rat is mediated by release of lysosomal material from PMN-leukocytes.

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The Leukocyte Response to Intraperitoneal Ehrlich Ascites Tumor In Mice.* (31308)

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The defenses of the host against malignant invasion are at present ill-defined, but include reactive or immune counter measures by leukocytes. The qualitative and quantitative aspects of this relationship require further definition. The amount of tumor presented in an artificial situation such as transplantation does not necessarily reflect the natural circumstance, but host response can be evaluated thereby.

Ehrlich ascites tumor (EAT) has been shown to be a successful invader of many mouse strains; it appears to possess foreign antigenicity, since previously immunized mice will reject otherwise tumor-producing doses. Furthermore, McCarthy has shown that C_3H mice with intraperitoneally growing EAT will accept foreign skin grafts, which are retained until the death of the animal(1). Thus, the acceptance of the neoplasm resulted in an "acceptance" of the homografted skin, an outcome known to be dependent at least in part on lymphocytic functions.

The peritoneal cavity may be a rather specialized site for tumor cell growth. Not only are the structural, nutritional, and transport aspects somewhat unique, but the free and close contact between invading tumor cell and resisting leukocyte may allow an evaluation of factors, mechanical or biological, determining neoplastic invasion(2,3). Old *et al* have reported on their interest in the leukocyte/tumor cell ratio in the inva-

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sion by ascites tumor and have indicated the importance of the leukocyte in host resistance (4). The role of leukocytes in the peritoneal cavity in tumor rejection is as yet controversial, however(5,6).

An investigation has, therefore, been undertaken concerning host white blood cellular responses in malignancy, using as a model the outpouring of leukocytes into the peritoneal cavity of the Swiss mouse upon the introduction of Ehrlich ascites tumor cells. An animal whose peritoneal cavity has been filled acutely with viable tumor cells is defined here as "instant" tumor. A difference is demonstrated in host cellular responses to neoplastic cells related to timing and the amount of tumor introduced.

Materials and methods. Two hundred white Swiss HA/ICR mice from the Charles River Mouse Farms, Brookline, Mass., were used, fed autoclaved Purina chow, and kept in an air conditioned environment. Ehrlich ascites tumor cells were obtained from Dr. T. Hauschka, Roswell Park Memorial Institute, and maintained by serial passage of 6×10^6 cells every 10-14 days in the test animal. The mice were 6 to 8 weeks old and weighed 20-25 g when used in these experiments.

Quinacrine solution was made in a concentration of 200 mg per 20 cc of normal saline. Quinacrine, 0.1 mg, was determined to be optimal for labeling one cc of whole blood or tumor fluid for observation of cellular fluorescence. An AO Fluorolume unit with a 200-watt mercury vapor light source was utilized with a standard Pathostar AO microscope and suitable filters. Technics of fluorescent leukocyte labeling and observation have been presented in detail(7-10).

Routine hematologic and immunologic technics were used for preparing, counting, transferring, staining, and observing the cells in question.

Immunized mice were prepared by 4 intraperitoneal injections of 0.5, 1.0, 2.0, 2.5 $\times$ 10^6 lyophilized Ehrlich ascites tumor cells (EAT) at weekly intervals. These animals were challenged by EAT injections 4 to 5 weeks after the last dose. Non-viable cells were obtained by freezing and thawing. X-ray

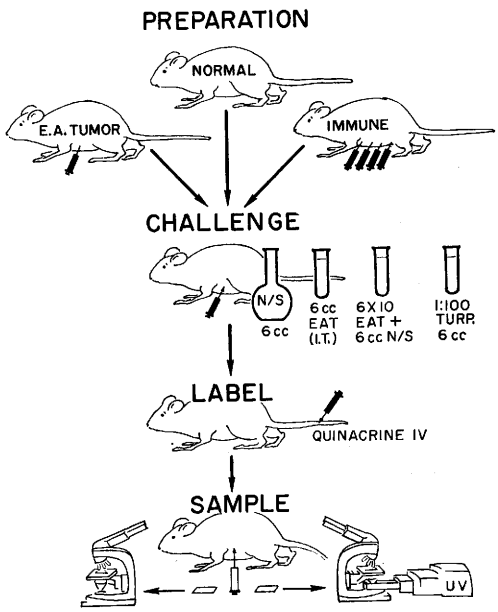

FIG. 1.

was given as whole body irradiation in a cage by a cobalt unit. Turpentine was obtained from a commercial source.

