DIFFERENTIAL STIMULATION OF MURINE LYMPHOMA GROWTH IN VITRO BY NORMAL AND BCG-ACTIVATED MACROPHAGES

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In vitro study of the interaction of macrophages with lymphocytes has led to an abundance of reports in which macrophages are said to exert either stimulatory or inhibitory influences (1–4; additional references cited in 5). In contrast, the voluminous literature on the in vitro interaction of macrophages with malignant cells describes only inhibitory effects, or none at all. That is, macrophages "activated" in a wide variety of ways appear to kill or inhibit the growth of all malignant cells, but have little effect on nonmalignant cells; while "normal" macrophages appear to have only a slight inhibitory effect, if any, on malignant cells (reviewed in 6; 7–10).

If true, these generalizations would suggest: (*a*) that all malignant cells share some property, not possessed by normal cells, such that their interaction with activated macrophages leads to inhibition of their growth; (*b*) that there are only minor differences in the susceptibility of various types of malignant cells to the activated macrophage; and (*c*) that the acquisition by macrophages of antitumor activity is a stereotypic, all-or-nothing response to potential activating agents.

The activation of murine macrophages by Bacille Calmette-Guérin (BCG)[1] has been particularly well studied (11). However, the interaction of macrophages from BCG-treated mice with malignant lymphoid cells has been studied in vitro only rarely (12). The lack of more intensive work in vitro with such cell combinations is surprising, in view of the experimental use of BCG in patients with lymphoid malignancies (reviewed in 13), and the ability of BCG to promote resistance to the growth of lymphocytic leukemias or lymphomas in the mouse (14–18).

For these reasons, we have studied in detail the effects of macrophages from BCG-treated and normal mice on a variety of murine lymphomas in vitro. The results stand in contrast to previous generalizations concerning macrophage-tumor cell interactions in vitro.

Materials and Methods

Mice. Female DBA/2, BALB/c, C57BL/6, and C3H/HeN mice age 2–6 wk were obtained from the Jackson Laboratories (Bar Harbor, Maine) or NIH Animal Production.

[1] *Abbreviations used in this paper:* BCG, Bacille Calmette-Guérin; 2-ME, 2-mercaptoethanol; PPD, purified protein derivative.

TABLE I

Thymidine Uptake by Tumors Cultured with Macrophages, Day 2

Tumor*	Strain	No. of Exp.	Macrophages	Initial tumor cell no.‡				
				0	1×10^3	5×10^3	5×10^4	5×10^5
Group I								
L1210	DBA/2	4	Normal	128 ± 35	4,965 ± 281	22,885 ± 1,464	99,753 ± 4,539	55,487 ± 9,041
			BCG	134 ± 22	364 ± 145	520 ± 103	22,512 ± 1,495	58,116 ± 14,935
			None	114 ± 48	374 ± 224	325 ± 163	3,140 ± 510	58,888 ± 4,292
LSTRA	BALB/c	3	Normal	219 ± 51	12,756 ± 946	90,008 ± 5,568	170,953 ± 14,053	25,003 ± 2,872
			BCG	238 ± 100	624 ± 261	1,058 ± 396	15,518 ± 3,405	28,785 ± 2,944
			None	282 ± 81	455 ± 164	627 ± 286	321 ± 133	20,032 ± 2,996
PU5-14	BALB/c	2	Normal	140 ± 35	8,518 ± 240	37,079 ± 693	234,120 ± 6,438	20,538 ± 663
			BCG	103 ± 11	532 ± 177	2,996 ± 233	73,354 ± 3,916	46,279 ± 2,103
			None	205 ± 80	75 ± 24	121 ± 33	1,521 ± 676	55,564 ± 3,098
P1534	DBA/2	8	Normals	173 ± 63	1,410 ± 32	7,760 ± 657	77,624 ± 4,214	72,183 ± 7,695
			BCG	147 ± 62	377 ± 153	1,149 ± 330	24,805 ± 1,432	72,949 ± 16,883
			None	133 ± 44	92 ± 45	101 ± 32	5,594 ± 400	80,672 ± 7,865
PU5-1	BALB/c	2	Normal	143 ± 21	239 ± 60	1,062 ± 173	18,302 ± 2,274	17,327 ± 293
			BCG	83 ± 37	154 ± 63	162 ± 24	2,198 ± 117	19,289 ± 857
			None	45 ± 8	90 ± 34	71 ± 20	1,445 ± 295	68,095 ± 9,664
EL4	C57BL/6	3	Normal	175 ± 40	1,702 ± 108	7,665 ± 430	93,447 ± 5,722	29,857 ± 4,558
			BCG	97 ± 37	148 ± 37	472 ± 57	4,675 ± 287	48,399 ± 6,649
			None	144 ± 61	171 ± 36	316 ± 16	2,714 ± 56	64,314 ± 9,217
Group II								
TLX9	C57BL/6	1	Normal	77 ± 32	808 ± 62	3,461 ± 152	39,334 ± 484	121,164 ± 9,903
			BCG	36 ± 2	457 ± 53	2,002 ± 77	28,141 ± 1,737	186,271 ± 781
			None	103 ± 40	240 ± 29	1,295 ± 77	25,491 ± 1,131	215,156 ± 1,121

