Lymphatic Obstruction in Carcinomatous Ascites

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SUMMARY

The i.p. inoculation of C3H mice with $5 \times 10^4$ cells of a transplantable ovarian carcinoma invariably evokes accumulation of large amounts of ascitic fluid. Histological and pharmacotherapeutic studies indicate that obstruction to peritoneal lymphatic drainage is a key factor in the formation of carcinomatous ascites in this model. In the early stages of ascites formation, an intense inflammatory reaction appears to occlude the conduits that connect the peritoneal cavity to the subdiaphragmatic lymphatic plexus. This inflammatory reaction, elicited by the presence of tumor cells within the peritoneal cavity, can be inhibited with high-dose systemic corticosteroid therapy. Ascitic fluid accumulation in animals so treated is markedly retarded. Tumor cells do not gain access to lymphatic capillaries draining the peritoneal cavity until ascitic fluid accumulation is massive. Systemic anticoagulation with heparin or sodium warfarin does not prevent lodgment of tumor cells within these lymphatic capillaries, nor does it alter the pattern of ascitic fluid accumulation. Various considerations suggest that excess production of ascitic fluid is not a likely pathogenetic factor in murine carcinomatous ascites.

INTRODUCTION

In 1953, Holm-Nielsen (7) postulated that carcinomatous ascites might be due to tumor cell obstruction of the lymphatics that drain the peritoneal cavity. Recent work (4) involving the study of rates of egress into the peripheral bloodstream of i.p.-injected erythrocytes confirmed that lymphatic obstruction does indeed occur prior to the onset of ascitic fluid accumulation.

Fluid that is free in the peritoneal cavity usually returns to the intravascular compartment very rapidly. It is either absorbed directly into blood capillaries in the peritoneal membrane, or it takes a more tortuous route through the peritoneal lymphatic drainage system. The lymphatics that serve the peritoneal cavity originate in a plexus on the inferior surface of the diaphragm. Lymphatic channels within the diaphragm connect this plexus to another plexus on the pleural surface of the diaphragm. From there, fluid passes to upper mediastinal nodes by way of substernal lymphatic vessels. The bulk of lymphatic fluid originating from the peritoneal cavity returns to the intravascular space via the right lymphatic duct. Of course, to enter the lymphatic drainage system initially, fluid must pass through the peritoneal membrane lining the inferior surface of the diaphragm (3, 13).

The precise location of the lymphatic blockage associated with carcinomatous ascites could not be determined from the kinetic data of the erythrocyte-egress experiments cited earlier. The work reported here involves histological and pharmacotherapeutic studies intended to determine precisely where the obstruction occurs, and to shed light on its specific nature.

MATERIALS AND METHODS

Animals. All experiments were conducted with 1- to 3-month-old male and female C3H mice weighing 18 to 25 g.

Tumor. The tumor used in these experiments (generously supplied by Dr. R. Knapp and Dr. S. Order, Boston, Mass.) was an ovarian carcinoma that arose spontaneously in a C3H female mouse at The Jackson Laboratory (2). The tumor has been maintained by serial i.p. transplantation.

Implantation of Tumor Cells i.p. Fluid was withdrawn from the abdominal cavity of an ascitic mouse through a 20-gauge needle into a sterile syringe. A cell count was determined, the ascitic fluid was appropriately diluted with sterile 0.9% NaCl solution, and $5 \times 10^4$ cells were injected i.p. into test animals through a 27-gauge needle in a bolus of 0.1 to 0.3 ml.

Preparation of Diaphragms for Histology. Animals were sacrificed by exposure to ether. After removal of skin from the legs to the neck, 2 to 3 ml of Zenker-formalin were injected i.p. The lower extremities were then amputated, leaving the peritoneal cavity undisturbed. The neck was severed and the resultant block of tissue, which included the entire abdomen and thorax, was fixed in Zenker-formalin for 24 hr. After fixation and rinsing were complete, the diaphragm and its supporting bony and soft-tissue structures were carefully dissected free. The specimens were decalcified, mounted in paraffin, sectioned in step-serial fashion, and stained by the method of Fraser and Lendrum (9).