Fig. 1 illustrates the outline of experimentation. Normal mice, immunized mice, or mice with growing tumor initiated by I.P. injection of 6×10^6 EAT cells (challenged as designated) were weighed and prepared on the day of the experiment. A challenge was given by IP injection of 6 cc of normal saline, 6×10^6 EAT cells in 6 cc of normal saline, "instant" tumor (6 cc of EAT), or 6 cc of 1:100 turpentine in normal saline. At either 5 or 30 minutes later (as designated), 0.5 mg quinacrine was given I.V. to label the circulating population of leukocytes. Intraperitoneal samples were obtained at 1 to 300 minutes afterward and duplicate preparations made; one was observed as a direct smear under the fluorescent microscope, and one was stained with Wright's stain. Samples were obtained from blood and bone marrow and evaluated similarly. The numbers of leukocytes per 100 tumor cells in the various groups are rounded to the nearest 50 above 100 and to the nearest 0.1 below 10, indicating the gross differences observed.

We have assumed that the intraperitoneal number of EAT cells changes minimally

TABLE I. Acute Leukocytic Response of Mice Given EAT Challenge.

		Time after I.P. challenge (6×10^6 EAT)					
		6 min	9 min	10 min	15 min	25 min	35 min
		Time after I.V. quinacrine					
Animal	No. of mice	1 min	3 min	5 min	10 min	20 min	30 min
Normal	9	1000*	2000	2000	2000	1000-1500	500
Tumor-bearing (1-9 days)	10	neg†	neg	neg	neg	neg	neg
Immune	7	1000	2000	2000	2000-2500	2000	2000

* Numbers indicate leukocytes per 100 EAT cells. † Neg indicates zero.

TABLE II. Leukocytic Responses of Mice Given an EAT Challenge.

		Time after I.P. challenge			
		5 min	90 min	4½ hr	23½ hr
		Time after quinacrine I.V.			
Animal & challenge	No. of mice	35 min	2 hr	5 hr	24 hr
Normal mouse					
Instant tumor	5	neg	5-10	5-10	4
6×10^6 EAT + N/S	9	300	50-100	5	15*
Tumor-bearing (9 days)					
Instant tumor	3	neg	.1	1	died
6×10^6 EAT + N/S	6	neg	neg	.1	did not sample
No challenge	4	neg	.1	2-10	5
Immune mouse					
Instant tumor	4	.1	2	2*	.2
6×10^6 EAT + N/S	7	2000*	2000*	†	did not sample

N/S indicates normal saline. Neg indicates zero * With clumps. † Many clumps.

within a 6-hour period after inoculation of 6×10^6 cells, since the mitotic interval approximates 18-21 hours(11). Instant tumor amounts to approximately 360×10^6 EAT cells (absolute number by hemocytometer count), and a 7- to 9-day growing tumor after initial 6×10^6 EAT cells numbers approximately 600×10^6 cells (estimate by sampling)(11). The estimation of absolute cell numbers once inside the peritoneal cavity is inaccurate for several reasons: *i.e.*, surface attachment, sequestration, cell death, fluid changes, etc.

Results. Intravenous administration of 0.5 mg of quinacrine resulted in 100% intravascular leukocyte labeling within one minute as observed by blood samples. This same dose of quinacrine given intraperitoneally also labeled leukocytes intravascularly, although requiring a somewhat longer interval. Leukocytes labeled *in vitro* and reinfused intravenously appeared to survive only 3-5 minutes in the intravascular space, the majority sequestered in the lung as determined by smears.

Table I depicts the leukocytic response observed in normal, tumor-bearing, and tumor immunized mice at short intervals when given 6×10^6 EAT cells in 6 cc normal saline. The results in all Tables are expressed as the number of leukocytes per 100 Ehrlich ascites tumor cells. The initial response of the normal and immune mouse results in 1000-2000 leukocytes per 100 tumor cells during the first 20 minutes of the experiment compared to no observable leukocytes outpouring in the tumor-bearing mouse (1-9 days). The early leukocyte response involved members of both the lymphocytic and myelocytic series, rapidly becoming one mainly of lymphocytes. Six cc of normal saline injected into normal or immune mice resulted in 1500 leukocytes/cu mm I.P. fluid when withdrawn at 30 minutes. Leukocytic responses in the tumor-bearing animals were essentially the same when tested from 6 hours to 9 days after the initial live-tumor dose.

