POTENTIATION OF THE T-LYMPHOCYTE RESPONSE TO MITOGENS

I. The Responding Cell*

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A number of recent experiments, both in vivo and in vitro, have suggested that macrophages play a significant role in the immunologic responses of lymphocytes of both the T (thymus processed) and B (nonthymus processed) classes to antigen.[1]

Three cells, macrophages ("adherent cells") together with T and B lymphocytes, are required for the antibody response to certain antigens in intact animals (1). Similarly, in a variety of in vitro systems involving antibody responses to such antigens as sheep erythrocytes but not, for example, to polymerized flagellin, three cells are required (2–9). An early interpretation of this finding was that cell cluster formation, perhaps providing a means of antigen presentation, was the essential element in the reaction (2, 10, 11). More recent work, however, has shown that soluble factors released by the participating macrophages could be substituted for intact macrophages in these systems (5, 12, 13). Similarly, for the mitotic response of sensitized lymphocytes to specific antigen (14, 15) or of unsensitized lymphocytes to allogeneic cells (16), macrophages are essential but may be replaced by the soluble factors which they release (17, 18). With nonspecific mitogens such as phytohemagglutinin (PHA), the response of thymocytes or "purified" lymphocytes may also be enhanced by the presence of cells from peritoneal exudates, bone marrow, spleen, or indeed xenogeneic blood leukocytes (19–23). Adherent cells are identified as the active participant in some of these experiments (20, 22, 23), but soluble mediators have not thus far been described. Macrophages also play a role in the action of many adjuvants (24–26). Here a recruit-

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[1] *Abbreviations used in this paper:* ALS, anti-lymphocyte serum; B, nonthymus processed; BSA, bovine serum albumin; Con A, concanavalin A; LAF, lymphocyte-activating factor; LPS, lipopolysaccharide; MEM, minimal essential medium; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; SUP, supernatants; T, thymus processed; T-^{3}H, thymidine-^{3}H; XT, irradiation plus syngeneic thymocytes.

ment and mitotic response of T cells are observed elements of the adjuvant effect in vivo (27–28),[2] and the expression of adjuvancy requires the presence of T cells (28, 29). Here, again it is not clear whether a soluble mediator plays a role. Hanna et al., (30) have suggested that the effective microenvironment provided by tissue macrophages is a principal determinant of immune responsiveness and may dwindle with age.

In the present series of papers we present a detailed report of experiments, described elsewhere in preliminary form (31), which show that macrophages release one or more mitogenic substances. These act on T lymphocytes, greatly potentiating their response to standard mitogens such as phytohemagglutinin, but do not stimulate B cells in the absence of T cells. Their production is markedly enhanced by agents such as endotoxin. Data concerning the nature of the target cell are given in the present paper. Our study of the cell population responsible for release of potentiating factors and preliminary characterization of the factors are described in subsequent publications (32).[3]

Materials and Methods

Animals.—Mice were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine. CBA/J mice of both sexes, 6–12 wk old, were used in all experiments except that in which cells of mice of several strains were compared. Human peripheral lymphocytes were obtained by bleeding colleagues in the laboratory.

X-Ray.—A Siemens Stabilipan 250 kv machine (Siemens Corp, Iselin, N.J.) was used to deliver 850 R (85 R/min) to mice in a special rotating Lucite holder at a target distance of 70 cm. These were reconstituted by injection of 5–8 $\times$ 10^7 syngeneic thymus cells (XT) the same day, and used 4–7 days after treatment.

Cell Suspensions.—Mouse cell suspensions were prepared (31, 33) by teasing the lymphoid tissues with needles, allowing clumps to settle, and washing. The preparation of human peripheral blood lymphocytes (PBL) is described in reference 31. All manipulations were carried out in minimal essential medium (MEM-S, with l-glutamine and antibiotics, Microbiological Associates, Inc., Bethesda, Md.) with 8% pooled normal human serum added.

