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Factor(s) from Ehrlich Ascites Cells Responsible for Delayed Rejection of Skin Allografts in Mice and Its Assay on Lymphocytes *in Vitro* (38748)

ION GRESSER, FRANÇOISE VIGNAUX, CHANTAL MAURY, AND PERNILLA LINDAHL

Institut de Recherches Scientifiques sur le Cancer 94800 Villejuif, France

Our previous experiments on the effect of interferon on the multiplication of Ehrlich Ascites (EA) tumor cells in mice suggested the possibility that the tumor secreted factors which affected the function of host cells (1–3). Although it is known that animals and man with cancer often exhibit an impaired cell mediated immune responsiveness, it is not known to what extent tumor cells may liberate substances capable of depressing host response to the tumor. This possibility is clearly relevant to the efficacy of any antitumor therapy and particularly to one based on enhancement of the host's immune response. In mice it has recently been shown that transfer of cell free tumor ascitic fluid to normal mice results in an inhibition of the antibody response to sheep erythrocytes (4–6), abrogation of lymphocyte "trapping" (7) and enhancement of the growth of a transplantable tumor (5). That the lymphocytes of tumor bearing animals are probably potentially reactive, but are only temporarily depressed by an inhibitor is suggested by the findings that bone marrow, thymus and spleen cells from these mice exhibit immunologic competence when transferred to syngeneic irradiated recipients (8). In accord with this interpretation, it was found that serial aspiration of ascitic fluid from tumor bearing mice resulted in recovery of a large number of mice (not seen in untreated mice) suggesting again the presence in the ascites of factors inimical to the host (9).

The experiments to be reported were stimulated by the studies of McCarthy who showed that mice inoculated with EA cells exhibited a prolongation of skin allografts (10). Although he was unable to demonstrate a delay in rejection of primary skin allografts in mice treated with cell free Ehrlich ascitic fluid (10, 11), he could show that the usually accelerated rejection of second and third set allografts was delayed by continued administration of this material (11). We show herein that the cell free ascitic fluid from EA tumor bearing mice and the nutrient medium from Ehrlich ascites cells maintained *in vitro* contain a factor(s) which when transferred to mice does delay rejection of primary skin allografts and also enhances the growth of a transplantable tumor. Both cell free ascitic fluid from EA tumor bearing mice and the medium of EA cells *in vitro* inhibit DNA synthesis in mouse splenic lymphocytes stimulated with phytohemagglutinin (PHA) *in vitro*. These findings suggest that Ehrlich tumor cells secrete factors which depress the normal lymphocytic response in mice and that the biologic activity of such factors can be assayed *in vitro*.

Materials and Methods. Tumor cells. EA cells (hypotetraploid variant) obtained from Dr. J. Lindenmann were passaged by intraperitoneal (ip) inoculation of Balb/c mice (1). The Lewis Lung tumor (3LL) obtained from Dr. K. Hellmann was passaged by subcutaneous inoculation of C57Bl/6 mice (12).

Preparation of cell free ascitic fluid. One to 3 mo old male or female Balb/c mice were inoculated ip with $1–2 \times 10^6$ EA cells. Five days thereafter, the peritoneum of each mouse was washed with 5 ml of cold phosphate buffered saline (PBS), the washings pooled, centrifuged at 500 rpm for 15 min and then ultracentrifuged at 100,000 *g* for 1 hr. As a control, cell free peritoneal washings from uninoculated mice or mice inoculated ip with thioglycollate broth or triolein were substituted for the cell free Ehrlich ascitic fluid.

Skin grafted mice treated with ascitic fluid were kept for 4 mo to check for the presence of viable cells in the supernatant. It had been shown that 1 to 10 EA cells constitute 1 LD_{50} for Balb/c mice (1).

Preparation of nutrient medium from EA

cells maintained in vitro. Balb/c mice were inoculated ip with 1–2 × 10^6 EA cells. Four to 5 days thereafter the mice were sacrificed, the peritoneal cavity washed with 5 ml of RPMI 1640 (Gibco) medium containing 1% inactivated horse serum. The cells were washed twice in the same medium and usually resuspended at a concentration of approximately 5 × 10^7 cells/ml in RPMI 1640 medium with 2½% horse serum. After 24 hr the nutrient medium was collected and the cells sedimented by centrifugation at 2000 rpm for 15 min and the supernatant recentrifuged at 10,000 rpm for 1 hr at 4°. The cell free supernatant was used in the experiments to be described.

