Immunofluorescent Studies in Human Leukemia

David S. Yohn, Julius S. Horoszewicz, Rose Ruth Ellison, Arnold Mittelman, Lee S. Chai, and James T. Grace, Jr.

Section of Viral Oncology and Department of Medicine, Roswell Park Memorial Institute, Buffalo, New York 14203

SUMMARY

High-speed (80,000 × g) pellets of plasma from leukemic patients and from normal individuals were used to prepare antisera in rabbits. The leukemic plasma pellets contained virus-like particles as determined by electron microscopy, whereas similar particles were not observed in the normal pellets. Globulin fractions from six antisera to normal pellets (NPA) and six antisera to leukemic pellets (LPA) were labeled with fluorescein isothiocyanate and then absorbed with normal human peripheral blood constituents and liver or spleen powders. Fully absorbed NPA preparations did not react with either normal or leukemic leukocytes, whereas fully absorbed LPA preparations yielded positive cytoplasmic reactions with peripheral leukocytes from 10 of 29 myelocytic leukemic patients. Immunofluorescent-positive cells, members of the myelocytic series, were found in the bone marrow of 5/5 patients with positive peripheral cells. Lymphocytic leukemic cells and cells from normal individuals or from patients with other diseases were nonreactive, as were cultured leukocytes.

The antibody activity of the LPA preparations was removed by absorption with pooled or individual normal human bone marrow powders. It was concluded that the antigens detected in immature cells of certain myelocytic leukemia patients are also present in certain normal human bone marrow cells but in concentrations too low to be accurately detected by direct immunofluorescence. The evidence is not sufficient to support the hypothesis that these antigen(s) are viral-associated antigen(s).

INTRODUCTION

Antisera containing neutralizing antibody against murine leukemia viruses have been shown by Fink et al. (7) to yield useful diagnostic immunofluorescent reagents. These antisera were produced by immunization of rabbits with virus concentrated and partially purified from leukemic mouse plasma by differential centrifugation procedures.

The success obtained with these reagents in demonstrating viral-associated antigens in various murine leukemia tissues prompted parallel studies in human leukemia by these same workers (6, 8–10). Pellets which contained virus-like particles were obtained from the plasma of human patients with leukemia. Pools of these pellets were used to immunize rabbits and monkeys. Similarly prepared pellets from normal human plasma which contained no detectable virus-like particles were used to produce control antisera. Following extensive absorptions with normal human peripheral blood constituents and organ powders but not bone marrow powder, direct immunofluorescent tests were conducted on human bone marrow smears, peripheral leukocytes, and various cell cultures. These studies revealed that leukocytes of some patients with leukemia reacted with the labeled globulins, while leukocytes from normal individuals did not (6, 8, 9). The authors reported that the presence of virus-like particles in the plasma was correlated with the presence of reactive cells in the bone marrow (9). Furthermore, it was suggested that in several instances the stage of disease relative to relapse or remission was predictable on the basis of the degree of immunofluorescent reactivity (6, 8).

The importance of these observations to etiology, diagnosis, and disease prognosis of human leukemia was apparent. Accordingly, we prepared high-speed pellets from the plasma of patients with leukemia. Pellets which contained virus-like particles similar to those described by Porter et al. (17) and by Dalton et al. (3) were used to immunize rabbits. This report describes our observations with these antisera in immunofluorescent tests on human leukemia cells. It is our impression that the differences between the reactivity of leukemic and normal cells, as revealed by immunofluorescence with antisera produced in heterologous hosts to human leukemia plasma pellets, are not qualitative but are quantitative; observations in support of this concept are described.

MATERIALS AND METHODS

Preparation of Leukemic and Normal Plasma Pellets. Patients with leukemia, untreated or in relapse, were plasma-pherased. Plasmas were diluted with an equal volume of 0.3 M citrate and then submitted to differential centrifugation according to the method of Porter et al. (17). Pellets from the final high speed (80,000 × g) centrifugation were resuspended in 9 ml of buffered citrated saline, repelleted at 80,000 × g, and resuspended to one-tenth of original plasma volume. A total of 10 pellets were obtained which contained virus-like particles as determined by electron microscopy of thin sections (14). Photomicrographs of the virus-like particles were examined by Dr. A. J. Dalton of the National Cancer Institute, who confirmed their resemblance to mature murine leukemia “C-type” viruses (Fig. 1).
Ten freshly obtained normal human plasmas were processed in an identical manner. No virus-like particles were seen in pelleted material from these plasmas.

**Immunization Schedule.** Six rabbits, Numbers 10-15, were immunized with the leukemic material, and six control rabbits, Numbers 16-21, were immunized with normal plasma material. Inoculations were subcutaneous and intradermal at multiple sites, 0.1 to 0.2 ml per site. Complete Freund’s adjuvant was included in the 1st and 4th injections (Table 1). Final bleedings were obtained 16 weeks after the initial injection.

