TUMOR INDUCTION BY IMMUNOLOGICALLY ACTIVATED MURINE LEUKEMIA VIRUS*

BY MARTINE Y. K. ARMSTRONG, NANCY H. RUDDLE,†
MURIEL B. LIPMAN, AND FRANK F. RICHARDS

(From the Departments of Microbiology, Epidemiology and Public Health, and Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510)

(Received for publication 29 December 1972)

The association of a variety of immune disorders and human leukemia and lymphoma is well established. Aberrations such as anergy in active Hodgkin's disease (1) and hypogammaglobulinemia in chronic lymphocytic leukemia (2) are clearly the result of malignant disease of the lymphoreticular tissues. In other instances, however, the defect in immunity appears to precede by months or even years the subsequent neoplasia. Thus, leukemia and lymphoma have developed during the course of autoimmune hemolytic anemia (3), Sjögren's syndrome (4), and congenital agammaglobulinemia (5).

A mouse model has been used to study the mechanism by which an immune disturbance can lead to the development of lymphoreticular malignancy. In this experimental system, developed by Schwartz and Beldotti (6), a graft-vs.-host reaction (GVHR) is induced in young adult F1 hybrid mice by the administration of spleen cells from one of the parental strains. The recipients have the same antigens as the grafted cells and do not reject them, whereas immunologically competent cells in the inoculum are stimulated by those antigens in the F1 host which are derived from the other parent. After a latent period of some months, a proportion of these GVHR mice develop lymphoreticular tumors (7).

The experiments described here were designed to test the possibility that the immune disturbance associated with the GVHR activates a virus with oncogenic potential, and that this is the agent responsible for subsequent tumor development. There is precedent for this viral activation concept in the work of Kaplan (8), who established that thymic lymphomas can be induced in the low leukemia C57BL mouse strain by an irradiation-activated murine leukemia virus (MuLV).

In the present study, the GVHR was induced in (BALB/cJ × A/J)F1 and (BALB/cJ × C57BL/6J)F1 hybrid mice by the administration of BALB/cJ

* Supported by U. S. Public Health Service grant AI-08614, by American Cancer Society Institutional Grant IN-31-L-7, and by U. S. Public Health Service grant AI-09937.
† Fellow of the Damon Runyon Memorial Fund.

Abbreviations used in this paper: CAF1, (BALB/cJ × A/J)F1; CB6F1, (BALB/cJ × C57BL/6J)F1; CF, complement-fixing; CFE, cell-free extract; gs, group-specific; GVHR, graft-vs.-host reaction; MuLV, murine leukemia virus; NIH, National Institutes of Health; PFU, plaque-forming units; RCN, reticulum cell neoplasm.
spleen cells. Both the F1 hybrid strains, as well as the BALB/c strain, have a low spontaneous incidence of lymphoreticular malignancy (7, 9). Data are presented which demonstrate that (a) cell-free extracts (CFEs) prepared from GVHR mice are oncogenic; (b) these extracts contain infectious MuLV and/or group-specific (gs), complement-fixing (CF) MuLV antigen; (c) serially passed CFEs originating from a GVHR-induced tumor contain infectious MuLV and CF MuLV antigen. When these serially passed CFEs are injected into recipient mice, they cause lymphoreticular tumors, which also contain infectious MuLV and CF MuLV antigen, as well as characteristic C-type particles. Since CFEs from normal control mice matched for strain, age, and sex are not oncogenic and contain no CF antigen or infectious virus, it seems clear that the GVHR is "turning on" an MuLV which is tumorigenic.

Materials and Methods

Animals.—BALB/cJ, C57BL/6J, and (BALB/cJ × A/J)F1 hybrid mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. National Institutes of Health (NIH) Swiss mice were obtained from the Animal Production Section of NIH. (BALB/cJ × A/J)F1 and (BALB/cJ × C57BL/6J)F1 hybrid mice were bred in our own laboratory. (BALB/cJ × A/J)F1 and (BALB/cJ × C57BL/6J)F1 hybrid mice are hereafter referred to as CAF1 and CB6F1 mice, respectively.

Induction of GVHR.—A GVHR was induced in 6-wk-old male CAF1 and CB6F1 mice by the administration of spleen cells from 6-8-wk-old male BALB/cJ mice. Mice of each F1 hybrid strain received, in four weekly intraperitoneal doses, a total of approximately 200 × 10^6 spleen cells, prepared as previously described (10).

Preparation of CFEs.—CFEs were prepared from tumor tissue, or from a pool of reticular tissues, in non-tumor-bearing mice. This pool consisted of spleen, mesenteric and other lymph nodes, thymus, portions of liver and kidney, and bone marrow from the femurs. For in vivo inoculation, the tissues were processed according to the method used by Moloney to extract MuLV (11). A 10% suspension of finely minced tissue in 0.153 M potassium citrate containing 1.5 mg hyaluronidase/100 ml was allowed to digest at room temperature, then was homogenized in a Duall tissue grinder (Kontes Glass Co., Vineland, N.J.). The homogenate was cleared of nuclei and cell fragments by two 20-min centrifugations at 23,000 g/min. The supernatant was centrifuged at 10,000 g for 1 min to remove mitochondrial fragments and aggregated particulates, and at 30,000 g for 60 min to sediment the MuLV. The pellet was resuspended in a volume of 0.05 M pH 6.8 sodium citrate buffer adjusted so that each milliliter of suspension was equivalent to 1 g of tissue. The suspension was clarified by further centrifugation at 5,000 g and the supernatant was inoculated into a litter of syngeneic mice within 36 h of birth. Each animal received 0.05 ml of supernatant intraperitoneally. For in vitro testing, tissues were finely minced in Eagle’s minimal essential medium with 100 U penicillin and 100 μg streptomycin/ml. 10-20% suspensions were homogenized in a Duall tissue grinder and clarified twice by centrifugation at 2,300 g/min for 20 min. Supernatants were stored at −60°C.

