A Lipid Mobilizing Factor in Serum of Tumor-Bearing Mice

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ABSTRACT

There is considerable evidence that the growing tumor requires a source of unsaturated fatty acids, but the nature of this source and the mechanism of mobilizing the fatty acids from it are obscure. These experiments make use of AKR mice with implanted adipose tissue labeled with 1-^{14}C linoleic acid. With this experimental animal, it has been found that: (a) in the normal, fed mouse, fat is mobilized slowly and appears largely as respiratory CO_2, following oxidation, (b) in the normal, fasted mouse, fat is mobilized rapidly and appears largely as respiratory CO_2; (c) in the tumor-bearing, fed mouse, fat is mobilized rapidly and appears largely in the tumor; and (d) the serum from tumor-bearing mice, when injected into normal mice, produces an immediate massive fat mobilization that does not respond to feeding, whereas the serum from normal, fed mice does not. It is concluded that a mobilizing factor of unknown nature is present in the serum of tumor-bearing AKR mice.

INTRODUCTION

Many observations have indicated a growing tumor is able to derive the fatty acids necessary for membrane formation from the host tissues. For example, Smedley-Maclean and coworkers (1,2) reported implanted Walker tumors caused a decrease in polyunsaturated fatty acids in surrounding tissues. Mead and Decker (unpublished results) found that chronically fat-deficient mice died with acute fat deficiency symptoms following transplantation of an Ehrlich tumor. The source of the fatty acids was not clear from these and similar experiments. Studies specifically designed to answer this question have not been helpful, possibly because of the heterogeneity of the tumors used and the different reactions in different experimental animals. For example, Spector and Brenneman (3) reported little intraperitoneally injected glucose radioactivity appeared in the tumor lipid of Ehrlich ascites tumor-bearing mice, and Baker and his associates (4,5) have reported an impairment of lipid transport in these mice and have indicated the liver is not the direct source of the hypertriglyceridemia seen.

On the other hand, it seems evident that in mammals, at least, the major source of rapidly available fatty acid must be adipose tissue and many observations of tumor-bearing animals and patients have revealed a picture of fat depletion with advanced malignancies.

In preliminary experiments in this laboratory, it was found that in AKR mice with advanced lymphomas, injection or oral administration of [1-^{14}C]linoleic acid resulted in relatively increased incorporation into liver and thymus phospholipids and decreased incorporation into triglycerides as compared to controls (6). The hypothesis advanced at the time was that, in tumor-bearing mice, an increased transport of fatty acid to the tumor and increased incorporation into tumor cell membranes was occurring. Observations of this sort have been made previously (7).

Clearly, there is a need for a definitive study to identify the source of tumor lipid, the means of transport to the tumor and the mechanism by which the lipid transfer is induced. These experiments were designed to answer those questions, at least for one animal and type of tumor.

MATERIALS AND METHODS

Experimental Animals

Both male and female AKR mice were used. This strain has an almost 100% mortality from thymic lymphoma between the ages of 6 and 14 months with a peak incidence at 9-10 months (8). If injected with Gross Murine Leukemia Virus at 3 days, the onset of the disease is accelerated and peak incidence occurs at 2.7 months (9). Animals were housed in groups of 6-7 and fed laboratory mouse chow ad libitum.

Animal Procedures

[1-^{14}C]Linoleic acid was administered orally to a healthy AKR mouse and after 4 hr the mouse was sacrificed and the resulting labeled retroperitoneal and suprapelvic adipose tissue was removed and divided into pieces of about 0.1 g (100,000 cpm). These were transplanted into different locations of the peritoneal cavity of age-matched lymphoma-bearing and control AKR mice under light ether anesthesia (10). Vascularization of the transplanted adipose tissue occurred in about 2 days, as shown microscopically, and these mice were

used for the experiments described in the following text. In all, 8 lymphoma-bearing mice and an equal number of normal controls were used. In some cases, mice bearing radioactive grafts were used for several experiments and thus served as their own controls. Animals with both spontaneous and accelerated lymphoma were used as recipients of labeled adipose tissue grafts. The serum for lipid mobilization experiments was taken from 2 to 3-month-old mice with accelerated lymphoma and from 1-month-old healthy controls.

