Circulating Membrane Vesicles in Leukemic Blood

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ABSTRACT

Ultramicroscopic membrane vesicles were found in the plasma of 17 patients with various types of leukemia (acute promyelocytic leukemia, acute monocytic leukemia, acute myelomonocytic leukemia, and chronic myelogenous leukemia) and in guinea pigs with the L2C leukemia. Labeled vesicles were cleared from normal guinea pig plasma according to a two exponential function with a half-life for the second exponent of $>11$ h. By immunofluorescence, vesicles shared antigens with the L2C leukemic cells. Attempts to elucidate the cellular origin of the circulating vesicles in human leukemias were less definitive. However, vesicles did not react with the platelet membrane antigen GP IIb/IIIa, nor did the presence of circulating vesicles or vesicle-associated procoagulant activity correlate with the platelet count. In three patients studied serially, circulating vesicles paralleled disease activity. Vesicles were not detected in 16 other patients with leukemias including acute myelogenous leukemia and most lymphoid leukemias. Similarly, vesicles were not present in 29 normal plasmas or in 10 plasmas from patients with solid tumors or nonmalignant hematological disorders. In contrast to vesicles of similar appearance shed by a variety of solid and ascites tumor cells in vivo and in vitro, the vesicles circulating in leukemia patients and guinea pigs expressed variable and generally weak procoagulant activity and no tissue factor activity. Thus, although many of the patients with circulating vesicles expressed abnormal coagulation, we were not able to establish a close pathogenetic relationship between the procoagulant activity of circulating vesicles and clinical coagulopathies.

INTRODUCTION

An extensive but scattered literature suggests that, under appropriate conditions, a variety of mammalian cells shed portions of their plasma membranes in the form of membrane vesicles (1–5). Shedding of this type may be associated with cell development and differentiation. Examples include the maturation of reticulocytes to erythrocytes, the budding of retinal and thyroid cells and of platelets from megakaryocytes, and the shedding of matrix vesicles from chondrocytes (6–11). A common feature of these examples is that shedding is a characteristic of dividing cells and is subject to regulatory control, ceasing with maturation (12). Membrane shedding has also been described in a variety of malignant cells in both tissue culture and malignant ascites (13, 14). Malignant cells in a state of continuous activation and division might be expected to shed membrane vesicles continuously (1). Shedding, as used here, occurs under physiological conditions of high cell viability and needs to be distinguished from the plasma membrane blebbing which is attributable to cell injury, as after treatment of cells with cytotoxic agents such as formaldehyde (2, 15, 16).

The membrane vesicles shed by a variety of tumor cells in vivo in ascites fluid (13) and in vitro in tissue culture possess potent procoagulant activity. This activity is expressed in at least two distinct steps of the clotting cascade, i.e., as tissue factor and as a phospholipid surface that facilitates prothrombinase generation (13, 14). The work of Gasic and of others indicated that vesicles shed by tumor cells also activated platelets (17–20). In many instances, however, platelet activation was a secondary event that followed initiation of clotting; i.e., platelets were activated by thrombin generated through the agency of shed vesicles which triggered the clotting cascade (21).

The present studies sought to extend these observations to leukemia. Many patients with leukemia develop coagulopathies of various types and degrees of severity. Further, microscopists have described light and ultrastructural images suggestive of cell shedding in fixed preparations of leukemic blood (22–24). We reasoned, therefore, that circulating leukemic cells might shed membrane vesicles with procoagulant activities that could initiate clotting and lead to the coagulopathies commonly associated with leukemias. We here report that membrane vesicles do circulate in the blood in both guinea pigs with the L2C leukemia and patients with several types of leukemia, particularly APL,3 AMoL, AMIMoL, and CML. In contrast to our previous findings in animal and human cell lines derived from solid tumors, however, only some of the vesicle samples isolated from leukemic human blood expressed detectable procoagulant activity, and none expressed tissue factor activity.

MATERIALS AND METHODS

Guinea Pig L2C Leukemia. The L2C leukemia (a gift from Dr. Atul Bhan, Massachusetts General Hospital, Boston, MA) is a B-cell lymphocytic leukemia that is syngeneic in strain 2 guinea pigs (25). The tumor was passaged by injection of $5 \times 10^6$ leukemic cells s.c.; leukemia (leukocyte counts > $150,000/\mu l$) appeared 12–14 days later. Leukemic cells and vesicles were recovered from the blood following cardiac puncture under ether anesthesia (see below).

