Fetal Antigens in Human Leukemia

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Summary

Immunization of BALB/c male mice with human peripheral leukemic blasts effectively reduced the later formation of syngeneic fetal liver, but not bone marrow hematopoietic colonies in the spleen when these mice were lethally irradiated and challenged i.v. Fetal antigen was detected in 6 of 6 lymphocytic leukemic patients and in 4 or 8 myelocytic leukemia patients and was correlated with low cellular levels of sialic acid.

A rabbit antiserum to BALB/c 15-day fetal liver cells labeled only 0 to 2% of normal donor peripheral leukocytes in indirect immunofluorescence but reacted with 10 to 21% of leukemic peripheral blasts. Active disease bone marrow on the same patients gave 7 to 40% fluorescent cells. Two remission bone marrow samples were negative and 1 had 44% fluorescent cells. Using this antiserum coupled to sepharose, affinity column separation of KCI extracts from mouse and human fetal liver and from chronic lymphocytic leukemia has produced 4 common protein bands (identifiable on polyacrylamide gel electrophoresis).

Sera from mice immunized with leukemic blasts reacted with syngeneic fetal liver cells, but not with bone marrow or adult liver by immunofluorescence. While only 3 to 10% of the cells were positive in the unfractionated fetal liver, separation of cells by density on discontinuous albumin gradients gave 15 to 40% fluorescence in the 23% albumin fraction. This represented a 70 to 90% purification of the leukemia cross-reactive cell (recovery of fluorescent cells) and, concomitantly, 79% recovery of the hematopoietic stem cell, as determined by the spleen colony assay.

The data suggest that antisera raised against the purified fetal hematopoietic stem cells or the solubilized cross-reactive leukemia antigen may be valuable in monitoring the clinical status of leukemia patients.

Introduction

Fetal antigens have been demonstrated on the surface of a variety of human solid tumor cells by in vitro assays primarily using heterologous antihuman fetal serum (1, 4, 8, 9, 23, 29, 36). Harris et al. (17) and Bentwich et al. (6) have also suggested the presence of fetal antigen on human leukemic cells as determined by immunological criteria.

Hemoglobin F production has been reported in juvenile chronic myeloid leukemia, but the slight elevations noted in adult forms of leukemia were attributed to recovery from chemotherapy-induced bone marrow aplasia (37). Marked increases in ferritin concentration of leukemia serum (21) and cells (38) may reflect either a normal tissue ferritin (10) or an electrophoretically distinct isoferritin common to fetal liver and placenta (22, 28). Lewis et al. (24) have noted human bone marrow fluorescent labeling by an antifetal serum in nonneoplastic abnormalities, particularly in pernicious anemia and folic acid deficiency, as well as in several types of leukemia. This antiserum also binds to a number of human solid tumor cells (4).

The best available evidence for a human leukemia-associated fetal antigen is the demonstration by Hollinshead and Herberman (19) that the skin reactive antigens isolated from ALL blasts are electrophoretically similar to early (6 to 18 weeks) fetal thymus antigens that gave positive skin test reactions in ALL but not AML patients. Furthermore, in these studies, antigen preparations from 26- to 30-week fetal thymus, or from early gestation spleen and bone marrow, failed to elicit delayed hypersensitivity reactions in either ALL or AML patients.

Salinas et al. (34, 35) have developed a quantitative in vivo assay for tumor-associated fetal antigen based on the reduction of syngeneic fetal liver colonies by cytostatic antibody or sensitized lymph node cells. We have applied this model to the demonstration of fetal antigens on human tumor cells (12).

While interspecies cross-reactivity of fetal antigen has been shown in animal systems (2, 34), no studies to our knowledge have based the characterization and quantitation of human tumor-associated fetal antigens on the cross-reactivity of murine and human fetal antigens. In this report, we present evidence for such cross-reactivity and results of some biophysical and immunological studies on leukemia-associated fetal antigens.