**Absorption Materials and Procedures.** Materials employed to absorb the fluorescein-labeled antibody preparations included A and B Rh-positive erythrocytes, normal human platelets, pooled normal human plasma albumin, globulin and fibrinogen, normal human peripheral leukocytes ("buffy coat"), and normal human spleen and liver powder. Acetone-extracted (2) normal human and calf bone marrow powders were employed as absorbants in special experiments.

Constant ratios of absorbants per volume of labeled preparation were used. Fifteen mg (dry weight) of organ powder were employed to absorb each ml. In the case of leukocytes and platelets, the sediment from one ml of a 10% v/v suspension of “buffy coat” was employed to absorb each ml of conjugated globulin. One ml of washed packed erythrocytes was employed per ml of conjugate. Absorptions were performed at 25°C for one hour with constant agitation.

**Fractionation of Antisera and Labeling and Processing of Globulin Fractions.** Globulin fractions were precipitated at 0°C by dropwise addition of an equal volume of saturated (NH₄)₂SO₄ (pH 7.0). The globulin fraction was dissolved in borate buffer (pH 8.0) and adjusted to 10 mg per ml with 0.15 M NaCl at pH 9 prior to the addition of 0.5 mg of fluorescein isothiocyanate per ml. The mixture was stirred overnight at 4°C and then passed through a Sephadex G-25 column.

Preliminary serologic tests on the antileukemic and antinormal pellet sera revealed antibodies to human plasma fibrinogen, albumin, globulins, red blood cells, platelets, and normal leukocytes. These antibodies were removed by the absorption techniques described above.

The labeled absorbed reagents were chromatographed on diethylaminoethyl (DEAE)-cellulose columns according to the method of Goldstein (11). This procedure was used to eliminate: (a) unlabeled proteins in the original reagent and those employed as absorbants; (b) free dye, if any; (c) heavily labeled, strongly electronegative proteins; and (d) nonsedimented antibody-antigen complexes formed during absorption procedures.

Final concentrations of the labeled, absorbed, and chromatographed antibody preparations were adjusted to 10 mg protein/ml. Fluorescein to protein (F/P) ratios were determined spectrophotometrically with the aid of standard reference absorption curves (14).

**Types of Cells Examined.** Glass slide and cover slip smears were made of cells obtained directly from in vitro cultures of human leukemia cells. The growth of these cells in vitro has been described previously (12, 16). Bone marrow smears were made directly on glass slides without anticoagulant. Peripheral leukocyte smears were prepared from “buffy coat” of fresh citrated blood which had been allowed to settle or had been centrifuged at 100 × g for 10 minutes. Peripheral leukocyte and cell culture smears were fixed (see below) and stored at —60°C until used. Bone marrow smears were air dried, stored at —60°C, and fixed as needed.

**Selection of Fixing and Staining Methods.** Final fixation and staining methods were selected on the basis of preliminary experimental studies. When cell smears were fixed with cold acetone only, the physicochemical affinity of buffy coat cells and certain leukemia cell lines for fluorescein-labeled globulin proved to be an important problem. The problem, as recognized by others (2, 11, 19, 20, 25) is a function of the attraction of electropositive (acidophilic) cellular components for globulins rendered electronegative (acidic) by coupling to fluorescein. Highly acidic proteins were removed from the labeled globulins by DEAE-cellulose chromatography (11). Acidophilic activity in specimens was reduced by (a) drying specimens in air for 24 hours to permit oxidative deamination,

![Table 1](image)

<table>
<thead>
<tr>
<th>Immunization day</th>
<th>Rabbit number</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
</tr>
<tr>
<td>7</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
</tr>
<tr>
<td>15</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
</tr>
<tr>
<td>29</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
</tr>
<tr>
<td>35</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
</tr>
<tr>
<td>43</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
</tr>
<tr>
<td>102</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
</tr>
<tr>
<td>108</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
<td>AML</td>
</tr>
<tr>
<td>119</td>
<td>Final bleed</td>
<td>Final bleed</td>
<td>Final bleed</td>
<td>Final bleed</td>
<td>Final bleed</td>
<td>Final bleed</td>
<td>Final bleed</td>
</tr>
</tbody>
</table>

Immunization schedule for production of rabbit antibody to human leukemia plasma pellets. Each inoculum contained 2 ml of pellet material; an equal volume of Freund's complete adjuvant was included in the 1st and 4th injections. Remaining inocula were 2 ml and contained no adjuvant. Inoculations were subcutaneous and intradermal at multiple sites, 0.1 to 0.2 ml per site. AML, acute myelocytic leukemia pellet employed as inoculum; ALL, acute lymphocytic leukemia pellet employed as inoculum.
morphonuclear neutrophils; eosinophils were brilliant orange.

