The Effect of Hyperlipemia on Pulmonary Metastases of Walker 256 Carcinosarcoma in the Rat*

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SUMMARY

Rats made hyperlipemic by feeding a high fat diet for 21 days showed increased inhibition of fibrinolysis.

Tumor cells injected into these hyperlipemic animals were removed from the circulation rapidly as determined by direct cell counts and subcutaneous injection of blood into weanling animals.

Metastases in the lungs occurred much more frequently in hyperlipemic than control animals. This occurred with 100,000 cells/animal, but the results were not significant. With 25,000 cells/animal a significant increase in pulmonary metastases was noted. This increase in pulmonary metastases was seen in the animals that died before 9 weeks and in those that were sacrificed after surviving 9 weeks. Not only was the number of animals with metastases greater, but larger numbers of metastases were seen in the lungs of the individual hyperlipemic animals.

The capacity to metastasize is one of the characteristics of malignant tumors. Usual sites of metastases have been carefully documented, but the mechanism of production of metastases has not been clearly established. With the development of interest in the presence of cancer cells in the blood, this problem has again attracted attention.

If cancer cells are to produce metastases via the blood stream, they must first lodge and then grow and multiply. It seems logical to consider that fibrin formation might be of importance in this development. Alterations in the clotting mechanism in patients with cancer have been observed, but no great significance has been attached to these findings. We have undertaken to investigate the effects of alterations in the clotting mechanism on metastases of tumors.

We first endeavored to prevent or decrease the rate of tumor metastases by preventing fibrin formation with anticoagulants or by lysis of fibrin with fibrinolyisin (3, 6). It has been possible to decrease, significantly, the number of metastases and to decrease the rate of takes in secondary recipients by this means (3, 11, unpublished data).

To confirm the importance of the clotting mechanism and this theory it is also necessary to alter the clotting mechanism toward excessive coagulability and to determine the effects of these changes on the rate of metastasis. It has been shown by Wessler (15) and in our laboratory (9) that injection of serum will increase coagulability. Hyperlipemia also alters the clotting mechanism in the direction of increased coagulability or resistance of clots to lysis (4).

The present study was set up to determine the effect of altering the coagulation system by the hyperlipemic method and to test its effect on tumor metastases.

MATERIALS AND METHODS

Animals.—The animals were male white rats, Charles River Farm.

The donor and primary recipient animals were young males weighing 180–200 gm. The weanling animals used for subcutaneous inoculation of blood (secondary recipient) weighed 80–90 gm.

Tumor cells.—These were Walker 256 carcinosarcoma carried in the ascites form. This has been maintained for many years in this form in this institution.

High fat diet: contents.—Butter, 40 per cent; General Biochemicals Inc., Laboratory Park, Chagrin Falls, Ohio.
casein, 20 per cent; cholesterol, 5 per cent; sucrose, 21.5 per cent; sodium oxalate, 2.0 per cent; propylthiouracil, 0.3 per cent; Celluloflor (a cellulose flour), 5.0 per cent; salt mixture (Wesson’s), 4.0 per cent (described in the journal Science, 75:339, 1932); choline chloride, 0.2 per cent; and vitamin mixture, 2.0 per cent.

Vitamin mixture.—Vitamin A concentrate, 4.5 gm. (200,000 units/gm); Vitamin D concentrate, 0.25 gm. (400,000 units/gm); D-l-a-tocopherol, 5.0 gm.; ascorbic acid, 45.0 gm.; inositol, 5.0 gm.; menadione, 2.25 gm.; p-aminobenzoic acid, 5.0 gm.; nicotinic acid, 4.5 gm.; riboflavin, 1.0 gm.; pyridoxine hydrochloride, 1.0 gm.; thiamine hydrochloride, 1.0 gm.; calcium pantothenate, 3.0 gm.; biotin, 0.09 gm.; and folic acid, 0.09 gm.

Diet.—Control animals were fed a stock diet with water ad libitum. They ate about 10 gm/day. The animals fed the high fat diet ate about 10 gm of the diet/day for 20 or 21 days prior to tumor injection. Feeding of the high fat diet was continued for 3 days after the tumor inoculation, and the animals were then placed on a regular diet.

Anesthesia.—The anesthesia was ether, by inhalation.

Tumor.—Ascites fluid was aspirated from a donor animal, and a direct tumor cell count was made. The fluid was then diluted with saline to give the desired counts (100,000 cells/ml and 25,000 cells/ml). Fresh transplantable tumor cells were used for each injection. The viability of these cells was established by tumor growth following subcutaneous injection in weanling animals.

Intravenous tumor injection: (primary recipients).—The femoral vein was exposed, and 1 ml. of the tumor cell suspension (100,000 or 25,000 cells/ml) was injected directly into the vein (11). Counts of cells in the blood were made by Seal’s flotation technic (13).

Injection into secondary recipients.—Five cc. of blood was drawn by femoral vein puncture from each primary recipient. Blood from two animals was pooled (10 ml). One (1) ml. of this was injected subcutaneously into each of ten weanling rats. The recipients were observed 9 weeks for tumor growth. Post-mortem was performed when they died or when sacrificed at 9 weeks.

