EVIDENCE FOR TRANSFORMATION OF SPLEEN CELLS ONE DAY AFTER INFECTION OF MICE WITH FRIEND LEUKEMIA VIRUS

AUTONOMOUS GROWTH POTENTIAL AND EXPRESSION OF HYBRID-RESISTANCE GENES*

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Established Friend virus-induced leukemic cell lines present the striking characteristic of differentiating along the erythrocytic pathway in tissue culture while retaining neoplastic properties and replicating small amount of virus (1). When injected into lethally irradiated mice, cells of these lines produce erythroid spleen colonies (2). During the course of quantitative studies on such colony-forming units (CFU)1, it was observed that the yield of CFU was greater in syngeneic DBA/2J mice than in (BALB/cJ X DBA/2J)F1 hybrids of both sexes. A similar phenomenon has been reported by McCulloch and Till with normal C57BL bone marrow cells injected into syngeneic and (C57BL X C3H)F1 hybrid mice (3). The phenomenon was termed "repression" to signify that deficient host colonization was presumably due to factors other than histoincompatibility. On the contrary, studies by Cudkowicz and Stimpfling with the same and several other mouse strains, while confirming the observation, indicated that deficient hemopoietic colonization of F1 hybrid mice by parental C57BL marrow grafts was a phenomenon of histoincompatibility controlled by alleles of a gene closely associated with, or part of, the complex.

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1 The following abbreviations were used: CDF1, (BALB/c X DBA/2) F1 hybrid mice; CFU, colony-forming units; FLV, Friend leukemia virus; H, histocompatibility gene(s); Hh, hybrid histocompatibility gene(s); IUdR, 5-iodo-2'-deoxyuridine.
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H-2 region in the murine linkage group IX (4). Successively, it was found that hybrid resistance to parental marrow grafts is a relatively frequent occurrence in mice, not restricted to the C57BL strain, and due to destructive reactions elicited by irradiated F1-host mice against grafted parental cells (5, 6). The phenomenon appeared to be analogous in many respects to allograft reactions elicited by irradiated mice against grafted parental cells (5, 6). The peculiarity of hybrid histoincompatibility resided in the controlling genes (symbol, Hh) which express themselves only in homozygous cells, presumably by governing the synthesis of specific surface structures. The latter could be recognized as foreign by the heterozygous F1 hybrids in which Hh alleles are not expressed, and elicit allograft reactions. The parental-specific substances possessed, therefore, the properties of transplantation antigens, although Hh alleles were not codominant. Alleles of classical histocompatibility genes (H) are codominant and, hence, expressed in homozygous as well as in heterozygous cells.

To obtain an understanding of the mechanism of hybrid resistance to virus-induced leukemic cells and of the possible relationship with leukemogenesis, normal and leukemic cells were tested in a variety of DBA/2 sublines and their respective F1 hybrids with BALB/c mice. The results revealed that hybrid resistance to virus-induced leukemic cells (of DBA/2J origin) occurred even when other DBA/2 sublines had been used to breed the F1 hybrids. In contrast, a rather weak hybrid resistance to normal DBA/2J bone marrow cells was observed in (BALBcJ × DBA/2J)F1 mice, but not in the other hybrid sublines. Thus it appeared that virus-induced leukemic cells express Hh genes more fully than normal hemopoietic cells do and, therefore, are more likely to be recognized as “nonsel” by genetically resistant hybrids.

When DBA/2 spleen cells of the Jackson subline were infected in vivo with Friend leukemia virus (FLV) and similarly tested at various intervals, enhanced expression of Hh genes could be detected as early as 6 hr after virus infection. At 24 hr the infected spleen cells displayed the newly acquired capacity to grow in spleens of nonirradiated syngeneic hosts, and to a lesser degree in those of unirradiated hybrids. Both the expression of Hh genes and the capacity of autonomous growth were not detectable in the corresponding normal spleen cells transplanted into animals of the Haenschka subline. It appeared that these characteristics are the earliest signals of the viral-induced transformation, thus far detected.

Materials and Methods

Mice.—Animals aged 6-14 wk of the following strains and sublines were used: DBA/2J, Jackson Laboratory, Bar Harbor, Maine; DBA/2Ha-DD (intense-brown), West Seneca Animal Production Unit of Roswell Park Memorial Institute, Buffalo, N.Y.; DBA/2Cr, Animal Production Section, National Cancer Institute, Bethesda, Md.; DBA/2Cum, Cumberland View Farms, Clinton, Tenn.; and B10.D2J-N, Jackson Laboratory. They were used either as graft recipients or as the male parents of the following F1 hybrid sublines: (BALBcJ ×
DBA/2J)F1 and (BALB/cj × DBA/2Ha-DD)F1 mice bred at the Mount Sinai School of Medicine of the City University of New York; (BALB/cCr × DBA/2Cr)F1 and (BALB/cCum × DBA/2Cum)F1 hybrid mice obtained from the Animal Production Unit of the National Cancer Institute, and Cumberland View Farms, respectively. The hybrids designation will be abbreviated hereafter as CDF1. Animals of both sexes were used throughout the study, as noted in the tables.

Irradiation Procedures.—For experiments of marrow and spleen cell transplantation, prospective host mice were given 750–800 R of X-rays in one exposure, using a General Electric Maxitron-250 Unit with the following specifications: 250 kv, 30 ma, 1 mm of Cu-added filtration, half-value layer of 0.95 mm of Cu, 190 R/min in air at 50 cm from the source. For spleen colony studies, the animals were given either 950 R of X-rays or 1200 rad of γ-rays. X-irradiation was split in two exposures (550 + 400 R), using two assembled Maxitron-300 Units. Physical factors were: 200 kv, 20 ma, 1 mm of Cu filter, half-value layer of 0.9 mm of Cu, 124–146 R/min in air at 50 cm from the source. Gamma rays were also split in two exposures (600 rad from the bottom and 600 from the top), using a 60Co Unit (Theratron, Atomic Energy Commission, Ottawa, Canada) with an exposure rate in air of 26.5 rad/min at 120 cm from the source. In all instances the animals were kept in Lucite boxes, surrounded by bolus material. Under these conditions the incidence of endogenous colonies was, on the average, less than 0.2 colony per spleen.

