Inflammation and Tumor Growth

I. Tumor Growth in Mice with Depressed Capacity to Mount Inflammatory Responses: Possible Role of Macrophages

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Studies were made of the effects of various treatments on the growth in mouse feet of isografts of two methylcholanthrene-induced fibrosarcomas: C-4, of CBA/J mice, and A-2, of A/J mice. The isografts were prepared by pronase digestion of subcutaneous tumors and were injected as unseparated cell suspensions or as tumor-cell-enriched suspensions after depletion of infiltrating host inflammatory cells. The recipient mice were untreated or treated with reserpine, sublethal whole body irradiation, cyclophosphamide, or corticosteroids. Depletion of host cells from the inoculum resulted in increased growth from the same number of tumor cells. Reserpine treatment decreased the growth of both tumors, whether unseparated or tumor-cell-enriched, and whether injected into the foot or the flank. Irradiation, cyclophosphamide pretreatment, and corticosteroid pretreatment decreased the growth of normal inocula or enriched inocula or both. The effects of cyclophosphamide and corticosteroids were apparently not due to cytotoxicity to tumor cells. Normal resident peritoneal cells increased tritiated thymidine uptake by tumor cells *in vitro*. Sedimentation velocity separation showed the largest cells to be the most potent. It is suggested that some host inflammatory reaction is necessary for optimal tumor growth and that murine hosts produce not only cells with antitumor effects but also cells, possibly a subpopulation of macrophages, that potentiate tumor growth. (Am J Pathol 1981, 104:114–124)

IN THE COURSE of experiments on the mechanism of expression of immunity to syngeneic tumors we observed that reserpine treatment depressed the ability of immune mice to reject challenge tumor inocula. Together with the inhibitory effects of irradiation, niridazole, and carrageenan this was taken as evidence that reactions akin to those of delayed-type hypersensitivity could be involved in rejection.[1,2] Paradoxically, however, although immune mice treated with reserpine permitted the growth of challenge tumors, tumors failed to grow in reserpine-treated normal mice. This suggested that some reaction, possibly inflammatory, on the part of a normal host might be important in allowing the establishment of a syngeneic tumor graft. Our interest in this possibility was heightened by the results of the experiments reported in the accompanying paper,[3] which indicated that local inflammatory reactions did not necessarily lead to a reduction in tumor growth in normal hosts. There were several reasons for a particular interest in the role of macrophages or a subpopulation thereof. Progressively growing tumors frequently contain macrophages.[4] Depletion of macrophages from a suspension of "tumor" cells used as an isograft has been reported to increase the growth of the transplanted tumor.[5] Likewise, increasing the proportion of macrophages in the isograft can result in decreased tumor growth.[6,7] On the other hand, Evans[8] reported decreased growth of tumors in mice that had been irradiated and depleted of monocytes, suggesting that host macrophages, at least in small number, might potentiate tumor growth. Finally, these findings and concepts appear to be relevant to

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consideration of what Prehn[9] has described as the lymphodependent phase of neoplastic growth.

Materials and Methods

Mice

Male CBA/J mice and female A/J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. The mice were aged 3–6 months at the time of use, and 5 animals were included in each experimental group.

Tumors

The fibrosarcomas had been induced by subcutaneous injection of 1 mg of 3-methylcholanthrene in olive oil. They were maintained by subcutaneous passage of suspensions of 10^7 cells prepared by means of pronase digestion and stored in the frozen state as previously described.[10] Viability was estimated by means of trypan blue exclusion. All tumor cell suspensions were 90–95% viable; all cell counts herein refer to viable cells. The tumor designated C-4 had been induced in a CBA/J mouse; A-2 had been induced in an A/J mouse. Both tumors were immunogenic.[2,10] In these experiments the tumors had undergone 2–10 passages since retrieval from frozen storage.

Tumor Challenge and Measurement of Tumor Growth in the Footpad

Mice were challenged on Day 0 with 5×10^5 viable cells injected beneath the footpad in a volume of 50 μl. The degree of tumor growth was followed by daily measurement of the increase in footpad thickness by means of a Schnelltaster dial gauge. The uninjected hind foot was used as a control.

Purified Tumor Cell Suspensions

Cell suspensions from pronase digested solid tumors were enriched for tumor cells in the following manner. Three to 5×10^7 viable cells were incubated overnight at 37 C in an atmosphere of 5% CO_2 in air in Falcon No. 3024 tissue culture flasks (75 sq cm area) in 15 ml of Dulbecco's modified Eagle's medium (DME; Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS; Australian Laboratory Services, Melbourne). The flasks were shaken gently, the supernatants aspirated, and the adherent cells washed twice with 30 ml of 0.9% NaCl. Ten milliliters of 0.1% trypsin (Difco, 1:250) in DME was added, and the flasks were incubated for 10 minutes at 37 C. After gentle tapping, the suspensions of trypsin-sensitive cells were removed and FCS was added to a concentration of 10%. The cells were collected by centrifugation at 800*g* for 10 minutes and resuspended in DME alone. The proportion of tumor cells in the suspension was assessed on smears stained with May-Grünwald-Giemsa, and the concentration of the suspension adjusted with DME to the same concentration as that of the tumor cells in the normal tumor digest.