Patient Data Accrual. A total of 42 Beth Israel Hospital patients were studied after informed consent had been obtained from both patient and physician, according to a protocol approved by the hospital’s Committee on Clinical Investigations. Thirty-two patients had leukemia, and 10 had other disorders. One patient was first tested when he had myelofibrosis and as a phospholipid surface that facilitates prothrombinase generation (13, 14). The work of Gasic and of others indicated that vesicles shed by tumor cells also activated platelets (17–20). In many instances, however, platelet activation was a secondary event that followed initiation of clotting; i.e., platelets were activated by thrombin generated through the agency of shed vesicles which triggered the clotting cascade (21).

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pertinent laboratory and treatment data were recorded. Most patients with acute leukemia were studied at the time of diagnosis and, unless otherwise noted, prior to treatment. At the conclusion of the study, each patient’s record was again reviewed for confirmation of diagnosis and further clarification of any coagulation disorders.

Isolation of Vesicles from Blood Samples. Preliminary experiments on guinea pigs with the L2C leukemia led to the development of a satisfactory methodology for isolating from guinea pig or human leukemic blood circulating vesicles that were free of contaminants, such as platelets or vesicle aggregates. Blood samples (15 ml, guinea pig; 25 ml, human) were drawn atraumatically into sufficient chilled anticoagulant to provide final concentrations of heparin (10 units/ml) and 0.01 M EDTA; blood was then diluted immediately in an equal volume of PBS (pH 7.4) supplemented with heparin (10 units/ml) and 0.01 M EDTA. Samples were centrifuged twice at 4°C at 2000 x g for 20 min in plastic tubes to remove cells. Supernatants were then centrifuged at 100,000 x g for 90 min or 150,000 x g for 45 min. The resultant pellets were resuspended in PBS and filtered through 1.0-μm and 0.8-μm Nucleopore (Pleasanton, CA) polycarbonate filters to remove any remaining platelets and debris. Filtres were ultracentrifuged a second time, and the resulting pellets were resuspended in PBS and filtered through 0.8- and 0.6-μm Nucleopore filters. The sample was then divided for a final ultracentrifugation, generally 40% for clotting studies and 60% for electron microscopy. Leukemic samples were run in parallel with equal volumes of blood taken from normal controls.

Transmission Electron Microscopy. Ultracentrifuge pellets were fixed immediately at room temperature for 1 h in dilute paraformaldehyde/gluteraldehyde and processed for embedding in Epon and thin sectioning as previously described (26). To ensure adequate sampling, blocks were oriented so that sections were cut vertically through the entire thickness of the pellet. In this way, all levels of the pellet were examined, avoiding sampling problems that might arise from layering of heavy and light pelletable material. Vesicles were said to be undetectable if no vesicles were visualized by electron microscopy or if an ultracentrifuge pellet was not visible or was < 1 mm in diameter, making it impossible to process.

Immunofluorescence Studies of Leukemic Cells and Circulating Vesicles. L2C leukemic cells and circulating vesicles were isolated from the plasma of strain 2 leukemic guinea pigs and subjected to immunofluorescence study with antibodies directed against known L2C cell surface markers. Platelets isolated by centrifugation from strain 2 guinea pig platelet-rich plasma and washed 3 times in PBS served as controls. L2C cells, circulating vesicles, and normal strain 2 platelets were also studied by direct immunofluorescence, using several fluorescent-labeled antibodies: IgG and F(ab’)2 fractions of goat anti-mouse IgG and IgG fractions of goat anti-rabbit IgG, both heavy and light chain specific (Cappel Laboratories, West Chester, PA). L2C cells, circulating vesicles, and normal strain 2 platelets were also studied by direct immunofluorescence, using several fluorescent-labeled antibodies: IgG and F(ab’)2 fractions of goat anti-guinea pig IgG (heavy and light chain specific); IgG fractions of goat anti-guinea pig C3; and F(ab’)2 fractions of rabbit anti-mouse IgG (heavy and light chain specific; Cappel).