For collection of respiratory CO_2, special plexiglass cages holding a single mouse were used. (These cages were generously furnished by Dr. M. Ookhtens of the Tumor Lipid Laboratory, Wadsworth Veterans Administration Hospital, Los Angles, CA) (11). The cages had one air inlet allowing air to enter from the rear and one outlet directly in front of each mouse to permit collection of expired CO_2. Dimensions of the cages were such that the mice could not turn around throughout the period of breath collection. Air was swept at a rate of 500 ml/min/cage (dead space swept at least 10 times per min) and bubbled through 40 ml of hydroxide of hyamine 10-x (Rohm and Haas) solution. The 0.032 M solution was prepared in absolute ethanol containing phenolphthalein as an indicator. Each CO_2 collection funnel was supplied with 40 ml of this solution. Disappearance of the pink color of the solution indicated the completion of the titration, i.e., the saturation of the hyamine by expired CO_2. Four separatory funnels were arranged to collect CO_2 from a pair of mice simultaneously. Aliquots of hyamine solution were collected after the pink color of the hyamine solution disappeared and were used for ^{14}C assay.

Liquid Scintillation Counting

Aliquots (5 ml) of the hyamine solution were taken after disappearance of the pink color and were mixed with 10 ml of Dimilume-30 in glass counting vials and kept in the dark at 4 C overnight. Appropriate blanks and experimental samples were counted on a Beckman LS 8100 liquid scintillation spectrometer.

Extraction and Analysis of Lipids

Tissue samples, after weighing, were extracted by the method of Bligh and Dyer (12). Total lipid was separated into nonpolar and polar fractions by elution from small silicic acid columns with chloroform and methanol, respectively.

Radio Gas Liquid Chromatography

Methyl esters of component fatty acids were prepared from the separated lipids by methanolysis with 4% HCl in methanol for 1 hr at 80 C. The methyl esters were extracted with pentane and were analyzed for mass and radioactivity on the Packard, Model 824, gas chromatograph with gas proportional counter, Model 894, fitted with a 6 ft x 4 mm glass column with a liquid phase of Apolar 10 C.

RESULTS

Findings after Transplantation of Adipose Tissue

Figure 1 shows transplanted adipose tissue in a lymphoma-bearing mouse. There are intact blood vessels containing red blood cells. New vascularization has taken place between the peritoneum and transplanted adipose tissue. In this mouse, the transplanted adipose tissue had decreased in size during 3 days. About 80% of the radioactivity from the transplanted tissue was found incorporated in the various tumor tissues – liver, thymus, spleen, mesenteric

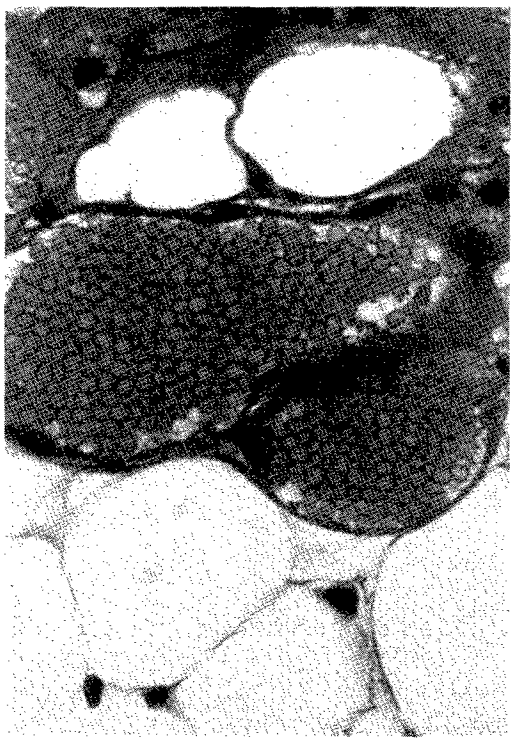

FIG. 1. Transplanted adipose tissue from a lymphoma-bearing mouse. (Data shown in Table I). Hematoxylin and eosin are used for staining; figure is magnified x 400. The graft in this lymphoma mouse survived as well as that in control mice.

lymph nodes, inguinal lymph nodes, and cervical lymph nodes, for example.