Treatment of Mice Before Tumor Challenge

Mice were subjected to the following treatments before tumor challenge in the foot on Day 0:

Irradiation

On Day −3, mice were given 500 rads of whole body irradiation from a ^{60}Co source at a rate of approximately 1 rad/sec.

Cyclophosphamide

On Day −3, mice were injected intraperitoneally with cyclophosphamide (Endoxan-Asta, Mead Johnson, Sydney, Australia, 10 mg/ml) at doses of 200 or 100 mg/kg.

Cortisone

CBA/J mice receiving normal C-4 tumor cell suspensions were given cortisone acetate (Cortisyl, Roussel Labs Ltd., London, England, 25 mg/ml) intraperitoneally on Day −1 in a single dose of 500 mg/kg.

Hydrocortisone

CBA/J mice receiving enriched C-4 tumor cell suspensions and A/J mice receiving normal or enriched suspensions were given hydrocortisone acetate (Merck, Sharp and Dohme, Sydney, Australia, 25 mg/ml) intraperitoneally on Day −1 in a single dose of 500 mg/kg.

Reserpine

Mice were injected intravenously with reserpine diluted in saline (Serpasil, Ciba, Basel, Switzerland, 1 mg/ml) on Days 0 and +4 at doses of 1, 0.5, 0.25, or 0.125 mg/kg.

Blood Leukocyte Counts

Total counts and differential counts (on smears stained with May-Grünwald-Giemsa) were made on tail vein blood from normal mice, mice irradiated or treated with cyclophosphamide (200 mg/kg) 3 days

earlier and mice treated with hydrocortisone 1 day earlier. Similar counts were made on blood obtained under ether anesthesia from the brachial artery of mice treated with reserpine (1 mg/kg) 4 hours earlier and from a control group of anesthetized untreated mice.

Effects of Irradiation or Drug Treatment on an Inflammatory Response in the Peritoneal Cavity

The mice that had been bled from the tail vein and a second (nonbled) group of reserpine-treated mice received intraperitoneal injections of 1 ml saline containing 50 μg concanavalin A (Con A, Calbiochem-Behring, Sydney, Australia). The peritoneal cavities were washed out 2 days later, each with 2 ml of DME containing 10% FCS and 10 U/ml preservative-free heparin. Total and differential counts were made on the peritoneal cell population obtained.[11]

Tritiated Thymidine Uptake by Cultured Tumor Cells

Tritiated thymidine uptake by cultured tumor cells was measured as described by Hopper et al.[12] Enriched tumor cells (10^6) were cultured in Falcon Microtest II plates for a total of 42 hours. Normal peritoneal cells, or fractions of the cell population separated by sedimentation velocity, were added at ratios of from 3:1 to 50:1 for the last 24 hours and tritiated thymidine (2 μCi of methyl-^{3}H-thymidine, specific activity 5 Ci/mmole, Radiochemical Centre, Amersham, England) in fresh medium for the last 6 hours.

Peritoneal Cells: Separation by Sedimentation Velocity

Resident cells were obtained from the unstimulated peritoneal cavities of normal mice, which were killed by cervical dislocation. They were washed out with 2 aliquots of 2 ml Hanks' solution without Ca^{2+} or Mg^{2+} and containing 10 U/ml preservative-free heparin. They were washed twice in DME. Fractionation by sedimentation velocity was carried out in a Sta-put apparatus[13] (Johns Scientific, Toronto, Canada) as described elsewhere.[12]

Statistical Evaluation

The Student t test was used.

Results

Drug Treatments and Irradiation

Figure 1 shows that the growth of A-2 tumor in the feet was abolished by treatment with reserpine (1 mg/kg) on Days 0 and 4. It was almost abolished by treatment with 0.5 mg/kg. The growth of C-4, however, was abolished only by treatment with 1 mg/kg. Mice treated with these large doses of reserpine were cold and somnolent.

As the skin of the mouse foot is reportedly richer in mast cells than that of the flank,[14] the effect of reserpine treatment on the growth of subcutaneous tumors was examined. CBA/J and A/J mice receiving subcutaneous isografts of 10^7 C-4 and A-2 tumor cells were treated with reserpine (1 mg/kg intravenously) on Days 0 and 4. On Day 10 the mice were killed, and the tumors were excised and weighed. Table 1 shows that the growth of both tumors was depressed in treated mice. Differential counts were made on May-Grünwald-Giemsa-stained smears of pronase-digested tumor cell suspensions. Table 1 also shows that the ratio of host:tumor cells was unchanged by reserpine treatment in CBA/J mice bearing C-4, but that A-2 tumor in treated mice showed a higher proportion of neutrophils.

In A/J mice (Figure 2) irradiation, treatment with cyclophosphamide, and treatment with hydrocortisone acetate all retarded A-2 tumor growth. Irradiation and hydrocortisone had prolonged inhibitory effects. In all cases the differences were statistically significant ($P < 0.005$) up to 10 days. In CBA/J mice, however, only irradiation retarded the growth of C-4.