Cell Separation.—Discontinuous bovine serum albumin (BSA) density gradients (34) were used to separate thymocytes into four bands, designated A, B, C, and D (from least to most dense) plus a pellet, P. As in earlier work (35, 36), larger cells were found almost exclusively in A, B, and C, and macrophages in A and B. For removal of adherent cells (2, 6), 1-ml aliquots of cell suspension, containing 10^7 cells in MEM with 10% normal human serum or fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.), were incubated in Petri dishes (No. 3002, Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.) for 40 min, and the nonattached cells separated. The process was repeated twice more, and the final suspension of nonadherent cells was used for culture. Purified human lymphocytes were obtained as described in references 14 and 37. About 4 $\times$ 10^7 leukocytes in MEM with 10% human serum were incubated in a Pasteur pipette (15 cm) with washed nylon fibers for 30 min at 37°C, and nonattached cells were washed out with the same medium. Human adherent cells were prepared (16) by incubating PBL in MEM with 30% autologous human

[2] Spiesel, S. Z., R. K. Gershon, and B. H. Waksman. Adjuvant effects on mouse thymus-derived cells. I. A survey of various classes of adjuvants. Manuscript in preparation.

[3] Gery, I., and R. E. Handschumacher. Potentiation of lymphocyte responses to mitogens. III. Characterization of mediators. Manuscript in preparation.

serum in Petri dishes for 4 hr, and decanting the nonadherent cells. The attached cells were washed three times with MEM plus 10% human serum before incubation.

Tissue Culture.—The materials employed and culture techniques, as well as the use of scintillation counting to measure thymidine-^{3}H incorporation, are described in detail in references 31 and 33. Mouse cultures contained 5×10^6 thymus or 2×10^5 spleen or lymph node cells in 1.0 ml of medium. Different numbers of human cells were employed. All were cultured in 5% CO_2 and harvested at 72 hr; unless otherwise indicated, thymidine-^{3}H(T-^{3}H) was given as a 1.0 μCi pulse during the last 24 hr of incubation. Data are reported as uncorrected counts per minute and always represent the average of values obtained in duplicate tubes. 1 μl of phytohemagglutinin-P (PHA; Difco Laboratories, Detroit, Mich.), 3.0 or 6.0 μg of concanavalin A (Con A; Nutritional Biochemicals Corporation, Cleveland, Ohio), and 100 μg lipopolysaccharide W from *Escherichia coli* strain 055:B5 (LPS; Difco Laboratories) were routinely used as mitogens (31, 33).

Supernatants.—Human leukocytes or mouse (syngeneic) lymphoid cells (1.5×10^6 or 4×10^6 cells, repectively, in 2.0 ml of medium) were cultured 24 hr without stimulation or in the presence of PHA or LPS. Human adherent cells (from 5×10^7 PBL) were incubated in 5 ml of MEM with 20% human serum without mitogen for 48 hr. All supernatants (SUP) were cleared by centrifuging at 1000 rpm for 10 min and again at 1800 rpm for 30 min and

TABLE I

Effects of Human SUP on the Response to PHA of Lymphocytes from Various Mouse Lymphoid Organs

Stimulant	Thymus	Lymph nodes	Normal spleen	XT spleen
None	87*	474	1,504	41
PHA	337	39,189	34,491	1,522
PHA + SUP	21,237	41,544	32,291	10,354

* T-^{3}H incorporation (counts per minute).

were stored at −20°C for up to 2 months before being used. SUP was added at various dilutions to mouse cell cultures. When not otherwise indicated, SUP was produced with LPS.

RESULTS

Effect of Human SUP on Mouse Lymphoid Cell Responses.—Supernatants, prepared by incubating human peripheral blood lymphocytes with LPS for 24 hr, markedly increase the mitotic response of adult CBA mouse thymocytes cultured with PHA but not the response of lymph node or spleen cells (Table I). Spleen cells of mice harvested 5–7 days after whole body irradiation and reconstitution with syngeneic thymocytes (XT cells) responded to SUP and PHA in a manner intermediate between thymocytes and spleen cells (Table I). Similar results are obtained in experiments with human SUP prepared with PHA rather than LPS (31, 32) and with SUP prepared from syngeneic mouse spleen or bone marrow (see below). The possibility that mitogen carried over from the donor cell culture may be responsible for the potentiating effect of SUP is ruled out by the demonstration of a similar effect with SUP from human adherent cells incubated without stimulant (Table II). Such SUP is mitogenic

alone and increases the response to either PHA or a suboptimal (unpublished data) concentration of Con A but does not affect the response to LPS.

Kinetics of Mouse Thymocyte Response to Human or Mouse (Syngeneic) SUP and PHA.—Thymidine-^{3}H uptake values in thymocyte cultures har-

TABLE II

Potentiation of Thymus Cell Responses to Various Mitogens by SUP from Unstimulated Human Adherent Cell Culture

Mitogen*	T-^{3}H uptake (cpm)	
	Without SUP	With SUP
None	104	5,911
PHA	879	27,613
LPS	353	7,837
Con A	9399	23,642

* PHA, 1.0 μl/culture; LPS, 100 μg/culture; Con A, 3.0 μg/culture.