Additional measures employed to reduce nonspecific reactions included (a) pretreatment of all smears prior to actual staining with a 24 mg/ml solution of bovine albumin containing 1 mg/ml of rhodamine-labeled bovine albumin and (b) addition of 1.5 M glycine to the final rinse in pH 7.0 buffer. The latter was employed to dissociate any nonantigen-antibody complexes formed during the final staining procedure (20).

The pretreatment with rhodamine-labeled albumin produced a definite orange fluorescence in the cytoplasm of normal polymorphonuclear neutrophils; eosinophils were brilliant orange. Cultured leukemic cells occasionally contained very pale orange or red fluorescence in the nucleoli and cytoplasm; no nuclear fluorescence was noted. In bone marrow smears, cytoplasmic granules in members of the myelocytic series were bright orange. Plasma cells were generally light yellow.

The final staining was performed at 37°C for 60 minutes in a humidified chamber. Preparations were examined with dark-field ultraviolet illumination in a Leitz Ortholux microscope. The filter system consisted of a UG-1 or UG-5 exciter filter and a 430 mμ barrier filter.

Reference Reagents. Aliquots of globulin obtained from the final bleedings of each rabbit injected with leukemic pellet material were sent to Dr. Mary Fink, who, after preliminary tests, prepared a labeled globulin from a pool of Rabbits 10 and 11 and tested it against known positive and negative bone marrow specimens. Aliquots of this material were returned to us, along with some of Dr. Fink's own labeled monkey antihuman leukemic pellet (LPA) preparation. These two LPA reagents were employed as reference antibodies to standardize our labeled LPA globulins.

Criteria for Positive Reactions and Positive Specimens. The foregoing fixing and staining procedures were found to yield reproducible findings with the same specimen observed under coded conditions by at least two observers. Cells which contained only green cytoplasmic fluorescence (Figs. 2, 3) and did not exhibit rhodamine fluorescence were considered positive.

In many slides the contrast between these positive cells and those with only rhodamine fluorescence was remarkably distinct. However, in other slides the cytoplasm of some cells, particularly the comparatively large mononuclear cells found in peripheral leukocyte and bone marrow smears from acutely ill myelogenous leukemia patients, often contained both fluorescein and rhodamine fluorescence. These specimens were initially recorded as questionable. But if in subsequent tests with additional labeled antibodies the same cell types always contained fluorescein label, the specimen was designated as probably positive. The final criterion by which a specimen was designated positive required that the immunofluorescent reaction not be blocked by unadsorbed antibody to normal human plasma pellet (NPA) but that it be blocked by unadsorbed unlabeled LPA. This requirement eliminated many questionable reactions.

The proportion of reactive cells varied from patient to patient and from specimen to specimen from the same patient. Generally, an index of 1% or more of clearly positive cells of the same morphologic type was employed as the minimal basis for designating a specimen as positive. Specimens containing 0.01 to 0.1 percent positive cells were recorded as questionable. The occasional specimen with only a few weakly positive cells on the entire smear was recorded as negative. The number of cells examined on each specimen varied with leukocyte density. Never less than 100 cells, usually at least 1000, and often many thousands were viewed.

RESULTS

Reactivity of Fluorescein-labeled Antinormal Plasma Pellet Antibodies. Undiluted (10 mg/ml) aliquots of the six (Numbers 16-21) labeled NPA preparations which had been absorbed with all absorbants except buffy coat prior to DEAE-cellulose chromatography yielded reactions graded + to ++ when tested with normal and leukemic leukocytes (Table 2). Three preparations, Numbers 17, 19, and 20, also reacted when diluted 1:2. Number 17 yielded nuclear reactions with virtually all leukocytes; the 5 others produced cytoplasmic reactions only. One step blocking tests with homologous unadsorbed unlabeled NPA preparations prevented reactions with normal and leuko-

<table>
<thead>
<tr>
<th>NPA number</th>
<th>F/P ratio (ug/mg)</th>
<th>Highest dilution of labeled NPA reactive with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peripheral leukocytes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not BC absorbed</td>
</tr>
<tr>
<td>16</td>
<td>6.62</td>
<td>Undiluted</td>
</tr>
<tr>
<td>17</td>
<td>3.72</td>
<td>1:2</td>
</tr>
<tr>
<td>18</td>
<td>7.24</td>
<td>Undiluted</td>
</tr>
<tr>
<td>19</td>
<td>7.14</td>
<td>Undiluted</td>
</tr>
<tr>
<td>20</td>
<td>10.45</td>
<td>Undiluted</td>
</tr>
<tr>
<td>21</td>
<td>7.10</td>
<td>Undiluted</td>
</tr>
</tbody>
</table>

Immunofluorescent reactivity of fluorescein-labeled antinormal plasma pellet antibody (NPA) preparations. nr, no reactivity; F/P, fluorescein to protein; BC, buffy coat.