Cell Preparations.—Normal bone marrow cells for transplantation were harvested from femurs and tibias of adult male donors. The ends of the bones were severed with bone-cutting scissors and the marrow cavities were flushed with chilled Eagle's medium through a 23-gauge needle. Spleen cells (before and after FLV injection) were also taken from male donors and harvested by piercing the capsule of the spleen with an 18-gauge needle and then expressing the cells with blunt forceps, while gently flushing with Eagle's medium. Each cell suspension was filtered through a 200-mesh/inch stainless-steel cloth to remove particulate matter. Spleen cells were also washed three times in medium. Nucleated cell counts were made with a Model B Coulter counter (Coulter Electronics, Inc., Hialeah, Florida) fitted to a 100μ aperture, after erythrocytes had been lysed with saponin. Leukemic tissue-cultured cell lines have been described in detail elsewhere (1). Cells from cultures were centrifuged for 10 min at 700 rpm, washed once, and resuspended in Ringer's solution. Cell counts were made in a hemocytometer. The cell suspensions were then diluted in Ringer's solution to the desired concentration. All preparations were kept in ice water throughout the procedure.

Virus Preparations.—Filtrates of 20 % homogenates of leukemic spleens were prepared as previously described (7). Each mouse was injected intraperitoneally with 0.2 ml of a 10−1 dilution of the filtrate. The animals were then sacrificed at intervals after infection.

Assays for Proliferation and Differentiation of Normal and Virus-injected Cells.—Marrow and spleen cell preparations, before and after FLV injection, were transplanted by tail vein injection into irradiated or unirradiated recipient mice, or into both. 5 days later, the mice were injected intraperitoneally with 0.5 μc of the deoxyribonucleic acid precursor S-iodo-2′-deoxyuridine (I UdR) labeled with 125I. A detailed description of this isotope assay for the assessment of cells entering mitosis has been published elsewhere (8). In most instances, as noted in the text, mice were given a mixture of 0.5 μc of 125I UdR and 0.2 μc of 59FeCl3 to label cells engaged in synthesis of DNA and/or heme. The two radioactive labels neither interact nor interfere with the processes governing their incorporation into regenerating spleens of recipients. Mice were killed by cervical dislocation 17 hr after injection of the radioisotopes and the spleens removed. Radioactivity retained in spleens was measured individually in a two-channel, well-type crystal scintillation counter; splenic uptake was expressed.

2 Cudkowicz, G., and M. Bennett. Unpublished observation.
as the percentage of the total radioactivity injected into the animals. Uptake values recorded in the spleens of radiation control mice, i.e. irradiated mice not injected with cells, were subtracted from those of experimental animals to obtain net values of isotope retention. Mean splenic uptake values of $^{125}$IUDR and $^{59}$Fe will be given for each group of recipient mice with standard errors or 95% confidence-intervals.

**Colony-formation by Leukemic Cell Lines.**—Mice of the F$_1$ hybrid sublines were sacrificed on the 10th day after cell inoculation, and DBA/2 mice on the 11th day. The spleens were placed in Telleyesnitzky's fixative (4.5-ml glacial acetic acid, 4.5-ml formaldehyde, 100-ml 70% ethanol) for 5 min to allow a precise demarcation of the colonies which were then counted. The 1-day difference in sacrifice time between F$_1$ hybrid and DBA/2 mice was necessary for the following reason. 11 days after cell inoculation spleen colonies were confluent in F$_1$ hybrid mice, while 10 days after inoculation they were too small to be detected in DBA/2 mice. It is possible that the doubling time of transplanted leukemic cells was shorter in F$_1$ hybrid than in DBA/2 mice, once hybrid resistance had been overcome by transplantation of the appropriate cell number.

**Solid Tumor Formation by Leukemic Cell Lines.**—Graded numbers of tissue culture cells suspended in 0.2 ml of Ringer's solution were inoculated into the subcutaneous tissue of DBA/2J and CDF$_1$J mice. Survivors were regarded as tumor-exempt 100 days after cell inoculation.

**Statistical Analysis.**—Results of at least two different cell dilutions were used to calculate CFU ratios with standard errors. The mean numbers of colonies per spleen were subjected to regression analysis. Since the relationship between number of cells inoculated and number of colonies observed is linear with an intercept not significantly different from zero (2), the intercept value itself has been used as the third data point to perform regression analyses when results of only two cell dilutions were available.

Mean splenic uptake values of $^{125}$IUDR and $^{59}$Fe in DBA/2 and CDF$_1$ mice were compared, using the Student's test of significance. Since the variance of mean per cent uptake values increased with the number of cells injected, the logarithm of individual splenic uptake values was used to compute means and to stabilize the variances. The $t$ tests were, therefore, made on logarithmic values. The data were then converted to antilogarithms with 95% confidence intervals for tabulation.

