even a presumably *de novo* one like BCG, than existed prior to immunization, i.e., that no memory cells were produced. A further im-

plication is that no memory occurs in the absence of antigen and that the proliferative response initiated by antigen is self-limiting and returns to a preexisting state when the stimulus to cell division is gone. This is clearly not the case in spleen cell populations where antibody formation by individual cells is assayed in vitro as a response to immunization (67). Here, an easily detected increment of memory cells accrues in the spleen after an antigenic experience with SRBC stroma. No similar comparison in the case of BCG and alloantigens has been made. The cell population which responds to SRBC stimulation by making and secreting antibody appears to represent a memory system, and the population which is stimulated to divide and to mediate delayed hypersensitivity-type phenomena are antigen driven, but do not increase the numbers of progeny having similar properties. A corollary of this line of speculation would be that the latter population must be replenished regularly from stem cells by thymic derivation. It is possible, and difficult to rule out, that the increment of added antigen-sensitive cells required for memory—a more rapid and augmented response to reencountered antigen—is small, and the system employed here is too insensitive to detect it. More information is needed on sensitivity before this question can be resolved, and the credibility of the memory concept stated above can be evaluated critically.

SUMMARY

The media and culture conditions required for in vitro stimulation of mouse lymphoid cells are described. The medium was arginine-rich and contained heat-inactivated human serum. A component of the human sera necessary for stimulation of the cells was a natural mouse cell agglutinin, which affected both background stimulation and the degree of induced stimulation with phytohemagglutinin (PHA). Absorption of the agglutinin from the human serum rendered the medium incapable of sustaining DNA synthesis in the presence of PHA.

The response to PHA of mouse spleen and thymus cells was age-dependent and, although this response was not present at birth, it rapidly rose to adult levels. Spleen cells from mice immunized with bacillus Calmette-Guérin (BCG) or sheep erythrocytes (SRBC) showed increased in vitro reactivity to added purified protein derivative (PPD) or SRBC stroma, dependent on the time of immunization.

The dose response curve for the SRBC stroma stimulated, immune spleen cells is compatible with a theory of cell to cell interaction being necessary for an in vitro reaction to antigen.

The possible role of the mouse cell agglutinin (AMLG) is discussed.

We would like to thank Mrs. Penny Marquette for her help in the preparation of this manuscript.

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