Tumor Cell Enrichment (Host Cell Depletion) and Reserpine Treatment

As shown in Figure 3, an inoculum of 3.7×10^5 viable C-4 cells, 98% pure, grew more extensively than did the same number of viable tumor cells in a suspension containing 26% host cells, mostly macrophages ($P < 0.005$ to Day 8, $P < 0.025$ to Day 10). In mice treated with reserpine at 0.5 or 1 mg/kg the growth of the enriched tumor cells was profoundly inhibited. In A/J mice host cell depletion, leaving an inoculum containing 98% tumor cells, had a similar enhancing effect on tumor growth and treatment with reserpine (0.5 or 1 mg/kg) depressed (though not totally) the growth of enriched tumor inoculum.

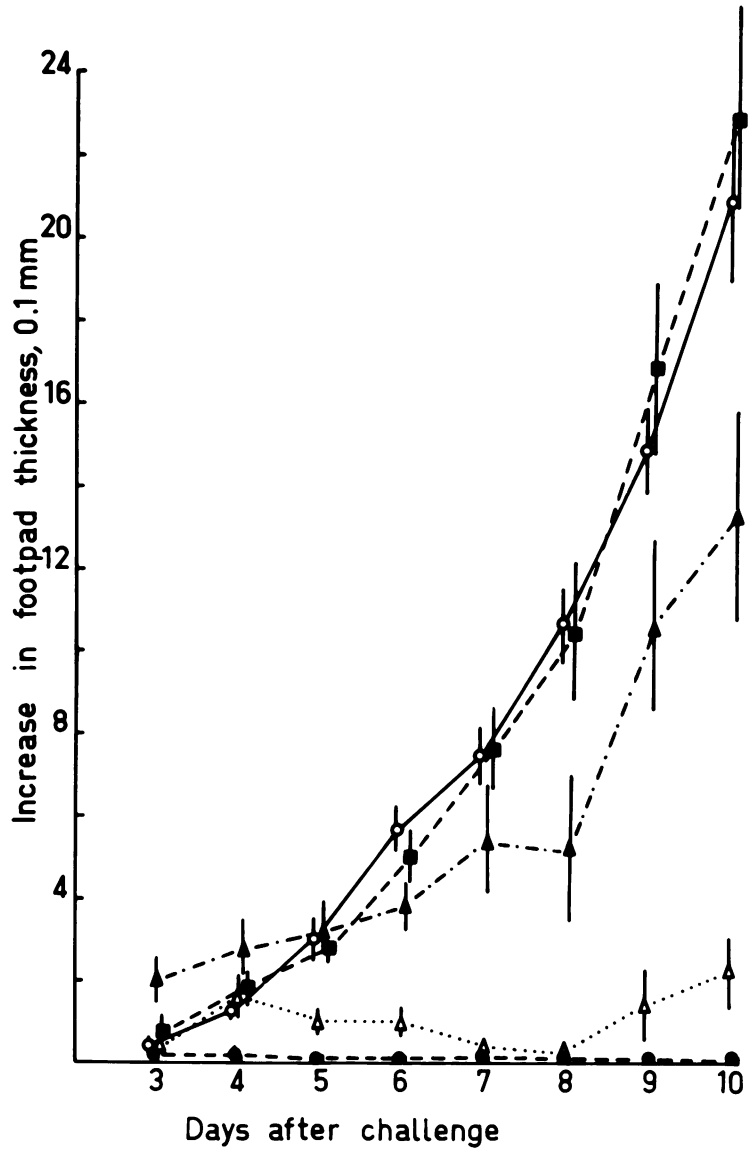

Figure 1—Effect of reserpine treatment (Days 0 and 4) on the growth of A-2 tumor. Untreated control mice: O——O. Mice treated with reserpine on Days 0 and 4: 1 mg/kg, ●---●; 0.5 mg/kg, △···△; 0.25 mg/kg, ▲—·—▲; 0.125 mg/kg, ■---■.

Effect of Other Agents on the Growth of Enriched Tumor Cells

Figure 4 shows that irradiation, hydrocortisone, and cyclophosphamide treatment all retarded the growth of C-4 tumor cell inocula ($P < 0.025$ at 8 days in each case). Cyclophosphamide treatment was especially inhibitory, reducing growth below that of the normal inoculum, in contrast to the effects of these agents on the growth of normal inocula, when only irradiation was inhibitory. With macrophage-depleted A-2, on the other hand, only cyclophosphamide had a notable inhibitory effect, which was to diminish tumor growth to a level equivalent to that of the normal inoculum. Irradiation, which was profoundly inhibitory to the growth of a normal inoculum, had only a transient depressive effect on the growth of the macrophage-depleted inoculum. Cyclophosphamide, which inhibited the growth of the normal inoculum, had no effect on the macrophage-depleted inoculum.