*Tests conducted with reagents prior to absorption with peripheral leukocytes.

+Tests conducted after all absorptions and chromatography were completed.

Undiluted reagents contained 10 mg globulin per ml.

NPA-17 reacted with leukocyte nuclei; all others yielded cytoplasmic reactions only.
Table 3

<table>
<thead>
<tr>
<th>LPA number</th>
<th>F/P ratio (µg/mg)</th>
<th>Reactivity on peripheral leukemic leukocytes (0.1 ml of each dilution)</th>
<th>Antibody units/mg of LPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>8.61</td>
<td>+++++</td>
<td>+ + +</td>
</tr>
<tr>
<td>11</td>
<td>5.19</td>
<td>+ + + + + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>12</td>
<td>7.28</td>
<td>+ + + + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>13</td>
<td>3.9F</td>
<td>+ + + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>14</td>
<td>4.55</td>
<td>+ + + + + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>15</td>
<td>12.48</td>
<td>+ + + + + + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>Ref. 1a</td>
<td>nt</td>
<td>nt + + + +</td>
<td>nt + + + + + +</td>
</tr>
<tr>
<td>Ref. 2b</td>
<td>nt</td>
<td>nt + + +</td>
<td>nt + + + + + +</td>
</tr>
</tbody>
</table>

Titration of the immunofluorescent reactivity of labeled absorbed antileukemic plasma pellet antibody (LPA) preparations. nt, not tested.

a Monkey reference globulin supplied by Dr. Fink.
b Rabbits 10 and 11 reference globulin prepared by Dr. Fink.

Immunofluorescence in Leukemia

Standardization of fluorescein-labeled antileukemic plasma pellet antibodies. Aliquots of the six (Numbers 10-15) labeled LPA preparations which had been absorbed with all absorantes except buffy coat prior to DEAE-cellulose chromatography required 2 to 3 sequential absorptions with normal buffy coat to remove all reactivity for normal peripheral and bone marrow leukocytes. Positive leukemic peripheral leukocyte smears, as predetermined in direct immunofluorescent tests with the two labeled LPA reference preparations, were employed as test cells to titrate the fully-absorbed, labeled LPA preparations (Table 3). The intensity of the reactions was graded ± to +++ relative to the reactions given by the reference LPA. All six labeled LPA preparations yielded positive reactions but varied in their antibody content from 2 units (Number 13) to 8 units (Numbers 10, 12, 14, and 15) per 0.1 ml. One antibody unit was defined as contained in 0.1 ml of that dilution of labeled LPA which yielded a reaction comparable to a one plus positive reaction by the reference reagents. The unit dilution was generally 2-fold less than the end-point of reactivity. Two units of labeled LPA were employed in initial screening tests on peripheral leukocyte and bone marrow cells.

One step blocking tests with 0.1 ml of undiluted homologous unlabeled unabsorbed LPA effectively inhibited reactions with 1:2 dilutions of labeled LPA preparations (Table 4). Reciprocal blocking tests between LPA preparations revealed common antibodies. However, no one unlabeled LPA blocked the reactivity of all six labeled LPA. This result should be considered no more than suggestive that different antibodies were present in the various LPA preparations. Detailed analysis of the specific antibody differences, if any, in the LPA preparations was not within the scope of the present study. However, one step blocking tests performed with undiluted, unabsorbed, unlabeled NPA against two units of each LPA did not inhibit immunofluorescent reactions. Undiluted preimmunization sera were without effect also. Thus, the LPA preparations contained antileukemic leukocyte antibodies for which no blocking antibody was present in the NPA preparations.

Immunofluorescent screening tests on peripheral leukocytes. A total of 101 peripheral leukocyte specimens from 62 individuals (Table 5) were tested with two units of antibody from two different labeled LPA preparations. Positive and negative reactivities were rechecked by undiluted homologous unlabeled unabsorbed LPA. One positive patient had no Philadelphia chromosomes. One positive and one negative patient had additional chromosomal abnormalities.

Summary of immunofluorescent tests on peripheral leukocytes with labeled antileukemic pellet antibody.

a Specimens obtained during therapy with active disease, and obtained during blastic crisis. One positive patient had no Philadelphia chromosomes. One positive and one negative patient had additional chromosomal abnormalities.
b Specimens obtained after therapy when the disease was under apparent control.
ineconclusive specimens were retested under coded conditions with 2 or 3 additional LPA preparations. Because of the low antibody concentration in LPA-13 (Table 3) necessitating its use undiluted, it was not routinely employed. All positive cells were tested with LPA Numbers 10, 12, 14, and 15, and, if sufficient slides were available, with LPA-11. Positive reactions were confirmed by their ability to be blocked with unlabeled unabsorbed LPA and not with NPA preparations. The latter blocking procedures eliminated many of the questionable reactions noted in the initial screening tests. A total of 25/60 specimens from 10/29 different myelocytic leukemia patients were designated positive. None of 12 specimens from 12 different lymphocytic leukemia patients were positive. Likewise, no specimens from 10/29 different myelocytic leukemia patients were designated positive. None of 12 specimens from 15 normal individuals were positive.