Technique of skin grafting. Skin grafts were taken from the tail of male or female C_3H or C57Bl/6 mice and transplanted to the dorsum of 8- to 10-wk-old male or female Ajax or Balb/c mice. Isografts were also performed as a control in each experiment. Grafts were read daily and scored. The time of rejection was taken as the day when 50% or more of the graft appeared necrotic and began to separate from the graft bed.

Assay of DNA synthesis in lymphocyte suspensions. Suspensions of C_3H or C57Bl/6 mouse splenic lymphocytes were prepared by the method of Mangi and Mardiney (13) at a concentration of 2.5 × 10^6 cells/ml in RPMI 1640 medium with 20% heat-inactivated horse serum. One ml cell suspensions were incubated in a 5% CO_2–air mixture at 37° and 0.1 ml of a 100-fold dilution of stock PHA P (Difco) was added (14). In most experiments dilutions of the material to be tested were added together with PHA and incorporation of tritiated thymidine (C.E.A., France, specific activity 15 Ci/mmole) was measured 48 and 72 hr thereafter using a 4 hr pulse with 1 μCi/ml in quadruplicate cultures at 37°. The cells were washed three times with PBS and the radioactivity of the acid-insoluble material was measured in a liquid scintillation counter. In each experiment the incorporation of radioactive thymidine was also determined in unstimulated suspensions of lymphocytes. Under the conditions used, DNA synthesis was maximal 2–3 days after the addition of PHA, and ^{3}H–thymidine incorporation was 7- to 40-fold (average 20-fold) that of unstimulated control cell suspensions.

Results. Delayed rejection of skin allografts in mice treated with cell free ehrlich ascitic fluid or with nutrient medium from ehrlich ascites cells maintained in vitro. As can be seen from experiments 1, 2 and 3, Table I, treatment of Ajax (H-2^a) or Balb/c mice (H-2^d) with cell free Ehrlich ascitic fluid (prepared in Balb/c mice) resulted in a delayed rejection of skin allografts from C_3H (H-2^k) or C57Bl/6 (H-2^b) mice. A comparable delay in rejection time of skin allografts was also observed when C57Bl/6 (H-2^b) mice were pretreated with cell free ascitic fluid and then grafted with skin from CBA mice (H-2^k).

Ehrlich cells were harvested from the peritoneal cavities of Balb/c mice and resuspended *in vitro* (see Methods and Materials). Ajax mice were pretreated with the cell free supernatant from 24-hr Ehrlich cell suspensions and then grafted with skin from C_3H mice (experiment 3 and 4, Table I). A significant delay in graft rejection was observed for treated mice compared to control mice.

Mortality in mice inoculated with lewis lung carcinoma and treated with nutrient medium from ehrlich ascites cells maintained in vitro or ehrlich ascitic fluid. Six-week-old male C57Bl/6 mice were injected ip with supernate from EA cells maintained *in vitro* and 3 days thereafter inoculated subcutaneously with Lewis Lung carcinoma cells. An earlier appearance of tumor, an increased growth rate of the tumor and an earlier mean day of death (Table II) was observed in mice treated with nutrient medium from Ehrlich cells compared to untreated tumor inoculated mice.

Likewise, inoculation of C57Bl/6 mice injected ip with cell free ascitic fluid also resulted in an enhancd growth of the 3LL carcinoma implanted subcutaneously with a decrease in the mean day of death.

Neither control cell free peritoneal washings nor control nutrient medium affected the growth of tumor.

Activity of cell free ascitic fluid or the nutrient medium from EA cells maintained in vitro as assayed by inhibition of DNA syn-

TABLE I. DELAYED REJECTION OF SKIN ALLOGRAFTS IN MICE TREATED WITH CELL FREE EHRLICH ASCITIC (EA) FLUID OR WITH NUTRIENT MEDIUM FROM EHRLICH ASCITES CELLS MAINTAINED *in vitro*.