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PU-45	BALB/c	1	Normal	76 ± 3	1,137 ± 193	6,171 ± 343	128,278 ± 5,838	26,395 ± 706
			BCG	131 ± 72	399 ± 71	1,541 ± 62	40,615 ± 3,173	52,471 ± 19,470
			None	66 ± 48	217 ± 48	817 ± 252	13,522 ± 645	53,242 ± 7,682
Group III								
L5178Y	DBA/2	2	Normal	147 ± 24	9,388 ± 525	57,102 ± 5,155	185,465 ± 6,453	29,367 ± 7,355
			BCG	283 ± 94	6,454 ± 275	51,600 ± 2,720	186,622 ± 7,378	46,792 ± 11,515
			None	121 ± 40	6,182 ± 424	46,151 ± 2,882	190,573 ± 7,765	13,381 ± 81
SL2	DBA/2	1	Normal	73 ± 10	8,849 ± 203	74,501 ± 394	102,860 ± 17,711	24,860 ± 13,010
			BCG	116 ± 47	7,487 ± 129	51,452 ± 8,550	93,404 ± 15,764	31,227 ± 5,697
			None	39 ± 7	3,302 ± 274	49,070 ± 1,532	92,216 ± 4,752	41,777 ± 17,452
Line 10 (hepatoma)	Guinea pig	2	Normal	106 ± 14	1,025 ± 102	3,604 ± 328	6,442 ± 2,002	ND§
			BCG	98 ± 17	1,129 ± 295	4,058 ± 378	10,617 ± 959	ND
			None	107 ± 42	976 ± 61	4,395 ± 343	11,711 ± 1,316	ND

* Tumors were induced as follows: carcinogen: L1210, EL4, L5178Y, Line 10; virus: LSTRA; X-irradiation: TLX9; spontaneous: P1534, PU5-1, PU5-14, PU5-45, SL2.

‡ Mean counts per minute ± standard error of the mean, from the number of experiments indicated, each in triplicate, using 5×10^4 initial DBA/2 peritoneal cells. Nonadherent cells were removed before addition of tumor cells, leaving approximately 2×10^4 adherent cells. 2-mercaptoethanol not added.

§ ND, not done.

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BCG. Phipps strain fresh frozen BCG (Trudeau Institute no. 1029, Saranac Lake, N. Y.) (9) was stored at −70°C. Approximately 10^7 viable organisms in 0.1 ml were injected i.p. into DBA/2 mice unless otherwise indicated. Macrophages from mice injected with the vehicle [Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) with Tween 80 (Sigma Chemical Co., St. Louis, Mo.)] gave the same results as from uninjected mice; the latter were routinely used as controls.

Tumors. Tumors were passed as ascites in the strains indicated in Table I. The following were obtained at the National Cancer Institute: L1210 from Mr. S. Yancey; EL4, LSTRA from Dr. J. Wunderlich; P1534 IV a′/95 from Mr. N. Greenberg; the L51784 subline of L5178Y from Mrs. H. Porter; and Line 10 hepatoma from Dr. B. Zbar. The latter was maintained only in tissue culture. PU5-1, PU5-14, and PU5-45 were from Doctors R. Asofsky and R. Tigelaar, NIAID. SL2 and TLX9 were the kind gift of Dr. J. Krahenbuhl, Palo Alto Medical Research Foundation. L929 cells were obtained from Microbiological Associates, Bethesda, Md.

Basic Assay. 2–7 wk after BCG injection, injected and control mice were killed by cervical dislocation. Two 4-cc aliquots of cold Hank's balanced salt solution with 50 U penicillin and 50 mg streptomycin/100 ml were injected through the exposed peritoneum and withdrawn with a no. 25 needle, yielding 1–6 × 10^6 cells per mouse, depending on age. Cells were washed twice at 200 *g* and resuspended in RPMI 1640 (NIH Media Unit) made 20% in heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.) and 2 mM in glutamine, with antibiotics as above; pH was adjusted with 7.5% sodium bicarbonate ("medium"). In cytocentrifuge preparations (Sakura Fine Technical Co., Ltd., Tokyo), the peritoneal cells from both BCG-injected and normal mice appeared to contain approximately 40% macrophages; the remainder were mostly lymphocytes. 5 × 10^4 cells in 0.2 cc medium were added to each well of flat-bottomed MicroTest plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and placed in a humidified 37°C incubator in 5% CO_2. After 3 h, nonadherent cells were aspirated with a capillary pipette attached to wall suction, and 0.2 cc medium was added, containing dilutions of thrice-washed tumor cells, with or without 2-mercaptoethanol (2-ME) (Sigma). Each day, 0.04 cc of fresh medium was added, including 2-ME where appropriate. [^{3}H]Thymidine (Schwartz/Mann, Div. Becton, Dickinson & Co., Orangeburg, N. Y., spec act 3Ci/mmol or New England Nuclear, Boston, Mass., spec act 20 or 2Ci/mmol) was brought to a final spec act of 2Ci/mmol with cold thymidine (Sigma), and 0.02 cc containing 1 μCi in medium was added to each well for a 4-h pulse. These conditions were selected to make cpm a linear function of labeling time and of tumor cell number at high numbers, relationships which did not obtain at higher specific activities. Wells were aspirated onto glass fiber filters (Reeve-Angel, Clifton, N. J.) with a MASH II harvester (Microbiological Associates), and washed copiously with normal saline. Use of cold 5% trichloroacetic acid did not affect results. Filter discs were punched out with a 12-mm cork borer for liquid scintillation counting. For adherent target cells, the labeling medium was discarded. 0.2 cc of 0.25% trypsin (Worthington Biochemicals, Freehold, N. J.) in saline was added to each well for 20 min before harvesting as above. No visible target cells and no cpm remained in the wells.