Autopsies.—All animals killed for preparation of CFEs were autopsied, as were all recipients of CFEs. The latter were killed at 18 mo of age or, if tumors developed, before then. The reticular tissues of all animals autopsied were routinely sampled for histological examination, as were other tissues showing macroscopic abnormalities. Tissue samples were fixed in 10% buffered formalin, and sections were cut and stained with hematoxylin and eosin. Lymphoreticular neoplasms were classified according to Dunn (12).

Infectious MuLV Assay.—CFEs were tested for infectious MuLV following the UV-XC
procedure described by Rowe and Pincus (13). NIH Swiss and BALB/cJ mouse embryo cells, prepared in our laboratory and seeded into 60 × 15 mm Falcon plastic Petri dishes (Falcon Plastics, Oxnard, Calif.), were treated with 25 μg/ml DEAE-dextran, then inoculated with 0.1 ml of serial dilutions of the test extracts. 1 wk later, the mouse embryo cells were irradiated with 1,000-1,500 ergs/mm² of ultraviolet light, and 1 × 10⁶ XC cells were added. The cultures were fixed and stained with Giemsa 3-4 days later. Areas of syncytial formation with giant cells, in the XC cell sheet overlying foci of MuLV infection, were counted as plaques under the microscope. Titers were expressed as log₁₀ plaque-forming units (PFU)/0.2 g of tissue. Each CFE was tested on both NIH Swiss and BALB/cJ mouse embryo cells. Each set of XC assays included two positive controls, namely, N-tropic virus WN1802N and B-tropic virus WN18023, kindly provided by Dr. Wallace P. Rowe.

Complement Fixation Test for gs MuLV Antigen.—CFEs were tested in Dr. Robert J. Huebner’s laboratory for the presence of gs antigen using a micro-complement fixation test (14). The tissue extracts were incubated with murine sarcoma virus pool 30 serum (pooled sera from Fischer rats with transplanted sarcomas induced by Moloney murine sarcoma virus) in the presence of complement. The amount of complement fixed was measured in a sheep erythrocyte hemolytic system. Double dilutions to 1 in 16 were carried out for each extract.

Electron Microscopy.—Portions of splenic tumor and normal control spleen were minced into 1 mm cubes. These were fixed in 2.5% glutaraldehyde with 0.2 M sucrose in 0.1 M sodium phosphate buffer, and postfixed in buffered 1% osmium tetroxide. After dehydration, the samples were embedded in Epon-Araldite (15). Sections from the resulting blocks were stained with 3.5% uranyl acetate in 50% ethanol and lead citrate (Reynolds’ stain) (16). Control spleen and tumor cells were examined at × 20,000 magnification and micrographs were taken at both this and × 36,000 in a Philips EM-200 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.) operating at an accelerating voltage of 60 kV.

RESULTS

Oncogenicity of CFEs from Mice with the GVHR.—

Experimental group: A total of 29 CAF₁ mice with the GVHR were killed at intervals from 3 days to 15 mo after the first injection of parental spleen cells (Fig. 1). A CFE prepared from each of these animals was inoculated into a litter of newborn syngeneic mice, which were then observed for tumor development over the ensuing 18 mo. Of the 29 CFEs prepared, 12 (41.4%) were positive; i.e., lymphoreticular tumors developed in one or more littermate recipients of each CFE within the 18 mo observation period. The oncogenic CFEs were from donors killed at all stages of the GVHR, the earliest from an animal 10 days after the first injection of parental cells (Table I). However, the percent of positive CFEs was greater among donors killed late in the GVHR. Thus, 5 of 16 (31.3%) CFEs from mice killed within 5 mo of GVHR induction were positive; 1 of 5 (20%) CFEs from mice killed 6-11 mo after GVHR induction was positive; and 6 of 8 (75%) CFEs from mice killed 12-15 mo after GVHR induction were positive (Table II). 25 of the 29 animals used as CFE donors showed no evidence of lymphoreticular tumors, four had a reticulum cell neoplasm (RCN), type B, involving the spleen, and/or lymph nodes, and Peyer’s patches. Three of the four CFEs prepared from tumor-bearing mice were oncogenic (Table I).

Lymphoreticular tumors developed in recipients of CFEs only after a protracted latent period; with one exception, all tumors were detected in recipients
**Fig. 1.** Tumor induction by CFEs from CAF₁ mice with the GVHR. The blocks represent the number of CFEs inoculated into newborn syngeneic mice; the hatched portions indicate the number of CFEs associated with the subsequent development of lymphoreticular tumors in one or more littermate recipients. The number above the blocks is the total number of weaned recipient mice. The abscissa gives the time in months at which the donors of the CFEs were killed after induction of the GVHR.