Materials and Methods

Preparation of Immunogens. Single cell suspensions were prepared from the liver of 15-day BALB/c fetuses and...
from adult femoral bone marrow by several passages of the tissue through an 18-gauge needle. Adult liver cells were prepared by lysozyme digestion of tissue fragments and Ficoll gradient separation (32). Human fetal liver was obtained from a therapeutically aborted nonviable 19-week fetus. Single cell suspensions were prepared by mechanical dispersion of the teased fetal liver through a 50 mesh screen. Human leukemia cells were collected by leukapheresis, as previously described (15). Antigen was solubilized from tumor cell suspensions with 3 M KCl by Meltzer’s modification of the procedure of Reisfeld et al. (26). The extracts were stored at -70°, while FLC and tumor cells were stored in liquid N2 as viable cell suspensions with glycerol or dimethyl sulfoxide. Peripheral WBC freshly drawn from normal donors were separated from defibri-
nated venous blood by sedimentation with dextran and by
lysis of residual RBC. Lymphocytes stimulated with PHA
(Difco Laboratories, Detroit, Mich.) were incubated with 0.05 ml PHA/106 cells for 48 hr (13).

Spleen-Colony Assay. BALB/c male mice (8- to 10-weeks old, specific pathogen free) were immunized i.p. 3 to 5 times in a 2-week period with 106 viable (trypan blue dye-excluding) test cells or with an equivalent dose of KCl extract (0.3 to 0.5 mg) for each injection. There were 15 or 20 mice/group. Test cells used for immunization included various human leukemic or normal cells and human or mouse FLC. Syngeneic cells were irradiated with 5000 rads (137Cs). Nonimmunized controls were given injections of Hanks’ balanced salt solution.

On the 4th week, all mice received a lethal dose (750 R) of whole-body radiation from an Atomic Energy of Canada, Ltd. Eldorado 8 teletherapy unit. There was no spontaneous hematological recovery. The mean background count from endogenous colony formation after irradiation was 0.04 CFU per spleen. Furthermore, such mice are immunologically incompetent, but still have circulating antibody.

Twenty-four hr after irradiation of the mice, a freshly prepared colony-forming inoculum was injected i.v. Ten mice/group were challenged with syngeneic FLC from 15-
day fetuses (2.5 or 5 x 104 cells); the remaining mice were challenged with 0.5 or 1 x 106 syngeneic adult bone marrow cells as controls. Immunized groups received injections before the Hanks’ group to offset any loss in viability of the colony-forming cells. Spleens were collected 8 days later and fixed in Bouin’s solution. The number of surface colonies was counted, and the mean values for immunized and nonimmunized animals were compared by the 2-tailed Student’s t test. Reduction of FCFU in normal mouse bone marrow cells available was limited, the serum incubations were carried out on acetone-fixed cells mounted in wells on Teflon-coated glass slides.

Affinity Chromatography. The DEAE-purified IgG from the rabbit antiserum to BALB/c fetal liver was bound to Sepharose 4B (Pharmacia, Uppsala, Sweden) by the cyanogen bromide-coupling method of Porath et al. (31). The antibody-linked sepharose was packed in a Pharmacia K 16/ 20 column and attached to a “cyclum” device (Oak Ridge National Laboratories, Oak Ridge, Tenn.) for automated affinity chromatography (3). After thorough washing and equilibration of the column with PBS (pH 7.0), 3 M KCl extracts of mouse or human fetal liver and of a single CLL were processed through the column in small (3- to 4-ml aliquots at room temperature at a flow rate of 1.5 ml/min, with spectrophotometric monitoring at 280 nm. Elution of absorbed material was accomplished with 10 ml of 3 M NH4SCN. All fractions emerging from the column were continuously dialyzed against PBS, collected at 4°, concentrated with a Diaflow PM 10 membrane, and filtered with a Millipore filter.

Polyacrylamide Gel Electrophoresis. We followed the procedure described by Hollinshead et al. (20) for gradient polyacrylamide gel electrophoresis separation of tumor antigens. The gel solutions were layered as follows: 12% solution to the 6-cm mark, 7% to 7.5 cm, 4.75% to 3 cm, and 3.5% to 10 cm. Fetal and leukemic antigen extracts were
analyzed electrophoretically before and after affinity chromatography separation to identify the components that were absorbed by the antifetal serum.

