Brief Definitive Report

IMMUNOSTIMULATORS INDUCE GRANULOCYTE/MACROPHAGE COLONY-STIMULATING ACTIVITY AND BLOCK PROLIFERATION IN A MONOCYTE TUMOR CELL LINE*

By PETER RALPH, HAL E. BROXMEYER,‡ and ILONA NAKOINZ

(From the Sloan-Kettering Institute for Cancer Research, Rye, New York 10580)

The monocyte/macrophage is the normal producer in marrow and blood of colony-stimulating activity (CSA) required for progenitor cells to differentiate into granulocytes and monocytes in culture (1–3). Bacterial lipopolysaccharide (LPS) activates macrophages to increased CSA production (3–5), as well as synthesis and release of various enzymes, and nonspecific toxicity for tumor cells (6–8). Other activating agents associated with increased CSA levels in monocyte populations include dextran sulfate (9) and polyinosinic-polycytidylic acid (5).

LPS and other macrophage activators specifically inhibit the growth of tumor cell lines of the monocyte series without affecting other hematopoietic tumor types-erythroleukemia, mastocytoma, T lymphoma, myelomas, or fibrosarcoma (10, 11). We describe here induction of CSA in monocyte line PU5-1.8, specifically by immunostimulators that block proliferation of tumor cells.

Methods and Materials

PU5-1.8 Cell Line. Monocyte cell line PU5-1.8, derived from a spontaneous tumor in a BALB/c mouse, actively phagocytoses zymosan and latex beads, bears receptors for immunoglobulin and complement, secretes lysozyme (11), phagocytoses sheep erythrocytes, and kills tumor targets in the presence of specific antisera. Cells grow in RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum with a doubling time of 16–20 h.

Substances Tested for Induction of CSA. Dialyzed latex beads 0.81 μm diameter, LPS (*Salmonella typhosa* W0901, Difco Laboratories, Detroit, Mich.), tuberculin purified protein derivative (PPD) (gift from Dr. A. Gray, Merck Sharp & Dohme, West Point, Pa.), *Mycobacterium* Bacillus Calmette-Guérin (BCG) (Tice strain, University of Illinois, Chicago), zymosan, dextran sulfate (mol wt 500,000), dibutyryl cyclic AMP (Sigma Chemical Co., St. Louis, Mo.), and silica (gift from Dr. B. Bloom, Albert Einstein College of Medicine, Bronx, N.Y.) were dissolved or suspended in phosphate-buffered saline, pH 7.4. Phorbol myristic acetate (Consolidated Midland Corp., Brewster, N.Y.) was dissolved in ethanol. Lipid A was derived from LPS by mild acid hydrolysis (12), and free polysaccharide obtained by hydrolysis in 1% acetic acid at 100°C for 2h.

Assay for CSA (13). Mouse bone marrow cells (7.5×10^4) were suspended in 1 ml 0.3% Difco agar culture medium containing enriched McCoy's medium plus 10% fetal calf serum. PU5-1.8 conditioned medium was assayed at 0.1 ml. Colonies (>40 cells per aggregate) and clusters (3–40 cells per aggregate) were scored after 5–7 days. For maximum intracellular CSA, cell pellets were suspended in saline to the original volume and lysed by two cycles of freeze thaw. Supernates were

* Supported by National Science Foundation grant BMS 75-19734, National Cancer Institute grants CA 17353, CA 17085, and the Gar Reichman Foundation.

‡ Special Fellow of the Leukemia Society of America.