Stored, frozen (~80°C) vesicles and cells from several leukemic patients who had had cell typing performed at the time of diagnosis were later studied for the presence of certain previously identified myeloid antigens, as well as for the platelet membrane antigen GP IIb/IIIa. First antibodies were F(ab’)2 fractions of mouse monoclonal anti-human My 4, My 7, My 8, My 9 (28, 29), and IgG fractions of rabbit anti-human platelet GP IIb/IIIa (the generous gift of Dr. Jacek Hawiger, prepared according to the method of Jennings and Phillips) (30). The second antibodies were fluorescent-labeled F(ab’)2 fractions of goat anti-mouse IgG (Cappel) and F(ab’)2 fractions of goat anti-rabbit IgG (Cappel). As normal strain 2 guinea pig platelets and L2C vesicles, were similarly studied with antiplatelet glycoprotein IIb/IIIa antibody.

For indirect staining, 5 x 106 freshly isolated and washed L2C leukemia cells or normal platelets were incubated with 0.3 ml of first antibody for 30 min at 4°C. The cells were then washed 3 times with PBS and resuspended in 0.3 ml of second antibody for 30 min at 4°C. The cells were washed 3 times in cold PBS and fixed with 3.7% formalin in PBS for 30 min at 4°C. After 3 final washes, the cells were resuspended in PBS:glycerol:ethanol (1:1) at room temperature and examined in a Zeiss fluorescence microscope. Controls included the use of PBS or a nonspecific antibody in place of either the first or second antibody. For direct fluorescence, cells were incubated with appropriate concentrations of fluoresceinated antisera and washed as above.

Normal platelets and vesicles isolated from L2C leukemic guinea pigs or from leukemic patients were stained in suspension (direct immunofluorescence) or after drying on microscopic slides (both indirect or direct fluorescence).

Leukocyte isolation from Leukemic Blood. Approximately 7 ml of human or guinea pig blood were collected in anticoagulant (0.1 ml of 1% EDTA per ml of blood). The blood was mixed with 3.5 ml of 3% freshly prepared gelatin in PBS in a plastic tube and left upright in a 37°C water bath for 40 min to sediment erythrocytes. The supernatant containing leukemic cells was removed. Total cell and differential counts were performed, and cell viability (always >95%) was determined with trypan blue.

Coagulation Studies. One stage recalcification times were performed at 37°C in an automated Coagulation Profiler (Bio/Data Corp., Willow Grove, PA) as previously described (13, 14). Pooled homologous (human or guinea pig) platelet-poor plasma was prepared from 4 or more normal donors (12). Samples and control buffer were assayed in random sequence to control for random drift in the assay. Values from 2 to 4 replicate points were averaged. The percentage of shortening induced by experimental samples, as compared to buffer, was calculated as

\[
\frac{1 - \text{Test sample clotting time (s)}}{\text{Buffer clotting time (s)}} \times 100 = \% \text{ of shortening}
\]

Shortening of less than 10% was considered insignificant. Samples with shortening greater than 10% were tested in a two-stage clotting test. A two-stage clotting test to measure tissue factor activity was performed according to the method of Pitlick and Nemerson (31), using buffer as control, homologous platelet-poor plasma, 0.03 M CaCl₂, homologous (human or guinea pig) Factor VII-X as prepared by Pitlick and Nemerson (31), and rabbit brain cephalin (Sigma, St. Louis, MO). Activity of test samples was read from a standard curve, prepared by sequential dilution of rabbit brain thromboplastin (Simplex; General Diagnostics, Morris Plains, NJ) for human samples or of guinea pig brain extract for guinea pig samples (31).

Clearance of Radioactively Labeled Vesicles from the Guinea Pig Circulation. It was desirable to determine the rate of clearance of circulating vesicles from the blood. Because L2C vesicles were difficult to obtain in the large numbers required for radioactive labeling, vesicles were prepared instead from cultured guinea pig line 10 tumor cells, a line that sheds abundant vesicles (13, 14). Vesicles isolated as ultracentrifuge pellets from conditioned medium were resuspended in 0.4 ml of 0.5 μ phosphate buffer, pH 7.4, and incubated with 1 μCi of Na125I (New England Nuclear, Boston, MA) and one iodocad (Pierce, Rockford, IL) for 10 min at room temperature (32). Labeled samples were dialyzed against PBS supplemented with the protease inhibitors EDTA, iodoacetic acid, N-ethylmaleimide, and phenylmethylsulfonyl fluoride, all at final concentrations of 2 μM. Ninety-five % of the radioactivity of the labeled vesicles was precipitated with 10% trichloroacetic acid.