Effect of Serum From Treated Mice

Cyclophosphamide and corticosteroids are widely used in cancer chemotherapeutic regimens. It seemed possible that some of their effects in these experiments might be due to a direct antitumor cell action. To test this hypothesis, groups of mice were treated with cyclophosphamide or hydrocortisone. Some

Table 1—Effect of Reserpine on the Growth and Composition of Subcutaneous Tumor Isografts

Tumor	Treatment of mice	Tumor* weight (mean ± SE)	Cells identified in smears of tumor digest (%)			
			Tumor cells	Macrophages	Lympho-cytes	Poly-morphs
C-4	Untreated	301 ± 32 mg	77	18	4	1
	Reserpine†	108 ± 16 mg	76	20	4	1
A-2	Untreated	196 ± 12 mg	71	20	6	3
	Reserpine	17 ± 1 mg	55	18	4	24

* On Day 10.
† 1 mg/kg intravenously, Days 0 and 4.

were challenged with tumor cells (enriched C-4 and normal A-2), and some were bled, the serum being injected intraperitoneally into other mice 4 hours before challenge. As before, tumor growth was depressed in mice that had been actively treated, but serum from treated mice either caused no depression (with A-2) or much less depression (with C-4) than occurred in the actively treated mice.

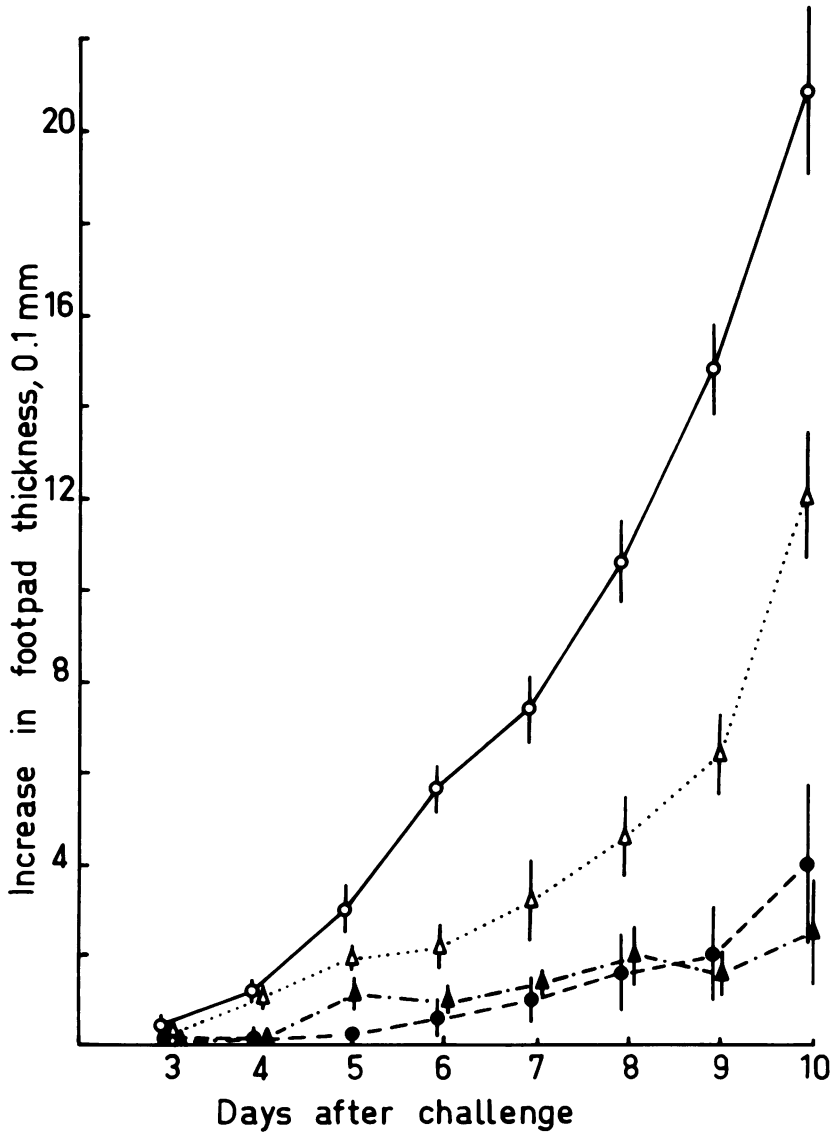

Figure 2—Effects of host treatment on the growth of A-2 tumor. O——O, untreated control; ●---●, irradiation (500 rad, Day −3); Δ · · · Δ, cyclophosphamide (200 mg/kg, Day −3); ▲—·—▲, hydrocortisone acetate (500 mg/kg, Day −1).