Antifibrinolytic activity (APF) was determined by a previously reported method (4). Cholesterol was determined by the method of Abell et al. (1).

RESULTS
Series I

Effect of high fat diet on fibrinolytic activity.—To determine that the diet could produce increased antifibrinolytic activity in the animals, the following experiments were performed.

1. Control rats (twenty animals) were fed a regular diet.
2. Fat-fed rats (fifteen animals) were fed the high butter-oil diet.

Each animal was given an infusion containing 3000 units of streptokinase on the 1st, 10th, 14th, and 20th days of the feeding experiment. Fifteen (15) minutes after each infusion blood was drawn for determinations of fibrinolytic and antifibrinolytic activity of the blood. The total cholesterol level was determined on days 1 and 20.

The antifibrinolytic (APF) activity of the blood of the control animals remained essentially unchanged during the course of the experiments. It was 352 min. on the first day and 379 min. on the 20th day. Cholesterol also remained essentially the same, being 52 and 66 mg. per cent with a mean of 62 mg. per cent (Chart 1).

With Group II (fat-fed animals), the APF and cholesterol levels rose significantly. APF on day 1 was 368 min., essentially the same as the controls. On day 10 it was 940 min., and on the 21st day it was 1440 min. Cholesterol rose from 60 mg. per cent to 1250 mg. per cent in the 20 days (Chart 1). Gross lipemia was also apparent at this time. The animals remained healthy.

It was apparent that maximum lipemia, cholesterol levels, and APF levels were present at 20 days. In previous experiments it had been shown...
that clot formation and lysis were markedly altered by lipemia and APF levels of this magnitude (4, 9). It, therefore, seemed logical to choose this time interval as the one at which the most marked changes in formation of metastases would result, if increased APF and lipemia were important.

**Series II**

**Metastases with 100,000 cells.**---

Control (twenty rats). These animals were fed a normal diet.

Fat-fed (twenty rats). These animals received the high fat diet for 21 days.

After 3 weeks on the control or high-fat diet, 100,000 tumor cells were injected into each animal (direct injection into the exposed femoral vein).

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**Series III**

**Effect of lipemia on circulating cancer cells.**---To better understand the mechanism of metastases we consider it important to know how long the cells remain in the circulation. This can be determined by direct count of the number of cells remaining in the circulation (A) or by the ability to transmit tumor by injection of blood into recipient animals (B).

(A) Fifty rats (25 control and 25 fat-fed) were used for the direct cell count method (Chart 2).

Each rat was given injections of 25,000 cells (directly into the femoral vein). At intervals of 1, 5, 10, 15, 20, and 30 minutes and at 1, 2, 3, and 4 hours, blood was withdrawn either from the vena cava or aorta of an individual animal for each time interval. The animals were then sacrificed. Tumor cell counts of the blood were made by the Seal flotation technic (13).

The results of the cell counts of blood from the vena cava and aorta of primary recipients are shown in Chart 2. There were fewer circulating cells in the fat-fed animals than in the controls, even at 1 minute. At 1 hour no cells could be found in the vena cava blood of the fat-fed animals, whereas cells could still be found in the controls. In the aortic blood no cells were found in the fat-fed animals after 15 minutes. In the control animals, by contrast, a few cells were still found after 3 hours in both the vena cava and aorta.

(B) The viability of cells in the circulating blood was determined by secondary subcutaneous transplant with the use of 62 primary recipient animals (31 control and 31 fat-fed) and 310 secondary recipient (weanling animals). As before, 50,000 cells were injected into the femoral vein of each primary recipient. Five ml. of blood was withdrawn from either the vena cava or aorta of individual animals at the times stated in (A) above. Blood was pooled from the vena cava of two animals, or the aorta of two animals at each time interval. One ml. of this pooled blood was then injected subcutaneously into a single weanling animal. For each time interval blood from the
aorta was injected into ten animals and blood from the vena cava into ten animals. The number of takes in secondary recipients (Chart 3) were much fewer in the treated than in the control animals. The curves are similar to the curves of the cell counts (Chart 2). In other studies we have observed that, with this particular preparation and with our counting techniques, approximately 500–1000 cells/ml must be present in blood to obtain a take in a secondary recipient.

Series IV

Metastases with 25,000 cancer cells (Table 1).—The smaller numbers of cancer cells were used in this series to observe fewer takes in the controls. With 100,000 cells the controls had so many takes that a significant differential could not be obtained. A large enough group of animals to give significant results was also used.

One hundred control and 100 fat-fed animals were used in this series of experiments (total, 200 animals).

After 20 days' feeding of either the control or high fat diet 25,000 tumor cells were injected into the femoral vein of each rat under direct vision.

Of the 100 control rats, 44 survived for 9 weeks, and 26 of the 100 fat-fed animals survived 9 weeks (Table 1). From this table it is apparent that death occurred somewhat earlier and that metastases were more common early in the hyperlipemic than in the control animals. In the individual animals there were usually more metastases per lung in the hyperlipemic than in the controls. It should be noted that the animals which died without metastases before 9 weeks succumbed because of large local tumor growths at the site of inoculation. This was probably due to spillage. It should also be noted that 26 control animals died of local tumor as compared with 21 hyperlipemic animals. This would indicate that the high fat diet and hyperlipemia do not favor tumor growth.

