Blood Coagulation Changes in Mice Bearing Lewis Lung Carcinoma, a Metastasizing Tumor

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SUMMARY

In view of the possible role of platelets and coagulation mechanisms in the growth and dissemination of solid tumors, a number of hematomal parameters were followed during development of an experimental syngeneic tumor in mice, Lewis lung carcinoma. This tumor, when transplanted i.m. in C57BL/6 mice, grows locally and spontaneously metastasizes to the lungs. The transplanted animals survive for about 4 weeks. Metastases are visible from the third week. A slight but constant increase in plasma fibrinogen level and marked thrombocytopenia were first observed during the second week after tumor implantation. No other significant changes in coagulation and fibrinolysis parameters were detected. Moreover, the animals developed marked hemolytic anemia, possibly microangiopathic in origin. $^{125}$I-labeled fibrinogen survival was decreased by about 20% during the second week after tumor implantation and was not further reduced later. Fibrinogen turnover was progressively accelerated, being more than doubled by the end of the third week. Labeled fibrinogen accumulated in the primary tumor and in the lungs (its rate of disappearance from the tumor was much slower than that from lungs or blood). $^{51}$Cr-labeled platelet survival did not change throughout the observation period, whereas platelet turnover was markedly reduced from the end of the second week, suggesting defective platelet production. $^{51}$Cr-labeled RBC survival was drastically reduced to about 30% of the controls starting from the second week.

The occurrence of low-grade, localized intravascular coagulation could be suggested on the basis of these data. Moreover, when Lewis lung carcinoma cells were abruptly injected i.v. through the tail vein, more impressive signs of intravascular coagulation could be seen. Indeed, there was a rapid decrease in the number of platelets, a reduction in fibrinogen, and an increase in fibrin-fibrinogen degradation products. The effects of i.v. injection of Lewis lung carcinoma cells indicate a relevant interference of cancer cells with the hemostatic system. In contrast, the tenuous evidence for coagulation disorders in animals receiving injections of tumor cells i.m. seems to indicate a limited effect on hemostasis of the same cells during i.m. tumor growth.

INTRODUCTION

It has been suggested that factors involved in hemostasis and thrombus formation play a role in tumor growth and metastasis formation (13, 14). In 1958, O’Meara (24) proposed that infiltrative tumor growth could be facilitated by the deposition of a fibrin network at the tumor periphery. Platelet aggregates have also been found to assist the passage of blood-borne cancer cells into extravascular spaces (12, 13). This view received indirect support from experiments showing that, in animals treated with antiaggregating, anticoagulant, or fibrinolytic agents, the number of circulating cancer cells and/or their persistence in the blood could be reduced; however, the beneficial effect of such a therapeutic approach has been questioned (1, 5, 12, 14, 16, 26, 30). Moreover, much criticism can be rendered against most of these investigations (29); in many studies all gardens were used, and dissemination after acute i.v. tumor cell injection instead of spontaneous metastasis followed; in addition, treatments with antithrombotic agents were evaluated without accounting for possible modifications of the hemostatic parameters in tumor-bearing animals and the possible effects of these drugs on other functions of the host or on the tumor cells (3).

The aim of this investigation was to study the evolution of some hemostatic parameters in mice bearing 3LL; in this experimental model spontaneous and progressive metastases after i.m. injection of cancer cells have been obtained under syngeneic conditions (18, 27, 28). To better understand the mechanism(s) by which 3LL might interfere with the hemostatic parameters, acute experiments were also performed by injecting 3LL cells i.v.

MATERIALS AND METHODS

Animals and Tumor. Six hundred male C57BL/6 mice, obtained from Charles River Breeding Laboratory, Calco, Italy, and weighing 22 to 24 g at the start of the experiments, were used. The 3LL was maintained i.m. by passages every 3 weeks. Single-cell suspensions were obtained by mechanical homogenization of tumor fragments in a Virtis homogenizer in phosphate-buffered saline (Dulbecco) without cal-

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2 Recipient of a Wellcome Italian Research travel grant. To whom requests for reprints should be addressed.