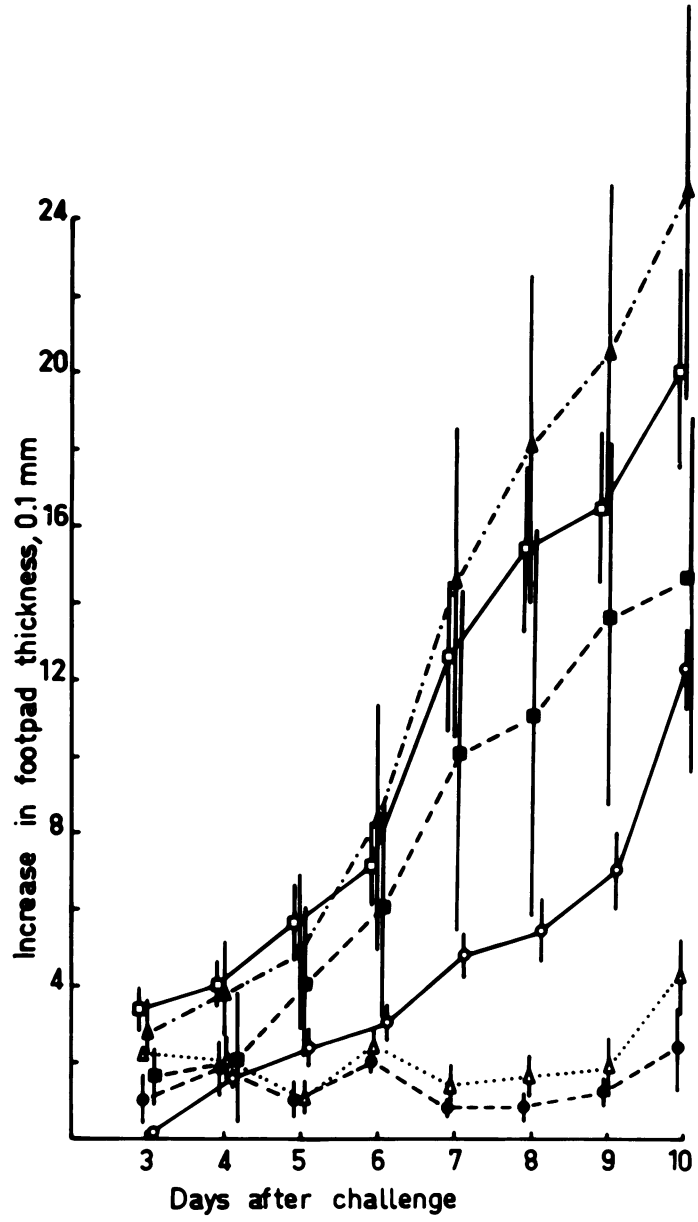

Figure 3—Effect of reserpine treatment (Days 0 and + 3) on the growth of enriched C-4 tumor. O——O, normal inoculum, untreated controls; □——□, enriched tumor, untreated controls; ● - - - ●, enriched tumor, reserpine 1 mg/kg; △ · · · △ enriched tumor, reserpine 0.5 mg/kg; ▲—·—▲, enriched tumor, reserpine 0.25 mg/kg; ■ - - - ■, enriched tumor, reserpine 0.125 mg/kg. The normal inoculum contained 74% tumor cells and the enriched inoculum 98% tumor cells.

Effects of Irradiation and Drugs on Blood Leukocytes and Peritoneal Exudate Cell Counts

Figure 5 shows that cyclophosphamide, hydrocortisone, and irradiation all caused large falls in the counts of monocytes and macrophages, lymphocytes and granulocytes in blood, and, with the exception of irradiation and granulocytes, pertioneal exudates. Reserpine, on the other hand, caused a neutrophil leukocytosis in blood but no change in the numbers of other cells in blood or in the composition of peritoneal exudates.

Effects of Normal Resident Peritoneal Cells on the Proliferation of Tumor Cells in Culture

It seemed possible that some of the effects observed might be due to depletion of a host helper cell that promotes tumor growth. Because the peritoneal cavity is a convenient source of diverse cells, peritoneal cells were obtained from CBA/J mice and separated by sedimentation velocity. Figure 6 shows the profile of the separated cells and the fractions that were pooled. C-4 tumor cells were cultured with the pooled fractions and a sample of the unseparated cells.