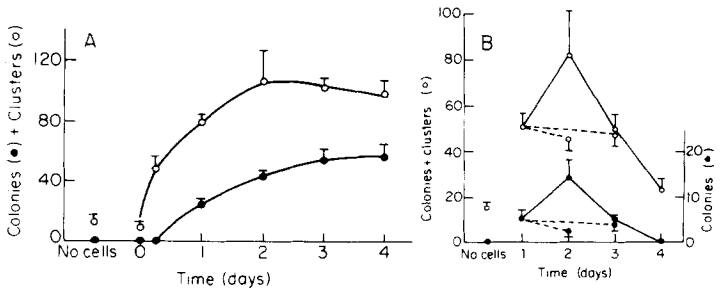

FIG. 1. Induction of CSA in PU5-1.8 cells by LPS. A. Cumulative production. Cultures at 3 × 10^5 cells/ml were incubated with 1 μg/ml LPS, and supernates assayed at various times for CSA (± 1 SEM). PU5-1.8 cultures without LPS reached 32 × 10^5 cells/ml after 3 days; these supernates did not contain CSA. B. Incremental production. Cultures were initiated at 3 × 10^5 cells/ml, 1 μg/ml LPS. At day 1, supernate was collected and cells reincubated with the same volume of fresh medium containing 1 μg/ml LPS. This was repeated at day 2 and day 3. CSA secreted from day 0 to 1, 1 to 2, 2 to 3, and 3 to 4 is shown. In parallel, cells were incubated 1 day with LPS, centrifuged, washed once and reincubated 1 or 2 days without LPS (dashed lines). Continual presence of inducer is necessary for maximal production of CSA.

dialyzed extensively to remove actinomycin D or puromycin before assay for CSA. Control cultures received drugs at the end of incubation with LPS and were similarly dialyzed.

Results

Kinetics of CSA Production by PU5-1.8 Cells in the Presence of LPS. PU5-1.8 cells were incubated with 1 μg/ml LPS which inhibited their growth more than 90%. Supernates of untreated cultures did not contain CSA. Small amounts of CSA were detected by 6 h incubation with LPS (Fig. 1 A). Supernates of PU5-1.8 treated with LPS for 24 h caused colony formation with plateau levels of CSA reached after 3–4 days. The plateau represented cessation of CSA synthesis, not steady-state synthesis and decay, since the daily production of CSA declined after the second day and was undetectable after the third day (Fig. 1 B). Colonies contained granulocytes and macrophages, similar to that stimulated by WEHI-3 CSA (14).

CSA of cell lysates was less than 40% that of the corresponding supernates from 6 h to 4 days of LPS treatment. Lysates did not contain inhibitors of granulopoiesis; they did not interfere with colony formation stimulated by CSA from WEHI-3, a myelomonocytic leukemia cell line secreting CSA constitutively (14). Although PU5-1.8 cell growth was inhibited during incubation with LPS, there was less than 10% cell death by trypan blue (11). Induced CSA appears to be actively secreted. Synthesis of lysozyme, continuously secreted by PU-5-1.8, was not affected or was slightly enhanced during LPS treatment (11); overall protein synthesis was partially inhibited (12). Actinomycin D at 0.01 μg/ml or 3 μg/ml puromycin during LPS treatment blocked CSA production more than 80%, implying a requirement for RNA and protein synthesis. Concentrations of colchicine (0.1 μg/ml) and mitomycin C (1 μg/ml) that inhibit PU51.8 growth did not interfere with LPS induction of CSA.

Dose-Response of LPS. LPS at 0.001 μg/ml caused 40% inhibition of tumor cell growth but no activation of CSA (Fig. 2). 0.01 μg/ml LPS caused some significant production of CSA. Maximal induction occurred with 1 μg/ml, which

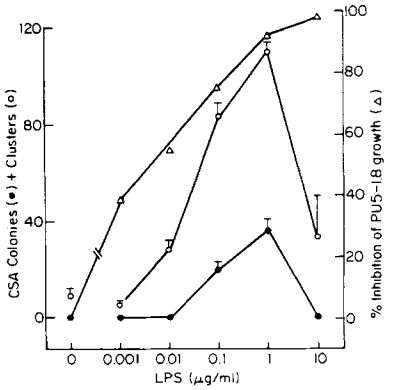

FIG. 2. Dose response of CSA induction by LPS. Cultures were initiated at 2×10^5 cells/ml plus final concentrations of LPS. After 2 days, tumor cells were counted (percent inhibition of growth shown) and supernate CSA determined. Control medium, medium incubated 2 days with 1 or 10 μg/ml LPS, and 2 days' control cultures of PU5-1.8 to which 1 or 10 μg/ml LPS was added when preparing supernates had only background levels of CSA (8–14 clusters).