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3 The abbreviations used are: 3LL, Lewis lung carcinoma; FDP, fibrin-fibrinogen degradation products; IVC, intravascular coagulation.
The cells were then passed through stainless steel filters and washed in isotonic 0.9% NaCl solution at 4°C. Viability was determined by trypan blue exclusion. After an i.m. injection of 2 × 10^6 cells, the primary tumor was palpable on Days 10 to 11 and was accurately measurable by Day 14, when it had a mean weight of approximately 0.2 g; a plateau in weight increase was reached after Days 21 to 22. In the group of tumor-bearing mice used in this study, the mean survival time was 28.6 ± 1.6 days, and the mean primary tumor weight at death was 7.1 ± 0.2 g.

Under these conditions lung metastases became detectable around Days 18 to 19 and at death reached a mean number of 19.2 ± 0.8 per animal with a mean metastatic total weight of 141.5 ± 11.2 mg (Chart 1). Tumor cells were present in the circulation from the 9th day, as detected by a biologic assay. In some experiments 3LL cells, prepared as described above, were resuspended in 0.1 ml buffer and were rapidly injected into the tail vein of normal mice at a concentration of 4 × 10^6 per animal.

**Blood Coagulation Assays.** For each series of experiments, groups of at least 5 tumor-bearing and control animals were sacrificed at 2- to 3-day intervals during the whole observation period. Blood was collected by intracardiac puncture with a Luer Lock 25 G needle from open-chested animals under slight ether anesthesia. For anticoagulation, 9 parts of blood were mixed directly in a disposable plastic syringe with 1 part 0.126 M trisodium citrate.

Platelets were counted by phase microscopy after dilution of blood with ammonium oxalate by means of a capillary-standardized pipeting system (Unopette; Becton-Dickinson Italia, Novate Milanese, Italy). Fibrinogen concentration was measured using a commercially available reagent (Nygard, Immuno, Pisa, Italy). The spontaneous fibrinolytic activity was measured by the fibrinolytic potential assay of Fearnley (11) using 0.4 ml total blood.

Platelet-poor plasma was obtained by centrifugation of anticoagulated blood at 3000 × g for 15 min. Prothrombin time was measured using commerically available rabbit brain thromboplastin (Hyland, Profarco, Milano, Italy). Factor V plasma concentration (25) was measured using as a substrate the plasma of a congenitally deficient patient. The plasma was kindly provided by Dr. J. Vermlegen, University of Leuven, Leuven, Belgium. FDP were measured in the serum obtained by clotting platelet-poor plasma according to the method of Merskey et al. (22); the staphyloccocal clumping test was performed as described by Donati et al. (9), with the use of a kit generously provided by Biochemia, Milano, Italy.

**Hematological Studies.** The following tests were performed using standard hematological procedures as described by Dacie and Lewis (8): leukocyte, reticulocyte, and RBC counts, tests for total and plasma hemoglobin, packed RBC volume, RBC osmotic fragility, blood film inspections, bone marrow smears, and the Coombs tests.

**Platelet, RBC, and Fibrinogen Kinetic Studies.** Mouse platelets from pools of 10 to 15 normal animals were labeled with chromium-51 (the Radiochemical Centre, Amersham, England) as described by Dacie and Lewis (8); 0.2 ml plasma containing 2 × 10^6 platelets/μl was injected i.v. into control mice as well as Day 9 and Day 16 tumor-bearing animals (20 mice per group). At different time intervals, starting 30 min after injection, groups of 4 animals were anesthetized; blood was collected by intracardiac puncture and rapidly mixed with Na_2-EDTA in disposable plastic tubes. Platelet recovery after infusion, survival, and turnover were calculated as described by Harker and Slichter (15). Platelet recovery ranged from 34 to 37%, and blood volume varied between 1.6 and 1.8 ml; no significant differences were noted for these parameters between control and tumor-bearing animals.

Immediately after blood collection, both hind legs were removed from the animals that received injections of labeled platelets on Day 16 after tumor transplantation. After the limbs were dried with blotted paper, their weight was determined. Radioactivity of blood and tissue samples was measured in a Packard Model 578 gamma counter.