**RESULTS**

**Splenic Uptake of Radioisotopes in Control Mice.**—Splenic uptake values of $^{59}$Fe and $^{125}$IUDR in various control animals are reported in Table I. It is of interest to note that no difference was detectable between uptake values in spleens of irradiated mice given no other treatment (radiation controls) and those in spleens of irradiated animals given $6 \times 10^6$ washed and killed spleen cells from FLV-infected DBA/2J mice. The cells were killed by freezing and thawing three times. Donors provided cells from 6 hr to 8 days after infection, but the data were pooled because no time-dependent differences were observed. It is apparent that FLV still present in the spleen cell suspensions after three washings did not promote isotope uptake upon injection into irradiated animals. In contrast, in the spleens of the nonirradiated DBA/2 and CDF$_1$ animals given the same frozen and thawed cell preparation, the availability of target cells to the residual virus resulted in an increase in the uptake of both radioisotopes. The small amounts of virus had induced proliferation and erythroid differentia-
### Table I

**Uptake of ^59Fe and ^3HdR in Spleens of Control Mice**

| Treatment* | Strain† | Mean splenic uptake of isotope |  |  |  |  |
|------------|---------|--------------------------------|---|---|---|---|
|            |         | Irradiated mice                |  |  | Nonirradiated mice |  |  |
|            |         | ^59Fe  | ^3HdR  | ^59Fe  | ^3HdR  |  |  |
|            |         | % ± SE | % ± SE | % ± SE | % ± SE |  |  |
| None       | DBA/2   | 0.40 ± 0.05 (10)§ | 0.04 ± 0.003 (33) | 8.34 ± 0.86 (8) | 0.55 ± 0.06 (8) |  |  |
|            | CDF₁    | 0.59 ± 0.05 (20) | 0.02 ± 0.003 (40) | 10.68 ± 0.87 (5) | 0.74 ± 0.09 (5) |  |  |
|            | B10.D2-N| 0.88 ± 0.26 (5)  | 0.02 ± 0.003 (14) | 14.85 ± 2.39 (8) | 1.09 ± 0.10 (8) |  |  |
| 6 × 10⁸ noninfected DBA/2 spleen cells | DBA/2 | 8.81 ± 0.67 (18) | 8.82 ± 0.09 (18) |  |  |  |  |
|            | CDF₁    | 7.17 ± 0.98 (19) | 0.73 ± 0.09 (19) |  |  |  |  |
|            | B10.D2-N| 14.13 ± 1.23 (4) | 1.50 ± 0.17 (4) |  |  |  |  |
| 6 × 10⁸ infected DBA/2 spleen cells frozen-thawed three times|||  | |  | | |
|            | DBA/2   | 0.39 ± 0.05 (15) | 0.04 ± 0.003 (15) | 12.47 ± 0.65 (29) | 1.04 ± 0.09 (29) |  |  |
|            | CDF₁    | 0.52 ± 0.07 (12) | 0.06 ± 0.020 (12) | 14.25 ± 0.65 (21) | 0.99 ± 0.08 (21) |  |  |
|            | B10.D2-N| not done | not done | 13.77 ± 1.40 (5) | 0.55 ± 0.11 (5) |  |  |

* Cells were injected 5 days before radioisotopes.
† Data obtained from different DBA/2 and CDF₁ sublines were pooled.
§ Number of mice tested in parentheses.
∥ Data obtained at different intervals from infection were pooled.
tion of the unirradiated hematopoietic spleen cells. The comparison was made with splenic uptake values of DBA/2 and CDF$_1$ mice given $6 \times 10^6$ noninfected viable spleen cells. The isotope uptake values thus derived were regarded as the baseline for experiments in which nonirradiated mice were given FLV-infected viable spleen cells. Finally, no difference was detected between the uptake values observed in spleens of nonirradiated B10.D2-N mice given either viable noninfected spleen cells, or FLV-infected frozen-thawed cells, indicating these mice were not susceptible to FLV. This was to be expected since the B10.D2-N strain is congenic with the highly FLV-resistant C57BL/10 strain.

### TABLE II

| Recipient strain | Number of mice | Sex | Mean splenic uptake of $^{125}$IUDR* % ± SE |
|------------------|----------------|-----|-------------------------------------------|
| DBA/2J           | 21             | M, F| 0.30 ± 0.02                               |
| CDF$_1$J         | 15             | F   | 0.13 ± 0.02                               |
| CDF$_1$J         | 7              | M   | 0.14 ± 0.04                               |
| DBA/2Ha          | 9              | M, F| 0.69 ± 0.05                               |
| CDF$_1$Ha        | 11             | F   | 0.81 ± 0.09                               |
| DBA/2Cr          | 15             | M, F| 0.47 ± 0.04                               |
| CDF$_1$Cr        | 14             | M, F| 0.45 ± 0.04                               |
| DBA/2Cum         | 5              | M   | 0.82 ± 0.03                               |
| CDF$_1$Cum       | 5              | F   | 1.04 ± 0.09                               |
| B10.D2-N         | 7              | F   | 0.38 ± 0.04                               |

* Mean radiation control values (see Table I) were subtracted from each experimental value.

Growth of Marrow Grafts from DBA/2 Sublines into their F$_1$ Hybrids with BALB/c.—In view of the deficient growth of FLV-induced leukemic cells in irradiated CDF$_1$J mice (2), it was of interest to determine the patterns of growth of normal bone marrow cells from different DBA/2 sublines upon transplantation into mice of syngeneic sublines and into their corresponding F$_1$ hybrids. Results are shown in Table II for cells of DBA/2J donors, and in Table III for cells of the other sublines.

Splenic uptakes of $^{125}$IUDR promoted by grafts of DBA/2J bone marrow cells into syngeneic and F$_1$ mice of the Jackson and other sublines indicated that hybrid resistance to normal cells was detectable only in CDF$_1$J mice. The differences were statistically significant at the 0.01 level. Mice of the DBA/2Ha and CDF$_1$Ha, DBA/2Cr and CDF$_1$Cr, and DBA/2Cum and CDF$_1$Cum sub-
lines supported well the growth of grafts of DBA/2J origin. Furthermore, it was of interest that also B10.D2-N mice supported well the growth of such grafts. The splenic uptakes promoted by marrow grafts of DBA/2 donors other than DBA/2J indicated that the transplants were generally well accepted by DBA/2 hosts of different sublines and by the corresponding F1's. The exceptions were DBA/2Cum marrow cells in CDF1Ha and CDF1Cr mice (P < 0.001), but in both instances resistance was rather weak. The data from Tables II and III also indicated that DBA/2Ha recipients supported the growth of all marrow grafts better than recipients of any other DBA/2 sublines. We were aware, since 1963, of immunogenetic differences among DBA/2 sublines; skin grafts exchanged either between DBA/2J and DBA/2Ha or between DBA/2Cr and DBA/2Ha mice were rejected 20–60 days after grafting, due to non-H-2 histoincompatibility. However, DBA/2J to DBA/2Cr skin grafts, as well as intrasubline skin grafts, were consistently accepted.3 Such intersubline differ-