Exp	Mouse strain: Donor	Mouse strain: Recipient	Treatment	No. of mice	Mean day of survival of allograft		Confidence interval 0.95
1	C_3H♂	Ajax♂	None	8	13.8	+[a]	10.8–16.7
			Ehrlich ascitic fluid	8	17.5		16.1–18.9
2	$C_{57}Bl/6$♀	Balb/c♀	None	8	13.8	N.S.	12.8–14.8
			Washings from peritoneum of normal mice	9	14.0	++[b]	13.0–15.0
			Ehrlich ascitic fluid	9	17.9		15.9–19.9
3	C_3H♀	Ajax♀	None	14	13.2	++	11.5–14.9
			Ehrlich ascitic fluid	13	17.7		15.1–20.2
			Nutrient medium from EA cells *in vitro*	15	18.4	++	15.0–21.8
4	C_3H♂	Ajax♂	None	11	13.0	N.S.	11.2–14.8
			Nutrient medium alone	11	13.2		12.1–14.2
			Nutrient medium from EA cells *in vitro*	11	16.2	+	13.3–19.0

[a] + $P < 0.05$.

[b] ++ $P < 0.01$.

NS not significant.

Skin graft was performed on day 0.

In experiment 1. Ehrlich ascitic fluid was injected (0.25 ml) ip on days −2, −1, 0 and daily thereafter for 18 days.

In experiment 2. Ehrlich ascitic fluid, or control peritoneal washings was injected (0.25 ml) ip on days −3, −2, −1, 0 and daily thereafter for 15 days.

In experiment 3. Ehrlich ascitic fluid or nutrient medium from EA cells *in vitro* was injected (0.25 ml) ip on days−2, −1, 0 and daily thereafter for 5 days.

In experiment 4. Nutrient medium from EA cells *in vitro* or control medium was injected (0.25 ml) ip on days −3, −2, −1, 0 and daily thereafter for 4 days.

TABLE II. MORTALITY IN C57Bl/6 MICE INOCULATED WITH LEWIS LUNG CARCINOMA AND TREATED WITH NUTRIENT MEDIUM FROM EHRLICH ASCITES CELLS MAINTAINED *in vitro*.[a]

Treatment	No. of mice	Mean day/death of		Confidence interval 0.95
None	10	34.4	$P < 0.01$	30.8–38.0
EA cell nutrient medium	10	26.7		22.7–30.7

[a] 0.2 ml of EA cell supernate inoculated ip on days −3, −2, −1, and 0.

thesis in lymphocyte suspensions. When cell free Ehrlich ascitic fluid was incubated at a 1:10 dilution with mouse splenic lymphocytes, an evident inhibition of the incorporation of radioactive thymidine was observed in the acid-insoluble fraction of unstimulated and PHA stimulated lymphocytic suspensions compared to control suspensions (Table III).

Likewise, the nutrient medium from EA

TABLE III. EFFECT OF CELL FREE ASCITIC FLUID ON DNA SYNTHESIS IN UNSTIMULATED AND PHA STIMULATED C57Bl/6 MOUSE SPLENIC LYMPHOCYTES.[a]

Group	Day 2		Day 3	
	Unstimulated	PHA added	Unstimulated	PHA added
Control	2056 ± 350[b]	16,103 ± 502	2585 ± 402	18,971 ± 589
Incubated with cell free ascitic fluid	1395 ± 286	10,912 ± 2,170	1622 ± 122	10,606 ± 1,299

[a] PHA was added to all cultures at 0 hr: incorporation of ^{3}H—thymidine was measured by 4 hr pulse labelling of five cultures per group.

[b] Figures indicate cpm/culture.

cells maintained *in vitro* also inhibited the incorporation of radioactive thymidine in stimulated lymphocytes. The preparations were still active on dilution and as shown in Fig. 1, this test provided a reproducible assay since a given lot of Ehrlich cell supernate tested on four different occasions on different preparations of C_3H mouse spleen lymphocytes gave comparable results. Over the past year, 20 different lots of Ehrlich cell supernate have been prepared and all have proven active using this assay.

Incubation of control cell free peritoneal washings or control nutrient medium did not affect the response of mouse lymphocytes to PHA.

Inhibition of DNA synthesis was not due to any obvious cytotoxic effect of the preparations. No significant difference in the percentage of viable lymphocytes was observed between control and Ehrlich cell supernate treated lymphocyte suspensions using trypan blue dye exclusion as a criterion of viability.

The factor responsible for inhibition of DNA synthesis in mouse splenic lymphocytes (of different mouse strains) was not species specific as evidenced by its activity on human lymphocytes stimulated with PHA.