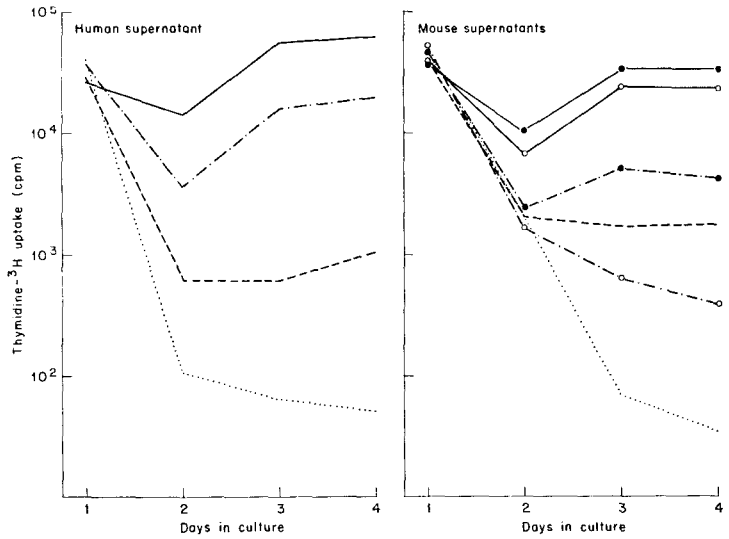

FIG. 1. Thymidine-^{3}H uptake by mouse thymocytes, cultured for different periods in the presence or absence of SUP derived from human PBL (left) or from syngeneic mouse cells (right), either spleen (open symbols) or bone marrow (closed symbols). Unstimulated, ... ; PHA alone, – – – –; SUP alone, –·–·–; SUP + PHA, ——.

vested after various intervals (Fig. 1) show that spontaneous DNA synthesis has largely ceased by the third day and that there is relatively little increase in uptake in stimulated cultures after the third day. SUP prepared from syngeneic mouse spleen or bone marrow by incubation with LPS was almost as effective in potentiating the thymocyte response to PHA as SUP from similarly

stimulated human cells. In each case, the SUP itself showed mitogenic activity in the absence of PHA, also with a maximal response by the third day.

Responsiveness of Lymphocytes from Different Mouse Strains to Human SUP and Mitogens.—Mice of the four strains examined in the present study showed significant differences in lymphocyte reactivity toward human SUP produced with LPS (Tables III and IV). SUP itself serves as a mitogen for thymocytes in each case (much better, in fact, than PHA) and appeared to act synergistically with PHA in CBA, NZB, and SJL mice. A synergistic effect with Con A at the concentration used here (6 μg/ml) was seen only with AKR and C57BL thymocytes which reacted less well to the mitogen alone than thymocyes of the other strains. With SUP + LPS, the response was no greater than with SUP alone, and it is probable, as shown in the next paper, that the

TABLE III

Effect of Human SUP on Responses of Thymus Cells from Various Mouse Strains to Different Mitogens

Mouse strain	CBA/J	AKR/J	SJL/J	C57BL/6J	NZB/J*
Stimulant					
None	135	99	452	87	24
PHA	843	572	6,298	516	723
LPS	958	697	1,348	847	183
Con A‡	143,285	92,148	120,606	76,470	21,794
SUP§	17,453	14,090	12,896	11,881	2,531
SUP + PHA	60,568	19,729	44,205	10,254	5,971
SUP + LPS	13,945	11,107	10,681	10,544	—
SUP + Con A‡	156,579	134,490	108,633	136,354	—

* NZB mice were studied in a separate experiment.

‡ Con A, 6.0 μg/culture.

§ SUP, human PBL, stimulated with LPS (1:2).

response seen with LPS alone was due to the formation of SUP from thymus macrophages (32). Essentially similar results were obtained in other experiments making use of SUP from unstimulated PBL or PBL exposed to PHA.

Human SUP was highly mitogenic for spleen cells, often more so than PHA (Table IV), but showed no synergy with PHA or Con A on cells of any strain tested. Indeed, an inhibition of the mitotic response was apparent in several instances.