In advanced lymphoma cases, thymus, lymph nodes and spleen cells were largely replaced by lymphoma cells, and in the liver extensive invasion by lymphoma cells was seen, particularly around the blood vessels. In control mice, the grafts in general had more extensive vascularity and better survival than those in the lymphoma-bearing mice. The control grafts did not decrease noticeably in size and little radioactivity was found in tissues other than the transplanted adipose tissue.

Analysis of Tissue Lipids

As shown in Tables I and II, after sufficient time for vascularization of the transplanted tissue, significant amounts of radioactivity were found in all tumor-containing tissues – liver, thymus, spleen and lymph nodes. Considerable variability occurred in the incorporation of radioactivity into the tissues of these mice because of several factors such as stage of lymphoma and vascularization of graft. Only small amounts of radioactivity were found in the liver and thymus of control mice. The most extensive mobilization was found in those animals in which the best vascularization occurred, as seen microscopically. In the tumor tissues, most of the radioactivity was found in the phospholipid fraction, as reported previously, following [1-^{14}C]linoleic acid administration (5). Radio gas chromatography showed the fatty acid radioactivity pattern of the phospholipid from tumor tissue was 85% linoleic acid and 15% arachidonic acid (Fig. 2).

Fasting Experiments

Since lipid mobilization also is caused by fasting, a comparison was made of the transfer of radioactivity induced by fasting or by the growing tumor. In a sense, this had already been done since it had been found that, in the normal mice, radioactivity from adipose tissue was not extensively transferred to other tissues, whereas in the tumor-bearing mice much of the radioactivity was found in the tumor or in the tissues, such as the liver, which were extensively invaded by tumor. These findings take on additional significance since it was found that the tumor-bearing mice ate approximately the same amounts as the controls (3-4 g/day).

For the fasting study, mice with the radioactive grafted adipose tissue were placed in the metabolism cages, radioactive CO_2 was collected and the radioactivity per mmol CO_2 was measured. In the cases of both fed control and lymphoma-bearing mice, a low level (less than 100 cpm/mmol CO_2) was produced throughout the period. Prolonged fasting in the normal mouse with radioactive adipose tissue graft, however, produced an increase in expired $^{14}CO_2$ with a peak value of 1700 cpm/mmol (which corresponds to about 3000 cpm/hr). This was confirmed by collecting CO_2 for 1 hr with a more concentrated hyamine solution. When the fasting was interrupted by feeding a minimal amount of diet (0.5-2 g), the radioactive CO_2 fell to baseline values precipitously and then slowly rose as fasting was resumed.

TABLE I-A

Radioactivity Incorporated into Various Tumor Tissues in Lymphoma-Bearing Mouse

	Radioactivity in total lipid (cpm)	Radioactivity in phospholipids (cpm)	Radioactivity in neutral lipid (cpm)
Liver	57340	41930 (80%)	10810 (20%)
Thymus	7620	6610 (89%)	820 (11%)
Spleen	11830	10870 (97%)	290 (3%)
Inguinal and cervical lymph nodes	8280	5017 (67%)	2460 (33%)
Mesenteric lymph node	2150		

TABLE I-B

Distribution of Radioactivity in Neutral Lipids of Thymus and Liver of Lymphoma-Bearing Mouse

	FFA cpm	FFA/TL%	Chol. cpm	Chol./TL%	TG cpm	TG/TL%	Chol. E cpm	Chol. E/TL%
Liver NL	50	0.1%	438	0.8%	7770	14%	730	1.3%
Thymus NL	7	0.1%	74	1.0%	395	5%	200	2.6%

TABLE II

Radioactivity Incorporated into Tissues of Lymphoma-Bearing and Control Mice

Mice		Sex	Days after transplantation	Radioactivity in liver TL (cpm)	Radioactivity in thymus TL (cpm)	Radioactivity in mesenteric lymph node TL (cpm)
Lymphoma	(1)	F	6	6650	473	1195
	(2)	F	4	9330	1660	910
	(3)	M	3	57340	7620	2150
	(4)	F	5	23970	2810	9850
	(5)	M	3	3920	733	
	(6)	F	4	8030	680	
	(7)[a]	M	4	3635	530	
	(8)[a]	F	3	5610	540	
Control	(1)	F	5	560	44	
	(2)	M	6	380	12	
	(3)	M	3	840	33	
	(4)	F	4	385	260	
	(5)	M	5	665		
	(6)	F	4	200		
	(7)	M	3	380		
	(8)[b]	F	4	665		

M = Male; F = Female.