Table 1

<table>
<thead>
<tr>
<th>Survival time (weeks)</th>
<th>Hyperlipemic rats</th>
<th>Control rats</th>
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<tr>
<td></td>
<td>Total no.</td>
<td>No. pulmonary metastases</td>
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<tr>
<td>1</td>
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<tr>
<td>8</td>
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<tr>
<td>9*</td>
<td>26</td>
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Total group

100           | 61          | 100          | 34          |

* Sacrificed at 9 weeks.

In the total series 61 per cent of the hyperlipemic animals developed metastases, compared with only 34 per cent of the control animals. It is in the animals which survived to be sacrificed that the greatest difference occurred. Only 26 hyperlipemic animals survived, and of these 30 per cent had gross pulmonary metastases. Forty-four of the control animals survived, and of these only 9 per cent had gross pulmonary metastases.

Discussion

Many investigators have shown that malignant tumor cells can be present in the blood without producing metastases. The metastatic rate has been shown to be affected by the malignancy of the tumor and host susceptibility. These alone do not seem to explain completely the variations in metastatic rate seen in controlled experiments.
when similar animals with identical/tumor inoculum were used.

It is obvious that fixation or at least stoppage of tumor cells in the capillaries or other small vessels will be necessary before metastases can occur. It also logical to presume that clumping of cells into aggregates large enough to block the vessels would be a significant factor. Since fibrin formation has been shown to be involved in cell clumping and fixation to vessel walls, it seemed logical to investigate the importance of this mechanism in the formation of metastases.

In previous experiments it was shown that decreasing the coagulability of blood by the use of heparin or dissolving fibrin by use of fibrinolysin decreases the metastases of VX2 (6), Brown-Pearce (6), and Walker 256 (11) tumors. This was not owing to direct effect on the tumor by the agent, as shown by incubation of tumor cells with the agent prior to subcutaneous inoculation.

To establish more definitely that the clotting mechanism itself is a significant factor it seemed important to show that an increased clotting tendency would result in an increase in metastatic rate. Increased clotting may result from an actual hypercoagulability of the blood or from increased resistance to the dissolution of fibrin by the normal fibrinolytic mechanism. It has been shown that hyper-fibrinogenemia and hyperlipemia both result in formation of resistant thrombi (4, 9), and a temporary hypercoagulable state can be produced by injection of serum into animals (9, 15). Any one of these mechanisms might be used to test the hypothesis that metastases would be accelerated by increasing the clotting tendency. Hyperlipemia was used for this series of experiments. It was first shown that by feeding it was possible to produce hyperlipemia in rats and that this resulted in an inhibition of fibrinolysis.

When an injection mixture was used containing 100,000 cells/ml there was an increase in metastases in the fat-fed animals, but this was not significant ($P > .025$). A smaller number of cells (25,000) was then used in a larger number of animals, and here the data were more convincing.

Additional studies were made to determine that the high lipide diet affected the circulation of the tumor cells. It was shown that the number of cells in the circulating blood decreases much more rapidly in lipemic animals than in the controls. This would indicate that the cells were being removed from the circulation probably by clumping and fixation in the capillaries. These findings contrast with the experiments with heparin and fibrinolysin where the cells remained in the circulation as long or longer in the treated animals than in the controls.

Explanation of the decreased number of secondary recipient takes following subcutaneous injection of blood is necessary. When anticoagulants were used there was also a decrease in these takes, which was thought to be due to failure of the cells to clump into groups of cells of sufficient mass to produce growth. It might also be considered that nonviable cells were circulating while the viable cells were filtered out in the animals’ capillaries. With the hyperlipemic animals the most likely explanation is that the cells clump, stick, and are filtered out of the circulation rapidly. Fewer cells remain in the circulation. After 15 minutes few or no cells are circulating, and it would be expected that few subcutaneous tumors could be produced by the injection of blood.

It might be considered that the high fat diet increases tumor growth, to explain the greater number of metastases in the fat-fed animals. The fact that more control animals had local tumor growth would tend to discount this idea.

Certain clinical observations may be correlated with these experimental results. Patients with moderately advanced cancer usually have high fibrinogen levels and high fibrinolytic inhibitor levels. When they are eating well, their lipides may also be higher than normal. Operative procedures result in a rise in the fibrinogen and fibrinolytic inhibitors. It is conceivable that apparent rapid increase in metastases occasionally seen after operation may be related to this mechanism. It is hoped that, with additional animal experiments, a method of diminishing the metastatic tendency by alterations of the clotting mechanism can be devised which will be practical in clinical situations. If these results can be confirmed they will warrant a more careful evaluation of the clotting mechanism in all patients with malignancy.

ACKNOWLEDGMENTS
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REFERENCES
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The Effect of Hyperlipemia on Pulmonary Metastases of Walker 256 Carcinosarcoma in the Rat

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