Table II illustrates the leukocytic responses of the 3 tested groups at longer intervals when given either 6×10^6 EAT in 6

TABLE III. Peritoneal Leukocytic Response 48 Hours After 1500 r.

Challenge	No. of mice	Time after I.P. challenge (6×10^6 EAT) 15 min / Time after quinacrine 10 min	35 min / 30 min	125 min / 120 min
6 cc normal saline	2	500 WBC/cm	500 WBC/cm	200 WBC/cm
6 " instant tumor	2	neg	neg	.5
6×10^6 EAT + 6 cc N/S	2	100	neg	neg

N/S indicates normal saline. Neg indicates zero.

TABLE IV. Peritoneal Leukocytic Response After a Second Challenge Dose.

Animal	No. of mice	Initial challenge I.P.	Time between challenge (hr)	Second I.P. challenge	Result, 30 min
Normal	5	6×10^6 EAT (viable)	6	6×10^6 EAT + N/S	0-2
	2	6×10^6 EAT (non-viable)	6	*Idem*	500
	2	6×10^6 EAT	6	1:100 turpentine 6×10^6 EAT in N/S	500-1000
	2	6 cc normal saline	6	6×10^6 EAT in 6 cc N/S	200- 500
	2	Turpentine	6	Turpentine	*
Immune	4	6×10^6 EAT (viable)	6	6×10^6 EAT + 6 cc N/S	0-2

* Many clumps. N/S indicates normal saline.

cc normal saline or "instant" tumor. The immune animals sustain the leukocytic response longer, and more clumps of leukocytes and tumor cells are noted. Fluorescent labeled leukocytes were sparse to absent in the tumor-bearing animals (9 days) challenged with 6×10^6 EAT in 6 cc normal saline, nor was an outpouring demonstrated by simply sampling the peritoneal cavity. "Instant" tumor showed a minimal response in all these situations.

Normal mice were irradiated with 1500 r whole body X-ray and tested 48 hours later. Their leukocytic response to the challenges as listed in Table III was blunted, and their peripheral blood leukocyte counts were in the range of 1000-3000 compared to 10,000-20,000 per cu mm in the normal. The negative results in this experiment reinforce the conclusion that the intravenous quinacrine does not rapidly or selectively label cells in the peritoneal cavity, in contrast to those in the circulating blood.

In animals initially injected with 6×10^6 EAT intraperitoneally, a repeat injection of 6×10^6 EAT in 6 cc normal saline did not evoke the expected response (Table IV). This effect was noted as early as 6 hours after the initial injection, and on all later occasions from one day until the death of the mouse (approximately 21 days). The initial intraperitoneal injection of 6×10^6 nonviable EAT, normal saline or 1:100 turpentine solution did not result in this blunting of leukocyte response. However, in the above situations when turpentine 1:100 was added to the 6×10^6 EAT with normal saline or merely in 6 cc normal saline, a dramatic leukocytic outpouring was observed. The addition of 1:100 turpentine to "instant" tumor also elicited a dramatic leukocytic outpouring. These responses in the presence of turpentine demonstrated that the organism is capable of leukocytic response at these times.

It is to be noted that "instant" tumor results in a rapid progression to death, usually within 7-10 days as compared to 15-20 days with 6×10^6 EAT intraperitoneally, and that "instant" tumor makes the mouse appear very lethargic. Administration of 1 cc of the fluid from centrifuged EAT to a normal mouse 3 to 4 times over a 24-hour period did not inhibit the leukocytic response. The use of 6×10^6 normal human leukocytes in 6 cc normal saline into the peritoneal cavity did not provoke a mouse leukocytic response

greater than saline alone in the tumor-bearing mouse when tested as above.

Discussion. A method has been presented for measurement of host leukocytic response to an intraperitoneal challenge, using quinacrine intravenously as a fluorescent leukocytic label. The advantages of this technic include the time-proven and stable experimental animal-tumor cell model, the rapidity of labeling and observation, and the convenience of sampling procedures. The results obtained may be related to the special circumstances of EAT growth in the peritoneal cavity(2,3). This method does not differentiate between a mechanical effect of including or excluding leukocytes from the peritoneal cavity and a biological effect in terms of absolute cell numbers. However, it does demonstrate a difference in the number of leukocytes compared to the number of tumor cells, a finding which may be of some significance in terms of tumor propagation. The results of these experiments are expressed as number of fluorescent leukocytes per 100 tumor cells in the peritoneal fluid. These findings are presented in this form because intraperitoneal absolute cell counts involve considerable sampling errors, *i.e.,* dilution, surface attachment, sequestration, cell death, or other loss.