[a]Lymphoma (7) and (8) are spontaneous lymphoma mice. (Lymphoma [7] = 9 months old, Lymphoma [8] = 7 months old).

[b]Control (8) is a 1-year-old healthy mouse. The rest of the lymphoma mice are virus accelerated (2-3 months old). The rest of the control mice are age-matched (2-3 months old).

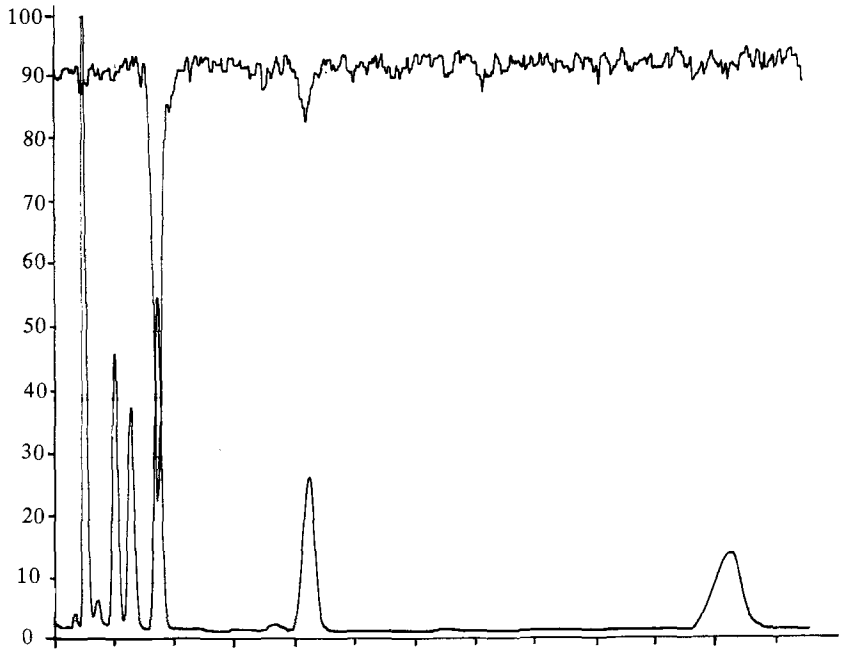

FIG. 2. Liver phospholipid fatty acid pattern from lymphoma-bearing mouse. Mass peaks (lower curves) are from a standard mixture. Radioactivity is present only in 18:2 and 20:4.

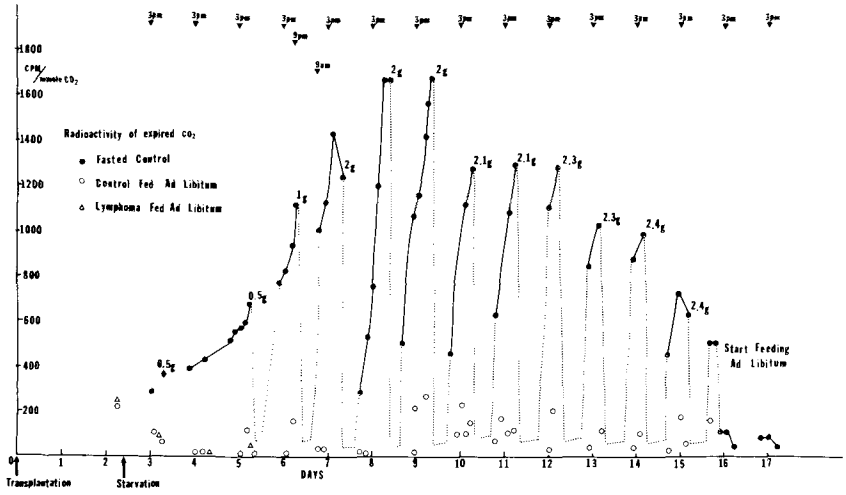

FIG. 3. Radioactivity of expired CO_2. The abscissa shows days after transplantation. Fasting started 2½ days after transplantation of labelled adipose tissue. The ( )g shows the amount of food given to the fasted mouse.