RBC from a group of 10 normal mice were labeled with

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**Chart 1.** Evolution of primary tumor and metastasis weight and of some selected hematological parameters in mice transplanted with the 3LL tumor on Day 0. As compared to control animals studied in parallel, platelet count decreased significantly (p < 0.01) in tumor-bearing mice, starting from Day 17 after tumor implantation. Mean fibrinogen level throughout the duration of the follow-up was significantly higher (p < 0.01) in tumor-bearing animals than in controls; however, no statistically significant difference between the 2 groups could be found at any single time. Thrombotest and FDP values were occasionally higher (Days 18, 21, and 23) (p < 0.05) in tumor-bearing animals than in controls, the numbers always remaining, however, within the normal range. Both prothrombin time and fibrinolytic activity were unchanged. Each point represents mean ± S.E. of results obtained from 10 animals. Statistical analyses were performed by Tukey's test (unconfounded means) (4, 19).
chromium-51 at a concentration of 10 μCi/2 × 10⁶ cells. Fifty animals received injections of 0.2 ml labeled RBC on the day of tumor transplantation, corresponding to about 0.5 μCi/animal. At 2- to 3-day intervals after injection, groups of 5 mice were sacrificed, and blood radioactivity was measured as described for platelets.

1²⁵I-Labeled human fibrinogen (Radiochemical Centre) was reconstituted in sterile bidistilled water at a concentration of 0.2 μCi/ml; 0.2 ml of labeled fibrinogen was injected i.v. into the control group, as well as Day 7, Day 15, and Day 20 tumor-bearing animals (20 mice per group). Immediately after blood collection, both lungs and hind legs were removed from the animals that received injections of labeled fibrinogen on Day 15 after transplantation and were processed as described above. Fibrinogen survival and turnover were calculated according to the method of Harker and Slichter (15). It was assumed that the ¹²⁵I-labeled human fibrinogen used for these studies would behave functionally like mouse fibrinogen and that, because of the short half-life of proteins in small animals, no immunological problems would arise.

RESULTS

Injection i.m. of 3LL Cells. In the control animals not receiving 3LL cells, no significant modifications of any of the parameters followed were observed throughout the whole observation period. Chart 1 shows the evolution of platelets and of other hemostatic parameters during the 30-day course of 3LL tumor development. The number of platelets remained almost constant during the 1st 2 weeks after tumor transplantation; starting from Day 17, circulating platelet levels progressively dropped and were approximately 75% lower than the initial values at the death of the animals. Blood fibrinogen concentration increased slightly during the 2nd week and markedly thereafter until death, by which time it had approximately doubled compared with the initial values. Serum FDP increased transiently during the 3rd week. Spontaneous fibrinolytic activity oscillated during the whole observation period. Prothrombin time and Factor V were unchanged, whereas the Thrombostest activity tended to increase in the 2nd half of the observation period, never exceeding, however, the upper limit of the normal range.

Chart 2 shows that a marked anemia developed in tumor-bearing animals starting from the 3rd week; the RBC count dropped progressively, paralleling the drop in total hemoglobin and concomitant with the appearance of reticulocytosis. Plasma hemoglobin only occasionally increased. The mean corpuscular volume and the mean cell hemoglobin remained unchanged, with a slight tendency to increase during the final days of observation; the mean cell hemoglobin concentration did not change (for the sake of simplicity, the latter parameters were not reported in the chart).

During microscopic examination the RBC in the peripheral blood appeared almost constantly normal; fragmented erythrocytes were only occasionally observed. A slight increase in leukocyte count was recorded during the 3rd week.

Examination of bone marrow from 3LL-bearing mice (15 to 26 days after transplantation) revealed a normal or moderately reduced degree of cellularity; the type of erythropoiesis and the general maturity of the erythropoietic and leukopoietic cells did not differ markedly from that observed in smears from normal animals. The myeloid:erythroid ratio tended to decrease during the last days before the animals' death, due to a moderate normoblastic hyperplasia. The number of megakaryocytes was normal or reduced, but few platelets were seen around them. The presence of tumor cells could not be detected.