**TABLE I**

**Oncogenic CFEs: Details of Donor and Recipient Mice**

| Donor of CFE          | Time at which donor was killed after induction of GVHR* | No. of mice developing tumors in total no. of weaned recipients | Age at which tumor developed in recipient(s) |
|-----------------------|--------------------------------------------------------|-----------------------------------------------------------------|---------------------------------------------|
| Non-tumor-bearing CAF₁| 10 days                                                 | 1/5                                                             | 15 mo                                       |
| Non-tumor-bearing CAF₁| 19 days                                                 | 2/9                                                             | 17 mo, 18 mo                               |
| Non-tumor-bearing CAF₁| 22 days                                                 | 1/7                                                             | 18 mo                                       |
| Non-tumor-bearing CAF₁| 40 days                                                 | 1/4                                                             | 18 mo                                       |
| Non-tumor-bearing CAF₁| 3 mo                                                    | 1/8                                                             | 18 mo                                       |
| Non-tumor-bearing CAF₁| 9 mo                                                    | 1/2                                                             | 18 mo                                       |
| Non-tumor-bearing CAF₁| 12 mo                                                   | 3/6                                                             | 16 mo, 16 mo, 18 mo                        |
| Tumor-bearing CAF₁    | 13 mo                                                   | 1/3                                                             | 18 mo                                       |
| Tumor-bearing CAF₁    | 13 mo                                                   | 1/2                                                             | 18 mo                                       |
| Tumor-bearing CAF₁    | 14 mo                                                   | 2/4                                                             | 15 mo, 15 mo                               |
| Non-tumor-bearing CAF₁| 14 mo                                                   | 1/4                                                             | 12 wk                                       |
| Non-tumor-bearing CAF₁| 15 mo                                                   | 2/11                                                            | 17 mo, 18 mo                               |

* Day 0, 1st injection of parental spleen cells.
### TABLE II

**Oncogenicity of CFEs from GVHR and Control Mice**

|                      | GVHR group | Control group |
|----------------------|------------|---------------|
| No. of positive CFEs | 12 (41.4%) | 1 (6.3%)      |
| Total no. of CFEs    | 29         | 16            |
| No. of positive CFE recipients | 17 (11.5%) | 1 (1.2%)     |
| Total no. of CFE recipients | 148     | 84            |
| No. of positive CFEs with tumors developing in two or more littermates | 4 (33.3%) | 0 (0%) |
| Total no. of positive CFEs | 12      | 1             |
| No. of positive CFEs using donors up to 7 mo of age | 5 (31.3%) | 1 (14.3%) |
| Total no. of CFEs using donors up to 7 mo of age | 16      | 7             |
| No. of positive CFEs using donors between 8 and 13 mo of age | 1 (20%) | 0 (0%) |
| Total no. of CFEs using donors between 8 and 13 mo of age | 5       | 5             |
| No. of positive CFEs using donors between 14 and 18 mo of age | 6 (75%) | 0 (0%) |
| Total no. of CFEs using donors between 14 and 18 mo of age | 8       | 4             |

between 15 and 18 mo of age (Table I). In addition, the tumors were usually found in only one or two members of a recipient litter, giving a significant but low overall tumor incidence for all recipients (11.5%). However, tumors arose in two or more littermates in one-third of positive CFEs (Table II). All recipient lymphoreticular tumors were RCNs of the same type which occur in the GVHR mice (7) and involved the same primary tissues, namely, spleen, lymph nodes, and Peyer's patches.

**Control group:** A total of 16 normal CAF1 male mice, ranging in age from 2 to 18 mo (Table II), were used as donors of CFEs, which were then tested for oncogenicity in exactly the same way as those prepared from GVHR animals. Only 1 of a total of 84 (1.2%) recipients developed a lymphoreticular tumor. This animal was one of a weaned litter of six that received at birth a CFE prepared from a 3-mo-old control donor. The tumor developed when the recipient was 12 mo old.

**Presence of Infectious MuLV and CF MuLV Antigen in CFEs from Mice with the GVHR.**

**Experimental group:** 14 CFEs prepared from a total of 23 CAF1 mice killed at intervals after the induction of the GVHR were tested for infectious MuLV using the XC assay (Table III). Each extract was assayed on both NIH Swiss and BALB/cJ mouse embryo cells. Nine of these extracts were obtained by
pooling tissue from two donors, five were prepared from single donors. The XC test was positive in 8 of the 14 (57.1%) CFEs. Four of eight (50%) CFEs prepared from mice killed within 9 mo of GVHR induction contained N-tropic virus in low titer (1.0–1.3 log_{10} PFU/0.2 g tissue). Four of six (66.7%) CFEs from mice killed 11–14 mo after induction of the GVHR contained B-tropic virus in higher titers (1.3–3.9 log_{10} PFU/0.2 g tissue). 7 of the 14 CFEs were also tested for the presence of CF MuLV antigen (Table IV), and 3 of these

