POTENTIATION OF THE T-LYMPHOCYTE RESPONSE TO MITOGENS

II. The Cellular Source of Potentiating Mediator(s)*

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The first papers of the present series establish that mouse thymocytes and peripheral thymus-processed (T)[1] lymphocytes are stimulated to mitosis and their response to such agents as phytohemagglutinin greatly potentiated by factor(s) designated "lymphocyte-activating factor" (LAF), which are produced in cultures of syngeneic or xenogeneic lymphoid cells (1, 2). In this paper, we present data showing that adherent cells, probably macrophages, are the principal source of LAF and that its production is increased by agents which stimulate these cells.

Materials and Methods

Animals.—Male or female CBA/J mice, 6–12 wk of age, were used without treatment or after irradiation (850 R) and reconstitution with 5×10^6 syngeneic bone marrow cells (XBM), 5–8 $\times$ 10^7 thymocytes (XT), or both bone marrow and thymus cells (XBMT). Young adult New Zealand albino rabbits of both sexes were purchased from a local dealer and used without treatment.

Cell Preparation and Culture.—All the materials and techniques employed for cell preparation and culture are fully described in our previous papers (1–3). The separation of adherent from nonadherent cells was carried out both on plastic (4, 5) and by the use of nylon columns (6, 7). While 8% pooled normal human serum was routinely used for lymphocyte culture, the adherence technique required higher concentrations, 10% human serum being used routinely and 10% fetal calf serum (Grand Island Biological Company, Grand Island, N.Y.) in one experiment.

RESULTS

Release of Potentiating Factors by Cells from Different Sources.—Factors stimulatory to normal CBA thymocytes are released by normal human, rabbit,

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[1] *Abbreviations used in this paper:* B, nonthymus processed; Con A, concanavalin A; LAF, lymphocyte-activating factor; LPS, lipopolysaccharide; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; SUP, supernatant; T, thymus processed; T-^{3}H, thymidine-^{3}H; XBM, irradiation plus syngeneic bone marrow cells; XBMT, irradiation plus syngeneic bone marrow cells and thymocytes; XT, irradiation plus syngeneic thymocytes.

or syngeneic mouse lymphoid cells into the supernatant (SUP) (Table I). Human peripheral blood lymphocytes (PBL), acted on by phytohemagglutinin (PHA) or lipopolysaccharide (LPS), produce SUP which is mitogenic alone and which greatly potentiates the thymocyte response to PHA. The production of SUP activity bears no relation to DNA synthesis in the donor culture, which is marked with PHA and negligible with respect to LPS. Concanavalin A (Con A), which was mitogenic for the donor culture, gave no SUP activity in this experiment.

With syngeneic spleen cells, all three mitogens stimulate mitosis and production of SUP activity but at a lesser level than with human cells. Only Con

TABLE I

Response to Stimulants and Production of Potentiating Factors by Cells of Different Species

Experiment	Donor culture			Recipient culture* T-^{3}H uptake	
	Cells	Stimulant	T-^{3}H uptake	SUP alone	SUP + PHA
I	None	—	—	29	242
	Human blood leukocytes	None	63	28	488
		PHA	64,492	14,880	38,418
		LPS	747	13,426	53,898
		Con A	2,426	73	368
	Mouse spleen cells	None	355	42	417
		PHA	17,003	109	1,224
		LPS	55,697	147	14,811
		Con A	85,200	1,091	9,090
II	None	—	—	147	665
	Rabbit spleen cells	None	1,391	265	1,305
		PHA	6,154	519	1,551
		LPS	1,856	520	4,637

* Mouse thymocytes.

A-SUP stimulates DNA synthesis in recipient thymocytes without added PHA. Rabbit spleen cells give similar findings but a still lower level of SUP production. However, rabbit cells were agglutinated to some extent by human serum in the medium, and SUP production may have been affected by this. LPS was clearly the best stimulant of SUP production with all the cell types tested.