Anticoagulation with Heparin. Sterile Depo-Heparin (Upjohn Co., Kalamazoo, Mich.) was injected s.c. every 8 hr in doses of 40 to 60 units. The level of anticoagulation achieved was monitored by measurement of the partial thromboplastin time of blood taken from the tails of test animals. Each dose of heparin produced an indefinitely prolonged partial
thromboplastin time for several hr. The partial thrombo-
plastin time then gradually decreased, but never fell below
1.5 to 2 times normal during the active course of the
experiment. Mortality directly attributable to heparin anticoagulation with this schedule was 35% (8 of 23) over 9
days. Test animals were inoculated with tumor only after
adequate levels of anticoagulation were achieved.

Anticoagulation with Warfarin. Sodium warfarin (Cou-
madin; Endo Labs, Inc., Garden City, N. Y.) was injected
s.c. daily in doses of 0.075 to 0.195 mg. Prothrombin time
was measured daily using samples of tail blood taken from
test animals. Several days were required to bring the
animals into an adequate range of anticoagulation. The
prothrombin times of test animals were kept from 1.5 to 4
times normal throughout the active course of the experi-
ments. Mortality resulting from Coumadin anticoagulation
was 45% (9 of 20) over 20 days. Test animals were
inoculated with tumor only after adequate levels of anticoagulation were achieved.

Administration of Hydrocortisone. Hydrocortisone so-
dium succinate (Solu-Cortef; Upjohn) was injected s.c.
every 8 hr in doses of 3.3 mg, for a total of 10 mg/day.
Tumor cells were injected 2 hr after the 1st dose of corticosteroid. In 1 experiment, hydrocortisone was discon-
tinued 8 days after tumor cells had been inoculated i.p. This
allowed observation of the pattern of ascites accumulation
after a period of steroid treatment. In a 2nd experiment, the
administration of hydrocortisone was not discontinued.

India Ink. The absorption of India ink into diaphragmatic
lymphatics was observed both in normal mice and in mice
previously inoculated with tumor. India ink, 0.75 ml, was
injected i.p. 24 hr prior to sacrifice of the test animal.

RESULTS

Absorption of India Ink from the Abdominal Cavity. Fig. 1
shows a representative area of mouse diaphragm 24 hr after
i.p. injection of 0.75 ml of India ink. Ink has passed readily
through the peritoneal membrane and filled the subdia-
phragmatic lymphatic plexus. Lymphatic vessels within the
diaphragmatic musculature are filled with ink as they
course toward a very markedly distended lymphatic on the
pleural surface. Retrosternal lymphatic vessels and upper
mediastinal nodes, not shown, are also heavily laden with
ink.

The thoracic duct contained no ink, corroborating the
long-standing observation that the thoracic duct plays no
significant role in the drainage of particulate matter from
the abdominal cavity (13).

Fig. 2 shows a corresponding area from the diaphragm of
a mouse that had been inoculated i.p. 48 hr previously with 5
x 10⁶ tumor cells. Again, 0.75 ml of India ink was injected
i.p. 24 hr prior to sacrifice. Although penetration of the
peritoneal membrane by ink is evident, it is grossly less
pronounced than in the normal animal. Some of the
lymphatics on the pleural surface contain ink, but they are
not at all distended. Some pleural-surface lymphatics
contain no ink at all, a circumstance not observed in the
normal animal. Substernal lymphatic channels and mediastin-
tnal nodes still show heavy blackening.

A representative area of diaphragm from a mouse
inoculated with tumor 5 days previously is shown in Fig. 3.
The tumor cells themselves do not prevent mechanical
contact between the ink and the diaphragmatic peritoneum.
Nevertheless, virtually no ink has penetrated through the
peritoneal membrane into the subdiaphragmatic lymphatic
plexus. No i.m. lymphatic channels can be found containing
ink, nor has any ink reached the plexus on the pleural
surface of the diaphragm. In this animal, the substernal
channels and mediastinal nodes were free of ink.

Animals studied later than 5 days after tumor inoculation
also show no penetration of ink from the peritoneal cavity
into the subdiaphragmatic lymphatic plexus.