| TABLE III: MuLV TITERS IN TISSUES OF CAF1 GVHR MICE |
|---------------------------------|
| CFE no. | No. of donors | Time at which donor(s) killed after GVHR induction | MuLV titer (log_{10} PFU/0.2 g) on mouse embryo cells of: |
|        |              |                                                      | NIH Swiss | BALB/cJ |
| 1      | 2            | 10 days                                               | Neg§      | Neg     |
| 2      | 2            | 10 days                                               | Neg       | Neg     |
| 3      | 2            | 21 days                                               | Neg       | Neg     |
| 4      | 2            | 6 wk                                                  | 1.0       | Neg     |
| 5      | 2            | 6 wk                                                  | Neg       | Neg     |
| 6      | 2            | 3 wk                                                  | 1.0       | Neg     |
| 7      | 2            | 5 mo                                                  | 1.3       | Neg     |
| 8      | 1            | 9 mo                                                  | 1.0       | Neg     |
| 9      | 1            | 11 mo                                                 | Neg       | Neg     |
| 10     | 1            | 11 mo                                                 | Neg       | 1.3     |
| 11     | 2            | 13 mo                                                 | Neg       | 3.9     |
| 12     | 2            | 13 mo                                                 | Neg       | 2.5     |
| 13     | 1            | 14 mo                                                 | Neg       | Neg     |
| 14     | 1            | 14 mo                                                 | Neg       | 3.1     |

* More susceptible to infection by N-tropic virus.
† More susceptible to infection by B-tropic virus.
§ Neg, <10^1.
|| Tumor-bearing animal.

(42.3%) contained significant titers of the antigen (1:4 and over). All three CFEs positive for CF antigen were prepared from tumor-bearing mice late in the GVHR and all three were also positive in the XC test.

Control group: Five CFEs from normal CAF1 male mice were tested for infectious virus on both NIH Swiss and BALB/c mouse embryo cells, and for CF antigen. One of these extracts was prepared from two 9-wk-old animals; the rest came from single donors, two of which were 18 mo old and two others, 24 mo old. In none of these was either infectious virus or CF antigen detected (Table IV).

---

2 Growing preferentially on NIH Swiss mouse embryo cells.
3 Growing preferentially on BALB/cJ mouse embryo cells.
TABLE IV

| CFE no. | No. of donors | Age of donor (mo) | CF antigen titer/0.2 g | MuLV titer (log10 PFU/0.2 g) on mouse embryo cells of: |
|---------|---------------|------------------|-----------------------|--------------------------------------------------|
|         |               |                  |                       | NIH Swiss | BALB/cj |
| GVHR group |               |                  |                       |          |        |
| 4       | 2             | 3 mo             | <1:2                  | 1.0      | Neg*   |
| 7       | 2             | 7 mo             | <1:2                  | 1.3      | Neg    |
| 8       | 1‡            | 11 mo            | 1:8                   | 1.0      | Neg    |
| 9       | 1             | 13 mo            | <1:2                  | Neg      | Neg    |
| 10      | 1‡            | 13 mo            | 1:4                   | Neg      | 1.3    |
| 13      | 1             | 16 mo            | <1:2                  | Neg      | Neg    |
| 14      | 1‡            | 16 mo            | 1:16 or >§            | Neg      | 3.1    |
| Control group |         |                  |                       |          |        |
| N1      | 2             | 9 wk             | <1:2                  | Neg      | Neg    |
| N2      | 1             | 18 mo            | <1:2                  | Neg      | Neg    |
| N3      | 1             | 18 mo            | <1:2                  | Neg      | Neg    |
| N4      | 1             | 24 mo            | <1:2                  | Neg      | Neg    |
| N5      | 1             | 24 mo            | <1:2                  | Neg      | Neg    |

* Neg, <10^1.
‡ Tumor-bearing animal.
§ Dilutions only taken out to 1 in 16.

Serial Passage In Vivo of CFEs from Mice with the GVHR. —The oncogenicity of the GVHR-activated MuLV has been reproduced in two separate serial passage experiments. In the first experiment, a CFE prepared from a non-tumor-bearing CAF1 mouse 15 mo after induction of the GVHR was injected into a litter of newborn syngeneic mice. An RCN developed at 17 and 18 mo of age, respectively, in 2 of 11 (18.2%) weaned recipients. A CFE prepared from the 17-mo-old tumor-bearing mouse caused the same type of tumor in second-passage CAF1 mice, also injected at birth. Three of six (50%) weaned littermate recipients developed an RCN, after 11 mo in one, and at 18 mo of age in the other two.

In the second experiment, involving CB6F1 mice, RCNs have arisen in three transplant generations of syngeneic animals injected at birth with CFEs (Fig. 2). A cell suspension prepared from an RCN arising in a CB6F1 mouse 12 mo after GVHR induction was injected into a litter of 2-wk-old syngeneic mice. The tumor grew out readily in all recipients within 5 wk, and a CFE of this transplanted tumor was prepared from a 6-wk-old male animal. The CFE was inoculated into a litter of newborn CB6F1 mice and caused RCNs in at least six of the eight weaned recipients after a latent period ranging from 10 to 18 mo (mean, 15 mo). Extracts from two of these second-transplant generation tumors were tested for CF antigen, which was found in both in a titer of 1:8. In addition, one of these extracts was assayed for infectious virus, and was shown to contain B-
Fig. 2. Oncogenicity of GVHR-activated MuLV in serial passage in vivo. Mouse with lymphoreticular tumor, ■; normal mouse, □; mouse dead of undetermined cause, △; mouse dead of nontumor cause, ∆. A tumor cell suspension prepared from a reticulum cell neoplasm arising in a CB6F1 mouse 12 mo after GVHR induction was injected intraperitoneally into a litter of 2-wk-old syngeneic infant mice (first-passage recipients). The RCN grew out readily in all recipients and a CFE of the transplanted tumor was prepared from a 6-wk-old male mouse. This extract caused RCNs in at least six of eight syngeneic mice injected at birth after a latent period ranging from 10 to 18 mo. CFEs prepared from two tumor-bearing second-passage mice both caused RCNs in third-passage recipients; at least 9 of 14 mice developed tumors after a mean latent period of 13 mo. RCNs also developed in six of seven fourth-passage recipients of a CFE prepared from a non-tumor-bearing 3-mo-old third-generation animal, and in two of five mice that received a CFE of a third-generation RCN. CFEs prepared from representative RCNs arising in second-, third-, and fourth-passage recipients were examined for CF antigen and/or infectious MuLV and C-type particles as indicated. CF, complement-fixing MuLV antigen titer; XC(N), titer of infectious MuLV on NIH Swiss mouse embryo cells; XC(B), titer of infectious MuLV on BALB/cJ mouse embryo cells. Titers of infectious MuLV are expressed as log_{10} PFU/0.2 g of tissue. N, normal mouse; UC, mouse dead of unrelated cause; EM +, C-type particles seen during electron microscopy.