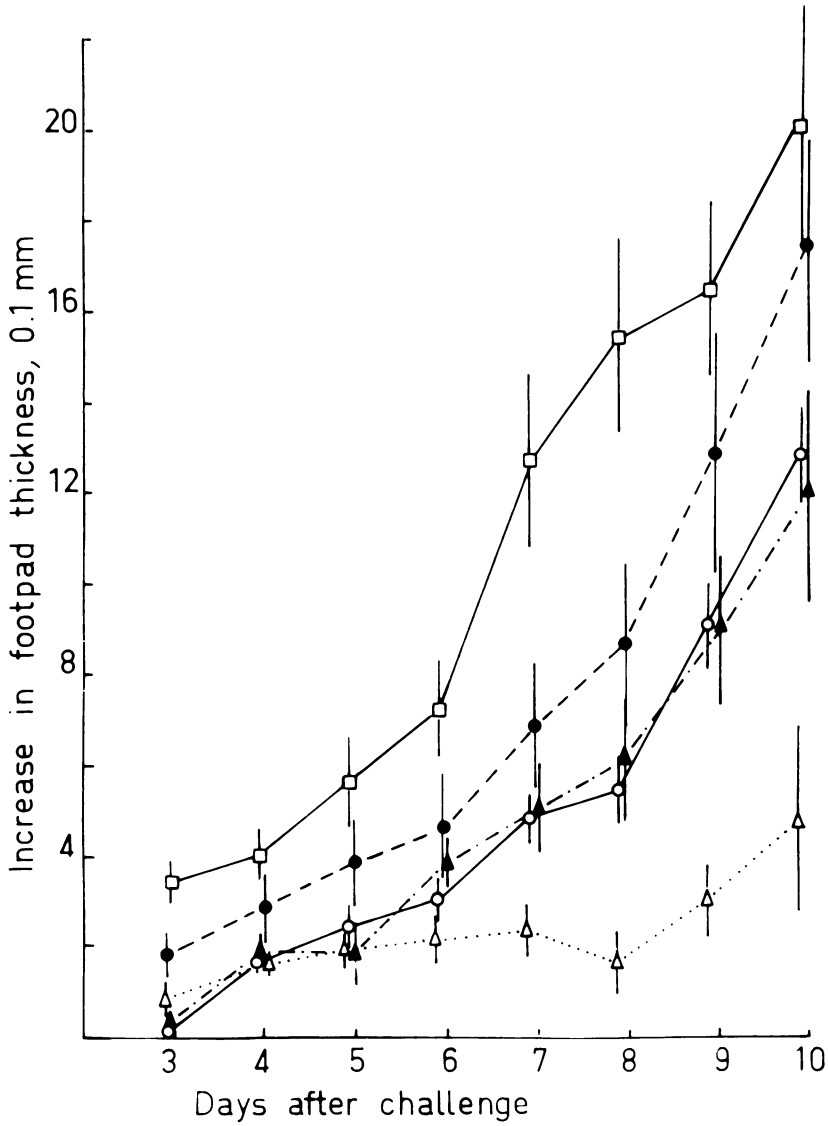

Figure 4—Effect of host treatment on the growth of enriched C-4 tumor. O——O, normal inoculum, untreated controls; □——□, enriched tumor, untreated controls; ●---●, enriched tumor, irradiation (500 rad, Day −3); △···△, enriched tumor, cyclophosphamide treatment (200 mg/kg, Day −3); ▲—·—▲, enriched tumor, hydrocortisone acetate (500 mg/kg, Day −1). The normal inoculum contained 74% tumor cells and the enriched inoculum 98% tumor cells.

Table 2 shows the incorporation of tritiated thymidine by tumor cells cultured with peritoneal cells at a ratio of one tumor cell to 25 peritoneal cells. The whole peritoneal cell population caused some increase in proliferation, mainly a function of the largest cells (Fractions 4 and 5). Two fractions (2 and 3) of smaller cells caused a slight decrease in proliferation. The results were essentially the same at other peritoneal:tumor cell ratios. In a similar experiment with A/J mice and A-2 tumor large cells again potentiated tumor cell proliferation, as reflected by tritiated thymidine uptake.

Discussion

The first in this series of experiments confirmed our earlier findings[1,2] that systemic treatment of normal (nonimmune) mice with reserpine resulted in decreased growth of tumors inoculated into the feet. Scott et al[15] also found that pretreatment of rats with reserpine reduced the percentage of "takes" of a fibrosarcoma in rats. There appeared to be a parallel between this effect of reserpine (and other agents) and the disruption of mast cells.[15,16] Serotonin has been found to suppress the growth of some tumors *in vivo*.[17-19] Mast cells themselves can be cytotoxic to tumor cells *in vitro*.[20,21] This cytotoxicity is, however, inhibited by reserpine.[21] Because reserpine treatment can enhance tumor growth in immune mice[1,2] and, when appropriately timed, in rats,[15] simple cytotoxicity by mast cell products does not explain its effects. Effects on blood flow and depletion of other amines may be important.