Of the ten individuals with positive peripheral leukocytes, four were acute myelocytic patients and six were chronic myelocytic patients. In general, the presence of positive cells, particularly when a high percentage of cells were reactive, was associated with active or blastic disease. In only a single instance was a specimen judged positive from a patient in apparent clinical remission. All reactions were cytoplasmic (Figs. 2, 3). Nuclear immunofluorescence was not observed.

Immunofluorescent reactions varied in intensity and in the percent of cells involved. Generally, the more intense reactions occurred when only one to five percent of the cells were involved. In other instances when a high percentage of similar cells reacted, the staining was less intense. The latter occurred mainly in patients whose peripheral WBC was 25,000/cu mm or more (Table 6) and consisted almost exclusively of relatively large mononuclear cells. The intensity of the staining reaction in these cells was found to be proportional to the units of antibody employed in the immunofluorescent tests.

**Immunofluorescent Screening Tests on Bone Marrow Cells.** A total of 74 bone marrow specimens from 28 individuals (Table 7) were examined in the same manner as were the peripheral leukocyte specimens. Thirteen positive specimens from five different patients were identified. All were obtained from patients with myelocytic leukemia whose peripheral leukocytes were also positive (Table 6). The positively reacting bone marrow cells resembled those seen in buffy coat smears and were designated as immature mononuclear members of the myelocytic series.

None of the 18 normal bone marrow smears or 24 specimens from patients with diseases other than myelogenous leukemia contained myelocytic cells which were sufficiently reactive by immunofluorescence to be judged positive. Very low intensity staining, generally graded as suggestive at best, was noted in a few cells of the myelocytic series in normal bone marrow smears. The cell types yielding these questionable reactions ranged from myeloblasts through metamyelocytes, but they most often were myelocytes. Generally, when this reaction was present, 1 to 5% of the myelocytes were involved. These observations are at most only suggestive that some of the antibody in our LPA reagents may be directed against an antigen.

### Table 7

<table>
<thead>
<tr>
<th>Specimen source</th>
<th>Number positive/total specimens</th>
<th>Number positive/total patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelocytic leukemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>10/22</td>
<td>4/6</td>
</tr>
<tr>
<td>Chronic</td>
<td>3/11</td>
<td>1/3</td>
</tr>
<tr>
<td>Total</td>
<td>13/33</td>
<td>5/9</td>
</tr>
<tr>
<td>Lymphocytic leukemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute</td>
<td>0/8</td>
<td>0/5</td>
</tr>
<tr>
<td>Chronic</td>
<td>0/3</td>
<td>0/1</td>
</tr>
<tr>
<td>Total</td>
<td>0/11</td>
<td>0/6</td>
</tr>
<tr>
<td>Other diseases</td>
<td>0/13</td>
<td>0/8</td>
</tr>
<tr>
<td>Normal individuals</td>
<td>0/17</td>
<td>0/5</td>
</tr>
</tbody>
</table>

Summary of immunofluorescent tests on bone marrow cells with labeled antileukemic pellet antibody.

### Table 8

<table>
<thead>
<tr>
<th>Specimen source</th>
<th>IF+, IF−, Total IF+</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>3/3 3/1 0/4</td>
</tr>
<tr>
<td>CML</td>
<td>3/3 3/0 4/4</td>
</tr>
<tr>
<td>ALL</td>
<td>0/0 4/0 0/4</td>
</tr>
<tr>
<td>CLL</td>
<td>0/0 1/0 0/1</td>
</tr>
<tr>
<td>Normal</td>
<td>0/0 3/0 0/3</td>
</tr>
<tr>
<td>Other</td>
<td>0/0 2/0 0/2</td>
</tr>
<tr>
<td>Total</td>
<td>6/6 19/32 12/32</td>
</tr>
</tbody>
</table>

Comparison of immunofluorescence of peripheral leukocytes and presence of virus-like particles in plasma pellets obtained from the same specimen. IF, immunofluorescence test on buffy coat cells; EM, electron microscopic examination of plasma pellets (thin section); AML, acute myelocytic leukemia; CML, chronic myelocytic leukemia; ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia.

Peripheral WBC, bone marrow immunofluorescence tests, and presence of virus-like particles in the plasma of patients with immunofluorescent-positive peripheral leukocytes. nt, not tested; AML, acute myelocytic leukemia; CML, chronic myelocytic leukemia.
present during some stage of myelocytic morphogenesis. If, indeed, an antigen of this latter nature was being detected, its concentration in normal myelocytes was markedly less than in cells from myelocytic leukemia patients.