Fig. 2. Oncogenicity of GVHR-activated MuLV in serial passage in vivo. Mouse with lymphoreticular tumor, ■; normal mouse, □; mouse dead of undetermined cause, △; mouse dead of nontumor cause, ∆. A tumor cell suspension prepared from a reticulum cell neoplasm arising in a CB6F1 mouse 12 mo after GVHR induction was injected intraperitoneally into a litter of 2-wk-old syngeneic infant mice (first-passage recipients). The RCN grew out readily in all recipients and a CFE of the transplanted tumor was prepared from a 6-wk-old male mouse. This extract caused RCNs in at least six of eight syngeneic mice injected at birth after a latent period ranging from 10 to 18 mo. CFEs prepared from two tumor-bearing second-passage mice both caused RCNs in third-passage recipients; at least 9 of 14 mice developed tumors after a mean latent period of 13 mo. RCNs also developed in six of seven fourth-passage recipients of a CFE prepared from a non-tumor-bearing 3-mo-old third-generation animal, and in two of five mice that received a CFE of a third-generation RCN. CFEs prepared from representative RCNs arising in second-, third-, and fourth-passage recipients were examined for CF antigen and/or infectious MuLV and C-type particles as indicated. CF, complement-fixing MuLV antigen titer; XC(N), titer of infectious MuLV on NIH Swiss mouse embryo cells; XC(B), titer of infectious MuLV on BALB/cJ mouse embryo cells. Titers of infectious MuLV are expressed as log_{10} PFU/0.2 g of tissue. N, normal mouse; UC, mouse dead of unrelated cause; EM +, C-type particles seen during electron microscopy.

tropic virus in a titer of 3.4 log_{10} PFU/0.2 g of tissue. CFEs prepared from two other second-transplant generation RCNs were inoculated into separate newborn litters. Third-transplant generation RCNs have developed among recipients of each extract. At least 9 of a total of 14 weaned third-passage recipients
developed lymphoreticular tumors within 17 mo, the earliest tumor being detected at 5 mo (mean latent period, 13 mo). Two third-transplant generation tumors (one from each litter) were tested for infectious virus and CF antigen. Both contained B-tropic virus in titers of $2.6$ and $1.3 \log_{10}$ PFU/0.2 g tissue, respectively, and both had high titers of CF antigen (1:16 or greater). CFEs prepared from two third-passage mice for in vivo inoculation and injected into two separate newborn litters have also caused lymphoreticular tumors in both groups of mice (mean latent period, 12 mo). One of these third-into-fourth-generation experiments is of particular interest in that the CFE injected was prepared from a third-passage CB6F1 mouse killed at the age of 3 mo, at which time no macroscopic or microscopic evidence of tumor was detected. Inoculation of a CFE prepared from the reticular tissues of this animal has resulted in the development of RCNs in six of seven weaned recipients within the 13 mo that have since elapsed (mean latent period, 12 mo). Extracts from two of these fourth-generation tumors were tested for CF antigen, which was detected in both extracts in high titer (1:16 or greater). One of these extracts was also tested for infectious virus, but was negative on both NIH Swiss and BALB/cJ mouse embryo cells. However, extracts from two other RCNs arising in littermates contained B-tropic MuLV in titers of $3.5$ and $2.8 \log_{10}$ PFU/0.2 g tissue, respectively.

Splenic tumors from the two fourth-passage recipient mice just described were examined in the electron microscope. Although most of the cells were in good condition, they contained swollen and internally disrupted mitochondria (Fig. 3 a). Some cells showed granular electron-opaque inclusions (Fig. 3 b).

Particles with the structural characteristics of MuLV (17, 18) were readily found and fairly uniformly distributed throughout the tissues examined. The majority of the virions were found extracellularly (Figs. 3 a, 3 b, 4 a, 4 e). Particles were seen in various stages of budding from the cell membrane (Figs. 3 b, 4 b, 4 c) and a few were found budding into or completely bounded by cytoplasmic vacuoles (Figs. 3 a, 4 d). The virions were round with a diameter ranging from 90 to 115 nm (mean, 104 nm). The budding or free virions were bounded by a trilaminar envelope with small, irregular projections on its surface (Figs. 4 a, 4 e). The nucleoid of the virions was surrounded by two moderately electron-opaque shells, the inner shell being more electron-opaque than the outer shell (Figs. 4 b, 4 e). The center of newly formed "immature" particles was electron-lucent (Figs. 4 a, 4 e). In the more "mature" virions, the central area was condensed and denser (Fig. 4 d). Virions were not found in the nucleus, and no other types of viral particles were seen in any of the tissues examined.