It can be inferred from these results that excess produc-
tion of ascitic fluid cannot by itself account for the
accumulation of ascites. Were excess production of fluid the
only cause of ascites, ink would flow even more rapidly than
usual into the lymphatic drainage pathways. Clearly, the
flow of ink out of the peritoneal cavity is obstructed. It
appears, from Figs. 1 to 3, that the obstruction occurs in the
conduits between the peritoneal cavity and the subdia-
phragmatic lymphatic plexus. A significant negative observation
is that tumor cells do not by themselves directly cause
mechanical obstruction in the early stages of ascites accu-
mulation.

Subdiaphragmatic Reaction to i.p. Tumor Cells. With
attention focused to the subdiaphragmatic lymphatic plexus
and the peritoneal membrane lining it, the next set of
histological studies was undertaken. Animals were sacri-
ficed at regular intervals after tumor inoculation, without
any i.p. injection of India ink.

Fig. 4 shows the diaphragm of a mouse 2 days after i.p.
tumor inoculation. Tumor cells adhere to the diaphragmatic
peritoneum. Nuclei of very thin mesothelial cells of the
peritoneal membrane can barely be seen. A pleural-surface
lymphatic is visible; it is not distended and it contains no
nucleus cells. Blood capillaries within the diaphragmatic
musculature are visible, but not prominent, and they are
uniformly distributed.

Fig. 5 shows the subdiaphragmatic lymphatic plexus and
adjacent peritoneal membrane from a mouse 7 days after
tumor inoculation. By this time, no India ink would
penetrate into the lymphatic lumen. The significant differ-
ces to note when comparing this section to that in Fig. 4
are the changes involving the mesothelial cells of the
peritoneal membrane. So thin as to be nearly invisible in
previous sections, these cells have become thickened and
crowded, and their nuclei have become much more pro-
nounced. Upon examination of a section from an animal 9
days after tumor inoculation (Fig. 6), the progression of a
dramatic inflammatory response can be appreciated. Large
numbers of mononuclear cells with elongated, spindle-
shaped nuclei have invaded the area. These cells, most
probably lymphocytes, are accompanied by some mature
lymphocytic forms. Very few polymorphonuclear leuko-
cytes are observed. The area of mononuclear cell invasion is
studded with an intense proliferation of blood capillaries.
Although not readily apparent from the reproduced micrograph, the entire reaction occurs on the diaphragmatic side of the mesothelial cell layer of the peritoneal membrane.

Fig. 7, from an animal 12 days after tumor inoculation, shows a continuation of the inflammatory reaction. Proliferation of blood capillaries into the inflammatory zone is marked. Lymphocytes are now "backed up" into lymphatic capillaries within the diaphragmatic musculature. No tumor cells have left the confines of the peritoneal cavity, nor have they implanted on the peritoneal surface. The inflammatory reaction of the peritoneal membrane is limited to that portion of the peritoneal membrane lining the diaphragm. The visceral peritoneum is essentially normal. Examination of parietal peritoneum from the anterolateral abdominal wall (Fig. 8) reveals only a moderate thickening of mesothelial cells. By this time after tumor inoculation, the animals are very noticeably ascitic.

Fig. 9 shows the beginning of an involution of the inflammatory response. The thickness of the inflammatory zone of mononuclear cells and new blood capillaries is diminished in this section of diaphragm from a mouse 15 days after tumor inoculation. The lumen of the lymphatic capillary shown is free of tumor invasion. However, elsewhere in the diaphragm, tumor invasion into lymphatics has begun. This invasion progresses while the inflammatory response recedes. By 18 days after tumor inoculation (Fig. 10), the entire inflammatory zone is replaced by tumor cells. The subdiaphragmatic lymphatic plexus and intradiaphragmatic lymphatic capillaries are solidly impacted with tumor cells, and mononuclear cells have disappeared from the scene entirely.

It would appear, speaking teleologically, that the host mounts a defense against the dissemination of tumor cells from the peritoneal cavity. Although successful for many days, the defense eventually withers and tumor cells gain access to the lymphatic drainage system.