For clearance experiments, 125I-labeled line 10 vesicles were injected i.v. into normal strain 2 guinea pigs. Ear blood samples were taken at regular intervals over 48 h for radioactive counting. Curve fitting was accomplished with a Hewlett-Packard computer using a modified...
RESULTS

Procoagulant Vesicles of L2C Leukemic Guinea Pigs Shedded In Vitro and In Vivo

Cell-free conditioned medium prepared from 4-h cultures of L2C leukemia cells contained membrane-bound vesicles that could be sedimented in the ultracentrifuge (Ref. 14; Fig. 1A). L2C conditioned medium, and the vesicles purified from such medium, significantly shortened the one-stage recalcification time as previously reported (14). However, we detected no tissue factor activity in such preparations, in contrast to studies with vesicles derived from solid tumor lines.

Vesicles having a similar ultrastructure were also isolated from the circulating blood of strain 2 guinea pigs bearing L2C leukemia (Fig. 1B); in contrast, vesicles were never found in normal guinea pig blood. The vesicle preparations isolated from L2C leukemic blood significantly shortened the one-stage recalcification time (by 18 to 41% in 7 leukemic animals) but did not express tissue factor activity as measured by a two-stage assay. Thus, both in cultures of L2C cells and in the blood of L2C leukemic guinea pigs, vesicles were isolated with an ultrastructure similar to that observed in cultures or ascites fluid of solid-growing tumor cells (13, 14). Further, these vesicles exhibited one-stage procoagulant activity but no tissue factor activity.

In order to identify the cellular source of the circulating plasma vesicles, we reacted vesicles with antibodies directed against known L2C cell markers. Previous studies (33, 34) have demonstrated la and IgM antigens on the surface of L2C cells; surface IgM is reactive with antibodies to the μ-chain of IgM and also to antibodies with specificity for IgG light chains. We confirmed these findings and also demonstrated that these same antibodies stained vesicles purified from the plasma of leukemic guinea pigs (Table 1). However, these antibodies did not stain guinea pig platelets. Antibodies to human platelet GP IIb/IIIa, antibody stained neither guinea pig platelets nor vesicles.

Half-Life of Circulating Vesicles

125I-labeled line 10 vesicles were cleared from the blood of three normal strain 2 guinea pigs according to a two exponential function: $Y = 0.613 e^{-0.017t} + 0.387 e^{-0.081t}$. The mean half-life of vesicle clearance was approximately 45 min and 11.4 h for the first and second exponents, respectively. These data stand in contrast to studies of phospholipid vesicles (liposomes), 99% of which are cleared from the circulation within 4 h of i.v. injection (35, 36). Vesicles of the type circulating in leukemia may therefore be useful for applications such as targeted drug delivery (37, 38), where rapid reticuloendothelial clearance greatly limits the utility of presently available liposomes.

Human Studies

We next looked for circulating vesicles in the blood of patients with leukemia or other disorders. Several distinct groups of patients were identified.

Group 1: Leukemia Patients with Circulating Vesicles. In 17 of 33 leukemia patients, circulating vesicles were recovered from 25-ml blood samples (see Table 2 and Fig. 1, C to E). Nine of these 17 samples shortened the one-stage recalcification time by at least 10%. None of the samples tested expressed detectable tissue factor activity.

Vesicles from Patients 7 and 9 to 11 were thawed after prolonged storage at -80°C and tested by indirect fluorescence with antibodies against myeloid surface antigens. The leukemia cells from each of these four patients had originally reacted with at least two of these antibodies. However, none of the vesicle preparations reacted with any of these antibodies. Frozen cells from Patient 10, stored in parallel with her circulating vesicles, also failed to stain for the myeloid markers with which they had formerly reacted. Vesicles from Patients 2, 7, 9, 11, 16, and 17 were also tested with anti-platelet membrane GP IIb/IIIa antibody, and in every case, the results were negative. In contrast, freshly isolated human platelets, as well as platelets which had been disrupted by sonication and/or by repeated freezing and thawing, reacted strongly with anti-GP IIb/IIIa antibody.