When mouse organs are compared (Table II), bone marrow, spleen, and thymus, in that order, are found to contain cells active in producing SUP. Some is released by unstimulated cells, more by cells exposed to PHA or Con A, and the most by cells treated with LPS. Only LPS was effective in stimulating marrow to produce an active SUP. While all the supernatants tested acted synergistically with PHA, only Con A-SUP of spleen and LPS-SUP from

marrow were mitogenic alone, in each case more so than PHA. Again there was no correlation between donor cell mitosis and production of SUP.

Characterization of Cells which Produce Potentiating Factors.—Lethal irradiation of mouse donors and reconstitution with bone marrow alone did not significantly affect the ability of their spleen cells to produce SUP, when incubated without stimulant or with LPS (Table III). With PHA and Con A, on the other hand, there was some loss of ability to form SUP, and this was restored by the additional injection of thymocytes. These data and those of

TABLE II

Response to Stimulants and Production of Potentiating Factors by Cells of Different Mouse (Syngeneic) Organs

Experiment	Donor culture			Recipient culture T-^{3}H uptake	
	Cell source	Stimulant	T-^{3}H uptake	SUP alone	SUP + PHA
I	—	—	—	55	557
	Spleen	None	1,763	80	1,367
		PHA	42,061	183	3,919
		LPS	60,635	419	13,568
		Con A	112,606	5040	12,984
	Thymus	None	35	76	803
		PHA	456	146	1,141
		LPS	337	247	3,892
		Con A	7,108	199	886
II	—	—	—	69	1,693
	Spleen	None	231	56	3,046
		PHA	35,780	364	4,285
		LPS	61,310	632	23,529
		Con A	158,447	7125	25,406
	Bone marrow	None	3,336	45	2,741
		PHA	11,143	209	4,073
		LPS	10,663	5008	33,676
		Con A	11,328	369	4,883

Table II imply that T lymphocytes may contribute to the formation of active SUP with PHA and Con A but not with LPS.

When mouse spleen cells were separated into adherent and nonadherent populations by incubation in plastic Petri dishes, the ability to produce mitogenic and potentiating SUP, after stimulation with Con A and especially with LPS, was found to reside almost entirely in the adherent cells (Table IV). When the adherent cells were cultured for 2 days and washed repeatedly to remove cells other than macrophages, they remained active as sources of SUP. At this time contamination with granulocytes and lymphocytes is minimal.

Human PBL were similarly freed of adherent cells by passage through nylon

columns. These cells retained their reactivity with PHA, as shown by thymidine-^{3}H (T-^{3}H) uptake after 3 days in culture, yet lost to a considerable degree their ability to produce mitogenic or potentiating factors when stimulated with either PHA or LPS (Table V). Titrations indicated that column-purified

TABLE III

Response to Stimulants and Production of Potentiating Factors by Syngeneic Normal, XBM, and XBMT Spleen Cells

Donor culture			Recipient culture T-^{3}H uptake	
Treatment of mouse	Stimulant	T-^{3}H uptake	SUP alone	SUP + PHA
—	—	—	45	879
Normal	None	221	23	1,015
	PHA	22,025	136	2,132
	LPS	38,201	125	8,432
	Con A	115,712	3413	14,364
XBM	None	1,151	44	1,608
	PHA	6,484	95	2,458
	LPS	1,582	179	11,829
	Con A	10,653	641	5,137
XBMT	None	2,552	38	2,746
	PHA	21,705	233	6,227
	LPS	4,872	1048	13,832
	Con A	50,981	3028	13,702

TABLE IV

Production of Potentiating Mediators by Adherent and Nonadherent Mouse Spleen Cells

Stimulant of donor culture	PHA in recipient culture	Mouse spleen fraction			
		Original	Nonadherent	Adherent (1 hr)	Adherent (2 days)
None	−	30*	48	37	ND
	+	238	157	789	ND
LPS	−	76	42	3,112	173
	+	2258	759	15,326	7992
Con A	−	401	92	541	ND
	+	1662	258	3,665	ND

* Values for T-^{3}H uptake (counts per minute) in recipient thymocyte cultures without added SUP were unstimulated, 55, and PHA-stimulated, 293.

cells produced less than 4% of the SUP activity released by the original unfractionated PBL. The residual activity did not disappear on dilution and may differ qualitatively from that obtained with unpurified cells.