Long-Term Tumor Culture. Approximately 1 × 10^6 tumor cells from ascites passage were seeded in 10 cc of medium in 250-ml flasks (Falcon) with or without 2-ME, monitored by daily hemocytometer counts with 0.4% trypan blue, and passaged to maintain density at about 2–10 × 10^5/ml.

Esterase Staining. Peritoneal cells adherent to 35-mm plastic dishes (Falcon) were stained for nonspecific esterase as described (19). With both BCG-injected and normal mice, adherent peritoneal cells were 100% esterase positive (500-cell differentials); 1–3% were polymorphonuclear. The adherent peritoneal cell population will be called "macrophages".

Adherence. The percentage of peritoneal cells adhering to plastic was determined by measuring the initial and residual adherent protein and DNA in saline-washed 35-mm dishes as described (20, 21). Values for DNA and protein content per cell confirmed the previous report of Stubbs et al. (22). Finally, nonadherent cells in microtest wells were counted by hemocytometer 3 h after adding a known number of cells.

Glucose-1-^{14}C Oxidation. Glucose carbon-1 oxidation by adherent peritoneal cells was measured as described previously (20), with and without the addition of approximately 2 × 10^8 polystyrene beads (Difco) per dish.

Results

Culture of Macrophages with L1210. During the first 4 h of culture, the

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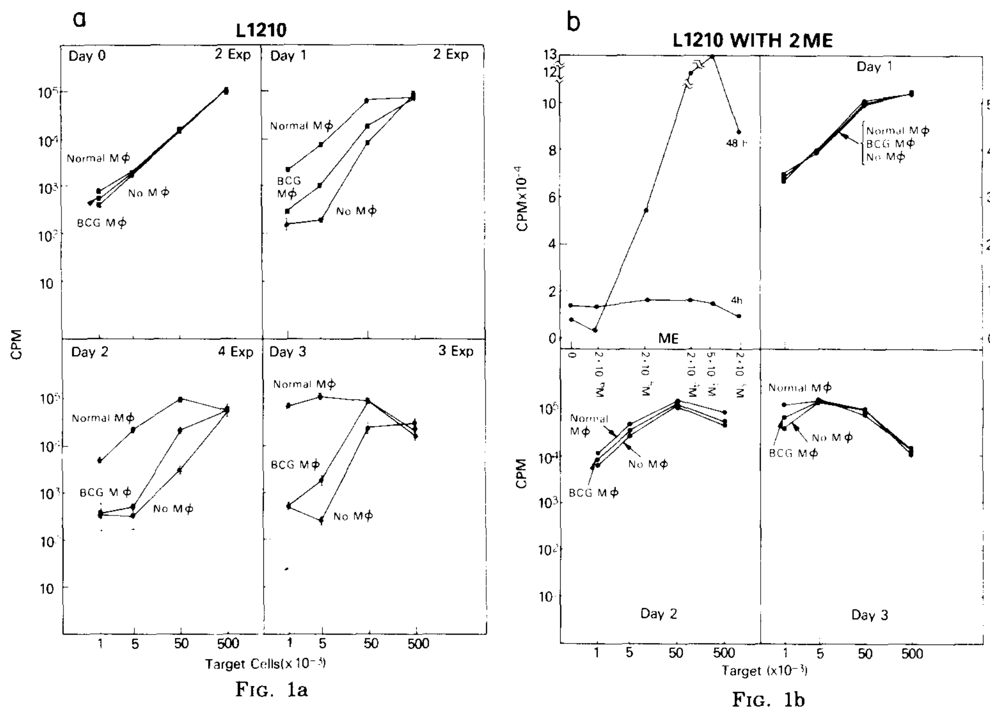

FIG. 1a

FIG. 1b

FIG. 1. (*a*) [^{3}H]thymidine uptake by L1210 cells cultured alone or with normal or BCG macrophages (mϕ). For cultures with macrophages, 5×10^4 peritoneal cells were added for 3 h, then nonadherent cells removed, before lymphoma target cells were added. Means ± SEM are shown for the number of experiments indicated, each in triplicate. Results on day 4 were similar to day 3. (*b*) Upper left panel: [^{3}H]thymidine uptake by 5×10^4 L1210 cells cultured in varying concentrations of 2-ME in the absence of macrophages. Remaining panels: varying numbers of L1210 cells cultured alone or with normal or BCG macrophages (mϕ) as in (*a*), but in the presence of 2×10^{-4} M 2-ME.

amount of thymidine taken up by L1210 DBA/2 lymphoma cells was a linear function of the number of lymphoma cells added, over the range 1×10^3 to 5×10^5 per well (Fig. 1 *a*). At this early time, there was no effect on thymidine uptake when the lymphoma cells were cultured together with 5×10^4 (initial number) adherent peritoneal cells from BCG-infected mice (called BCG macrophages) or from normal mice (called normal macrophages).