Table 1 shows the kinetic data obtained with labeled platelets; platelet survival did not significantly change, whereas platelet turnover markedly decreased. Labeled RBC survival was unchanged as compared with that of control animals during the 1st 2 weeks but was drastically reduced in the subsequent period (Table 2). Labeled fibrinogen disappeared from the circulation more rapidly in tumor-bearing than in control animals as early as Day 7 after transplantation; a slight, further increase in the disappearance rate was later detectable. By Day 7, fibrinogen turnover was higher than in the controls, and it subsequently increased further, more than doubling on Day 21 (Table 3).

In the animals that received ⁵¹Cr-labeled platelets, radioactivity did not appear to localize at the tumor site, in comparison with the contralateral limb; in addition, the disappearance rate of radioactivity from the tumor was in the same range as that from the blood (t₁/₂, 50.1 hr with 95% confidence limits ranging between 18.6 and 81.7 hr). In contrast, in the animals that received ¹²⁵I-labeled fibrinogen during the 3rd week, radioactivity appeared to localize in the lungs and in the tumor-bearing limb to a greater extent than in the contralateral leg. The rate of disappearance of ¹²⁵I-labeled fibrinogen from the tumor was slower than that
from the lungs or blood, the $t_{1/2}$ values being 26.0, 4.7, and 13.6 hr for tumor, lungs, and blood, respectively (Chart 3).

**Injection i.v. of 3LL Cells.** After i.v. injection of 3LL cells, the following acute changes were observed (Chart 4): a marked drop both in platelet count and in blood fibrinogen concentration and an increase in serum FDP. Both thrombocytopenia and hypofibrinogenemia were observed as early as 3 min after i.v. injection and did not reverse within 50 min. Later there was an increase in FDP. The RBC count remained unchanged.

**DISCUSSION**

The effect of 3LL cells on the hemostatic system in mice has been studied using 2 different experimental conditions. (a) Cancer cells were transplanted i.m., giving rise to the formation of a primary solid tumor which metastasized spontaneously to the lungs. In this experimental condition the animals could be observed for approximately 1 month, at which time they died. (b) Cancer cells were injected i.v., and the modifications of some hemostatic parameters were observed within a period of 1 hr. The former system was chosen because it parallels the growth pattern of some solid tumors that occur in humans, whereas the latter system was used because it represents a standard test for investigating the relationship between experimental cancer and hemostasis. The main features observed during the i.m. growth of 3LL were an increased in blood fibrinogen concentration, thrombocytopenia, and hemolytic anemia. The acute i.v. injection of 3LL cells also provoked a marked thrombocytopenia, but the blood fibrinogen concentration decreased, whereas FDP markedly increased.

Despite the sustained increase in the circulating levels of fibrinogen, experimental data were obtained suggesting an increased consumption of this protein in the long-term experiments; in particular, there was a shortening of fibrinogen survival, an acceleration of fibrinogen turnover, and an accumulation in the tumor of this protein in the absence of an overt hemorrhagic diathesis.

Fibrinogen consumption is 1 of the main signs of the IVC syndrome (21, 31). Overt IVC has been observed in rats that received an i.v. injection of Walker carcinoma cells (16) or that were transplanted with an i.m. inoculum of these cells leading to death in 9 to 10 days (17). The fact that after i.m. transplantation of 3LL tumor cells, fibrinogen consumption was of low grade and was not associated with the complete spectrum of changes in the IVC syndrome may be due to the small number of disseminating cancer cells present in the circulation at any single time. The abrupt introduction into the circulation of a high number of tumor cells by i.v. injections, possibly through a sudden release of procoagulant material, caused the appearance of overt signs of IVC (thrombocytopenia, hypofibrinogenemia, and appearance of FDP). It may be relevant that Cooper et al. (7) found that the injection of dilute thromboplastin in dogs was followed by a compensatory increase in plasma fibrinogen, whereas a decrease was seen after treatment with large thromboplastin doses. Increase in blood fibrinogen levels during chronic dissemination could therefore result from a compensatory increase in the hepatic synthesis of this protein; however, other nonspecific factors (such as chronic inflammation) could have contributed to the observed increase in blood fibrinogen. Another possible explanation for the absence of an overt IVC in chronic experiments could be the occurrence of localized rather than systemic fibrinogen consumption. This hypothesis seems likely in view of the observed accumulation of fibrinogen in the tumor and of its persistence there for markedly longer times than in blood. It would also account for the transient and moderate increase in circulating FDP observed during development of the tumor.