Correlation between Immunofluorescence of Peripheral Leukocytes and Presence of Virus-like Particles in Plasma Pellets. Electron microscopic examination was performed on 32 plasma pellets for which immunofluorescent data on peripheral leukocytes were available (Table 8). Seven pellets contained virus-like particles; peripheral leukocytes were positive in 6/7 specimens. Twelve peripheral leukocyte specimens were immunofluorescent positive; virus-like particles were detected in six of the corresponding pellets. This result suggests that positive immunofluorescence predicted a 50% chance that virus-like particles would be present in the plasma. Positive findings for both tests were associated in 5/6 instances with WBC >25,000/cu mm, no virus-like particles were seen in the plasma pellets. Thus high WBC do not appear to be a reliable index to predict the presence of virus-like particles. The absence of immunofluorescent-positive cells in peripheral blood appeared to predict a negative electron microscopic finding, since only one of twenty immunofluorescent-negative specimens yielded virus-like particles (Table 8).

Effect of Absorption of Labeled LPA Preparations with Normal Human Bone Marrow Powders. The possibility that the antigens revealed in leukemic cells by immunofluorescence were present in normal human bone marrow but in low concentration was further explored by absorbing the labeled LPA preparations with normal human bone marrow powder. Undiluted aliquots of six LPA preparations, including LPA reference 1 supplied by Dr. Fink, were absorbed with the hematologic powders listed in Table 9. The mg of each powder per mg of labeled globulin required to remove all reactivity from each undiluted LPA was determined. Immunofluorescent tests were conducted on known positive cells from the same patient. Sixteen mg of normal calf bone marrow powder per mg of labeled LPA failed to remove reactivity, even though the intensity of the staining reactions was somewhat diminished by this procedure. The amount of normal human bone marrow powder required to remove all reactivity was of the order of 4 to 8 mg per mg of labeled reagent, whereas essentially 1 mg of leukemic buffy coat powder completely extinguished reactivity. Four to eight units of antibody were removed by these absorption procedures. These data indicate a 4- to 8-fold greater antigenic content in the leukemic absorbant than in the normal absorbant.

One cannot ascribe all the absorption by normal human bone marrow powder to serologic reaction, since inherent physical entrapment occurs during absorption. However, the fact that absorption of a labeled hamster antiadenovirus-12 T-antigen (8 units per mg) preparation with 20 mg of normal human bone marrow powder per mg of labeled globulin did not reduce its reactivity for adenovirus-12 T-antigen is further evidence that a significant proportion of the absorptive capacity of normal bone marrow powder was due to specific serologic binding.

Further evidence of specific serologic absorption was obtained by absorbing undiluted aliquots of LPA-10, 11, and 12 with 23 individual normal human bone marrow powders at 1 mg per mg of labeled globulin (Table 10). In this study the intensity of the staining reaction following absorption was estimated visually, an admittedly subjective procedure. However, the tests were conducted under coded conditions. Four powders were without apparent absorptive effect. The remaining 19 removed various amounts of labeled antibodies, five of these (boxed in Table 10) consistently removed almost all reactivity. These five powders, when employed at the 1 mg/mg ratio, did not diminish the immunofluorescent reaction of the hamster anti-Ad-12 T-antigen system.

Individual bone marrow materials for absorption were obtained postmortem and supplied through the auspices of the Tissue Resources Unit, Viral Carcinogenesis Branch, National Cancer Institute. There was no apparent correlation of age, sex, race, and cause of death to absorptive capacity of individual bone marrow powders. None of the specimens employed was from leukemic or cancerous patients.

<table>
<thead>
<tr>
<th>LPA number</th>
<th>Antibody units per mg</th>
<th>Pooled normal calf bone marrow powder</th>
<th>Pooled normal human bone marrow powder</th>
<th>CML human buffy coat powder</th>
<th>Ratio of normal to leukemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>8</td>
<td>&gt; 10</td>
<td>2-4</td>
<td>0.8-1.0</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>&gt; 10</td>
<td>4-8</td>
<td>0.8-1.0</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>&gt; 10</td>
<td>8-16</td>
<td>1.0-2.0</td>
<td>8</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>&gt; 10</td>
<td>4</td>
<td>1.0</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td>8</td>
<td>&gt; 10</td>
<td>8-16</td>
<td>1.0-2.0</td>
<td>8</td>
</tr>
<tr>
<td>Ref. 1a</td>
<td>4</td>
<td>&gt; 10</td>
<td>4</td>
<td>0.8-1.0</td>
<td>4</td>
</tr>
</tbody>
</table>

*Control: hamster anti-adenovirus-12T vs adenovirus-T antigen
8 > 10 > 20 nt

Effect of absorption of labeled LPA globulins with hematologic powders. nt, not tested; CML, chronic myelocytic leukemia; LPA, antileukemic plasma pellet antibody.

a Reference LPA supplied by Dr. Fink.
Lymphoma Cells. Twenty-one different cell cultures derived
from patients with leukemia or lymphoma (Burkitt) were
tested with labeled LPA preparations in the same manner as
buffy coat cells. Three cultures contained cells during early
passages which gave positive cytoplasmic immunofluorescent
reactions. However, once these cultures were in continuous
passage, they became negative as were other established leu-
kenic cell lines.