By day 1 of culture, thymidine uptake by the lymphoma cells cultured alone began to decrease (Fig. 1 *a*). In the presence of normal macrophages, however, thymidine uptake was markedly stimulated, both in comparison to L1210 cells alone at the same time, and to L1210 cells in the presence of normal macrophages on the previous day. In contrast, very little stimulation of thymidine uptake was seen in the presence of BCG macrophages.

On days 2, 3, and 4, these trends were accentuated, so that at the lower initial target cell numbers, several hundred times as much thymidine was incorporated by L1210 cells in the presence of normal macrophages as in their absence, before correcting for background. While BCG macrophages appeared markedly cytostatic relative to normal macrophages, they exhibited no cytostatic effect

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when compared to the L1210 cells cultured alone. Similar results were obtained when cultures were made in flat-bottomed Linbro plates (Linbro Co., New Haven, Conn.) instead of microtest plates.

Tumors Behaving Like L1210. Five additional lymphomas, passed in DBA/2, BALB/c or C57B1/6 mice, gave results essentially the same as with L1210. For brevity, only the data for the second day of culture are shown in Table I, Group I. In addition, two lymphomas showed the same overall pattern as with L1210, but the degree of stimulation of tumor cell thymidine uptake in the presence of normal macrophages was less marked (Table I, Group II). Results like those described above were seen in 35 of 37 experiments done with the lymphomas in Groups I and II. In the remaining two experiments, the BCG macrophages were used more than 7 wk after BCG injection, and behaved like normal macrophages.

When the initial tumor cell number was as high as 5×10^5, tumor cells often appeared to exhaust the medium by day 1, so that thymidine uptake sometimes fell below that on day 0. Thus, the range of tumor cell concentrations covered the spectrum from what was detectable to what the medium could support.

Effect of 2-ME. In 1973, Broome and Jeng reported that the growth of many murine lymphomas in tissue culture is enhanced by the presence of certain thiols, including 2-ME (23). Moreover, the "helper" effect of macrophages for lymphocytes in several in vitro assays can be replaced wholly or in part by 2-ME (24, 25). We therefore examined the effects of 2-ME in the present assay. For each tumor studied, the effect of 2-ME on thymidine incorporation by the tumor cultured alone was examined in microtest plates on each day of culture, as shown for L1210 in Fig. 1 *b*. For the eight lymphomas in Groups I and II, a marked, dose-dependent stimulation of thymidine incorporation was evident beginning on day 1 and peaking between day 2 and day 4. For different lymphomas, the optimal doses of 2-ME ranged from 6×10^{-6} to 2×10^{-4} M.

When an optimal dose of 2-ME was then added to cultures of L1210 alone or in the presence of BCG macrophages or normal macrophages, the curves describing thymidine uptake vs. initial L1210 concentrations were made nearly identical, and were superimposable on those obtained in the absence of 2-ME but in the presence of normal macrophages on the same day of culture (Fig. 1 *b*). That is, 2-ME appeared to substitute fully for the stimulatory effect of normal macrophages on DNA synthesis by L1210 cells, and with the same time-course. Very similar results with 2-ME were obtained for the other seven tumors listed in Table I, Groups I and II.

In parallel with the above studies, the same lymphomas were grown in 250-ml flasks with and without 2-ME. The results of hemocytometer counts confirmed and extended the observations of Broome and Jeng (23). In general, the lymphomas whose thymidine incorporation was more markedly enhanced by normal macrophages demonstrated greater enhancement of their replication in bulk culture by 2-ME (Table II).

Culture of Macrophages with L5178Y. Not all lymphomas demonstrated stimulation of DNA synthesis by normal macrophages and by 2-ME. On the contrary, two lymphomas and a suspension-culture cell line derived from the Line 10 guinea pig hepatoma showed the same rapid rate of thymidine incorpora-

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TABLE II
Effect of 2-ME on Lymphoma Cell Number in Bulk Culture

Group	Tumor	Daily replication factor*		
		(A) without 2-ME	(B) with 2-ME‡	B/A
I	L1210	0.12	2.84	23.7
	LSTRA	0.23	4.76	21.1
	PU5-14	0.22	4.76	21.6
	P1534	0.27	2.21	8.19
	PU5-1	0.94	2.86	3.04
	EL-4	1.73	4.39	2.54
II	TLX-9	2.09	2.69	1.29
	PU5-45	1.93	2.11	1.09
III	L5178Y	3.37	4.01	1.19
	SL-2	3.11	2.61	0.84

* Daily hemocytometer counts with trypan blue were done on lymphomas in 250-ml T flasks. The daily replication factor is the viable cell count on day n+1 divided by the viable count on day n, adjusted for the dilution effect of passage, and averaged over all the days on which there were surviving lymphoma cells in the culture lacking 2-ME, which was at least 4 days. A daily replication factor less than 1 was associated with all tumors that died out, even though there may have been an initial period of growth. Medium was RPMI-1640 with 20% FCS, glutamine, penicillin, and streptomycin.

‡ 2×10^{-4} M.

tion whether or not macrophages were present, as shown for L5178Y in Fig. 2 *a*. 2-ME had no effect on the thymidine uptake of L5178Y, with or without macrophages (Fig. 2 *b*). SL-2 and line 10 showed the same pattern (Table I, Group III). Results like those seen in Fig. 2 were seen in eight of eight experiments with the tumors in Group III. 2-ME was also without effect on the growth of these tumors in bulk tissue culture (Table II).