**Control mice:** CFEs were prepared from each of four normal, uninjected, 18-mo-old male CB6F1 mice. All four extracts were negative for infectious virus on both NIH Swiss and BALB/cJ mouse embryo cells and negative (less than a titer of 1:2) for CF antigen.

Normal splenic tissue from a control 12-mo-old male CB6F1 mouse was also
Fig. 3. Reticulum cell neoplasm of spleen in 12-mo-old C86F1 mice. × 70,000. Bar represents 100 nm. (a) Two virus particles (v) are shown; one is in a forming cytoplasmic vacuole, the other is extracellular. A swollen and disintegrating mitochondrion (m), as well as an empty vacuole, can be seen in the cytoplasm. The cell nucleus (n) is in the upper right corner of the micrograph. (b) Extracellular virus particles, and virus budding from the cell membrane. A cytoplasmic inclusion (in) is present in an adjacent cell.
FIG. 4. C-type particles in the splenic reticulum cell neoplasm of 12-mo-old CB6F1 mice. $\times 120,000$. Bar represents 100 nm. (a) Group of at least three virions in the extracellular space. One is still in the process of budding (g). Small irregular projections can be seen on the surface of an adjacent particle (arrow). (b) Budding virion almost free of the cell membrane. (c) Particle in an earlier stage of budding, showing completion of the envelope (arrow) while the particle is still attached to the cell. (d) Virion formed in a cytoplasmic vacuole. The material in the center of the particle is beginning to condense. (e) This free but "immature" virion has an electron-lucent center surrounded by two concentric shells, the inner of which is more electron-opaque than the other. It is bounded by an envelope whose surface shows small, irregular spikes.

examined in the electron microscope. The cells contained normal mitochondria and no virions.

DISCUSSION

A substantial proportion of mice in which a protracted GVHR is induced subsequently develop lymphoreticular tumors (7, 9). It has been previously shown that MuLV is activated in the GVHR (19), and that CFEs prepared from GVHR mice are oncogenic (20). In order to establish the causal role of the virus, it is necessary to show that (a) there is an increase in MuLV titer in mice with the GVHR (b) CFEs prepared from GVHR animals both before and after the onset of tumors contain MuLV, and (c) such CFEs are capable of transmitting tumors to recipient mice.

The studies of Hirsch et al. (19) using the same CAF1 GVHR model as that described in this report, have demonstrated that MuLV may be recovered from the spleens of mice observed in the 4 mo after the induction of the GVHR. No
virus was recovered from normal control animals examined up to 6 mo of age. Since the presence of MuLV per se in the GVHR does not establish its causal role in the genesis of the lymphoreticular tumors, we prepared CFEs from CAF1 GVHR animals and tested these for their ability to reproduce the same tumors in normal syngeneic mice. Preliminary findings already reported (20) have been extended. 12 of 29 (41.4%) such extracts caused lymphoreticular tumors in recipient mice, while only 1 of 16 (6.3%) CFEs prepared from a control group of mice matched for strain, age, and sex did so. A striking feature of these experiments is that whereas less than 30% of CFEs from mice killed within 12 mo of GVHR induction were oncogenic, the percentage of oncogenic extracts from mice killed 12–15 mo after GVHR induction rose to 75. This contrasts clearly with the situation in normal uninjected mice of this low leukemia strain. CFEs were prepared from nine male CAF1 mice between the ages of 8 and 18 mo; none of these transmitted tumors to recipients.

We have sought to correlate oncogenicity in CFEs from animals with the GVHR with the presence in such extracts of infectious MuLV and CF MuLV antigen. Substantial titers of B-tropic MuLV and CF antigen were detected in 66.7 and 50%, respectively, of extracts from animals killed 11–14 mo after GVHR induction. During the first few months of the administration of parental cells, however, MuLV titers were low and CF antigen was absent. There is thus a good correlation late in the GVHR between the increased oncogenicity of tissue extracts and their content of substantial amounts of infectious MuLV and of CF antigen.

In two separate experiments, one involving CAF1 mice, the other CB6F1 mice, CFEs originally derived from a GVHR mouse have been serially passed in vivo. Lymphoreticular tumors of the same type that occur in GVHR mice have developed in two or more successive transplant generations of normal syngeneic recipients. The mean latent period of tumor development has been somewhat shortened by serial passage, but is still quite protracted (not less than 12 mo). However, in one experiment, the incidence of tumors among recipients rose from 18.2 to 50% in two successive transplant generations, and in the other experiment, at least 68% of all recipients in three successive transplant generations developed tumors. Such incidence figures make it very unlikely that the tumors arose spontaneously. Since all the lymphoreticular tumors tested in the serial passage experiments showed evidence of MuLV infection in the form of infectious virus and/or CF antigen and characteristic C-type particles, it seems highly probable that MuLV is the agent responsible for the development of lymphoreticular tumors in CAF1 and CB6F1 mice. Taken together, these data strongly support the concept that activated MuLV causes the lymphoreticular tumors which arise in GVHR mice.