The Indirect Action of Mitogens.—Removal of adherent cells from a normal CBA thymocyte population reduces the response of these cells in culture (T-^{3}H uptake) to PHA and Con A to less than one-half of control values

and virtually eliminates the response to LPS (Table VI). The supernatants after treatment of control cultures with any of the three mitogens show SUP activity, and comparable activity is much reduced in the supernatants of nonadherent cells in culture (data not shown).

Quantitative Comparison of Different Supernatants.—A comparative titration was carried out on two active SUP obtained from human PBL stimulated with PHA and LPS (Fig. 1). Both were mitogenic alone for mouse thymocytes, and both enhanced the response of these cells to PHA. Enhancement was signifi-

TABLE V

Effect of Nylon Column Purification on Ability of Human Blood Leukocytes to Produce Potentiating Factors

Experiment	Donor culture			SUP dilution	Recipient culture T-^{3}H uptake	
	Cells	Stimulant	T-^{3}H uptake		SUP alone	SUP + PHA
I	None	—	—	—	64	1,032
	Original	None	29	Undiluted	3,748	30,139
				1:5	202	12,853
		PHA	15,869	Undiluted	19,942	51,858
				1:5	1,214	20,073
				1:25	155	6,148
		LPS	25	Undiluted	13,656	56,126
				1:5	6,071	39,127
				1:25	1,511	21,831
	Purified	None	38	—	ND	ND
		PHA	7,859	Undiluted	125	1,900
				1:5	57	1,718
		LPS	21	Undiluted	122	3,655
				1:5	100	3,084
II	None	—	—	—	53	525
	Original	None	60	Undiluted	87	833
		PHA	45,855	Undiluted	27,529	31,518
	Purified	None		—	ND	ND
		PHA	26,210	Undiluted	200	1,409

cant at concentrations of SUP below the mitogenic threshhold. The maximal level of enhanced stimulation attained was only slightly greater in each case than that achieved with the optimal concentration of SUP alone. The titration curves, both for SUP alone and for SUP + PHA, were closely similar and suggested that the same active agent was present in the two preparations.

DISCUSSION

In the previous paper (2), evidence was presented that the target cell for LAF is the central or peripheral thymus-processed (T) lymphocyte. The data of the present paper show that LAF is probably produced by macrophages in

both human and mouse cultures. Active SUP were obtained from adherent cells, which could be maintained for 2 days attached to plastic and which withstood vigorous washing. Such preparations do not include significant numbers of T cells, nor do spleen cell suspensions from XBM mice, which also

TABLE VI

Stimulation of Mouse Thymus Cells by LPS: the Need for Adherent Cells

Stimulant	T-^{3}H uptake by thymus cells		Ratio original/nonadherent
	Original	Nonadherent*	
None	130	20	6.5
LPS	343	81	4.2
PHA	441	178	2.5
LPS + PHA	1527	274	5.6
Con A	5844	2606	2.2

* Collected after two incubations in Petri dishes.

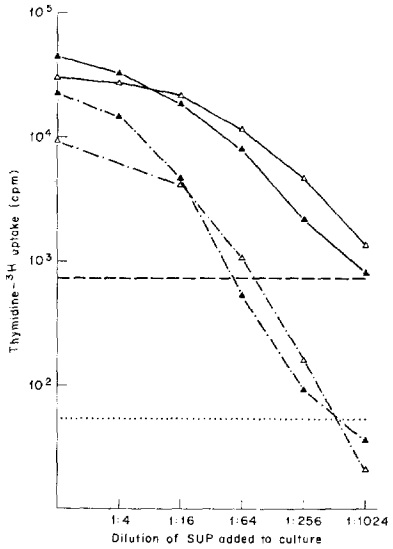

FIG. 1. Titration of mitogenic and potentiating activity of SUP prepared by incubating PBL with PHA (▲) or LPS (△). T-^{3}H-incorporation of mouse thymocytes without additives ....., with PHA – – – –, with SUP –·–·–, or with SUP + PHA ——.

produced SUP as active as that from normal spleen. T cells may play an indirect role, nevertheless, when PHA or Con A is used to stimulate LAF production by macrophages (see below), and this may account in part for differences in LAF production by different organs stimulated with the various agents. Nonthymus processed (B) cells appear to be ruled out as a source of LAF since active SUP could be obtained from thymus cell suspensions and from

XBM spleen at a time when no B cells reactive to LPS are present (3). The most active SUP were obtained with LPS, a well-recognized stimulant of macrophages (8–11).