Correlation of Changes in Thymidine Uptake and Target Cell Number. The data suggested an unexpected stimulation of thymidine uptake of many mouse lymphomas cultured with normal macrophages, but less so with BCG macrophages. Further studies were done to learn whether increased thymidine uptake reflected increased lymphoma cell replication in the presence of macrophages, and not macrophage replication in the presence of lymphoma cells. Hemocytometer counts were done in parallel with [^{3}H]thymidine pulsing of cultures of PU5-14 plated alone or with normal or BCG macrophages. Because up to half the macrophages became detached from the culture well by the second day, it was necessary to distinguish floating macrophages from lymphoma cells. This was accomplished by esterase staining of the nonadherent cell mixture. There was extremely close correlation (regression coefficient = 0.996) over a wide range (five orders of magnitude) between the numbers of nonadherent, esterase-negative cells, and the thymidine uptake by the cultures. In contrast, the

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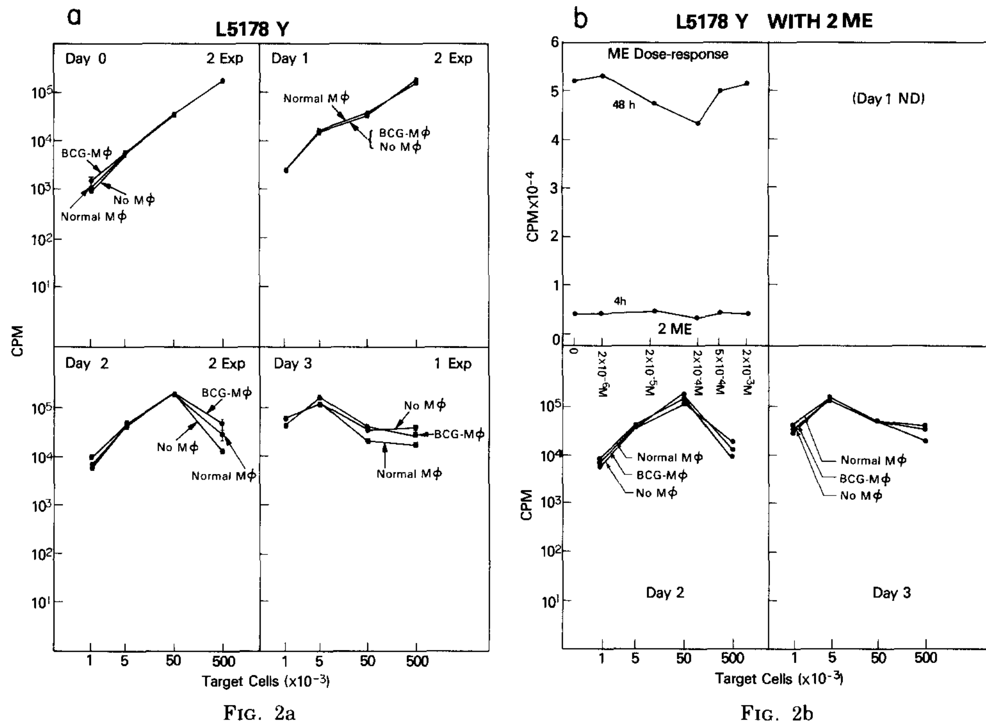

FIG. 2a FIG. 2b

FIG. 2. (*a*) [^{3}H]thymidine uptake by L5178Y lymphoma cells cultured alone or with normal or BCG macrophages (mϕ) as described in the legend of Fig. 1. (*b*) Top panel: [^{3}H]thymidine uptake by 5×10^3 L5178Y cells cultured in varying concentrations of 2-ME in the absence of macrophages. Lower panels: varying numbers of L5178Y cells cultured alone or with normal or BCG macrophages (mϕ) as in (*a*), but in the presence of 2×10^{-4} M 2-ME.

number of nonadherent, esterase-positive cells was always less than the number of peritoneal cells added initially, and was the same in wells with no appreciable thymidine uptake (no lymphoma cells added) as in wells with low to high thymidine uptake. This makes it exceedingly unlikely that macrophages were replicating in the presence of lymphoma cells.

Effect of Peritoneal Lymphocytes. To rule out possible effects of small numbers of lymphocytes which might remain on the adherent macrophage monolayer, a comparison was made between the effects of unseparated, adherent, and nonadherent peritoneal cells on the thymidine uptake of PU5-14 (Fig. 3). The unseparated peritoneal cells showed the stimulation usually seen with normal peritoneal macrophages, and relative lack of stimulation with BCG macrophages. All of this activity was present in the adherent peritoneal cell population, and none of it in the nonadherent population. Thus it appears unlikely that lymphocytes directly affected the results. These data do not rule out the possible participation of adherent, esterase-positive, lymphoid cells.