### TABLE III

| Donor strain | Recipient strain | Number of mice | Sex | Mean splenic uptake of 125IUdR (% ± SE) |
|--------------|------------------|----------------|-----|---------------------------------------|
| DBA/2Ha      | DBA/2Ha          | 7              | M   | 0.72 ± 0.03                           |
|              | CDF1Ha           | 7              | F   | 0.83 ± 0.09                           |
|              | CDF1Ha           | 12             | M   | 0.63 ± 0.06                           |
|              | CDF1J            | 4              | M   | 1.08 ± 0.25                           |
|              | CDF1Cr           | 5              | M   | 0.91 ± 0.06                           |
|              | B10.D2-N         | 7              | F   | 0.66 ± 0.08                           |
| DBA/2Cr      | DBA/2Cr          | 12             | M,F | 0.50 ± 0.06                           |
|              | CDF1Cr           | 11             | F   | 0.79 ± 0.05                           |
|              | CDF1Cr           | 9              | M   | 0.50 ± 0.02                           |
| DBA/2Cum     | DBA/2Cum         | 6              | M,F | 0.37 ± 0.06                           |
|              | CDF1Cum          | 7              | F   | 0.46 ± 0.05                           |
|              | CDF1Cum          | 8              | M   | 0.57 ± 0.05                           |
|              | DBA/2Ha          | 5              | M   | 0.56 ± 0.08                           |
|              | CDF1Ha           | 7              | M,F | 0.31 ± 0.04                           |
|              | DBA/2J           | 5              | M   | 0.26 ± 0.01                           |
|              | CDF1J            | 16             | M   | 0.31 ± 0.04                           |
|              | DBA/2Cr          | 5              | M   | 0.74 ± 0.10                           |
|              | CDF1Cr           | 13             | M,F | 0.44 ± 0.04                           |

* Mean radiation control values (see Table I) were subtracted from each experimental value.

were DBA/2Cum marrow cells in CDF1Ha and CDF1Cr mice (P < 0.001), but in both instances resistance was rather weak. The data from Tables II and III also indicated that DBA/2Ha recipients supported the growth of all marrow grafts better than recipients of any other DBA/2 sublines. We were aware, since 1963, of immunogenetic differences among DBA/2 sublines; skin grafts exchanged either between DBA/2J and DBA/2Ha or between DBA/2Cr and DBA/2Ha mice were rejected 20–60 days after grafting, due to non-H-2 histoincompatibility. However, DBA/2J to DBA/2Cr skin grafts, as well as intrasubline skin grafts, were consistently accepted.3 Such intersubline differ-

3 Hauschka, T. S., and G. Cudkowicz. Unpublished data.
ences were actually exploited in studies of combined chemotherapy and immunotherapy of L1210 leukemia (9). We want to emphasize here that DBA/2 sublines are not only different with respect to antigenic constitution (genetic drift, variable expression of antigens), but may also differ for other genetic

TABLE IV
Colony Formation in Spleens of Irradiated Mice 10–11 Days after Grafting Cultured-Leukemic CIA Cells*

| Recipient strain | Number of mice | Sex | Number of cells grafted (X 10⁴) | Mean number of colonies per spleen | CFU/10⁴ cells grafted (M ± SE) |
|-----------------|----------------|-----|-------------------------------|-----------------------------------|-------------------------------|
| DBA/2J          | 5 F            | 0.4 | 3.20                          | 6.08 ± 1.02                       |
|                 | 4 F            | 0.85| 5.20                          |                                   |
| CDF₁J           | 4 M            | 3.0 | 5.00                          |                                   |
|                 | 4 M            | 6.0 | 8.00                          | 1.63 ± 0.29                       |
|                 | 8 F            | 3.0 | 5.00                          |                                   |
|                 | 9 F            | 6.0 | 11.06                         |                                   |
| DBA/2Ha         | 13 M           | 0.25| 2.18                          | 15.25 ± 1.15                      |
|                 | 10 M           | 0.5 | 6.80                          |                                   |
|                 | 6 M            | 1.0 | 14.80                         |                                   |
| CDF₁Ha          | 5 M            | 1.0 | 5.60                          |                                   |
|                 | 7 M            | 3.0 | 17.00                         | 5.09 ± 0.51                       |
|                 | 6 F            | 1.0 | 5.00                          |                                   |
|                 | 8 F            | 3.0 | 13.70                         |                                   |
| DBA/2Ha         | 16 M           | 0.1 | 4.81                          | 59.20 ± 6.41                      |
|                 | 13 M           | 0.2 | 11.84                         |                                   |
| CDF₁Cr          | 9 M            | 0.4 | 3.78                          | 9.73 ± 0.16                       |
|                 | 7 M            | 0.8 | 7.78                          |                                   |
| DBA/2Ha         | 3 M            | 0.2 | 5.00                          | 25.00 ± 0.01                      |
|                 | 3 M            | 0.3 | 7.50                          |                                   |
| CDF₁Cun         | 7 F            | 0.3 | 4.60                          | 15.58 ± 0.32                      |
|                 | 12 F           | 0.5 | 7.40                          |                                   |
|                 | 13 F           | 1.0 | 15.60                         |                                   |

* The same preparation of CIA cells was injected into DBA/2 and CDF₁ mice.

factors regulating allograft reactivity to hemopoietic cells in irradiated mice (10).