Group 2: Leukemia Patients Lacking Vesicles. A group of 16 leukemia patients lacked detectable circulating vesicles and sedimentable procoagulant activity (Table 3). None of these patients exhibited spontaneous bleeding. Patient 27 had thrombocytopenia and was said to bleed from shaving cuts.

Groups 3 and 4: Nonleukemic Patients and Normal Controls. Ten additional patients with a variety of nonleukemic illnesses were also studied (see Table 3 and Fig. 1F). None expressed detectable one-stage procoagulant activity in ultracentrifuged plasma, and only Patient 41 (Waldenstrom’s macroglobulinemia) had small numbers of circulating vesicles found after study of multiple thin sections. Also, 29 blood samples from 9 healthy volunteers lacked vesicles and procoagulant activity. Rare vesicles, smaller but otherwise similar to those found in abundance in leukemic blood, were occasionally found when much larger samples (e.g., 80 ml) of normal blood were processed (Fig. 1F).
CIRCULATING LEUKEMIC VESICLES

Table 2
Clinical profile of leukemia patients with circulating vesicles

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>WBC/µl</th>
<th>Platelet count/µl</th>
<th>% of shortening</th>
<th>Clinical coagulopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (leukemia patients with both circulating vesicles and procoagulant activity)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CML, accelerated</td>
<td>101,000</td>
<td>154,000</td>
<td>23</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>CML, chronic</td>
<td>461,000</td>
<td>441,000</td>
<td>11</td>
<td>Spontaneous bleeding from venipuncture/bone marrow sites</td>
</tr>
<tr>
<td>3</td>
<td>CML, chronic</td>
<td>115,000</td>
<td>460,000</td>
<td>30</td>
<td>Gastrointestinal bleeding</td>
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<td>4</td>
<td>CML, chronic</td>
<td>171,000</td>
<td>465,000</td>
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<td>None</td>
</tr>
<tr>
<td>5</td>
<td>CML, chronic</td>
<td>140,000</td>
<td>303,000</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>CML, chronic</td>
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<td>1,090,000</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>AMMoL</td>
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<td>28,000</td>
<td>21</td>
<td>Epistaxis, hemoptysis</td>
</tr>
<tr>
<td>8</td>
<td>AMMoL</td>
<td>11,000</td>
<td>50,000</td>
<td>10</td>
<td>Minor</td>
</tr>
<tr>
<td>9</td>
<td>AMMoL</td>
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<td>61,000</td>
<td>4</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>AMMoL</td>
<td>142,000</td>
<td>52,000</td>
<td>7</td>
<td>Minor</td>
</tr>
<tr>
<td>11</td>
<td>AMMoL</td>
<td>43,000</td>
<td>70,000</td>
<td>0</td>
<td>Minor</td>
</tr>
<tr>
<td>12</td>
<td>AMMoL</td>
<td>8,000</td>
<td>24,000</td>
<td>8</td>
<td>Epistaxis</td>
</tr>
<tr>
<td>13</td>
<td>AMMoL</td>
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<td>53,000</td>
<td>0</td>
<td>Minor</td>
</tr>
<tr>
<td>14</td>
<td>AMMoL</td>
<td>17,000</td>
<td>86,000</td>
<td>12</td>
<td>Protracted uterine bleeding</td>
</tr>
<tr>
<td>15</td>
<td>APL</td>
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<td>123,000</td>
<td>10</td>
<td>DIC</td>
</tr>
<tr>
<td>16</td>
<td>APL</td>
<td>20,000</td>
<td>51,000</td>
<td>56</td>
<td>DIC</td>
</tr>
<tr>
<td>17</td>
<td>ALL</td>
<td>172,000</td>
<td>81,000</td>
<td>14</td>
<td>Deep venous thrombosis</td>
</tr>
</tbody>
</table>

Serial Study of Patients with Circulating Procoagulant Vesicles

Three patients were followed serially (Table 4). Patient 1 had circulating vesicles with procoagulant activity while in the accelerated phase and blast crisis of CML. He was treated successfully with intensive chemotherapy and received frozen autologous marrow that had been stored during his chronic phase. During remission, circulating vesicles were no longer detectable. He relapsed shortly thereafter, and vesicles reappeared, but after further chemotherapy and sibling marrow transplant, vesicles again became undetectable. He died a month later of sepsis.