**Fetal Liver Fractionation.** Separation of hematopoietic stem cells from BALB/c 15-day fetal liver was accomplished using the discontinuous albumin density gradient centrifugation technique of Dicke et al. (7), with the final solutions corresponding to the osmolarity of mouse serum (320 mOsmoles/liter). Cells recovered from the 19, 21, 23, and 25% albumin fractions were then analyzed for stem cell capacity in the spleen colony assay and were tested in immunofluorescence with either antifetal or antileukemic serum.

**Results and Discussion**

**Cross-Reactivity of Human and Murine Fetal Antigen.** Table 1 shows the effect of FLC immunization on FCFU when mice were challenged with 2.5 × 10⁶ FLC. Preimmunization with either BALB/c or human FLC significantly reduced FCFU from the control value of 7.2 colonies. Xenogeneic fetus was more effective than irradiated syngeneic FLC. Limited availability of human fetal tissue precluded testing another group of mice against a bone marrow challenge. However, there was no reduction of bone marrow CFU in mice immunized against syngeneic FLC.

Controls for nonspecific immunization were conducted with irradiated BALB/c adult bone marrow, liver and brain, normal human liver and spleen, normal human peripheral WBC, and KCl extracts from normal human adult liver and pooled WBC. None of these immunization procedures reduced either bone marrow or fetal colony formation.

PHA-stimulated lymphoblasts (from 6 donors) were used as a control for cell cycle antigens that might be expressed on proliferating cells. Enhancement of fetal colonies was observed while CFU were slightly reduced. The former effect could be related to the production of colony-stimulating factor by the PHA-primed lymphocytes (30).

Immunization with syngeneic brain also enhanced FCFU development. Further investigation of this point is in progress, since our experiments with a heterologous antibrain serum (unpublished data) confirm Golub’s report (11) of reduction of both FLC and bone marrow colony formation.

These experiments demonstrated the interspecies cross-reactivity of human and murine fetal antigen, since specific reduction of fetal colonies was obtained in mice preimmunized with fetal but not adult tissue and since the immunity mediated immune response among allogeneous remission lymphocytes (13, 33).

**Detection of Human Tumor-associated Fetal Antigen.** All 6 lymphocytic leukemia patients tested demonstrated the cross-reactive fetal antigen on their cells (Table 2). This group of patients included those with ALL, CLL, and poorly differentiated lymphoma. The KCl extracts of ALL and CLL cells proved as effective as the viable cells from the same patient in inducing cross-reactive immunity. Only 4 of 8 of the AML patients, including 1 progranulocytic leukemia, were positive in this assay.

**Table 1**

<table>
<thead>
<tr>
<th>Immunization</th>
<th>CFU*</th>
<th>FCFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hanks’ solution</td>
<td>15.4 ± 0.5</td>
<td>7.2 ± 0.4</td>
</tr>
<tr>
<td>BALB/c 15-day fetal liver</td>
<td>15.9 ± 0.9</td>
<td>3.9 ± 0.6*</td>
</tr>
<tr>
<td>BALB/c adult liver</td>
<td>14.7 ± 1.0</td>
<td>8.7 ± 0.7</td>
</tr>
<tr>
<td>BALB/c bone marrow</td>
<td>16.2 ± 0.9</td>
<td>7.5 ± 0.8</td>
</tr>
<tr>
<td>BALB/c brain</td>
<td>16.4 ± 0.8</td>
<td>12.5 ± 1.7</td>
</tr>
<tr>
<td>Human 19-wk fetal liver</td>
<td>15.4 ± 0.8</td>
<td>6.6 ± 1.7</td>
</tr>
<tr>
<td>Human adult liver</td>
<td>15.3 ± 1.6</td>
<td>7.7 ± 0.8</td>
</tr>
<tr>
<td>Human spleen</td>
<td>14.4 ± 1.2</td>
<td>6.7 ± 0.9</td>
</tr>
<tr>
<td>Human WBC</td>
<td>15.7 ± 1.4</td>
<td>6.9 ± 0.5</td>
</tr>
<tr>
<td>Human WBC (KCI, 0.33 mg)</td>
<td>15.5 ± 1.0</td>
<td>6.6 ± 0.5</td>
</tr>
<tr>
<td>Human WBC (PHA)*</td>
<td>13.6 ± 0.7</td>
<td>9.4 ± 1.4</td>
</tr>
</tbody>
</table>

* 10⁶ viable cells or 3 μl KCl antigen extract from 10⁷ cells i.p., 3 to 5 times; syngeneic cells were irradiated with 5000 R, 10 mice/challenge group.