blocked tumor cell growth almost completely. Lower CSA of cultures incubated with 10 μg/ml LPS may not represent high dose inhibition of CSA formation. This LPS concentration partially inhibits colony formation stimulated by WEHI-3 CSA. PU5-1.8 cells may produce a factor without stimulation which requires LPS for CSA activity. Cells were incubated 2 days without LPS, and supernate was prepared immediately after addition of the drug. This was inactive. Thus tumor cells must be in contact with LPS for induction.

Other Macrophage-Active Agents Which Induce CSA. Zymosan, BCG, PPD tuberculin derivative from *Mycobacterium*, LPS, and derived lipid A specifically inhibit growth of PU5-1.8 cells but not other hematopoietic tumor lines in culture (11). CSA is induced in PU5-1.8 cells by all of these macrophage-activating substances that inhibit growth of PU5-1.8, as well as by phorbol myristate (Table I). Dextran sulfate, latex beads actively phagocytosed by the monocyte cells (11), and alkali-treated LPS do not inhibit PU5-1.8 growth or induce CSA. Not all conditions of tumor cell cytostasis lead to CSA production. Reversible inhibition of cell growth by high concentrations of thymidine or cyclic AMP, or by incubating cells without serum did not induce CSA.

Granulocyte Inhibitor of Constitutive CSA Production. Human granulocytes contain an activity that inhibits production of CSA by human and murine monocytes, CSA-producing cells resident in the marrow and by murine, WEHI-3 cells, (13). Granulocyte extract, at concentrations which inhibit WEHI-3 constitutive production more than 50%, had no effect on induction of CSA when added to PU5-1.8 cells together with LPS (three experiments).

Discussion

Induction of CSA in PU5-1.8 tumor line is similar to that with normal mouse macrophages (4) and human monocytes (5) in that 10–100 ng/ml LPS is effective, and requires several days for maximal stimulation. This is characteristic of the induction period of mature characteristics in erythroleukemic cells (15), induc-

TABLE I
Induction of CSA in PU5-1.8 by Macrophage-Active Agents

Agent	Concentration	Inhibition of PU5-1.8 growth	CSA (colonies)
	(μg/ml)	%	
None		0	0
LPS	1	90	55 ± 8
Lipid A	10	98	12 ± 2
Alkali LPS	1	22	0
	10	44	0
	100	53	0
PPD	10	68	12 ± 4
	50	92	18 ± 3
BCG	10^6/ml	100	14 ± 2
Zymosan	50	87	58 ± 10
	100	90	9 ± 1
Phorbol myristate	0.01	11	0
	0.1	53	17 ± 3
	1	59	27 ± 3
Dextran sulfate	1	0	0
	10	0	0
	100	0	0
	1,000	44	0
DMSO*	2%	60	0
Silica	200	90	0
Latex beads	5×10^7/ml	0	0
Dibutyryl cAMP	10^{-3} M	79	0
	10^{-4} M	33	0
Thymidine	10^{-3} M	89	0
No serum		87	0

Drugs were added as shown to PU5-1.8 cultures initiated at 2×10^5 cells/ml. After a 3-day incubation, tumor cells were counted and CSA in supernates determined as in Fig. 1. Control culture medium containing these concentrations of drugs (except phorbol myristate) neither caused colony formation, nor inhibited the activity of preformed CSA in the assay system. Cell culture and control solutions of preformed CSA in the assay system. Cell culture and control solutions containing phorbol myristate had to be extensively dialyzed before assay since the drug was extremely active in stimulating CSA in a cell population resident in the bone marrow (Unpublished observations).