The second clear-cut finding was that depletion of

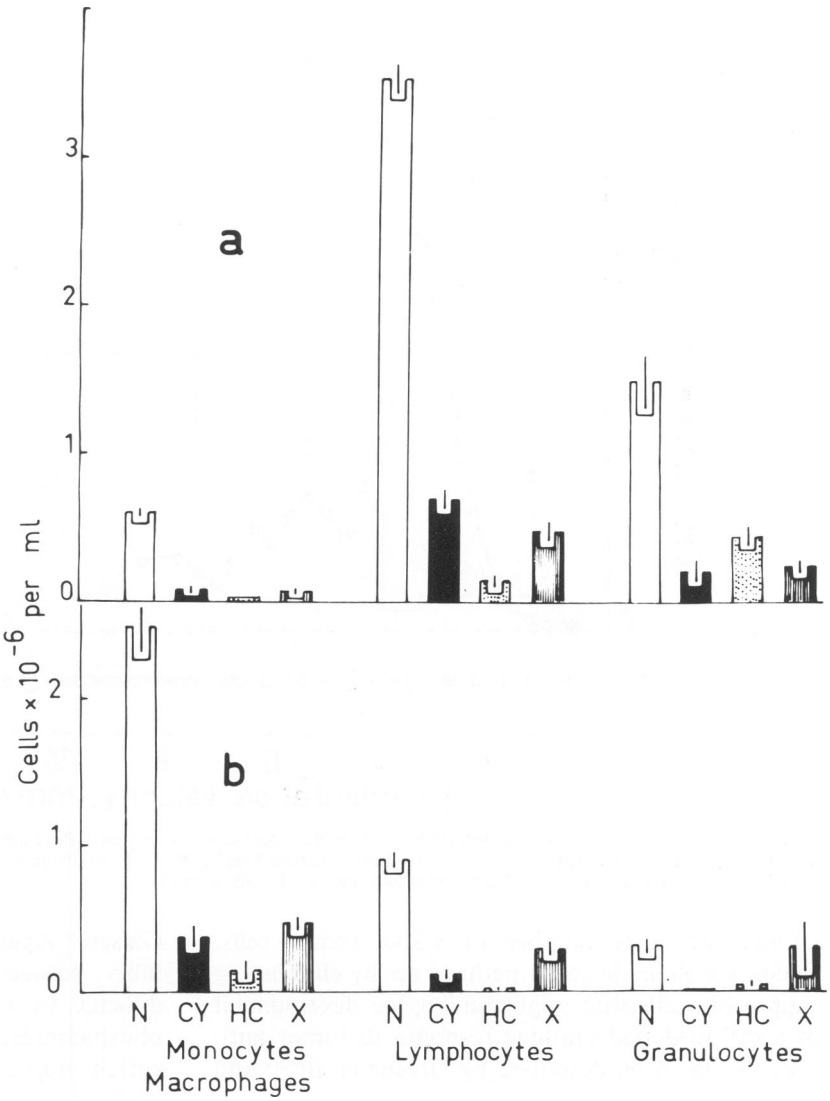

Figure 5—Counts of monocytes (blood) or macrophages (peritoneal exudate), lymphocytes and granulocytes in blood (**a**), and Con-A-induced peritoneal exudates (**b**) of CBA/J mice: normal (*N*), treated with cyclophosphamide, 200 mg/kg, Day −3 (*CY*), treated with hydrocortisone acetate, 500 mg/kg, Day −1 (*HC*), or irradiated, 500 rad, Day −3 (*X*).

infiltrating host cells from the tumor inocula led to increased growth of both tumors in normal hosts. This finding suggests that infiltrating host cells do in fact slow tumor growth and is consistent with the results of other experiments.[5-7] The host cells were mostly macrophages, which, in a stimulated state, have been shown in some[5,6] but not all[22] studies to depress tumor growth *in vivo* as well as *in vitro*. Host cells other than macrophages may also be active.[4,23,24]

Growth of the tumors was also inhibited by some or all of the other treatments of the host mice. Irradiation of the host diminished the growth of normal and enriched inocula of C-4 and of both types of inocula A-2, though the degree of depression of enriched A-2 tumor growth was slight. These results have also been reported for another chemically induced fibrosarcoma by Evans,[4,8,25] who has suggested that irradiation may act in this way by depressing macrophages that promote tumor growth (see below). Irradiation has, however, also been found to promote metastasis occurring naturally from a primary tumor[26] or artificially from intravenously injected tumor cells.[27]

Cyclophosphamide pretreatment led to diminished growth of enriched, but not normal, inocula of C-4 and of normal, but not enriched, inocula of A-2. Pretreatment with cortisone acetate or hydrocortisone acetate led to decreased growth of enriched, but not normal, inocula of C-4 and of both normal and enriched inocula of A-2. A direct chemotherapeutic effect of these agents seems unlikely to be responsible, both because serum from treated animals failed to transfer the effects and because of the differential susceptibility of normal and enriched inocula con-