Effect of Peritoneal Cell Dose and Culture Density. Because of the failure of BCG macrophages to inhibit lymphoma cell growth at the concentrations tested, the effect of increasing numbers of adherent peritoneal cells was examined. Fig. 4 indicates that even when confluent macrophage monolayers were employed,

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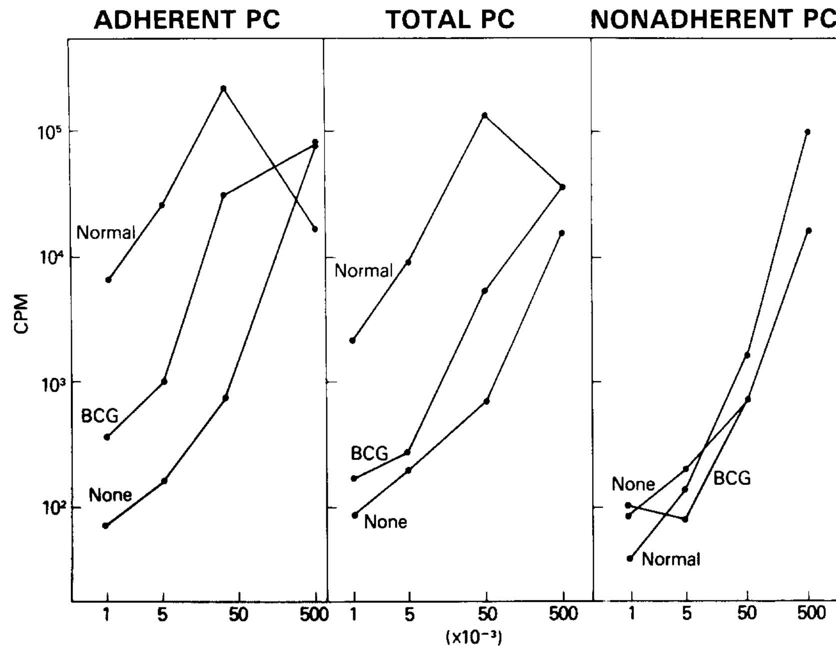

FIG. 3. [^{3}H]thymidine incorporation by various numbers of PU5-14 lymphoma cells cultured alone for 2 days or with 5×10^4 peritoneal cells (PC) (middle panel), with the adherent cells remaining after first adding 5×10^4 peritoneal cells for 3 h and then removing the nonadherent cells (left panel), or with the nonadherent peritoneal cells removed from the latter cultures (right panel). Hemocytometer counts indicated that 41% of the normal and 46% of the BCG peritoneal cells were adherent.

and regardless of the initial number of lymphoma cells over a 50-fold range, higher numbers of BCG macrophages continued to show a slight stimulatory effect on thymidine incorporation by L1210 or P1534. In contrast, increasing numbers of normal macrophages resulted in a dramatic corresponding increase in thymidine incorporation by the lymphoma cells.

Percent of Adherent Peritoneal Cells. Because of the steep dose-response curve for the effect of normal macrophages on lymphoma cell thymidine incorporation, it was necessary to know whether the apparently greater stimulatory effect of normal macrophages compared to BCG macrophages might be due merely to the presence of more adherent cells in the normal peritoneal population. This was ruled out as follows. The number of adherent cells was determined by assays for protein or for DNA content in 35-mm dishes, which were used to achieve detectable levels, and compared to the value for the initial cell inoculum. Alternatively, known numbers of peritoneal cells were added to microtest dishes, the nonadherent cells counted by hemocytometer, and the number of adherent cells calculated by difference. In six experiments, the mean number of adherent cells was 43.5% of those added for normal cells and 42.8% for BCG cells.

Effect of Macrophages Contaminating the Ascites Tumor. The ascites tumor could introduce a variable number of macrophages into the culture, raising the question whether the different requirements of various tumors for 2-ME could

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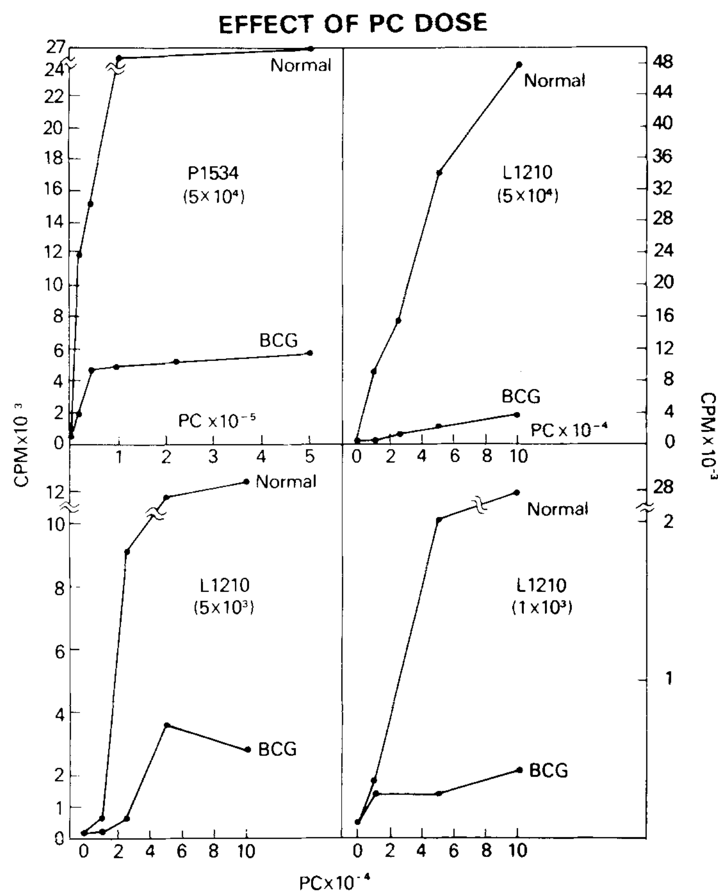