* No significant reduction of CFU by any immunization procedure.

* Mean ± S.E.

* Significant reduction from controls at the p < 0.001 level.

* PHA-stimulated lymphocyte blasts harvested after 48 hr of culture.

Greater reductions of FCFU were achieved when the mice were challenged with 2.5 × 10⁶ rather than 5 × 10⁶ FLC, so that the number of colonies in the control group (Hanks’ immunized) was lower. Reduction of fetal colonies at the optimal challenge dose were in the range of 50 to 100% (Experiments 1 to 3), whereas reductions of only 20 to 30% were obtained with 5 × 10⁶ challenge FLC (Experiments 4 to 7). Thus, for the CLL patient (T. C.), a 47% reduction was seen at the lower challenge dose compared with 30% at the higher challenge level. Repetitive testing of an ALL patient (P. C.) at the same challenge level gave reproducible results (21 and 25% reductions in FCFU). CFU controls were consistently negative at either the high- or low-dose challenge level.

Data on the amount of sialic acid released by neuraminidase treatment are also included in Table 2. Four patients’ AML cells that expressed fetal antigen all had sialic acid levels of <4 nmoles/5 × 10⁶ cells, while the 4 AML patients that were negative in the FCFU assay all had >5 nmoles/5 × 10⁶ cells. Reed et al. (33) have previously reported a wide distribution of sialic acid levels in AML cells that were both above and below the mean value of 12.9 g for normal mononuclear leucocytes. Low levels (<20) correlated with more vigorous stimulation of autologous remission lymphocytes in blastogenesis (33). Blasts from 4 ALL patients were found to express fetal antigen and to have lower than normal value of sialic acid, as previously reported. However, unlike AML blasts, leukemic lymphoblasts and their KCl extracts characteristically evoke a weak in vitro and in vivo cell-mediated immune response among autologous remission lymphocytes (13, 33).

The correlation of fetal antigen expression with low levels of sialic acid on leukemic cells is relevant to the observation by Hannon et al. (16) of increasing levels of sialic acid on
Table 2