DISCUSSION

Before considering the implications of these studies it should
be pointed out that the main differences between these results
and those of Fink et al. (6, 8–10) include our noting (a) positive
immunofluorescent reactions in myelocytic leukemia only, (b)
eytoplasmic reactions only, and (c) no immunofluorescence
with cultured human lymphoma and leukemia cell lines. Al-
though all six rabbits received lymphocytic leukemia pellet
material, only four were given it during the second month of
the 4-month immunization schedule (Table 1). It is possible
that these four rabbits were insufficiently sensitized to lympho-
cytic antigens during this interval. However, this seems un-
likely, since these antisera contained antibody for normal
lymphocytes and granulocytes. Absorption with normal
bone marrow powders (Table 1) was achieved by selected fixing procedures and prestaining with
rhodamine labeled albumin.) Immunofluorescent reactions be-
tween fully absorbed LPA and leukemic cells could thus be
attributable to antibody directed against antigen(s) present in
leukemic cells and not in normal leukocytes. This interpreta-
tion became untenable when it was shown that the anti-
body could be absorbed from the LPA preparations with
from a larger number of patients that positive reactions might
have occurred with other than myelocytic leukemia cells. How-
ever, at this point it is concluded that our fully absorbed
antisera were specific for myelocytic cells. Furthermore, the
presence of antibody for cytoplasmic antigens only in our fully
absorbed LPA preparations indicates a further degree of
specificity.

Accurate interpretation of an immunofluorescent reaction is
dependent upon precise knowledge of at least one of the sero-
logic participants. A complete understanding of the nature of
the antigen or antigens reactive in immunofluorescent tests
with antisera produced in heterologous species to pellets from
human leukemic plasma is difficult since these materials elicit
formation of antibodies to fibrinogen, albumin, globulins, red
blood cells, platelets, and normal leukocytes. Absorption with
the latter materials to the point that serologic reactions are
no longer detectable with the absorbant is a generally accepted
method of removing antibody specific for the absorbant. In the
present study, the absorption procedures employed were suffi-
cient to remove these antibodies, including those for normal
lymphocytes and granulocytes. (Elimination of nonserologic
binding of labeled globulin with granulocytes and eosinophils
was achieved by selected fixing procedures and prewashing with
rhodamine labeled albumin.) Immunofluorescent reactions be-
tween fully absorbed LPA and leukemic cells could thus be
attributable to antibody directed against antigen(s) present in
leukemic cells and not in normal leukocytes. This interpreta-
tion was supported when it was shown that (a) unabsorbed normal
plasma pellet antisera, which reacted with normal leukocytes,
did not block positive immunofluorescent reactions between
LPA and leukemic cells and that (b) these reactions were
blocked by unabsorbed LPA preparations. However, this inter-
pretation became untenable when it was shown that the anti-
body could be absorbed from the LPA preparations with

<p>| Bone marrow | Graded intensity of staining reaction after absorption (+++ to absorption) | Bone marrow | Graded intensity of staining reaction after absorption (+++ to absorption) |</p>
<table>
<thead>
<tr>
<th>source</th>
<th>(Age (years))</th>
<th></th>
<th>source</th>
<th>(Age (years))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>25</td>
<td>++</td>
<td></td>
<td>Sex</td>
</tr>
<tr>
<td>F</td>
<td>55</td>
<td>++++</td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>F</td>
<td>55</td>
<td>+</td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>F</td>
<td>60</td>
<td>++</td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>F</td>
<td>60</td>
<td>+</td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>F</td>
<td>65</td>
<td>++</td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>F</td>
<td>67</td>
<td>++</td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>F</td>
<td>67</td>
<td>+</td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>F</td>
<td>74</td>
<td>+</td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>F</td>
<td>76</td>
<td>++++</td>
<td></td>
<td>M</td>
</tr>
</tbody>
</table>
normal human bone marrow powders. It thus appears that the antigen(s) responsible for the immunofluorescent reactions in these studies are present in normal human bone marrow cells, but in concentrations too low to be accurately detected by direct immunofluorescent tests. They would appear to be absent in the mature leukocytes of the peripheral circulation of nonleukemic individuals.