There is no evidence that LAF produced by "unstimulated" adherent cells differs qualitatively from that produced after stimulation with PHA or LPS or that the latter are different from each other. All are mitogenic alone and show striking synergy with PHA or with Con A used at concentrations below those giving a maximal response. Their titration curves, alone or in the presence of PHA, are perfectly parallel (Fig. 1). Apparent qualitative differences in SUP from different animal species and different organs are most readily explained as depending on differences in LAF concentration. Also, as noted, differences in the number of T cells influence LAF production by PHA or Con A as compared with LPS. There may nevertheless be some heterogeneity of factors which act on lymphocytes. We have seen (Table V) that nylon column-purified PBL can be stimulated by LPS to produce weakly active SUP whose activity resists dilution. Tridente et al. (12, 13) and Winkelstein (14) have described synergistic responses of thymocytes with other cells having different kinetic properties from those under consideration here, and the former group reports that the adherent cells from bone marrow are less effective in stimulating thymocytes than unfractionated marrow cells.

The present observations suggest that LAF may play an essential role in many or all the immunologic responses in which T cells participate, certainly in each instance where a requirement for macrophages or "adherent cells" has been demonstrated. Such a requirement in the reaction of the sensitized cells of delayed hypersensitivity with eliciting antigen is well recognized, whether this be measured by proliferation and blast transformation (6) or by production of secondary mediators such as lymphotoxin.[2] Equally important is the primary response of unimmunized T cells, as in the graft-*versus*-host reaction and its in vitro equivalent, the mixed lymphocyte reaction (15, 16), or the response to an immunizing dose of soluble antigen with adjuvant (17).[3] In such primary responses, a further cooperation between T cells of different biologic types or specificities may be essential (18, 19). Finally, the cooperation of T and B cells (20, 21) in stimulating the latter to plasma cell transformation and antibody production requires macrophages both in vivo (22) and in vitro (4, 5, 23–27). In several of these instances involving T cells alone (16, 28) or T-B cell cooperation (24, 25, 29) it has already been recognized that soluble factors comparable to LAF serve as mediator. This conclusion does not necessarily conflict with other suggested mechanisms of macrophage action in the immune response. Supernatant factors described by other investigators appear in some instances to represent altered antigen (25, 29). There is per-

[2] Yoshinaga, M. Unpublished data.

[3] 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.

suasive evidence that antigen attached in or near the cell surface of macrophages may act as "superantigen" (30–33) or that antigen linked to a special RNA provided by the macrophage may play this role (34–38).

One of the most important implications of the present observations concerns the role of macrophages in the action of adjuvants, whether to increase delayed sensitization or to enhance antibody production. In the intact animal (10, 39) some adjuvant materials, such as mineral oil, simply promote wide dissemination and long persistence of the antigenic stimulus. Others increase the flux of lymphocytes into thymus-dependent areas of stimulated peripheral lymph nodes (40), a localization which may depend on specific as well as nonspecific elements in the local response to antigen and adjuvant and a release of mediators (41, 42). Adjuvant effects are also observed in vitro however (43), and clearly must be mediated by more direct cellular events. Enhanced antigen uptake by macrophages (44–46), whether as a result of the particulate character of the antigen (47, 48) or of stimulation directed to the macrophage (49, 50), was long felt to account for such effects. This mechanism accords well with the suggested role of the macrophage in providing superantigens to the host (30, 31, 33–37). More recently, however, the suggestion was made that adjuvants act by labilizing macrophage lysosomes (51–53), and an increased production of lysosomal enzymes does in fact result from the action of LPS on macrophage monolayers (11). A target for the released lysosomal contents has not been identified; it is possible that LAF may be a lysosomal factor the target of which is the T lymphocyte. T cells in XT mice synthesize DNA rapidly in response to in vivo stimulation with a variety of adjuvant materials, among them pertussis vaccine and purified LPS.[3] Our findings, presented in this and the preceding paper, establish that the stimulus to these cells must be mediated in part by macrophages and LAF production. These findings assume greater significance in the light of the recent demonstration that the adjuvant effect of pertussis, LPS, Freund's adjuvant, or retinol on antibody formation requires the participation of T cells (17, 42) and the in vitro demonstration that phagocytic cells and T lymphocytes together are required to "help" nonmitotic B cells respond to antigens such as sheep erythrocytes (24, 29, 54).