Thus, within 4 days after tumor inoculation, lymphatic drainage of i.p.-injected India ink is blocked. Swelling of the mesothelial cells of the peritoneal membrane is visible with light microscopy by Day 7 after tumor inoculation. Mesothelial cell swelling is the 1st sign of an intense inflammatory response which ultimately produces a marked thickening of the wall separating the peritoneal cavity from the lumen of the subdiaphragmatic plexus. A natural question emanating from these data is whether or not the observed inflammatory response might be directly related to the observed early blockage to lymphatic flow and the subsequent accumulation of ascitic fluid.

**Effects of Systemic Hydrocortisone.** To explore this possible relationship, an effort had to be made to alter the inflammatory response. To this end, 3.3 mg of hydrocortisone succinate were administered s.c. to test animals every 8 hr. Chart 1 depicts the effect of this regimen on the accumulation of ascitic fluid. Two control groups were used. One was a group of animals that received no tumor, but did receive corticosteroids. The 10.5% increase in their average weight over the course of the experiment is slightly greater than the weight gain observed in normal, untreated animals of the same age over the same span of time. Animals receiving \(4 \times 10^4\) tumor cells and no steroids constituted the other control group. First signs of an increase in weight occurred 6 to 10 days after tumor inoculation. Following the usual pattern, weight increased progressively. By Day 18, the average weight of animals in this group had increased by 24.6 g, or over 113%. Animals treated with the same dose of steroids and inoculated with the same number of tumor cells, respectively, as the controls, constituted the test group. These animals gained weight slightly more rapidly than the steroid-only controls. Nevertheless, their weight gain was dramatically less than that of the tumor-only control group, indicating that steroid therapy definitely retarded the accumulation of ascitic fluid.

In the animals of the test group, i.p. fluid was present. Roughly, 2 to 3 ml of a thick fluid rich in tumor cells was found at autopsy on Day 18 in these animals. By contrast, at Day 18 in the tumor-only group, over 20 ml of thin fluid with a relatively low tumor cell concentration had accumulated.

Chart 2 depicts an experiment in which hydrocortisone therapy was discontinued 8 days after tumor inoculation. After withdrawal of hydrocortisone, test animals began to
accumulate ascites. The rate at which test animals accumulated ascites after Day 8 closely paralleled the rate at which controls accumulated ascites after Day 0. In other words, hydrocortisone effectively retarded ascites accumulation throughout the duration of its administration, but had no lingering effect on ascites accumulation after it was withdrawn. Another experiment showed that ascites accumulates in a routine manner when the tumor cell inoculum is derived from ascitic fluid of animals treated with high-dose corticosteroids. These data tend to demonstrate that systemic hydrocortisone in high doses does not damage or alter i.p.-inoculated tumor cells.

The histological effect of hydrocortisone therapy is shown in Fig. 11. This is a representative section of diaphragm taken from a hydrocortisone-treated animal 13 days after tumor inoculation. Compared to Fig. 7, showing an untreated animal 12 days after tumor inoculation, the striking absence of an inflammatory response is evident. Peritoneal mesothelial cells are mildly enlarged, but neither infiltration of the area with mononuclear cells nor neovascularization is evident.

Obstruction of the lymphatic drainage of i.p.-injected India ink in steroid-treated animals is total by 5 to 7 days after tumor inoculation. This compares with total obstruction to ink by 4 to 5 days after tumor inoculation in untreated animals. The difference is only slight and may not be significant. Nevertheless, this observation is consistent with other data presented, suggesting that early obstruction to lymphatic flow in carcinomatous ascites is a by-product of the inflammatory response elicited by i.p. tumor cells.

Effect of Anticoagulation with Heparin and Warfarin. A large and growing body of literature (5, 8, 10, 11) has raised the possibility that tumor metastases can be significantly decreased by systemic anticoagulation. The decreased incidence of metastases observed in some systems has been speculatively attributed to an inhibition of the process by which tumor cells circulating in the bloodstream implant onto a capillary wall. Fig. 10 demonstrates that, in the latter stages of ascites accumulation, direct lymphatic obstruction by impacted tumor cells plays a role. Experiments were undertaken to determine whether systemic anticoagulation might inhibit ascites accumulation.