The observation that the immunofluorescent positive cells were generally immature cells of the myelocytic series could indicate that the reacting antigen(s) are characteristic of young cells of this series and that these antigens disappear with myelocytic maturation. This could explain the ability of normal human bone marrow to absorb the antibody, since immature myelocytic cells are normally present in bone marrow. Likewise, it could also explain the absence of reactivity with mature peripheral blood granulocytes. There were, however, two observations which may argue against this hypothesis. First was the failure to obtain specific fluorescence with nonleukemic bone marrow preparations which certainly contained immature myelocytic cells, and second was the failure to obtain immunofluorescence with established in vitro cultures of leukemic myeloblasts (12).

The first observation could perhaps be explained on a quantitative basis, i.e., the leukemic cells of the myelocytic series contained much higher levels of the "immature myelocytic antigen(s)" than did the normal immature myelocytic cells. This is suggested by the fact that a very low level of fluorescence (graded at best as questionable) was observed in some normal bone marrow preparations, and the cells showing this phenomenon were young cells of the myelocytic series. On the other hand, it might be that the responsible antigen(s) are present only briefly during normal myelomorphogenesis and, hence, are rarely encountered in normal bone marrow cells in a quantity sufficient to give bright fluorescence. The leukemic myelocytic cells might undergo "maturation arrest" during this period of antigen(s) production and thus continue synthesis. The fact that established cultures of leukemia myeloblasts do not give positive reactions detracts from this postulate but this could be due to cultural selection of stem cells which had not yet acquired the evanescent antigen(s) due to too little differentiation.

Fink et al. (6, 8) detected immunofluorescent-positive cells in remission bone marrows and noted that in some cases their appearance actually preceded relapse. This observation is in accord with the hypothesis that these antigens are associated with myelocyte maturation and are expressed in detectable amounts due to a control defect. The mechanism by which this hypothetical defect might be induced is not known. That it may be due to viral infection is within possibility. It is of interest that recent observations indicate that the avian leukemia COFAL antigen may be related to a normal embryonal chicken antigen (5). Furthermore, tumor antigens induced by chemical carcinogens have been demonstrated in embryonal tissues (18). These two observations support the concept that viruses may interfere with regulation of antigen synthesis during normal cellular maturation.

In order to attribute the antigen(s) involved in the immunofluorescent reactions obtained in these studies to the action of a virus, it is necessary to postulate that this virus has infected leukemic and at least some normal individuals whose bone marrow cells then maintain low concentrations of the antigens. At the present time there is little evidence to support this hypothesis in total. To designate the "virus-like particles" in our leukemic plasma pellets as actual viruses in the absence of any known biologic activity is tenuous (1, 4). The coincidental finding of positive immunofluorescence in leukemic cells and the presence of virus-like particles in the plasma may be related, not because these particles are virions, but because they represent a virus-mimicking segment of the cellular fragments present in these pellets (Fig. 1). Furthermore, even if the virus-like particles were virions, one cannot be certain that their concentration in the pellets was sufficient to be immunogenic.

It is, therefore, our belief that the antigen(s) demonstrated in leukemic cells in these studies cannot as yet be designated as viral or viral induced.

Demonstration of quantitative antigenic differences between normal and neoplastic tissues is not new (13, 21-24, 26). Designation of an antigen as tumor specific requires only in the narrowest sense that it not be found in normal tissue. In broader terms an excessive amount of antigen in neoplastic tissue compared to normal tissue may be defined as a "tumor-specific antigen" (24). The level at which quantitative differences are sufficient to be designated tumor specific is difficult to assess. In the present study the 4- to 8-fold greater antigenic content (antibody absorptive capacity) of leukemic buffy coat powder as compared to normal bone marrow powder may represent too low a quantitative difference to designate the antigen(s) as tumor specific. Perhaps more sensitive techniques such as the paired radiiodine-labeled antibody technic (26), will provide more accurate comparisons of quantitative antigenic differences between normal and leukemic leukocytes.

REFERENCES


Fig. 1. Electron photomicrograph of sections through pellets obtained from the plasma of four patients with myelogenous leukemia showing cross sections of virus-like particles (arrows). X 80,000. Bar indicates 0.5 μ.

Fig. 2. Photomicrograph of an immunofluorescent-positive reaction in relatively large mononuclear cells found in the bone marrow of a patient with active chronic myelogenous leukemia. Photograph slightly overexposed to show smaller negative cells in the background. X 520.

Fig. 3. Higher magnification of a positive immunofluorescent reaction in buffy coat cells of the same patient. Note the variation in distribution of cytoplasmic staining. X 1,200.
Immunofluorescence in Leukemia
Immunofluorescent Studies in Human Leukemia

David S. Yohn, Julius S. Horoszewicz, Rose Ruth Ellison, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/28/9/1692

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/28/9/1692.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.