Certain stimulatory effects observed in the intact animal and in vitro appear to be indirect. PHA and Con A produce less effective SUP from bone marrow than spleen, and its production in the latter is lessened in XBM animals as contrasted with XBMT animals (see Tables II and III). This implies that these mitogens, unlike LPS which is highly effective in producing LAF activity from bone marrow and in the presumed absence of T cells, act in part by stimulating T lymphocytes which then activate macrophages with their mediators and these in turn produce LAF, a striking circular mechanism. PHA and Con A act only on T cells (55–59). That stimulation of T cells may produce activation of macrophages indirectly by way of soluble mediators has been

demonstrated repeatedly (60–62). Thus the postulated circular mechanism may come into play in any situation where T cells are activated, for example in the interesting "allogeneic effect" described by Katz et al. (63). The fact that mouse spleen reacts maximally to a dose of Con A 5-10-fold lower than required for maximal stimulation of mouse thymus[4] may simply reflect the relative paucity of macrophages in the thymus and consequent weakness of the suggested circular mechanism. Removal of macrophages and other large cells from reactive thymocytes on bovine serum albumin gradients has been shown (2, 64) to diminish their reactivity to PHA. PHA and Con A, however, may also have direct effects on macrophages, and we have shown production of LAF from adherent cells by the former (59). In consequence, as one would predict, PHA acts as an adjuvant if administered at the correct time in relation to antigen (see references 65 and 66 for reviews).

The response to LPS also illustrates a second type of indirect effect. It is well recognized (8–10) that LPS is highly stimulatory to macrophages both in vivo (10) or in vitro (11). In the mouse, LPS also stimulates B lymphocytes but has no direct action on T cells (3, 59, 67). In accord with these findings, the stimulation of thymocytes by LPS is shown in the present paper to depend entirely on the presence of adherent cells, presumably macrophages. One must suppose that the vigorous proliferative response of peripheral T cells when LPS is given to XT animals[3] may also be dependent on production of small amounts of LAF which stimulate these cells.

SUMMARY

Effective supernatants (SUP), which potentiate mouse T-cell responses to phytohemagglutin (PHA), are obtained from cells of several species (human, rabbit, rat, mouse) and indeed from syngeneic spleen, thymus, or bone marrow cells. Unstimulated cells release some SUP activity but more is produced after stimulation. Lipopolysaccharide (LPS) produced very active SUP in all cultures tested. PHA was similarly active on human leukocytes only, whereas concanavalin A (Con A) gave highly efficient SUP only with mouse spleen cells. SUP production is not correlated with a mitotic response of the donor cells and is observed in cultures unable to respond mitotically to the stimulant. Adherent mouse spleen cell populations, consisting largely or entirely of macrophages, produce active SUP, while nonadherent cells do not. Similarly, purification of human peripheral leukocytes on nylon columns, with removal of macrophages and other adherent cells, destroys their ability to produce SUP. The importance of indirect effects in stimulating mitotic responses of T cells is emphasized by the fact that the mitotic response of mouse thymocytes to LPS and its ability to potentiate the response of these cells to PHA disappears with removal of adherent cells from the thymocyte population. Con-

[4] Gery, I. Unpublished data.

versely the production of SUP from spleen cells stimulated by Con A requires the presence of T cells.

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