* DMSO, dimethyl sulfoxide.

tion of granulocyte morphology, phagocytosis (16), receptors for immunoglobulin and complement, and lysozyme synthesis in a myeloid leukemia cell line (17).

The lipid A fraction of LPS induces CSA in PU5-1.8 cells, confirming the results of in vivo induction by Apte et al. (18). CSA inducers acting on macrophages are now expanded by these experiments to include BCG, tuberculin PPD, zymosan, and phorbol myristate. Failure of Cline et al. (3) to observe increased CSA production by macrophages cultured with heat-killed *Mycobacteria* may be due to toxicity since it is specifically toxic to some macrophage tumor lines in culture (11). Dextran sulfate neither inhibited growth of PU5-1.8 cells nor stimulated CSA production. Other macrophage cell lines sensitive to growth inhibition by dextran sulfate (11) are induced by the polyanion to synthesize CSA (manuscript in preparation). Other macrophage properties stimulated by activators in the macrophage tumor cell lines include LAF (19), prostaglandin release (E. Rietschel, personal communication), elastase, collagenase (Z. Werb, personal communication), plasminogen activator (J. Hamilton, manuscript in preparation), and antibody-dependent cellular immunity against erythrocyte and tumor targets (20).

CSA is induced by macrophage activators (but not other agents) in PU5-1.8

only at concentrations that block tumor cell proliferation more than 50%. Growth inhibition may be a consequence of induced terminal differentiation, as seen with erythroblast and myeloblast lines (15–17). Thus, extensive DNA synthesis or cell cycling is not necessary for induction. Presence of the inducer is necessary for a long time. Washing and resuspending cells in medium lacking LPS after 1 day greatly reduced their subsequent production of CSA (Fig. 1 B).

There are few studies on the necessity for other cell types in macrophage activation. Results herein and those mentioned with macrophage tumor lines in culture provide evidence for inductive events occurring in the complete absence of other cell types.

The granulocyte inhibitor of constitutive CSA production (13) failed to block LPS induction of CSA in PU5-1.8. Inhibition of constitutive CSA production by granulocyte extract can be overcome by stimulation of cells with androgenic and anabolic steroids and LPS (21). This may allow for increased granulopoiesis in the presence of high concentrations of mature granulocytes as in acute inflammatory reactions.

Summary

Monocyte tumor cell line PU5-1.8 does not normally produce colony-stimulating activity (CSA) required by granulocyte and macrophage progenitors to proliferate and mature in agar. However, CSA is induced in the culture line by as little as 10 ng/ml endotoxic lipopolysaccharide (LPS), with maximum CSA production and release to the medium between 2 and 3 days of incubation. Derived lipid A, but not alkali-treated LPS, is also active. Induction requires RNA and protein synthesis, but is not blocked by mitomycin C or Colcemid. Other inducers of CSA include *Mycobacterium* Bacillus Calmette-Guérin, tuberculin protein preparation purified protein derivative, zymosan, and phorbol myristate. All inducing agents are specific inhibitors of the monocyte tumor cell proliferation in vitro. Latex beads, another macrophage-activating agent, are rapidly phagocytosed by PU5-1.8 cells, but neither inhibit growth nor induce CSA.

We thank M. Moore for support and advice, N. Williams and M. Dexter for criticism, and S. Schrader for expert technical assistance.

Received for publication 11 April 1977.

References

1. Chervenick, P. A., and A. F. LoBuglio. 1972. Human blood monocytes: stimulators of granulocyte and mononuclear colony formation *in vitro*. *Science (Wash. D. C.)*. 178:164.
2. Moore, M. A. S., N. Williams, and D. Metcalf. 1973. *In vitro* colony formation by normal and leukemic human hematopoietic cells: interaction between colony forming and colony-stimulating cells. *J. Natl. Cancer Inst.* 50:591.
3. Cline, M. J., B. Rothman, and D. W. Golde. 1974. Effect of endotoxin on the production of colony-stimulating factor by human monocytes and macrophages. *J. Cell. Physiol.* 84:193.
4. Eaves, A. C., and W. R. Bruce. 1974. *In vitro* production of colony-stimulating activity. I. Exposure of mouse peritoneal cells to endotoxin. *Cell Tissue Kinet.* 7:19.