Systemic anticoagulation was achieved by means of s.c. injections of either warfarin or heparin. Warfarin anticoagulation was monitored to maintain the prothrombin time of test animals at 1.5 to 4 times normal. Heparin anticoagulation was adequate to keep the partial thromboplastin time at least 1.5 to 2 times normal levels. During most of the course of heparin anticoagulation, the partial thromboplastin time was prolonged indefinitely. The usual inoculum of $5 \times 10^6$ tumor cells was administered only after adequate anticoagulation was achieved. Neither modality of anticoagulation altered the pattern of accumulation of ascitic fluid in comparison to control animals that were not anticoagulated.

A high mortality rate resulting from complications of anticoagulation prevented continuing anticoagulant therapy indefinitely. However, anticoagulation was continued both with warfarin and heparin until gross ascites had clearly begun to accumulate in test animals. No delay in onset of ascites accumulation was noted. This is in contrast to the delay observed during a time-limited course of hydrocortisone administration (Chart 2).

DISCUSSION

The pathogenesis of carcinomatous ascites has been ascribed to production of ascitic fluid in excess of the capacity of the unimpaired lymphatic drainage system to remove it (6). The data presented in this series of experiments tend to refute that contention, at least in the murine model under discussion. A theory invoking increased fluid production as the sole cause of carcinomatous ascites would not explain the directly observed phenomenon of blockage of lymphatic drainage of i.p.-injected India ink. If no element of obstruction were present, ink would be swept into the lymphatics at an increased rate.

On the other hand, these experiments do not unequivocally establish obstruction to lymphatic flow as a sole cause of carcinomatous ascites either. Increased fluid production is not ruled out. However, a popular notion regarding “weeping” of fluid from sites of peritoneal tumor implants was not corroborated. Ascites accumulated long before tumor cells actually implanted on the peritoneal membrane. The inflammatory response noted during the initial phase of ascites formation might be cited as a more likely source of increased ascitic fluid production. However, this reaction did not involve the entire peritoneal membrane. It was essentially limited to that portion lining the diahragm.

Excess fluid produced in this region would be more likely to drain directly into diaphragmatic lymphatics than to transude into the peritoneal cavity.

A further hint regarding the possible role of increased fluid production is provided by studying the charts relating weight gain and time after tumor inoculation. The maximum rate of weight increase ever observed is 1.5 to 2 g/day. This translates directly to a maximum rate of ascitic fluid accumulation of 1.5 to 2 ml/day. Studies in humans (12) show that the normal flow of proteinaceous fluid through the lymphatics draining the peritoneal cavity is over 75% of the total blood volume. The total blood volume of a 30-g mouse is about 2.3 ml (11). If human data are relevant for the mouse, a normal daily flow of fluid through the peritoneal lymphatic drainage system of 1.5 to 2 ml would not be unthinkable. Thus, the maximum rate of ascitic fluid accumulation could be accounted for entirely by total obstruction of the normal flow of fluid through the draining lymphatics. Furthermore, total obstruction of lymphatics is observed histologically. Moreover, one might expect that the maximum rate of ascitic fluid accumulation would be much in excess of 1.5 to 2 ml/day if markedly increased production of fluid were also a significant pathogenetic factor.

Direct histological investigation reveals that mechanical obstruction of lymphatic capillaries by tumor cells does not occur until long after gross ascites is evident. However, obstruction of the conduits between the peritoneal cavity...
and the lumen of the subdiaphragmatic lymphatic plexus begins by the 2nd day after tumor inoculation. Various data presented suggest that this early obstruction is one of the first manifestations of an inflammatory reaction that becomes increasingly pronounced with the passage of time.

The precise nature of these obstructed "conduits" is controversial. Three layers separate the peritoneal cavity from the lumen of the subdiaphragmatic lymphatic plexus: peritoneal mesothelial cells, the collagenous peritoneal basement membrane, and endothelial cells of the lymphatic capillaries. The exact manner by which particulate material, corpuscles, or fluid traverses this 3-layered barrier remains unresolved (13). In any event, it seems apparent that small changes in the delicate architecture of this region could easily produce marked changes in its permeability. Early changes in the mesothelial cell layer are easily observed. Somewhat later, the collagenous layer thickens and becomes involved in a massive infiltration by mononuclear cells and blood capillaries. Systemically administered hydrocortisone markedly inhibits these changes and also retards the accumulation of ascitic fluid.

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REFERENCES

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