Patient 16 was followed through an induction course of chemotherapy. Vesicle-associated procoagulant activity progressively declined until he became aplastic, at which time neither vesicles nor significant procoagulant activity was found in the blood. He subsequently achieved a complete remission.

Patient 7 was initially studied when he had myeloid metaplasia (as Patient 43), and at that time he exhibited no circulating vesicles. Seventeen mo later, he developed a WBC of 163,000/µl with 90% blasts with nonspecific esterase activity characteristic of monoblasts. With the development of this leukemia, vesicles with procoagulant activity were isolated from his blood.

Relationship of Clinical Characteristics of Leukemic Patients to Circulating Vesicles

All but one of the Philadelphia chromosome (Ph1)-positive CML patient had circulating vesicles. The one exception (Patient 29, Table 3) was a boy who developed CML at age 15. Two Ph1-negative patients with CML-like syndromes lacked vesicles. No AML patient had vesicles. Patients with lymphoid leukemia lacked vesicles with one possible exception, Patient 17 (Fig. 1D). This patient was a 31-yr-old man with a marrow that included both immature lymphoid and myeloid cells. The lymphoid cells were positive for the common acute lymphocytic leukemia antigen, suggesting a diagnosis of ALL; however, he did not enter remission on first or second line ALL chemotherapy and died of a spontaneous intracranial hemorrhage.

The presence of circulating vesicles in leukemia patients could not be attributed to chemotherapy with resultant release of cell fragments into the blood. In a majority of cases, vesicles were isolated from blood obtained prior to the initiation of any chemotherapy (Table 2). Moreover, in a serial study of Patient 16, institution of chemotherapy was associated with the disappearance of vesicles from the blood (Table 4). Finally, in several instances, patients with AMMoL, AMoL, and AML received the same chemotherapy, but vesicles were present only in patients with AMMoL and AMoL, and not in AML patients.

No correlation was found between circulating vesicles and the platelet count. Analysis of the patients in Tables 2 and 3 for an association between the platelet count and the presence of circulating vesicles revealed a correlation coefficient of 0.13, indicating lack of association (39). Similarly, platelet counts did not correlate with vesicle-associated procoagulant activity; for the patients listed in Table 2, the Spearman rank correlation (39) between these variables was -0.08. We were also unable to correlate the presence of circulating vesicles with other laboratory clotting abnormalities including prothrombin time, partial thromboplastin time, fibrinopeptide A, and clinical bleeding. Several leukemia patients with circulating vesicles did demonstrate...
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Table 3
Clinical profile of leukemia patients and controls lacking vesicles