FIG. 4. Effect of the number of peritoneal cells (PC) added initially on the [^{3}H]thymidine uptake of P1534 or L1210 cells after 2 days in culture. 3 h after the addition of PC, nonadherent cells were removed, and lymphoma cells added in the numbers indicated.

be explained by the number or nature of macrophages in the ascites. This seemed extremely unlikely since multiple passages of the tumors in vitro did not diminish their 2-ME dependence nor independence, despite multiple dilution and adherence steps which would deplete macrophages. For example, the macrophage content of PU5-14 ascites was 1.3% by esterase staining, but was 0% (500-cell differential) after five passages in tissue culture; when both these PU5-14 populations were employed as targets in parallel experiments, the same marked stimulation of thymidine uptake was seen in the presence of normal macrophages and the same minor degree of stimulation with BCG macrophages. Considerations of dilution in these experiments would also mitigate against any role of ascitic lymphocytes, unless they were rapidly replicating.

Effect of Different Doses and Routes of BCG Administration. To learn whether a different manner of administering BCG might lead to macrophages with a definite cytostatic effect, macrophages were obtained from mice given BCG at the following times before harvest: 1 × 10^7 viable organisms i.p. at 2–7 wk; 1 × 10^7 i.p. at 2–7 weeks followed by 1 × 10^7 i.p. at 3 days; a single injection

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of 1×10^7 i.p. at 3 days; 2.5×10^7 intravenously at 5 days [reported to produce highly activated macrophages (26)]; and 1×10^7 i.p. at 2–7 wk followed by 2.5×10^7 i.v. at 5 days. When tested on P1534, L1210, or LSTRA, the results showed either the same minor degree of stimulation of lymphoma cell thymidine incorporation as with the first-listed treatment, or an even greater degree of stimulation.

Macrophages from Mice of Different Strains. The results described using DBA/2 mice were also seen with normal and BCG macrophages obtained from C3H/HeN, BALB/c, or C57BL/6 mice, tested on various tumors in both Group I and Group III.

Demonstration of Activation of BCG Macrophages. That the BCG macrophages used in this study were in fact "activated" was shown by morphologic, biochemical, and functional criteria. First, inspection under phase-contrast microscopy revealed that about 50% of the BCG macrophages were flattened (phase-dark) after 1/2 h of culture, while almost none of the normal macrophages were flattened at that time (26).

Second, in two experiments, glucose carbon-1 oxidation to CO_2 by resting macrophages averaged 21 nmol/mg-cell protein-hour for BCG cells compared to 9 for normal cells. During latex phagocytosis, values increased to 152 for BCG cells compared to 51 for normal cells.

Third, normal and BCG macrophages were cultured with L929 cells, an adherent murine fibroblast line. In contrast to the results seen with lymphoma targets, BCG macrophages caused definite inhibition of thymidine uptake by residual adherent L929 cells after 2 days in culture, whether compared to normal macrophages or compared to L929 cells alone. The mean results from six experiments, each in triplicate, were: 5,391 ± 429 cpm for 5×10^3 L929 cells alone, 7,989 ± 1,049 cpm in the presence of normal macrophages, and 2,005 ± 201 cpm in the presence of BCG macrophages. The minor degree of stimulation caused by normal macrophages could not be duplicated by adding 2-ME. In fact, 2-ME was toxic to L929 cells at all concentrations tested, from 2×10^{-6} M to 2×10^{-3} M.

Discussion

The data presented here unexpectedly revealed that the DNA synthesis of many (though not all) murine lymphomas was markedly stimulated in vitro by normal mouse peritoneal macrophages. Equally unexpected was the finding that macrophages from BCG-infected mice were not inhibitory to any of the 10 lymphomas tested; they were simply much less stimulatory than normal macrophages. This was not due to any major deficiency in the degree of activation of the BCG macrophages, because they were able to inhibit the thymidine uptake of an adherent fibroblast target cell line (L929), as has been described by others using macrophages activated in the same (9) or different ways (27–30). That the BCG macrophages were activated was also supported by their enhanced degree of glucose carbon-1 oxidation compared to normal macrophages, both at rest and with phagocytosis, as has been shown previously with guinea pig (20, 31) or human (32) mononuclear phagocytes incubated in media rich in migration inhibitory factor, or by infection of mice with BCG (33) or *Listeria monocytogenes* (33, 22).

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Stimulation of lymphoma cell DNA synthesis by normal macrophages was only evident with lymphoma lines which could also be stimulated by 2-ME, both in microculture and in bulk culture (23). The stimulation by normal macrophages and by 2-ME were both dose-dependent, and they had the same time-course. Addition of optimal doses of 2-ME to the lymphoma cell cultures overcame the relative lack of stimulation offered by BCG macrophages, but was not additive to that provided by normal macrophages. The degree of stimulation attainable with normal macrophages and with 2-ME were correlated for eight different lymphomas. On the other hand, two lymphomas and one xenogeneic suspension-culture hepatoma were unaffected in their growth or thymidine uptake by normal macrophages or by 2-ME. No stimulatory effect on ME-dependent lymphoma cells was seen when peritoneal lymphocytes, ME-independent lymphoma cells, L929 fibroblasts, or Line 10 hepatoma cells were substituted for normal macrophages (unpublished observations).