This process could be repeated continuously, each cycle bringing about an increase (fasting) or decrease (feeding) of radioactivity. In this manner, the radioactivity in the transplanted adipose could be, in a sense, titrated until all the radioactivity of the adipose tissue had appeared as $^{14}CO_2$ and this was confirmed, after sacrifice, since negligible radioactivity remained in the graft. One such series is shown in Figure 3. It should be emphasized that the conditions in this experiment are different from those that follow in that the former represents severe starvation and the latter mild fasting.

Test for Mobilizing Factor

Since, by the method outlined above, each mouse could be its own control, an experiment was initiated in which a normal mouse bearing a radioactive adipose tissue graft was tested for quantitative response to feeding or fasting. As usual, feeding 0.5 g food brought about a precipitous decline in radioactive CO_2 emission to very low values within 45 min. As fasting progressed, the radioactivity slowly increased, reaching 150 cpm/mmol in 3 hr (Fig. 4). A more gradual increase was seen following 1.0 or 1.5 g food; in the latter case, 150 cpm/mmol was reached in 6-7 hr (Fig. 4).

From the information gained in this experiment, the following studies were conducted after ¼-day of fasting – allowing liver glycogen to be largely depleted then giving 2 g food and additional food at appropriate times – in such a way that the effect of fasting was not a factor in the studies.

Two hr following the feeding of 2.0 g chow, when the radioactive CO_2 was at a low point, 0.3 ml of serum from a mouse with advanced lymphoma was injected by tail vein. There was an immediate dramatic increase in expired radioactivity (to 200 cpm/mmol) and a further increase to 300 cpm/mmol in 5 hr (Fig. 5). At 9 hr, 0.5 g food was given but did not result in a decrease of radioactivity. Injection of 0.3 ml serum from a normal, fed control mouse did not result in any change under the identical experimental conditions (Fig. 5).

This experiment was repeated using a mouse in which, following feeding, the expired CO_2 had an activity of 5.6 cpm/mmol. Injection of 0.3 ml of serum from a lymphoma-bearing mouse caused an increase to 120 cpm/mmol.

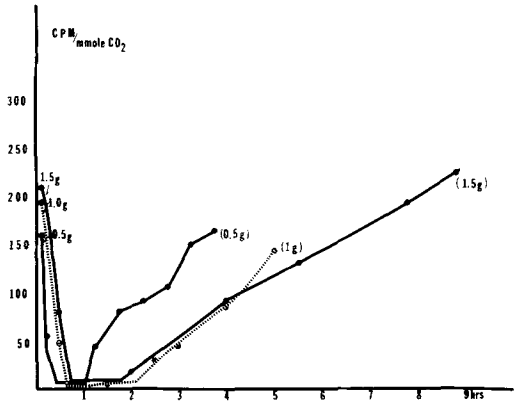

FIG. 4. Appearance of respiratory $^{14}CO_2$ in mouse with radioactive adipose tissue graft with time and treatment (see Results). (g) Indicates the amount of food given at time 0.

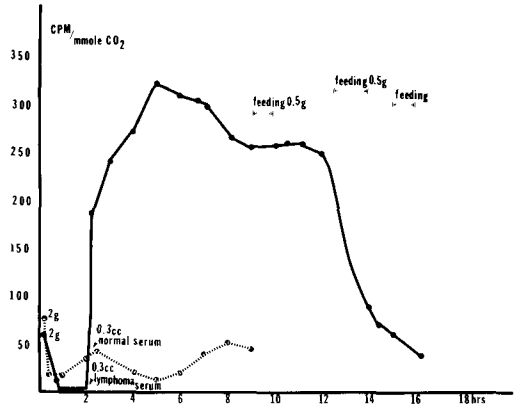

FIG. 5. Appearance of respiratory $^{14}CO_2$ in mouse with radioactive graft with time and treatment (see Results). ( )g Indicates the amount of food given at time 0 or the indicated time periods. Arrows show times of injections.