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Immunization cell type</th>
<th>Sialic acid* (nmoles/5 x 10^9 cells)</th>
<th>Spleen colony count</th>
<th>% reduction of CFU from control (p)</th>
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<tbody>
<tr>
<td>1</td>
<td>None†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALL (S. H.)</td>
<td>2.530</td>
<td>15.4 ± 0.5</td>
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<td>ALL (H. B.)</td>
<td>3.220</td>
<td>15.2 ± 0.5</td>
<td>0.0 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>None†</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AML (J. N.)</td>
<td>22.700</td>
<td>13.7 ± 1.1</td>
<td>8.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>ALL (L. S.)</td>
<td>5.400</td>
<td>14.1 ± 1.3</td>
<td>5.5 ± 1.2</td>
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<tr>
<td></td>
<td>Progranulocytic</td>
<td>637.300</td>
<td>13.7 ± 1.2</td>
<td>5.1 ± 1.2</td>
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<tr>
<td></td>
<td>leukemia (J. T.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CLL (T. C.)</td>
<td>ND</td>
<td>12.1 ± 1.0</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Poorly differeniated</td>
<td>3.700</td>
<td>11.0 ± 1.3</td>
<td>3.1 ± 0.5</td>
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<tr>
<td></td>
<td>lymphoma (M. T.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>None†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AML (G. L.)</td>
<td>0.188</td>
<td>6.2 ± 0.9</td>
<td>5.2 ± 0.4</td>
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<tr>
<td></td>
<td>ALL (D. M.)</td>
<td>9.020</td>
<td>8.0 ± 1.1</td>
<td>1.4 ± 0.6</td>
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<tr>
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<td>ALL (M. Z.)</td>
<td>13.150</td>
<td>8.1 ± 0.9</td>
<td>0.8 ± 0.2</td>
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<tr>
<td></td>
<td>AML (N. N.)</td>
<td>2.700</td>
<td>4.6 ± 2.1</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>AML (A. W.)</td>
<td>1.097</td>
<td>6.3 ± 1.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>None†</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>AML (R. O.)</td>
<td>11.780</td>
<td>12.3 ± 0.8</td>
<td>14.1 ± 1.7</td>
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<tr>
<td>5</td>
<td>None†</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALL (P. C.)</td>
<td>ND</td>
<td>15.2 ± 1.0</td>
<td>12.3 ± 0.6</td>
</tr>
<tr>
<td>6</td>
<td>None†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALL (P. C.)</td>
<td>10.7 ± 0.5</td>
<td>9.7 ± 0.8</td>
<td>14.7 ± 0.5</td>
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<tr>
<td></td>
<td>ALL (P. C.)</td>
<td>8.7 ± 0.5</td>
<td>8.7 ± 0.5</td>
<td>11.6 ± 0.8</td>
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<td></td>
<td>ALL (P. C.)</td>
<td>9.7 ± 0.5</td>
<td>9.7 ± 0.5</td>
<td>10.4 ± 0.5</td>
</tr>
<tr>
<td>7</td>
<td>None†</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>CLL (T. C.)</td>
<td>7.7 ± 0.7</td>
<td>10.4 ± 0.6</td>
<td>9.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>CLL (T. C.)</td>
<td>8.3 ± 0.8</td>
<td>10.3 ± 0.5</td>
<td>9.0 ± 0.5</td>
</tr>
</tbody>
</table>

* Challenge dose of FLC was 2.5 x 10^9 (Experiments 1 to 3) or 5 x 10^9 (Experiments 4 to 7); challenge dose of bone marrow cells was 0.5 x 10^9 (Experiment 3) or 1 x 10^9 (all other experiments).
† Released by 50 units V. cholerae neuraminidase (1 hr, 37°) measured by the Warren assay.
‡ No significant reduction of CFU by any immunization procedure.
§ None, Hanks’ alone; NS, no significant reduction of FCFU; ND, no data.
¶ Mean ± S.E.
†† Letters in parentheses, patients’ initials.
* Letters in parentheses, patients’ initials.
††† 3 M KCl antigen extract from 10^9 cells (0.5 mg).

Fetal tissue with gestation in the hamster. In their study, the SV40 cross-reactive fetal antigen was "phased out" after Day 10, when sialic acid levels increased dramatically.

The mouse fetal liver antigen most effective in the FCFU assay has been shown to be fairly phase specific to the 15th day of gestation (35). Since at this phase the liver is primarily a hematopoietic organ, it is of interest that the cross-reactive antigen was detected on certain solid tumor cells (melanoma and colon carcinoma) as well as on most of the leukemia cells tested (12, 18). The prevalence of antigen(s) in various histological tumors remains to be determined. The FCFU assay per se would not be practical for such a screening program due to the large tumor sample required, the tedious nature of the assay, and the lack of quantitative resolution, even at the optimal challenge dose. However, the human-murine immune cross-reactivity of the fetal antigen, as demonstrated by the FCFU assay, was utilized as the basis for further in vitro testing of human cells with an antiserum to BALB/c FLC.