The effects described above were independent of the *H-2* haplotype of the tumors and macrophages. It also did not appear to matter whether the tumors bear the theta antigen (EL4 (34), PU5-14[2], PU5-45[2]), surface immunoglobulin (PU5-1[2]), the EA receptor [LSTRA (35)], or none of these [L1210 (34)]. The stimulatory cell appeared to be the peritoneal macrophage, not the lymphocyte, and stimulation by macrophages or by 2-ME appeared to be unrelated to the small number of macrophages or lymphocytes present in the lymphoma ascites.

Evans and Alexander (12) previously described cultivation of TLX9 and SL2 lymphoma cells with macrophages from BCG-treated DBA/2 mice. Using lyophilized BCG, they observed no effect on the lymphomas unless purified protein derivative (PPD) was first added to the BCG macrophages. In that case, there appeared to be marked cytostasis compared to the lymphoma grown in the presence of normal macrophages. Comparison to the lymphoma cultured alone was not given. Effects on the lymphoma were followed with hemocytometer counts. We find the latter a difficult assay to interpret, since as many as half the macrophages may become nonadherent during culture. Using TLX9, we find that the addition of PPD at 1 or 10 μg/ml for the first 4 or 48 h of culture reduces the slight degree of stimulation seen with BCG macrophages, but in our hands it has not produced significant cytostasis compared to TLX9 cultured alone, as judged by tritiated thymidine uptake. In addition, PPD itself seemed to exert a toxic effect on the tumor (unpublished observations).

Olivotto and Bomford previously noted a slight increase in thymidine uptake by RI leukemia cells in the presence of normal mouse macrophages (36). Namba and Hanaoka reported that contaminating phagocytic cells appeared to enhance the growth in vitro of an explanted mouse myeloma (37). Stimulation of L1210 cell thymidine uptake in the presence of normal DBA/2 macrophages has also been observed by others.[3]

In contrast, the great majority of earlier workers have not reported stimulation of the growth of murine lymphomas or leukemias when cultured with normal macrophages, and have observed cytotoxic or cytostatic effects when the

[2] Asofsky, R. Personal communication. While PU5-14 originally consisted mostly of surface immunoglobulin-positive cells, the explant from which our ascites line was established contained mainly θ-positive cells.

[3] Papermaster, B. W. Personal communication.

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lymphomas were cultured with macrophages activated in ways other than BCG infection of the donor. Such means of activation have included use of lymphoma cells or skin grafting to immunize the donor of macrophages or the donor of lymphocytes cultured with the macrophages (38–44), use of macrophages from tumor-bearing mice (45), treatment of macrophages in vitro with double-stranded RNA or endotoxin (46), and infection of the macrophage donor with protozoa (30) or heat-killed *Corynebacterium parvum* (36).

It is of interest that most of the lymphoma lines used previously for in vitro studies with macrophages appear in retrospect to belong to the groups which in our hands are stimulated very little or not at all by normal macrophages nor by 2-ME. Very likely this reflects the tendency to select for in vitro studies those tumors which grow well when cultured alone. Such a selection artefact may explain why results such as those reported here have not been more widely described. In addition, stimulation of DNA synthesis would not be detected by assays using prelabeled target cells.

Previous generalizations concerning macrophage-tumor cell interactions in vitro (6) would appear to be circumscribed not only by the results reported here, but also by previous work showing the ability of macrophages to kill, or inhibit the proliferation of, a variety of nonmalignant cells, including fibroblasts (29, 47–49), erythrocytes (50), and even macrophages (29, 51).

Thus, the patterns of macrophage-target cell interaction in vitro appear to be highly dependent on the stimuli to which the macrophages are subjected, the target cells studied and the assays employed. Such patterns range all the way from profound cytotoxicity to equally intense stimulation.

The implications of the present studies for macrophage-tumor interaction in vivo are not clear. There is essentially no information about the biochemistry of the stimulation of lymphoma cell growth by normal macrophages or by 2-ME or the reason for the lack of stimulation by BCG-activated macrophages. Increased understanding of this system may shed light on the interactions between macrophages and normal lymphocytes.

Summary

Peritoneal macrophages from mice infected with Bacille Calmette-Guérin (BCG) and from normal mice were examined for their effects in vitro on thymidine uptake by 10 murine lymphomas, a murine fibroblast line, and a guinea pig hepatoma. Only the murine fibroblast line showed growth inhibition in the presence of BCG macrophages. For the majority of tumors, normal macrophages were profoundly stimulatory to tumor cell DNA synthesis, while BCG macrophages were much less stimulatory, without being frankly inhibitory. The effect of 2-mercaptoethanol on tumor cell growth was also studied. All lymphomas stimulated to grow more rapidly in vitro by normal macrophages were stimulated to a similar degree by 2-mercaptoethanol.

The authors are grateful to Ms. Virginia Hill for expert technical assistance, to Mssrs. Walter Lyles and Francis Jones for animal care, and to Mrs. Marilyn Schoenfelder for preparation of the manuscript.

Received for publication 15 May 1975.

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