The host leukocytic response to an intraperitoneal challenge with EAT cells has been shown to be consistently reduced in the presence of neoplastic proliferation. The outpouring of leukocytes into the peritoneal cavity in response to 6×10^6 EAT in 6 cc normal saline appears to persist longer in the immune mouse than in the normal, although the magnitude of the response is similar. The depression of the white cell reaction with malignancy could be demonstrated only in the presence of growing EAT cells, not with non-viable cells or tumor fluid. A proper stimulus, *i.e.,* turpentine, elicited a leukocytic response in the tumor-bearing animal at any stage, indicating the ability of the animal to react. The blunting of leukocytic responses with X-ray showed that a diminished leukocytic reserve resulted in fewer leukocytes responding to the intraperitoneal challenge, and that the intravenous quinacrine did not label the intraperitoneal cells brightly within a given time period.

Old *et al* have investigated the effects of leukocytes in normal and immune animals on peritoneal tumors(4). These studies indicate a difference of action of leukocytes against the tumor in immune compared to normal animals, and a variation in this reaction *in vivo* from *in vitro*. *In vivo* cells from the immune animal are necessary for inhibition of the tumor, whether or not immune serum is present (normal does as well), and the effect of these cells is not lost by repeated washing. A ratio of one immune leukocyte to one tumor cell appeared to be necessary for inhibition, a ratio obtained in our study only in normal or immune animals with 6×10^6 EAT cells injected.

The observation of clumps of leukocytes with tumor cells in this study, more pronounced in the immune animals, suggests a possible mechanism of action or functional state of these leukocytes. Specific functions of leukocytes from the various animal preparations were not measured in detail, however, and the activity of such cells can only be surmised.

Living or growing cells appear to be necessary to inhibit the leukocytic response to EAT, as shown in the above studies. The specificity of this phenomenon has not been determined, and specific antigens have not been directly implicated(11).

The deposition of an "instant" tumor in the peritoneal cavity of a mouse appeared to overwhelm the host defenses, resulting in rapid multiplication of the EAT and death of the animal. The "instant" tumor presents a very large amount of antigen to the mouse, thus an antigen excess could impair the immune response. To be considered also is a leukocytic response inhibitory substance emanating from the tumor, or possibly a protein overloading phenomenon(12). It is possible that metabolites or products of dead or aging tumor cells "turn off" the leukocytic response. The rapid growth of "instant" tumors, especially in immunized hosts, suggests the possibility of enhancement, although antibody *vs* tumor was not demonstrated directly.

McCarthy has shown that C_3H mice with

EAT tolerate skin grafts until the death of the animal(1). Patients with malignant disease have been shown to tolerate transplantations better than those without neoplasia (13). Southam has also shown an abnormal leukocytic response by the Rebuck skin window technic in patients with malignancy(15). Thus, it seems apparent that a subdued leukocytic reaction in the presence of malignant disease reduces the defenses of the host to various types of challenge. If neoplastic cells do inhibit the host leukocyte response, then the malignant tissue must possess the special property of induction of this phenomenon, thus aiding in its propagation and metastasis. Further studies are under way to clarify these observations.

Summary. A method has been described to demonstrate leukocytic responses in the peritoneal cavity of mice to challenge substances, which uses quinacrine to label the leukocytes for fluorescent microscopy. The leukocyte reaction to intraperitoneally inoculated Ehrlich ascites tumor cells was observed to be decreased in the presence of an already established ascites tumor. Massive tumor inoculation (6 cc EAT intraperitoneally) appears to suppress the outpouring of leukocytes in an immediate fashion, although a response does occur when turpentine is administered concomitantly. One explanation for these observations is the alteration of host defenses by the neoplastic cells.

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Effect of Purified Luteinizing Hormone-Releasing Factor (LH-RF) on Plasma LH Activity at Various Stages of the Estrous Cycle of the Rat.* (31309)

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An LH-releasing factor (LH-RF) has been found in hypothalamic extracts from several mammalian species(1-5). This factor has been purified and separated from the other hypothalamic neurohormones such as follicle stimulating hormone-RF, growth hormone-RF, corticotrophin-RF, and melanocyte stimulating hormone-RF which appear to control adenohypophyseal secretion(2,3,6). The factor is a small, basic polypeptide dissimilar

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