Responsiveness of Thymocyte Subpopulations to SUP + Mitogens.—When normal CBA thymocytes were separated on discontinuous BSA gradients, the lightest cells, in bands A and B, showed active spontaneous DNA synthesis continuing on the third day of culture (Table V). These cells, together with those in band C, were responsive to PHA, as reported in earlier work (35, 36). However, they were much more stimulated by SUP and still more by SUP + PHA. Yet they did not, even with the latter, reach the intensity of response

TABLE IV

Effect of Human SUP on Responses of Spleen Cells from Various Mouse Strains to Different Mitogens

Mouse strain	CBA/J	AKR/J	SJL/J	C57BL/6J	NZB/J*
Stimulant					
None	1,797	720	1,162	540	381
PHA	51,157	30,647	57,120	26,688	8,367
LPS	89,998	79,826	35,384	28,471	16,414
Con A‡	163,520	133,109	162,844	101,485	73,269
SUP§	70,040	53,060	28,854	28,953	6,854
SUP + PHA	57,354	44,942	51,801	40,336	14,335
SUP + LPS	79,475	77,519	41,370	43,885	—
SUP + Con A	133,149	119,034	141,773	101,365	—

* ‡ § As in Table III.

TABLE V

Response of Mouse Thymocyte Subpopulations to Mitogens and Human SUP

Experiment Stimulant*	Original	Fraction			
		A + B	C	D	P
I. *% of cells recovered*		*3.2*	*21.0*	*46.0*	*29.8*
None	44	2,661	181	13	68
PHA	794	29,916	4,198	91	62
SUP-2	13,085	50,796	19,882	2,630	375
SUP-2 + PHA	19,905	68,296	77,132	5,966	852
Con A	49,195	176,245	177,043	1,352	58
II. *% of cells recovered*		*1.7*	*15.1*	*39.7*	*43.5*
None	40	1,192	47	16	10
PHA	208	22,247	1,038	49	19
SUP-2	22,810		35,098	11,923	367
SUP-2 + PHA	24,437	57,151	56,161	9,101	659
SUP-1	54		135	11	
SUP-1 + PHA	3,474		27,214	366	

* SUP-1 was obtained from unstimulated human PBL; SUP-2 was obtained from PHA-stimulated PBL. The SUP preparation used in Exp. I was diluted 1:4; those in Exp. II were undiluted. Con A in Exp. I was used in a dose of 15 μg/culture.

attained with Con A. The denser small cells in band D and the pellet P, which made up four-fifths of the total cells recovered showed neither spontaneous DNA synthesis nor responsiveness to PHA. Yet these cells responded significantly to SUP or the combination of SUP and PHA, the response being greater than that elicited with Con A. In this experiment, SUP prepared from unstimulated PBL showed no mitogenic activity at the concentration tested, yet potentiated the response to PHA markedly.

Responsiveness of Nonadherent Spleen Cells to SUP + PHA.—Mouse spleen

cells, from which adherent cells were removed, required SUP for the expression of a PHA response at low cell concentrations (Table VI). At high cell concentrations, SUP produced a marked increase in the response over that given with PHA alone. The response of the nonadherent cells to PHA alone was greater than that of unpurified spleen cells, perhaps because of a relative increase in the proportion of T cells (see reference 14).

Responsiveness of Purified Human Lymphocytes to SUP + PHA.—It proved quite easy to demonstrate a potentiating effect of human SUP on the response of highly purified human peripheral blood lymphocytes to PHA when the concentration of cultured cells was lowered sufficiently (Table VII). The effect was not seen with unpurified cells and SUP by itself was not stimulatory.

TABLE VI

Role of Potentiating Factors in the Response of Mouse Spleen Cells to PHA

Experiment	Cells/culture × 10^3	SUP	PHA	T-^{3}H uptake	
				Original	Nonadherent
I	1000	−	−	227	122
		−	+	14,815	38,841
		+	−	6,413	7,163
		+	+	23,886	63,131
	50	−	−	9	7
		−	+	9	122
		+	−	44	21
		+	+	69	1,690
II	400	−	−	33	13
		−	+	3,165	7,469
		+	−	1,478	411
		+	+	10,465	15,952
	40	−	−	11	10
		−	+	145	233
		+	−	29	7
		+	+	274	1,167

DISCUSSION

The observations reported here establish that the proliferative response of T lymphocytes to nonspecific mitogens is enhanced by soluble factors released by other lymphoid cells. In the following paper, we show that macrophages are the probable source of such factors and that their production is markedly enhanced by stimulants like LPS. This finding extends the work of Dutton, Shortman, Bach, and others demonstrating the participation of similar factors in specific responses (5, 12, 13, 17, 18) and provides a plausible explanation

for the role of the macrophage in lymphoid cell interaction in responses to both antigen and nonspecific agencies (25, 38). We propose the term "lymphocyte-activating factor" or LAF for the potentiating activity in question, recognizing that LAF may prove to be heterogeneous.[4] Preliminary fractionation studies suggest,[3] however, that it is a single substance. This paper provides a simple, reproducible, and highly sensitive method for its assay.