**Immunofluorescence.** The data in Table 3 show the reactivity of peripheral leukemic blasts or leukemic bone marrow in an indirect immunofluorescence assay with rabbit anti-fetal serum. Peripheral leukocytes from normal donors were not significantly labeled. Various histological types of leukemia all showed reactivity with the antiserum that could be nearly completely absorbed out by BALB/c FLC but not bone marrow or ALC. The percentage of fluorescent cells did not correlate with the percentage of blasts in the sample. Since cap formation was prevalent under the conditions of the assay, further experiments are in progress to determine whether the fetal antigen is actually restricted to a subpopulation of cells or whether some cells scored as negative may have "capped off" their antigen. Two of 3 remission bone marrow samples were negative, but 1 patient exhibited 44% of her remission bone marrow cells positive for fetal antigen. Since Gutterman et al. (14) have demonstrated bone marrow stimulation of autologous peripheral remission lymphocytes prior to relapse in leukemic patients, the appearance of a fetal antigen in the bone marrow should be monitored to determine whether this is indicative of a preleukemic state.

**Affinity Chromatography.** Preliminary work has been done toward the purification of this cross-reactive fetal antigen. Chart 1 is a graphic representation of the electrophoretic separation of 3 M KCl extracts prepared from mouse or human fetal liver and from CLL cells. Each sample is paired
**Table 3**

Indirect immunofluorescence of human normal WBC or leukemic cells with rabbit antiserum to BALB/c fetal liver

<table>
<thead>
<tr>
<th>WBC of normal donors (%) F</th>
<th>Diagnosis</th>
<th>Remission bone marrow (%) F absorbed</th>
<th>Leukemic bone marrow (%) F absorbed</th>
<th>Peripheral blasts (%) F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>AML</td>
<td>3 (0)</td>
<td>0-1-4</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>AML</td>
<td>3 (0)</td>
<td>0-0-4</td>
<td>ND</td>
</tr>
<tr>
<td>1</td>
<td>ALL</td>
<td>44 (0)</td>
<td>1-0-15</td>
<td>ND</td>
</tr>
<tr>
<td>0</td>
<td>Acute monomyelocytic leukemia</td>
<td>ND</td>
<td>40 (56)</td>
<td>0-25-ND</td>
</tr>
<tr>
<td>2</td>
<td>AML</td>
<td>ND</td>
<td>19 (77)</td>
<td>3-22-14</td>
</tr>
<tr>
<td>0</td>
<td>AML</td>
<td>ND</td>
<td>7 (82)</td>
<td>1-5-ND</td>
</tr>
</tbody>
</table>

- % F, percentage of fluorescent cells labeled with antifetal serum (1:4, 37°, 1 hr) and goat fluorescein-conjugated anti-rabbit IgG (1:10, 37°, 30 min).
- Cells were reacted with antifetal serum that had been absorbed with FLC-, bone marrow-, or ALC; data after parentheses.
- Numbers in parentheses, percentage of leukemic blasts in bone marrow or in peripheral cells harvested by leukapheresis.
- ND, no data.

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with its "absorbed" (A) fraction recovered from affinity chromatography against the antifetal serum. Four protein bands of comparable electrophoretic mobility were identified in the BALB/c fetal liver and CLL preparations. The 2nd trimester human fetal liver preparation contained 3 of these bands.

Since the anodal component migrated in the region of albumin, countercurrent electrophoresis against goat antihuman albumin was used to check the fractions recovered from the column. It was found that recycling of the 1st human fetal A fraction removed any detectable albumin (sensitivity, 2.1 μg/ml) from the 2nd A fraction (tested at 100 μg/ml). Similarly, AFP radioimmunoassays performed by Dr. Stewart Sell (La Jolla, Calif.) detected 8 ng AFP per ml in the A fraction of human fetal liver (282 μg/ml). The A fraction of fetal liver and the CLL A fraction were less concentrated. Thus negative tests for AFP were not conclusive, but did establish that AFP, if present in these samples, would represent <0.005 and <0.009% of the sample protein, respectively. Hence, it is not likely that either albumin or AFP are significant components of the isolated cross-reactive fetal antigen(s). Similarly, in the rat sarcoma model, a phase-specific embryonic antigen of 65,000 to 70,000 molecular weight has been identified by Baldwin et al. (5). The absorbed antifetal serum used for the column did not react with ferritin in immunodiffusion. However, a more sensitive assay is required to determine whether ferritin is a component of the isolated A fraction of the CLL extract.