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>WBC/µl</th>
<th>Platelet count/µl</th>
</tr>
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<tbody>
<tr>
<td>Group 2 (leukemic patients)</td>
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</tr>
<tr>
<td>18</td>
<td>CLL</td>
<td>14,000</td>
<td>77,000</td>
</tr>
<tr>
<td>19</td>
<td>CLL</td>
<td>130,000</td>
<td>116,000</td>
</tr>
<tr>
<td>20</td>
<td>CLL</td>
<td>262,000</td>
<td>311,000</td>
</tr>
<tr>
<td>21</td>
<td>HCL</td>
<td>300</td>
<td>132,000</td>
</tr>
<tr>
<td>22</td>
<td>HCL</td>
<td>3,000</td>
<td>102,000</td>
</tr>
<tr>
<td>23</td>
<td>ALL</td>
<td>13,000</td>
<td>89,000</td>
</tr>
<tr>
<td>24</td>
<td>ALL relapse*</td>
<td>39,000</td>
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<tr>
<td>25</td>
<td>AML</td>
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<td>26</td>
<td>AML relapse</td>
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</tr>
<tr>
<td>27</td>
<td>AML relapse*</td>
<td>9,000</td>
<td>12,000</td>
</tr>
<tr>
<td>28</td>
<td>AML</td>
<td>13,000</td>
<td>101,000</td>
</tr>
<tr>
<td>29</td>
<td>CML, chronic</td>
<td>30,000</td>
<td>2,885,000</td>
</tr>
<tr>
<td>30</td>
<td>Myeloproliferative syndrome (Ph1-negative)*</td>
<td>103,000</td>
<td>53,000</td>
</tr>
<tr>
<td>31</td>
<td>Myeloproliferative syndrome (Ph1-negative)</td>
<td>7,000</td>
<td>675,000</td>
</tr>
<tr>
<td>32</td>
<td>Lymphoblastic lymphoma, leukemic (B-cell)</td>
<td>33,000</td>
<td>29,000</td>
</tr>
<tr>
<td>33</td>
<td>Immunoblastic lymphoma, leukemic (T-cell)</td>
<td>8,000</td>
<td>80,000</td>
</tr>
<tr>
<td>Group 3 (control patients)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Pancreatic carcinoma, microangiopathic hemolytic anemia</td>
<td>17,000</td>
<td>58,000</td>
</tr>
<tr>
<td>35</td>
<td>Recurrent venous thromboses</td>
<td>7,000</td>
<td>268,000</td>
</tr>
<tr>
<td>36</td>
<td>Immune thrombocytopenia, intracerebral hemorrhage, cerebellar subependymoma</td>
<td>12,000</td>
<td>15,000</td>
</tr>
<tr>
<td>37</td>
<td>Monocytosis</td>
<td>10,000</td>
<td>137,000</td>
</tr>
<tr>
<td>38</td>
<td>Leukemoid reaction</td>
<td>36,000</td>
<td>658,000</td>
</tr>
<tr>
<td>39</td>
<td>Hemochromatosis</td>
<td>6,000</td>
<td>nl</td>
</tr>
<tr>
<td>40</td>
<td>Polycythemia vera</td>
<td>8,000</td>
<td>nl</td>
</tr>
<tr>
<td>41</td>
<td>Waldenstrom’s macroglobulinemia</td>
<td>30,000</td>
<td>169,000</td>
</tr>
<tr>
<td>42</td>
<td>Pancreatic carcinoma, DIC</td>
<td>7,000</td>
<td>77,000</td>
</tr>
<tr>
<td>43/7</td>
<td>Myeloid metaplasia</td>
<td>8,000</td>
<td>42,000</td>
</tr>
<tr>
<td>Group 4 (normal control samples)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44–72</td>
<td>Normal*</td>
<td>nl</td>
<td>nl</td>
</tr>
</tbody>
</table>

*Chemotherapy had been started only in designated patients.

**Normal.

---

in the blood of normal guinea pigs or volunteers or in patients with AML. Vesicles were infrequently present in lymphocytic leukemias and were not found in blood samples taken from patients with nonmalignant bone marrow proliferations or extensive solid tumor burdens, even when associated with coagulopathies.

Because a variety of leukemia and solid tumor cells shed similar appearing vesicles when cultured in vitro or when growing in ascites form in vivo (13, 14), it seemed likely that these circulating vesicles might also be derived from leukemia cells. Spontaneous membrane shedding from malignant cells has been linked to alterations of membrane fluidity (5, 22, 40–42). Further, we found no simple relationship between platelet counts and the presence of circulating vesicles in human leukemias. Finally, circulating vesicles could not be attributed to chemotherapy since, in the majority of patients studied, blood samples for evaluating vesicles were drawn prior to the institution of chemotherapy.

On the other hand, George et al. (43) isolated microparticles with procoagulant activity from normal human serum and, in much smaller quantities, from normal plasma. By electron microscopy, these microparticles consisted of a heterogeneous array of subcellular components, pelleted with less than 10% of the centrifugal force required to sediment our membrane vesicles. Compared with the circulating vesicles reported here, those structures in the serum of patients with lymphocytic leukemia (22). Compared with the circulating vesicles reported here, those described by Petitou appeared heterogeneous and may have included cell and platelet fragments resulting from the blood coagulation required for his serum preparations.