Colony Formation by Leukemic CIA Cells (of DBA/2J Origin) in Spleens of Irradiated DBA/2 Sublines and their Corresponding F₁'s.—Although the occurrence of hybrid resistance to the FLV-induced leukemic cell lines had been
already described (2), we felt it necessary to repeat these experiments, owing to a change in irradiation procedures. Cell transplantation has been made in this study after the entire dose of radiation had been delivered to prospective host mice, whereas previously the cells had been transplanted between two successive exposures (2). The data are shown in Table IV. Hybrid resistance to FLV-induced leukemic CIA cells was detected in all hybrids, regardless of the parental DBA/2 subline used to produce F1 progeny. It thus looks as if all hybrids were potentially resistant to DBA/2J cells, and that the expression of hybrid resistance genes may have been variable in normal parental cells of different DBA/2 sublines. It also looks as if leukemic transformation may have enhanced the expression of Hh genes in DBA/2J cells.

**Tumor Formation by CIA Cells in Unirradiated DBA/2J and CDF1 Mice.**—To determine whether hybrid resistance would be expressed in sites other than the host spleen and whether nondifferentiating CIA cells would also express Hh genes, graded numbers of such cells were inoculated into the subcutaneous tissue of nonirradiated DBA/2J and CDF1 mice. Under these conditions CIA cells do not undergo detectable erythroid differentiation (1, 2).

The numbers of tumors arising within 100 days after cell inoculation are reported in Table V. Hybrid resistance was well demonstrated, in that 10^5 and 5 x 10^5 cells failed to give tumors in all but 1 of 35 CDF1 hosts. On the other hand, tumors developed in most of the DBA/2J mice inoculated with the same number of cells.

| Recipient strain | No. of mice with tumors/No. of mice grafted with: |
|------------------|--------------------------------------------------|
|                  | 10^5 cells | 5 x 10^5 cells | 10^6 cells |
| DBA/2J ♀ ♂       | 11/16      | 15/15          | 22/24      |
| CDF1 ♂           | 0/10       | 0/6            | 7/19       |
| CDF1 ♀           | 0/10       | 1/9            | 3/17       |

Expression of Hh Genes in FLV-infected DBA/2J Spleen Cells: A Time-Course Study.—Noninfected DBA/2J marrow cells grew without impairment in irradiated DBA/2Ha and CDF1Ha recipients (Table II). Apparently, histocompatibility differences between mice of the Jackson and Hauschka sublines were not strong enough to be detected, regardless of whether due to alleles at H or Hh genes. That such differences existed was indicated by rejection of DBA/2J skin grafts in nonirradiated DBA/2Ha host (H differences); by deficient growth of DBA/2J marrow grafts in irradiated CDF1J hybrids (Hh differences); and by deficient colony formation by CIA cells in CDF1J and CDF1Ha mice. Conceivably, Hh genes in DBA/2J marrow cells were expressed to an extent
which made them recognizable as foreign only by CDF1J hybrids. In leukemic CIA cells, such genes could have been expressed more fully so as to make the parental cells recognizable by CDF1Ha and CDF1J hybrids. These findings provided a tool to determine the time after FLV infection, at which hemopoietic DBA/2J cells acquire in vivo the property which is characteristic of leukemic CIA cells, i.e., deficient growth in CDF1Ha mice.

Spleen cells of infected mice rather than marrow cells were used for a time-course study since the spleen is the major target organ for FLV. The functional properties of hemopoietic stem cells in mouse marrow and spleen are known to be close enough not to affect the objectives of the present study (11). DBA/2J male mice infected with FLV \((LD_{50} = 10^{-2.97})\) were killed 6 hr, 1, 2, 3, and 8 days later. At each interval, spleens of five donors were pooled and 3 \(\times 10^6\) or 6 \(\times 10^6\) nucleated cells were grafted into individual mice of the following groups: irradiated DBA/2Ha, CDF1Ha, and B10.D2-N mice. Proliferation and erythroid differentiation of grafted cells was assessed after 5 days by measuring the uptake of \(^{125}\)I\(\mathrm{I}^{\text{dR}}\) and \(^{59}\)Fe in recipient spleens. Results are reported in Table VI.

Noninfected spleen cells grew without impairment in irradiated DBA/2Ha, CDF1Ha, and B10.D2-N mice, as normal marrow cells did (Table II). However, as early as 6 hr after infection, spleen cells grew and differentiated less efficiently in CDF1 than in DBA/2 and B10.D2-N mice. This observation was confirmed at later intervals until 8 days postinfection. Hybrid resistance to the growth of FLV-infected DBA/2 cells was best detected when 3 \(\times 10^6\) cells were transplanted. All comparisons of mean radioisotope uptake values in CDF1 and DBA/2 mice for this number of grafted cells were significant at the 0.01 level, except for the \(^{59}\)Fe uptakes 2 days after infection. This could be accounted for by the fact that in one of six CDF1 spleens the uptake was barely above control values, resulting in a mean with an unusually great variance.

Hybrid resistance was not as readily apparent at 6 hr when 6 \(\times 10^6\) cells were inoculated. This was presumably due to two factors: (a) even the strongest resistance to hemopoietic grafts can be overridden by increasing the number of transplanted cells (4, 6); (b) the hemopoietic cell population in infected spleens is composed of transformed and nontransformed spleen cells, both capable of proliferating in irradiated hosts. Only the transformed cells, though, would be resisted by CDF1 hybrids and, therefore, the ratio of transformed versus normal cells in the inoculum is critical as to whether a significant difference between the mean uptake values in DBA/2Ha and CDF1Ha mice can be detected. This ratio was probably not high enough at 6 hr after infection to detect deficient growth by relatively large grafts. However, from 1 day onwards, hybrid resistance to 6 \(\times 10^6\) cells was detected. Even with the less sensitive \(^{59}\)Fe uptake assay, differences between mean uptake values were significant at the 0.01 level, except for the 2-day \((P > 0.05)\) and 8-day \((0.02\)
> P > 0.01) assays. This is consistent with morphological and other biological data which indicate that virus-induced leukemic cells proliferate rapidly so as to constitute a progressively larger proportion of the transplantable spleen cell population (12).