TABLE VII

Effects of Human SUP on Reactions to PHA of Human Leukocytes, Before and After Column Purification

Experiment	Cells/culture × 10^3	SUP*	PHA	T-^{3}H uptake (cpm)	
				Unpurified	Column purified
I	500	−	−	49	38
		−	+	15,899	7,859
		+	−	44	65
		+	+	7,360	22,527
	40	−	−	33	37
		−	+	219	392
		+	−	33	45
		+	+	145	2,036
II	400	−	−	87	41
		−	+	32,164	42,287
		+	−	63	174
		+	+	25,979	42,984
	80	−	−	14	25
		−	+	7,036	14,824
		+	−	27	40
		+	+	4,823	16,758
	16	−	−	11	15
		−	+	215	416
		+	−	25	22
		+	+	211	2,983

* SUP, human PBL, stimulated with LPS (1:4).

The principal and perhaps only target of LAF appears to be the central or peripheral T cell, since it acts on thymocytes and on spleen cells of lethally irradiated mice reconstituted with thymus cells, both preparations largely devoid of B lymphocytes. LAF was active on preparations freed of macro-

[4] This term was proposed 2 yr ago on hypothetical grounds by B. A. Askonas and L. Jarošková (1970. *In* Developmental Aspects of Antibody Formation. J. Šterzl and I. Říha, editors. Academic Press, New York. 531.

phages by adherence to plastic or nylon fibers. It potentiated responses to PHA and to suboptimal concentrations of soluble Con A, mitogens which stimulate T lymphocytes exclusively (39–43). The failure of PHA to stimulate spleen cells of thymectomized XBM mice, even a month after reconstitution (33), showed that LAF and PHA are not active on B lymphocytes. Our SUP preparations did not potentiate the mitotic response of spleen cells to LPS, which stimulates mitosis only in B cells in the mouse (33).

The data show an apparent heterogeneity in responses of T cells to LAF and to PHA and Con A. In part this depends on the relative numbers of macrophages in the preparations tested and their consequent ability to produce endogenous LAF. Thus a potentiating effect was difficult to demonstrate in whole preparations of human PBL or mouse spleen, in contrast with thymocytes or with peripheral cell preparations after removal of adherent cells. Part of the difference in the PHA responses of different thymocyte subpopulations obtained on BSA gradients is attributable to the presence of macrophages in A and B and their absence in C, D, and P (36). There is also a heterogeneity of the target lymphocytes themselves, however. Within the thymus, there are clearly cells which respond to active SUP but not to PHA, and others which respond only to SUP + PHA. While the response to Con A by thymocytes in the A, B, and C fractions was always greater than the maximum attained with SUP + PHA, cells in D and P were stimulated more by SUP alone or SUP + PHA than by Con A. Similarly peripheral T cells, as shown by the results with nonadherent mouse spleen cells and column-purified PBL, show heterogeneity in that some respond to SUP or PHA while others appear to require both. These differences may reflect differences in the number of receptor sites for particular stimulants on individual cells. They are not related in any simple way to stages of maturation of cells within the thymus nor, in the present state of our knowledge, to the postulated subpopulations, T1 and T2, of the peripheral T-cell pool (44). The differences in response of thymocytes and spleen cells among several mouse strains tested reflect the type of heterogeneity discussed here, but may also depend on the relative numbers of macrophages in the different cell suspensions (see reference 32).

The data suggesting heterogeneity complement our earlier studies of immunocompetence in different subpopulations of thymocytes (36, 45), as well as those of other workers (46–53). In a number of species (rat, mouse, pig, chicken), the usual parameters of T-cell immunocompetence, among them ability to recirculate, to react against PHA or allogeneic cells, to produce a graft-*versus*-host reaction, or to cooperate with B cells in an antibody response, are found in a minor subpopulation (less than 10% of the total) of thymocytes, larger and less dense than the others. After separation on discontinuous density gradients (36, 53), the smallest and densest thymocytes, in band D and the pellet, which make up three-quarters to four-fifths of the total collected from the gradient, fail to show any of these forms of reactivity. However, a few of

the cells in D and possibly in P can be stimulated by antithymus serum, which reacts well with the surface of these cells, as judged by immunofluorescence and cytotoxicity (45), and a similar or slightly better response of these cells was stimulated by SUP + PHA. Yet when the numerical representation of the different subpopulations is used to calculate the contribution of each to the total T-^{3}H uptake, D and P contribute no more than about one-fifth even in the presence of this maximal stimulation. It is still correct, therefore, to regard the preponderance of D and P cells as inert.