The faster disappearance rate of labeled fibrinogen from the lung than from the tumor might be due to a higher fibrinolytic activity in the former tissue as suggested by
preliminary in vitro experiments. It should be considered that mechanisms other than IVC could play a role in conditions of accelerated fibrinogen catabolism (20); these mechanisms could involve the enhancement of the physiological processes of fibrinogen catabolism or the transformation of fibrinogen (either systemically or locally) by substances other than the clotting enzyme thrombin, such as basic proteins, lysosomal enzymes, or cell-derived proteases.

A decrease in platelet count was observed both during spontaneous metastasis of 3LL and after i.v. injection of tumor cells; in the latter case, this decrease could be due to a direct interaction between cancer cells and platelets and/or to aggregation of platelets induced by the thrombin generated during the development of the IVC syndrome. Neither of these 2 mechanisms can account for the thrombocytopenia observed during spontaneous metastasis of the 3LL tumor. Indeed, the thrombocytopenia occurring in these conditions was characterized by a normal survival time as well as a decreased platelet turnover. Platelet sequestration in tissues or organs can be excluded, since the recovery of labeled platelets was not significantly different between tumor-bearing and control mice. Moreover, radioactivity following labeled platelet injection in tumor-bearing mice did not accumulate at the tumor site from which it disappeared at a rate similar to that observed in the peripheral blood. Bone marrow examination revealed a slight reduction in the number of megakaryocytes with very few platelets around them. All of these findings strongly suggest that an impaired production rather than an enhanced destruction was the main cause of the reduction in circulating platelets.

In tumor-bearing animals a marked anemia was observed in conjunction with the appearance of thrombocytopenia. The anemia was normocytic and was accompanied by marked reticulocytosis and decreased RBC survival, all of which suggest a hemolytic origin. Bone marrow examination showed a moderate normoblastic hyperplasia. The negativity of the Coombs test would exclude an autoim-
mune phenomenon. In an experimental rat tumor (17) and in some cases of human cancers (2), the occurrence of a microangiopathic hemolytic anemia associated with IVC was reported; this could have been the consequence of damage to RBC passing through small vessels partially occluded by fibrin deposits and/or by cancer cells (2). The anemia observed in this study could be microangiopathic in origin, although fragmented RBC, the main sign of this form of anemia, were only occasionally observed. Whether RBC destruction occurred at the site of the tumor could not be investigated. The absence of changes in RBC number after acute i.v. injection of 3LL cells indicates that the anemia observed in long-term experiments was not due to a direct hemolytic effect of the cancer cells.

In conclusion, the effect of tumor cells on the hemostatic system studied by using 2 different experimental conditions has permitted observation of a different modification pattern. Clear signs of IVC were found to occur very rapidly after i.v. injection of 3LL cells, whereas the evidence for IVC in long-term experiments with the same cells was much weaker. This could be due to the presence, in the latter condition, of a lower number of circulating cancer cells. In the acute experiments the specificity of the trigger can be questioned. Indeed, nonviable tumor cells and tumor cell fragments induced a similar decrease in circulating platelets in acute experiments reported by Hilgard (16). That different mechanisms underlie the changes observed in the 2 experimental systems used in this study is further suggested by preliminary unpublished results obtained with an antiaggregating drug (Ditazole) and an anticoagulant (Warfarin). Both drugs protected the animals from the thrombocytopenia induced by i.v. injection of 3LL cells, whereas they were ineffective on thrombocytopenia developing after i.m. implantation of the tumor.

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