### TABLE VI

| Time after infection | Number of cells grafted (X 10^6) | Recipient strain* | Number of mice | Mean splenic uptake of isotope with 95% confidence intervals $^{*}$ | $^{14}$Fe | $^{14}$UdR |
|----------------------|----------------------------------|-------------------|----------------|-------------------------------------------------|--------|--------|
|                      |                                  |                   |                | Mean of mice                                    |        |        |
| Zero time, i.e. before infection | 3 DBA/2 14 | 0.55 (0.35-0.88) | 0.41 (0.32-0.52) |                                                |        |        |
|                      | 5 DBF1 | 0.11 (0.05-0.22) | 0.48 (0.40-0.57) |                                                |        |        |
|                      | 6 DBA/2 10 | 0.81 (0.42-1.53) | 1.10 (0.97-1.25) |                                                |        |        |
|                      | 3 CDF1 | 2.13 | 1.18 (0.53-2.62) |                                                |        |        |
|                      | 8 B10D2-N | 1.33 (0.53-3.34) | 1.51 (1.13-2.02) |                                                |        |        |
| 6 hr                 | 3 DBA/2 5 | 4.02 (2.99-5.42) | 0.65 (0.51-0.82) |                                                |        |        |
|                      | 4 CDF1 | 1.39 (0.46-4.21) | 0.19 (0.07-0.52) |                                                |        |        |
|                      | 6 DBA/2 6 | 5.21 (3.37-8.03) | 0.87 (0.62-1.24) |                                                |        |        |
|                      | 2 CDF1 | 5.38 | 0.74 (0.57-0.95) |                                                |        |        |
|                      | 8 B10D2-N | 5.14 (2.51-10.54) | 1.15 (0.70-1.82) |                                                |        |        |
| 1 day                | 3 DBA/2 5 | No uptake | 0.61 (0.35-1.06) |                                                |        |        |
|                      | 3 CDF1 | No uptake | 0.17 (0.05-0.63) |                                                |        |        |
|                      | 6 DBA/2 5 | 0.45 (0.07-2.81) | 1.03 (0.63-1.70) |                                                |        |        |
|                      | 4 CDF1 | 0.57 (0.19-1.68) | 0.73 (0.44-1.19) |                                                |        |        |
|                      | 2 B10D2-N | 1.17 | 1.47 |                                                |        |        |
| 2 days               | 3 DBA/2 5 | 0.86 (0.45-1.64) | 0.89 (0.60-1.33) |                                                |        |        |
|                      | 6 CDF1 | 0.43 (0.09-2.08) | 0.47 (0.23-0.94) |                                                |        |        |
|                      | 6 DBA/2 5 | 2.36 (0.97-5.76) | 1.72 (0.93-3.18) |                                                |        |        |
|                      | 4 CDF1 | 1.59 (0.38-6.73) | 0.93 (0.41-2.11) |                                                |        |        |
|                      | 2 B10D2-N | 1.96 | 1.27 |                                                |        |        |
| 3 days               | 3.5 DBA/2 4 | 3.22 (1.66-6.24) | 0.55 (0.36-0.83) |                                                |        |        |
|                      | 4 CDF1 | 1.60 (0.84-3.03) | 0.53 (0.21-0.50) |                                                |        |        |
| 8 days               | 3 DBA/2 5 | 6.34 (5.41-7.42) | 0.65 (0.51-0.82) |                                                |        |        |
|                      | 7 CDF1 | 3.77 (2.21-6.45) | 0.41 (0.27-0.63) |                                                |        |        |
|                      | 6 DBA/2 6 | 11.25 (7.49-16.90) | 1.06 (0.62-1.81) |                                                |        |        |
|                      | 6 CDF1 | 9.33 (7.14-12.19) | 0.82 (0.59-1.12) |                                                |        |        |

* DBA/2Ha and CDF1Ha throughout.

† 5 days after transplantation. Radiation control values (see Table I) were subtracted from experimental values. Confidence intervals were omitted for groups of three or less mice whenever the variability was exceedingly high.

§ Experimental values were not greater than radiation control values.
Autonomous Growth and Expression of Hh Genes in FLV-infected DBA/2J Cells: A Time-Course Study.—The major change in functional properties of hemopoietic cells upon leukemic transformation is the acquisition of autonomous growth potential in nonirradiated histocompatible hosts (12). A duplicate experiment was, therefore, set up in nonirradiated DBA/2Ha and CDF1Ha mice inoculated with samples of the same spleen cells harvested for the preceding experiment. Since normal hemopoietic cells do not grow appreciably in non-irradiated hosts (13), proliferation and differentiation of transformed cells could be assessed without the contribution of residual nontransformed spleen cells. This type of experiment could also verify whether changes in expression of Hh genes in infected cells would occur at the same time as the acquisition of