5. Ruscetti, F. W., and P. A. Chervenick. 1974. Release of colony-stimulating activity from monocytes by endotoxin and polyinosinic-polycytidylic acid. *J. Lab. Clin. Med.* 83:64.
6. Alexander, P., and R. Evans. 1971. Endotoxin and double-stranded RNA renders macrophages cytotoxic. *Nat. New Biol.* 232:75.
7. Hibbs, J. B., Jr. 1974. Heterocytolysis by macrophages activated by *Bacillus Calmette-Guérin*: lysosome exocytosis into tumor cells. *Science (Wash. D. C.).* 184:469.
8. Reed, W. P., and Z. J. Lucas. 1975. Role of soluble toxin in macrophage-inhibited cultures of tumor cells. *J. Immunol.* 115:395.
9. Gronowicz, E., P. Biberfeld, B. Wahren, and A. Coutinho. 1976. Characterization of dextran sulfate-sensitive cells. *Scand. J. Immunol.* 5:573.
10. Ralph, P., I. Nakoinz, and W. C. Raschke. 1974. Lymphosarcoma cell growth is selectively inhibited by B lymphocyte mitogens: LPS, dextran sulfate and PPD. *Biochem. Biophys. Res. Commun.* 61:1268.
11. Ralph, P., and I. Nakoinz. 1977. Direct toxic effects of immunopotentiators on monocytic, myelomonocytic, and histiocytic or macrophage tumor cells in culture. *Cancer Res.* 37:546.
12. Ralph, P., and I. Nakoinz. 1974. Lipopolysaccharides inhibit lymphosarcomas of bone marrow origin. *Nature (Lond.).* 249:883.
13. Broxmeyer, H. E., M. A. S. Moore, and P. Ralph. 1977. Cell-free granulocyte colony inhibiting activity derived from human polymorphonuclear neutrophils. *Exp. Hematol. (Copenh.).* 5:87.
14. Ralph, P., M. S. Moore, and K. Nilsson. 1976. Lysozyme synthesis by established human and murine histiocytic lymphoma cell lines. *J. Exp. Med.* 143:1523.
15. Friend, C., and W. Scher. 1975. Stimulation by dimethyl sulfoxide of erythroid differentiation and hemoglobin synthesis in murine virus-induced leukemic cells. *Ann. N. Y. Acad. Sci.* 243:155.
16. Ichikawa, Y., M. Maeda, and M. Horiuchi. 1975. Induction of differentiated functions which are reversibly suppressed by cytochalasin B. *Exp. Cell Res.* 90:20.
17. Krystosek, A., and L. Sachs. 1976. Control of lysozyme induction in the differentiation of myeloid leukemic cells. *Cell.* 9:676.
18. Apte, R. N., C. Galanos, and D. H. Pluznik. 1975. Lipid A, the active part of bacterial endotoxins in inducing serum colony stimulating activity and proliferation of splenic granulocyte/macrophage progenitor cells. *J. Cell. Physiol.* 87:71.
19. Lachman, L. B., and R. E. Handschumacher. 1976. Electromobility and purification of lymphocyte activating factor. *Fed. Proc.* 35:489.
20. Ralph, P., and I. Nakoinz. 1977. Antibody-dependent killing of erythrocyte and tumor targets by monocyte-related cell lines: enhancement by PPD and LPS. *J. Immunol.* In press.
21. Broxmeyer, H. E., and P. Ralph. 1977. In Vitro regulation of a mouse myelomonocytic leukemia line adapted to culture. *Cancer Res.* In press.