We used immunofluorescence methods in an attempt to identify the cellular source of these leukemic vesicles with greater certainty. We obtained strong evidence that the vesicles circulating in leukemic guinea pigs are of L2C cell origin. Both vesicles and tumor cells reacted with antibodies specific for the lα antigen of strain 2 guinea pigs, with antibodies specific for the guinea pig IgM μ-chain, and with antibodies and F(ab')2 fragments.
reactive with immunoglobulin light chains. In contrast, guinea pig platelets reacted with none of these antibodies.

Less conclusive results were obtained with antibodies selected to define the cellular origin of circulating vesicles in human leukemias. Such vesicles were clearly unreactive with antibodies to the GP IIb/IIIa platelet membrane antigen but also failed to react with antibodies to 4 myeloid markers. We attach some importance to the former negative result in that human platelet fragments retained undiminished reactivity with the anti-GP IIb/IIIa antibody even after sonication and repeated freeze-thaws. Failure to react with myeloid marker antibodies after prolonged storage in the frozen state is less definitive in that these antigens are relatively labile and were also not preserved on frozen cells which had earlier been positive. The small quantities of available vesicles made further analysis impossible on these patient samples.

Taken together with our studies of the guinea pig Lc2 leukemia, and with what is known of tumor cell shedding in vitro, it therefore seems likely that the circulating vesicles we observed in human leukemia are also derived from spontaneous shedding of leukemic cell membranes and not from platelets. To settle this issue conclusively, we plan to use quantitative immunological methods to study leukemia cells and circulating vesicles from the same patients in parallel, using a panel of antibodies directed against leukocyte and platelet markers. Such studies will require larger blood samples than were available to us in this study.

APL, AMMOL, AMOL, and even CML have been associated with DIC (44–46). Tissue factor has often been implicated in the pathogenesis of DIC in APL (47–48), particularly following chemotherapy. However, the circulating vesicles in our leukemic patients expressed modest or no one-stage procoagulant activity, and no sample tested expressed tissue factor activity. Parallel observations were made in guinea pigs bearing Lc2 leukemia where vesicles, shed in vivo and in vitro (14), expressed significant procoagulant activity, as measured by one-stage recalcification time assays, but also lacked tissue factor activity. The finding of shed leukemia vesicles that lack tissue factor or any significant procoagulant activity stands in contrast to earlier findings in solid tumors (13, 14, 17–19, 21), where vesicles shed either in vivo or in vitro regularly expressed substantial procoagulant activity of both types. It seems clear, therefore, that in the leukemias studied, shed vesicles do not have the capacity to initiate coagulation directly. However, these circulating vesicles could conceivably alter coagulation through other means, such as by an alteration in blood rheology.

ACKNOWLEDGMENTS

We thank Justine Osage and Patricia Estrella for technical assistance with the electron microscopy, V. Susan Harvey for assistance with the animal work, and Dr. Livingston VanDeWater and Dr. Jan McDonagh for critical review of the manuscript.

REFERENCES

32. Markwell, M. A. M. A new solid-state reagent to iodinate proteins. I. Conditions


Fig. 1. Electron micrographs of sedimented pellets isolated from conditioned tissue culture medium (12) or peripheral blood as described in "Materials and Methods." A, vesicles with procoagulant activity from conditioned medium of cultured guinea pig L2C cells. × 27,000. B, vesicles with procoagulant activity from plasma of an L2C-bearing guinea pig. × 27,000. C, vesicles with procoagulant activity from plasma of a patient (No. 14, Table 2) with AMoL. × 26,000. D, vesicles with procoagulant activity from plasma of a patient (No. 17, Table 2) with putative ALL but who did not respond to therapy and whose marrow also contained immature myeloid elements. × 20,250. E, vesicles that did not express detectable procoagulant activity from the plasma of Patient 13 (Table 2) with AMMoL. × 19,500. F, ultracentrifuge pellet from an 80-ml sample of blood from a normal volunteer. The pellet did not express procoagulant activity. Rare, tiny vesicles are included in a proteinaceous meshwork. × 83,600.
Circulating Membrane Vesicles in Leukemic Blood

Justine M. Carr, Ann M. Dvorak and Harold F. Dvorak


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