**Albumin Density Gradients.** While the FCFU assay detected cross-reactivity with a specific target cell, the fetal hematopoietic stem cell, the rabbit antiserum was raised against a heterogenous cell population in the fetal liver, representing primarily hematopoietic cells, but at various stages of differentiation. The absorbed rabbit anti-fetal serum labeled only 32% of the immunizing cell type, 15-day BALB/c fetal liver cells (Table 4). FLC fractions recovered from discontinuous albumin density gradient centrifugation were tested by immunofluorescence to characterize further the reactivity of the antiserum. Fluorescent cells were significantly concentrated in the 21 and 23% albumin fractions. The percentage of total cells positive in immunofluorescence was calculated from the number of viable cells and the percentage of recovery of cells in each fraction. The total of 33.4% was appreciably close to the immunofluorescence reading on the unfractionated fetal liver. A spleen colony assay was simultaneously conducted to determine the distribution of hematopoietic stem cells in the same fetal liver sample. Moore et al. (27) and Löwenberg (25) have demonstrated an increase in density of the stem cell compartment from about 20 to 25% albumin with progression from 10-day fetal liver to adult bone marrow. The overlap from such a progressive maturational shift is reflected in the data in Table 4. Colony-forming cells were enriched in the 21% albumin fraction and more so in the 23% albumin fraction. Repetition of these immunofluorescence and FCFU experiments has consistently revealed co-

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**Chart 1.** Polyacrylamide gel electrophoresis analysis of 3 M KCI extracts of I, BALB/c 15-day fetal liver; II, human 19-week fetal liver; III, human CLL. A, whole extracts; B, affinity chromatography fraction absorbed and eluted from antifetal column. I/II, obtained by recycling of the 1st cycle absorbed fraction. TD, tracking dye.
purification of fluorescent and stem cells. Greater enrichment was sometimes noted in the 21% fraction, probably due to variability encountered in the timed mating for collection of 15-day fetal liver.

Since the reactivity of the antiserum was shown to be about one-half against stem cells and one-half against other cells, this suggested another possible explanation for the low percentage of reactive leukemic cells in immunofluorescence versus their effectiveness in reducing spleen colony formation. It would seem that an antiserum raised against cells isolated in the 23% albumin fraction (with 73% fluorescence and 4-fold concentration of stem cells) should have greater reactivity against leukemic cells that were effective in the FCFU assay. If this reasoning is correct, then serum from mice immunized for the FCFU assay should, conversely, be most reactive against the purified stem cells.

Table 5 shows the reactivity of fractionated FLC (from the same gradients as in Table 4) with unabsorbed serum from syngeneic mice immunized with human WBC or leukemic cells. The percentage of total cells positive in immunofluorescence corresponds with data from an independent assay with a different sample of FLC. The percentage of fluorescent cells labeled with anti-WBC was not enriched in any individual fraction (% F) and probably reflects nonspecific staining, since similar activity was seen when bone marrow or ALC were used as target cells with any of the 5 antisera. In contrast, the low reactivity (3 to 10%) of the antileukemic antisera with unfractionated FLC was increased 3- to 5-fold in the 23% albumin fraction that had a 4-fold concentration of stem cells. There was also an appreciable enrichment of fluorescent cells in the 21% fraction that had a 2-fold concentration of stem cells. The percentage of total cells positive in each fraction (% T) is also listed in Table 5. From these values, if one derives the percentage of reactive cells, then only 29% of the cells reacting with anti-WBC were in the 23% albumin fraction whereas 63 to 91% of the cells reacting with antileukemic sera were in this fraction.

**Conclusion**

Experimental data from the spleen colony assay and affinity chromatography have demonstrated the presence of mut-
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rune cross-reactive human tumor-associated fetal antigens. Further studies utilizing immunofluorescence of leukemic cells with antifetal serum, or of fractionated FLC with anti-leukemic serum, suggest that leukemic cells express antigens common to fetal hematopoietic stem cells that are not expressed in normal adult bone marrow. Antisera to the purified fetal liver stem cells or to affinity chromatography purified leukemic-fetal antigen(s) may thus be useful in diagnosis and clinical monitoring of human leukemia.

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