Despite the reproducibility and ease of the *in vitro* assay procedure, we have encountered difficulties in the characterization of the factor(s) responsible for inhibition of stimulated lymphocytes. Our results to date may be summarized as follows: Biologic activity is not sedimented by centrifugation at 100,000 *g* for 1 hr. It appears to

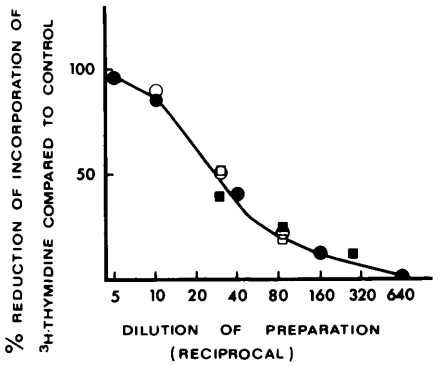

FIG. 1. Effect of nutrient medium from EA cells maintained *in vitro* on incorporation of radioactive thymidine in C_3H mouse splenic lymphocyte suspensions stimulated with PHA. A given lot tested on four different occasions, ie, ●, ○, ■, □.

be largely inactivated by heating at 60° for 30 min. The activity is not markedly affected by treatment with crystalline trypsin or chymotrypsin (500 γ/ml). It is resistant to ether. Although most of the activity is recovered after dialysis, some experiments suggest that part of the activity may be lost in dialysis. Its activity is lost following treatment with 0.02 *M* sodium periodate at 4° followed by dialysis.

Discussion. The results presented above show that the cell free Ehrlich ascitic fluid and the nutrient medium from Ehrlich ascites cells maintained *in vitro* for 24 hr contain a factor(s) which when transferred to normal mice delays rejection of primary skin allografts and enhances the growth of a syngeneic transplantable tumor. Since lymphocytes are considered of major importance for the rejection of allogeneic grafts

and resistance to tumors, the most likely explanation of the effects described herein is that these preparations inhibited normal lymphocyte activity. The finding that both Ehrlich ascitic fluid and the nutrient medium from EA cells inhibited DNA synthesis in lymphocytes stimulated by PHA (a test that has been widely used to measure T lymphocyte "reactivity") supports this interpretation and suggests the possibility that these factors may delay sensitization of lymphocytes to allogeneic or tumor antigens.

The preparations used in these experiments are crude and may contain many substances with different biologic activities. We cannot be sure that the factor(s) in the ascites and the one released by Ehrlich cells *in vitro* are the same (although this seems likely to us). Nor can we determine the similarity of the factors we have described to those tumor associated factors recently reported by other investigators which exert immunodepressive effects in experimental animals (4–8). The use of a reproducible *in vitro* assay for these factors (for example, inhibition of PHA stimulation of lymphocytes as presented herein) should greatly facilitate investigations and permit characterization of these inhibitors. Furthermore it is entirely possible that these tumor associated immunodepressive factors are not unique to tumor cells but are similar to factors from normal tissues that are also endowed with immunosuppressive properties (15–20).

Although it is usually believed that the Ehrlich Ascites tumor grows well in mice of almost any strain because this tumor expresses histocompatibility antigens weakly (21) it is tempting to suggest an alternative explanation—i.e. the Ehrlich tumor grows well in different mouse strains because it produces a factor that effectively inhibits the lymphocytic response of these mice to the implanted tumor.

Patients with cancer often exhibit diminished cell mediated immune responsiveness (22–29). Whereas it is possible that such deficiency may precede the development of cancer (failure of "immune surveillance" has been considered an important factor in the emergence of cancer (30–32)), it seems equally possible to us that in some instances the tumor itself may be the cause of the depressed immune response, perhaps by the liberation of substances similar in action to those described herein. Furthermore the finding that immunocompetence is restored to patients after successful chemotherapy (33, 34) seems to support our contention that the tumor may be the source of immunosuppressive factors. It would thus seem of therapeutic importance to understand the nature and mechanism of action of such tumor associated factors.

Summary. Cell free Ehrlich ascitic fluid from tumor bearing mice and the nutrient medium from Ehrlich cells maintained *in vitro* contain a factor(s) which when transferred to normal mice delays rejection of skin allografts and enhances the growth of a syngeneic transplantable tumor. Both types of preparations inhibit DNA synthesis in lymphocytes stimulated by PHA. These results suggest that Ehrlich tumor cells release a factor which impairs the normal lymphocytic response to allo and tumor antigens.

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