4 In a series of control mice killed in our laboratory at 18 mo of age, 1 of 91 (1.1%) CAF1 male mice and 6 of 82 (7.3%) CB6F1 male mice had lymphoreticular tumors.
The work of Rowe and Hartley and their colleagues (21, 22) has established that naturally occurring MuLVs fall into one of two categories with respect to their growth in mouse embryo cells of NIH Swiss (N) or BALB/c (B) mice. "N-tropic" viruses initiate infection 30-1,000 times more efficiently on NIH Swiss than on BALB/c mouse embryo cells, while "B-tropic" viruses show the reciprocal pattern. Furthermore, mouse embryo cells of other mouse strains resemble either NIH Swiss or BALB/c mouse embryo cells in their susceptibility to infection by either N- or B-tropic virus. The patterns of susceptibility to N- or B-tropic virus observed in F1 hybrid and progeny of backcross mice are compatible with a single genetic locus whose allelic genes are dominant for resistance to either N- or B-tropic virus (22). The apparent switch from low titers of N-tropic virus early in the GVHR to higher titers of B-tropic virus late in the GVHR is of interest. It has been shown (23) that normal BALB/c mice, which are mostly negative for infectious virus before 4 mo of age, yield both N-tropic and B-tropic virus as they age. It is possible that the GVHR activates both types of virus in the BALB/c hybrids, but that since CAF1 and CB6F1 mice are F1 hybrids of two B strains, only the B-tropic virus can achieve titers necessary for oncogenicity. Compatibility of host cell and viral tropism may also be an important factor in the transforming capacity of the virus. Other evidence (24) suggests that the efficiency with which MuLV is transmitted from cell to cell, and thus the ease with which an activated virus can become established in a given host, may be governed by other genetic factors in addition to the N-B locus.

Although the MuLV activated during the GVHR in CAF1 and CB6F1 mice undoubtedly has oncogenic potential in its host, that potential is clearly quite low. The incidence of lymphoreticular tumors in CAF1 GVHR animals is only 27% at 12 mo, rising to 55% at 18 mo (7). CFEs from GVHR animals induce tumors only after a protracted latent period (15 mo and more) and then usually in only a minority of littermate recipients. Serial passage of CFEs in syngeneic mice has somewhat enhanced the oncogenicity of the activated virus in that the percent of littermate recipients developing tumors has increased, and the latent period has been shortened. Other MuLV-host combinations are much more efficient in terms of in vivo tumorigenesis. The AKR strain, for example, is chronically infected from birth with MuLV, which causes lymphocytic leukemia in 92% of the animals, after an average latent period of 8 mo (25). However, like the other high leukemia mouse strain, C58, the AKR strain is of the N type and its virus is N-tropic (22). This suggests that the N allele of the N-B locus might also be a major determinant of spontaneous oncogenesis.

It is of interest that C-type particles with the structural characteristics of MuLV were so readily seen in both the CB6F1 tumors examined by electron microscopy in the present study. Previous attempts (7) to detect such particles in the tissues of CAF1 GVHR mice, both before and after the development of tumors, failed despite a fastidious search through many specimens. However,
in a recent study, a CAF, mice were selected for examination by electron microscopy early in the GVHR and only if infectious virus could be detected in their tissues by the XC test. Under these circumstances, C-type particles were seen in a majority of the animals. The CB6F, GVHR mice have not been previously examined for C-type particles, but it is noteworthy that the XC test was positive in both the animals we studied with the electron microscope. A number of lymphoreticular tumors arising in (BALB/c J X SJL/J)F1 hybrid mice after the administration of BALB/c spleen cells have also been examined in our laboratory, but only a very occasional C-type particle has been seen.

What are the possible mechanisms whereby viral activation and oncogenesis occur in the GVHR, in strains where MuLV normally exists in inactive or unexpressed form? It has been suggested that immunosuppression associated with the GVHR could permit virus-infected and/or virus-transformed cells to persist and multiply unchecked. However, experiments reported elsewhere have shown that in this particular GVHR model, cellular immune mechanisms that are probably responsible for the rejection of virus-infected and/or virus-transformed cells remain intact, and may even be enhanced. An alternative explanation is that the very large pool of lymphocytes stimulated during the GVHR present the virus with a previously nonpermissive population of cells, in which it can now replicate, and which it can also transform. It has been shown that tuberculin-sensitized lymphocytes activated in vitro by tuberculin-purified protein derivative are able to replicate Newcastle disease virus and vesicular stomatitis virus, whereas unstimulated cells do not (26). There is now some evidence that such a mechanism is important in the development of GVHR-associated tumors. This comes from the observation that in vitro stimulation of lymphocytes resulting from the cocultivation of normal BALB/c and CAF1 mice is associated with release of MuLV (27).

The demonstration that a normally inactive or unexpressed oncogenic virus can be activated by an immunological disturbance such as the GVHR, and that the activated virus is responsible for subsequent tumor development, has important implications. There is currently considerable interest in the role of viruses in cancer induction in man (28). Huebner and Todaro have suggested that the cells of many, and perhaps all, vertebrates contain in their genome the information for producing oncogenic RNA virus, and that this "virogene" has been acquired during the course of evolution, being transmitted vertically from cell to cell and parent to offspring. In their view, the occurrence of most cancers is a natural biological event determined by spontaneous or induced derepression.

---

6 Andre-Schwartz, J., R. S. Schwartz, M. S. Hirsch, S. M. Phillips, and P. H. Black. Activation of leukemia viruses by graft-versus-host and mixed lymphocyte culture reaction: electron microscopic evidence of C-type particles. Manuscript submitted for publication.