In considering the mechanism of action of LAF on T cells, two major possibilities can be envisaged. LAF may act simply as a mitogen. The synergy of LAF with PHA resembles that seen when nonspecific mitogens, such as PHA, anti-lymphocyte serum (ALS), or streptolysin, act together at suboptimal concentrations or when nonspecific mitogens act synergistically with specific soluble antigen (on sensitized cells) or allogeneic cells (on unsensitized cells) (54, 55). Frequently no mitogenic effect of SUP alone was seen at concentrations which synergized very well (see, for example, the data in Tables V, VI, and VII). The characteristic finding that two agents used simultaneously at optimal levels may produce a diminished response (54, 55) is duplicated at a number of points in our data, particularly with Con A (e.g., Table IV). This interpretation fits well with Greaves and Möller's suggestion that synergy simply represents stimulation of an increased number of receptor sites and that the cell can count (54, 55). It is of interest that thymocytes maximally stimulated with SUP + PHA gave as great a response as spleen cells. The possibility that carryover of mitogen, Con A in particular, from the donor cultures may account in part for the activity of SUP on thymocytes is small, since Con A–SUP prepared with PBL or with mouse thymus were virtually inactive and since the concentration of Con A in recipient cultures (0.75 μg/ml) was far below the mitogenic threshhold.[5]

An alternative hypothesis is that LAF may be a nutritional factor or an agent which affects the metabolic state of the cell, its level of reactivity, or some unidentified property of the cell membrane which affects the numbers or reactivity of its receptors for mitogens like PHA. Attention need only be called to studies which show that insulin greatly potentiates chicken thymocyte responses to allogeneic cells (49) and that reducing agents may increase the responses of human or rabbit PBL to PHA and ALS by two to three times (56). Whitfield and his collaborators have stressed the effect of cyclic adenosine monophosphate levels in potentiating the proliferative responses of mouse thymocytes to a variety of hormonal or other factors, such as cortisol (at low concentration), parathormone, vasopressin, bradykinin, cyclic guanidine monophosphate, or even Mg^{++} (see, for example, references 57 and 58), and adenyl cyclase triggering appears to be an early event in the response to PHA

[5] Gery, I. Unpublished data.

(59). A relationship of this sort is also implied by our results showing that LAF effects are much more apparent at low concentrations of peripheral T cells, which presumably result in lower production of "conditioning" factors, or at low concentrations of CO_2 (20). A considerable body of published work had suggested that, while the response of peripheral cells to antigen requires macrophage mediation, stimulation by such mitogens as PHA does not (see, for example, references 14 and 16). However, Oppenheim and his colleagues (14) have shown a macrophage requirement at low levels of PHA, i.e., with a reduction in the exogenous stimulus applied to the cells. Their finding and ours suggest that responses to antigen and to PHA may differ only in a quantitative sense.

SUMMARY

Human and mouse lymphoid cells, stimulated by phytohemagglutinin (PHA) or lipopolysaccharide W (LPS), release supernatant factor(s) which are mitogenic for mouse thymocytes and which potentiate their responses to PHA or concanavalin A (Con A). The term LAF (lymphocyte-activating factor) is proposed for this activity. LAF not only enhances the mitotic responses of the less dense thymus subpopulations (A, B, and C) separable on discontinuous bovine serum albumin (BSA) gradients but also gives substantial responses in the otherwise inert cells of the denser fractions D and P. LAF does not exert a potentiating stimulatory effect on the responses of unfractionated mouse spleen cells, but does act synergistically with PHA on nonadherent spleen cells and on spleen cells of mice of several strains 5 days after irradiation and injection of thymocytes. Similarly LAF, which has no visible effect on unfractionated human peripheral blood cells, strongly potentiates the PHA response of column-purified lymphocytes, when these are cultured at low concentration. We conclude that LAF stimulates both central and peripheral T lymphocytes and enhances their responses to other stimulants.

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