An extract of thymus from a lymphoma-bearing mouse caused an increase to 134 cpm/mmol, whereas an extract of pooled thymus glands from two control mice had no effect. Also, injection of conditioned medium from growth phase culture of an AKR SL3 lymphoma cell line (13) caused an increase to 134 cpm/mmol. No increase was observed with serum from a control mouse or control culture medium. (RPMI 1640 with 10% fetal calf serum).

DISCUSSION

The use of mice bearing transplanted radioactive adipose tissue permits the rapid and sensitive assessment of the state of mobilization of adipose tissue. It is evident that the graft behaved qualitatively similarly to the endogenous adipose tissue since under conditions of release of radioactivity from the graft, the endogenous adipose tissue decreased in size. A check on the measurement of $^{14}CO_2$ appearance was obtained after sacrifice, by measuring the radioactivity of all tissues. This indicated that in the fed normal mouse, mobilization is slow and much of the released fatty acid is oxidized. In the fasted normal mouse, mobilization is rapid and, again, most of the released fatty acid is oxidized. In contrast, in the fed, tumor-bearing mouse (these animals did not survive fasting), most of the fatty acid was mobilized but, in this case, it appeared in the tissues.

As discussed above, there is ample evidence that growing tumors require a source of unsaturated fatty acids for formation of membrane phospholipids. There is also a great deal of indirect evidence implicating fatty acids as growth promoting agents in carcinogenesis (14-15). In the same light, indirect evidence has supported the idea that mobilizing factors are produced by the growing tumor: the unsaturated fatty acids are made available for its growth. The above experiments furnish direct evidence that there is a potent mobilization inducing factor in the serum of tumor-bearing mice but not in that of controls. That it is different from the mobilization inducing factors (e.g., epinephrine) produced in fasting is evident, since feeding has little or no effect on the tumor serum induced mobilization, whereas it inhibits that produced by fasting. Since the factor is also present in tumor extract and in culture medium from lymphoma cell culture, it is evidently not produced indirectly from some other tissue but is a direct product of the tumor.

Future experiments will include attempts to isolate and identify the factor or factors. It is anticipated that these results will require time but will be immeasurably aided by the technique described in this paper.

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REFERENCES

1. Smedley-MacLean, I., and L.C.A. Nunn, Biochem. J. 35:983 (1941).
2. Smedley-MacLean, I., and E.M. Hume, Biochem. J. 35:996 (1941).
3. Spector, A.A. and D.E. Brenneman, in "Tumor Lipids: Biochemistry and Metabolism," Edited by Randall Wood, American Oil Chemists' Society Press, Champaign, Illinois, 1973, pp. 1-13.
4. Baker, N., C. Sandborg, D. Morris, and M. Ookhtens, Cancer Res. 37:2218 (1977).
5. Kannan, R., L. Wilson, and N. Baker, Lipids 13:887 (1978).
6. Kitada, S., L. Mwasi, J.F. Mead, and E.F. Hays, Lipids 13:464 (1978).
7. Littman, M.L., T. Taguchi, and E.H. Mosbach, Cancer Chemother. Rep. 50:25 (1966).
8. Hays, E.F., in "Virus Tumorigenesis and Immunogenesis," Edited by W.S. Ceglowski and H. Friedman, Academic Press, Inc., 1973, pp. 321-334.
9. Vredevoe, D.L., and E.F. Hays, Cancer Res. 29: 1685 (1969).
10. Peer, L.A., in "Transplantation of Tissues," Vol. II, Edited by L.A. Peer, Williams and Wilkins

Co., Baltimore, MD, 1959 pp. 165-244.
11. Ookhtens, M., and N. Baker, Cancer Res. 39:5118 (1979).
12. Bligh, E.G., and W.J. Dyer, Can. J. Biochem. 37: 911 (1959).
13. Nowinski, R.C., and E.F. Hays, J. Virol. 27:13 (1978).
14. Lavik, P.S., and C.A. Baumann, Cancer Res. 1:181 (1941).
15. Carroll, K.K., and G.J. Hopkins, Lipids 14:155 (1979).

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