### TABLE VII

**Time Course Study of the Growth and Differentiation of FLV-infected DBA/2J Spleen Cells in Nonirradiated Recipient Mice**

| Time after infection | Number of cells grafted (X 10^6) | Recipient strain* | Number of mice | Mean splenic uptake of isotope with 95% confidence intervals: |
|---------------------|----------------------------------|-------------------|----------------|---------------------------------------------------------------|
|                     |                                  |                   |                | ^5Fe 1^3HdR                                                   |
| 6 hr                | 3                                | DBA/2             | 5              | No uptake§                                                   | No uptake                                      |
|                     | 6                                | DBA/2             | 5              | “ “                                                        | “ “                                          |
|                     |                                  | CDF1             | 3              | “ “                                                        | “ “                                          |
| 1 day               | 3                                | DBA/2             | 4              | No uptake                                                   | No uptake                                      |
|                     | 6                                | DBA/2             | 4              | “ “                                                        | 0.53 (0.20-1.30)                              |
|                     |                                  | CDF1             | 3              | “ “                                                        | 0.07 (0.29-0.44)                              |
| 2 days              | 3                                | DBA/2             | 5              | 7.88 (3.06-20.31)                                           | 1.16 (0.47-2.86)                              |
|                     |                                  | CDF1             | 5              | 3.16 (0.08-6.24)                                            | 0.43 (0.03-0.88)                              |
| 3 days              | 17.8                             | DBA/2             | 2              | 17.23                                                       | 5.16                                          |
|                     |                                  | CDF1             | 2              | No uptake                                                   | 0.64                                          |
| 8 days              | 3                                | DBA/2             | 3              | 31.13 (24.31-39.86)                                         | 5.18 (2.24-12.00)                             |
|                     |                                  | CDF1             | 7              | 2.78 (0.02-5.54)                                            | 0.16 (0.19-0.47)                              |
|                     | 6                                | DBA/2             | 5              | 49.34 (29.88-81.46)                                         | 6.72 (4.94-9.14)                              |
|                     |                                  | CDF1             | 7              | 16.62 (10.78-25.62)                                         | 2.42 (1.52-3.83)                              |

* DBA/2Ha and CDF1Ha throughout.

§ 5 days after transplantation. Control values, i.e. splenic uptake in mice injected with frozen-thawed cells (see Table I), were subtracted from experimental values. Confidence intervals were omitted for groups of two mice.

§ Experimental values were not greater than control values.
autonomous growth potential. The results obtained with nonirradiated hosts are presented in Table VII.

As had been previously noted, noninfected spleen cells did not promote radioisotope uptake in spleens of nonirradiated mice (Table I). However, FLV remaining in washed infected spleen cells killed by repeated freezing and thawing did promote isotope uptake (Table I). If infected cells were to proliferate and differentiate upon transfer, the uptake values of $^{125}$IUDR and $^{59}$Fe in such recipient spleens should exceed those promoted by similar amounts of transferred virus without viable cells. Therefore, the data in Table VII are given as net per cent uptake, the values being obtained by subtracting the splenic uptakes of mice injected with frozen-thawed cells from those of mice injected with viable infected cells of the same preparation.

6 hr after FLV-infection, the inoculation of $3 \times 10^6$ or $6 \times 10^6$ spleen cells did not promote isotope uptake in recipient spleens. It is noteworthy that at this time hybrid resistance had been detected in irradiated host animals (Table VI). 1 day after FLV infection, the inoculation of $6 \times 10^6$ spleen cells did promote $^{125}$IUDR uptake in nonirradiated DBA/2 spleens and to a smaller extent in CDF1 spleens. This indicated that both changes, i.e. autonomous growth potential and expression of $Hh$ genes, had been detected at this time. The same observation was made 2, 3, and 8 days after infection for every cell dilution tested, both with the $^{125}$IUDR and $^{59}$Fe assays. The differences between mean uptake values in DBA/2 and CDF1 mice were significant at the 0.01 level, except for $^{59}$Fe uptake promoted by $6 \times 10^6$ cells 2 days after infection.

By transplantation assays in nonirradiated animals, autonomous growth potential of splenic hemopoietic cells and enhanced expression of $Hh$ genes could be detected simultaneously 1 day after FLV infection. However, the enhanced expression of $Hh$ genes could be detected even earlier, i.e., 6 hr after infection by transplantation assays in irradiated animals.

DISCUSSION

It had been previously shown that FLV-induced leukemic cells colonized less efficiently in the spleen of irradiated CDF1J mice than in those of syngeneic DBA/2J mice (2). To determine whether this was a manifestation of hybrid resistance (5, 6), studies were carried out with normal DBA/2 hemopoietic cells transplanted into mice of a variety of DBA/2 sublines and their respective (BALB/c × DBA/2)F1 hybrids. The results indicated that CDF1 mice of the Jackson subline, but not hybrids of other sublines, resisted the growth of grafted DBA/2J cells. The resistance was rather weak, for it was demonstrated only with small numbers of inoculated cells. Presumably, irradiated CDF1J mice could inactivate only a small number of parental cells so that hemopoietic colonization by surviving cells still took place. Furthermore, cells from DBA/2 sublines other than DBA/2J grew without impairment in most CDF1 tested. Apparently, there was considerable intrastrain (intersubline) variation either
in expression or in distribution of $Hh$ alleles, the genetic determinants of hybrid resistance.

It was then decided to investigate whether hybrid resistance to DBA/2J leukemic cells would also vary in different CDF$_1$ sublines. It was found that all CDF$_1$ mice tested were capable of resisting the growth of C1A leukemic cells of DBA/2J origin. Clearly, the DBA/2 sublines studied shared given $Hh$ alleles; but expression of these genetic determinants was subject to variation. Since CDF$_1$ hybrids could invariably recognize parental leukemic cells as foreign but not always normal parental hemopoietic cells, the major source of variation resided in the homozygous donor cells. In particular, the difference between normal and leukemic cells provided a tool to investigate whether Friend virus played a role in enhancing expression of $Hh$ genes in leukemic cells, and at what time after FLV infection this alteration could be detected. It was also possible to determine whether enhanced expression of $Hh$ genes in infected spleen occurred at the same time as acquisition of autonomous growth potential. The latter property was inferred from growth in nonirradiated host mice, as assessed by incorporation of the DNA-precursor $^{125}$IUDR in spleen cells entering mitosis and of $^{59}$Fe in hemoglobin-synthesizing spleen cells. The two parameters, hybrid resistance and autonomous growth, should be significant enough to detect FLV-transformed cells. The results of transplantation experiments, run concurrently in irradiated and nonirradiated mice, indicated that cellular changes occurred within 1 day after infection of DBA/2J mice with FLV. Such changes were reflected by deficient growth in CDF$_1$ mice as well as by autonomous growth.