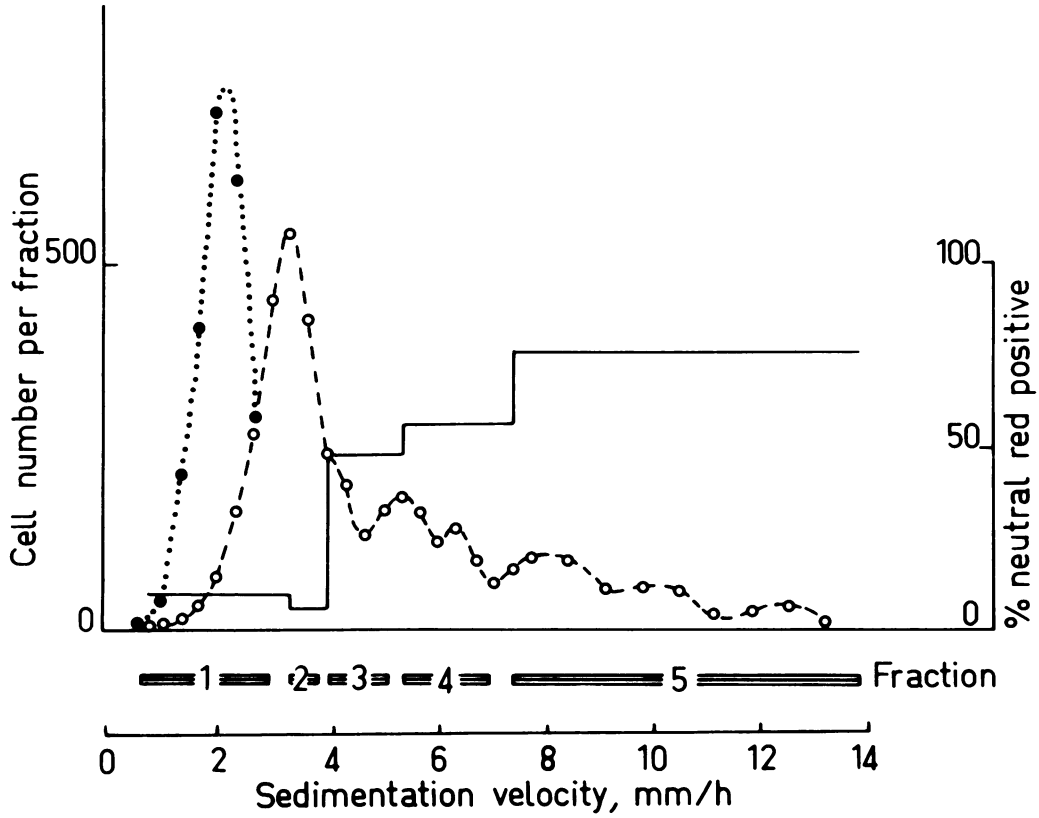

Figure 6—Separation by sedimentation velocity of resident peritoneal cells from CBA/J mice. The composition of the pooled fractions is indicated. Larger cells are to the right. O--- O, viable nucleated cells; ●· · ·●, erythrocytes; ——, percentage of cells that are Neutral Red positive, ie, show supravital cytoplasmic staining typical of macrophages.

taining the same number of viable tumor cells. Cyclophosphamide could perhaps act by eliminating suppressor cells that might inhibit the development of a cell-mediated immune response to tumor antigens, as has been described by Greene et al[28,29] and Glaser.[30] Again, however, the differences in susceptibility between normal and enriched inocula are difficult to explain on this basis. Unlike cyclophosphamide, corticosteroids have been found to enrich suppressor cell activity[31] and to suppress naturally occurring cytotoxic cells, some of which are macrophages.[32] Thus, potentiation of cell-mediated immunity or of mechanisms of natural resistance to tumors[33] seems unlikely to explain their effects.

Table 2—Proliferation of C-4 Tumor Cells Cultured With Normal Peritoneal Cells*

Tumor cells cultured with	Mean cpm ± SE†	Tritiated thymidine incorporation into DNA as percentage of incorporation by tumor cells alone
Nothing	12,800 ± 700	100
Unfractionated peritoneal cells	15,400 ± 1000	120
Fraction 1	13,100 ± 1000	103
Fraction 2	11,000 ± 1000	86
Fraction 3	9,400 ± 1000	73
Fraction 4	18,400 ± 3800	144
Fraction 5	17,500 ± 1500	137

* Enriched C-4 cells cultured with normal CBA/J peritoneal cells at a tumor:peritoneal cell ratio of 1:25; tritiated thymidine incorporation measured during the last 6 hours of a 42 hour culture period—see Materials and Methods.

† cpm (counts per minute) rounded to the nearest 100.

One mechanism that may be common to irradiation, cyclophosphamide, and corticosteroids is suppression of a host cell that could act as a tumor helper cell. Three lines of evidence are consistent with this idea. First, all three agents caused a diminution in circulating white blood cells of all types and of the accumulation of such cells in Con-A-induced peritoneal exudates (Figure 5). Second, certain resident peritoneal cells were in fact capable of potentiating tumor cell proliferation *in vitro*, with both C-4 (Table 2) and A-2. The most active fractions of the population were those containing the largest cells, ie, those with the highest proportion of macrophages. Finally, other tumors have also been found, or inferred, to be potentiated by macrophages. They include chemically induced fibrosarcomas of mice,[25] several

mouse lymphomas,[34-36] a rat lymphoma,[37] virus-transformed mouse fibroblasts,[38] SJL/J mouse reticulum cell sarcomas,[39,40] the Lewis lung carcinoma, which arose spontaneously,[41] human myeloma,[42] several human epithelial tumors,[43] and a human cancer-derived cell line.[4] There is, on the other hand, abundant evidence that appropriately stimulated macrophages have suppressive effects on tumor cells[45,46] and that even normal animals (especially rats) may offer natural resistance to tumor growth by means of macrophages.[47,48] There is no contradiction in this, since cells that can be classified as macrophages are highly heterogeneous[49] and their effects on target tumor cells, as well as their regulatory effects on normal cells, may be determined by the type and degree of activation or stimulation.[37,50,51] Such heterogeneity could well affect the degree to which macrophages can exercise, or be stimulated to exercise, surveillance over neoplasia.[52,53] Furthermore, the concept of helper macrophages, which may need some form of inflammatory response to become focused on developing tumors, is complementary to and consistent with the notion of immunostimulation or lymphodependence of neoplastic growth, evidence for which has been marshalled by Prehn.[9,54]

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