The expression of $Hh$ genes in FLV-infected spleen cells became detectable (by transplantation into irradiated CDF$_1$Ha mice) as early as 6 hr after infection and remained unchanged at the later intervals. At such an early time after infection, a large proportion of spleen cells must have been altered for deficient growth in hybrids to become measurable. Autonomous growth as well as expression of $Hh$ genes became detectable (by transplantation into nonirradiated hosts) 1 day after virus infection and also remained unchanged thereafter. The difference in the time of appearance of "deficient growth in hybrids" and autonomous growth could be attributed to the fact that the number of leukemic cells in spleens 6 hr after infection was not sufficiently great to be detected by our assays in nonirradiated mice. Whether or not changes in the expression of $Hh$ genes actually preceded the acquisition of autonomous growth potential is still to be determined. We conclude, therefore, that the data presented provide suggestive evidence for FLV-induced transformation at 6 hr after virus infection, and convincing evidence at 1 day. Had we also shown that FLV-infected spleen cells were serially transplantable in nonirradiated hosts at early intervals, the evidence would have been conclusive for the malignant nature of the transformed cells. This would not be an unlikely possibility in view of the fact that
no more than 5 hr were required for Polyoma virus to transform BHK/21 cells in vitro (14). The possibility that the time necessary for transformation may be more than the 6-24-hr period of interaction between FLV and the target cells in the inoculated donor animals cannot be excluded. The interaction could have proceeded over a longer period since some virus was transferred into the recipients along with the injected spleen cell preparations. In regard to the induction of cellular changes related to expression of \( Hh \) genes, however, the necessity for prolonged FLV action seems unlikely since rejection of parental cells by resistant hybrids does occur 24 hr after transplantation (5, 6, 10).

The deficient growth of infected spleen cells in CDF\(_1\)Ha mice could be explained by three mechanisms: (a) enhanced expression of one or more \( H \) genes, (b) appearance of new cell-surface antigens, possibly specific tumor antigen(s), and/or (c) enhanced expression of one or more \( Hh \) genes. In each case, infected cells would become more vulnerable than normal spleen cells to allograft reactions. The first possibility seemed unlikely, in that infected cells grew well in allogeneic B10.D2-N irradiated mice and in DBA/2 sublines capable of rejecting DBA/2J skin grafts. The second possibility could also be ruled out in view of the fact that lethally irradiated mice would hardly be competent to react rapidly and effectively to viral antigen(s) or tumor antigens in general. The third possibility is considered most likely since DBA/2 normal hemopoietic cells do possess the products of one or more \( Hh \) genes. The variable expression of such genes in DBA/2 mice and CDF\(_1\) hybrids has hampered and delayed immunogenetic analysis. Nevertheless, it is postulated that virus infection resulted in enhanced expression of \( Hh \) genes. An analogy can be found in the studies on other genes specifying cell-surface antigens that can be repressed in normal cells and become activated during leukemogenesis, as shown by Boyse et al. for the thymus-leukemia antigen system (15). Parental-specific substances specified by \( Hh \) genes may be redistributed or increase in amount upon FLV infection of hemopoietic cells. In either case, the phenotypic change of DBA/2J cells would have been quantitative rather than qualitative, a common occurrence during tumorigenesis (16, a review article). In this context, it would be of interest to determine whether FLV could induce full expression of \( Hh \) genes in hemopoietic cells of DBA/2 sublines which normally do not express such genes (e.g., DBA/2\( Ha \)), as well as changes in expression of tissue antigens (e.g., the thymus leukemia antigen) specified by other genes.

**SUMMARY**

Proliferation and erythroid differentiation of transplanted DBA/2 marrow cells and Friend virus-induced leukemic cells were assessed in syngeneic, allogeneic (\( H-2 \) compatible), and (BALB/c × DBA/2)\( F_1 \) hybrid mice (CDF\(_1\)). Measurements were made 5 days after transplantation of donor cells into non-irradiated or X-irradiated mice by the spleen colony or the \(^{125}\)I\( UdR \) and \(^{42}\)Fe uptake.
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take methods. Growth of DBA/2J (Jackson subline) marrow grafts was poor in irradiated CDF1J hybrids as compared with growth in syngeneic and allogeneic hosts. The DBA/2J transplants proliferated, however, without impairment in irradiated CDF1 hybrids which were the progeny of DBA/2 male parents of other sublines, e.g. DBA/2Ha, DBA/2Cr, and DBA/2Cum. In contrast, tissue-cultured Friend leukemic cells of DBA/2J origin grew deficiently in all CDF1 hybrids tested, regardless of irradiation and of the DBA/2 parent's subline. The growth pattern of transplanted DBA/2J cells was a manifestation of hybrid resistance. The results with DBA/2J and other DBA/2 subline grafts suggested that hybrid histocompatibility alleles were expressed to a greater extent in leukemic than in normal marrow cells, for the former were consistently recognized as "nonself" by CDF1 mice, but not the latter cells.

The property of deficient growth in irradiated CDF1Ha hybrids was acquired by DBA/2J hemopoietic cells within 6 hr from infection in vivo with Friend leukemia virus, and persisted during the following 8 days. It was ascribed to enhanced expression of hybrid histocompatibility gene(s) (Hh) induced by the virus. Autonomous growth potential of hemopoietic cells, manifested by proliferation in nonirradiated recipients, was first detected 24 hr from infection, and likewise persisted at the later intervals. At the same time, the infected cells grew deficiently also in nonirradiated CDF1Ha mice. The two irreversible cellular changes were regarded as the earliest signals of virus-induced transformation.

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