6 Heywood, P. Unpublished data.

7 Ruddle, N. H., M. Y. K. Armstrong, and F. F. Richards. Cellular immunity in mice developing graft-versus-host reaction-associated tumors. Manuscript submitted for publication.
of the "oncogene," that portion of the virogene which codes for a transforming protein (29). The oncogene theory has gained support from the observation that 5-iododeoxyuridine and 5-bromodeoxyuridine induce synthesis of MuLV in previously negative mouse embryo cell lines (30, 31). Activation of MuLV in the GVHR could represent an analogous in vivo situation.

The GVHR mouse model may have a counterpart in certain forms of human cancer where a viral etiology is likely. One such cancer is Burkitt's lymphoma (32) which occurs predominantly in children in geographic locations where the one single common factor so far identified is the occurrence of holoendemic malaria. Burkitt's lymphoma is also associated with a herpes-like DNA virus, the Epstein-Barr virus. Lymphoreticular hyperplasia is an important effect of chronic malaria and may provide Epstein-Barr virus with just those "lymphoblastoid" cells that it requires for its growth. The ability of Epstein-Barr virus to promote rapid and continued proliferation of lymphocytes in culture (33) and to bring about morphological transformation of human embryo fibroblasts in vitro (34) suggest that it may well be oncogenic in vivo.

SUMMARY

A graft-vs.-host reaction (GVHR) was induced in young male C57 and CB6F1 mice by the administration of BALB/cJ spleen cells. A proportion of such mice subsequently developed lymphoreticular tumors. Cell-free extracts (CFEs) prepared from the reticular tissues of CAF1 mice killed at intervals after the induction of the GVHR were tested for their capacity to produce the same tumors in a litter of syngeneic mice inoculated at birth. 12 of 29 (41.4%) such extracts were positive, causing lymphoreticular tumors in one or more littermate recipients. The positive CFEs came from donors killed at all stages of the GVHR, from tumor-bearing mice as well as from non-tumor-bearing mice. However, whereas less than 30% of CFEs from mice killed within 12 mo of GVHR induction were oncogenic, the incidence of oncogenic extracts from mice killed 12-15 mo after GVHR induction rose to 75%. None of the CFEs prepared from nine normal uninjected male CAF1 mice killed between the ages of 8 and 18 mo transmitted tumors to recipients. CFEs prepared from CAF1 mouse with the GVHR were tested for infectious murine leukemia virus (MuLV) using the XC assay and also for complement-fixing (CF) group-specific MuLV antigen. Substantial titer of B-tropic MuLV and CF antigen were detected in at least half the extracts from mice killed 11-14 mo after GVHR induction. During the first few months of GVHR induction, MuLV titers were low and CF antigen was absent. Neither infectious MuLV nor CF antigen were detected in CFEs prepared from normal control mice. Serially passed CFEs originating from a CB6F1 GVHR-induced RCN caused similar tumors in successive generations of syngeneic recipient mice. These lymphoreticular tumors were shown to contain infectious MuLV, CF MuLV antigen, and C-type particles. These data together provide evidence that MuLV is activated during the GVHR and that it is responsible for the eventual development of lymphoreticular tumors.
We are particularly indebted to Dr. Robert J. Huebner for his interest, and for doing the CF tests. We wish to acknowledge the excellent assistance of Miss Louise Camera, Mr. Jiri Jonak, and Mrs. Mary Garrison.

REFERENCES

1. Aisenberg, A. C. 1962. Studies on delayed hypersensitivity in Hodgkin’s disease. J. Clin. Invest. 41:1964.
2. Cone, L., and J. W. Uhr. 1964. Immunological deficiency disorders associated with chronic lymphocytic leukemia and multiple myeloma. J. Clin. Invest. 43:2241.
3. Schwartz, R. S., and N. Costea. 1966. Autoimmune hemolytic anemia: clinical correlations and biological implications. Semin. Hematol. 3:2.
4. Talal, N., L. Sokoloff, and W. F. Barth. 1967. Extrasalivary lymphoid abnormalities in Sjögren’s syndrome (reticulum cell sarcoma, “pseudolymphoma,” macroglobulinemia). Am. J. Med. 43:50.
5. Good, R. A., and J. Finstad. 1968. The association of lymphoid malignancy and immunologic functions. In Proceedings of the Internal Conference on Leukemia-Lymphoma. C. J. D. Zarafonidis, editor. Lea & Febiger, Philadelphia. 175.
6. Schwartz, R. S., and L. Beldotti. 1965. Malignant lymphomas following allogenic disease: transition from an immunological to a neoplastic disorder. Science (Wash. D.C.). 149:1511.
7. Armstrong, M. Y. K., E. Gleichmann, H. Gleichmann, L. Beldotti, J. André-Schwartz, and R. S. Schwartz. 1970. Chronic allogeneic disease. II. Development of lymphomas. J. Exp. Med. 132:417.
8. Kaplan, H. S. 1967. On the natural history of the murine leukemias: presidential address. Cancer Res. 27:1325.
9. Gleichmann, E., H. Gleichmann, and R. S. Schwartz. 1972. Immunologic induction of malignant lymphoma: genetic factors in the graft-versus-host model. J. Natl. Cancer Inst. 49:793.
10. Lewis, R. M., M. Y. K. Armstrong, J. André-Schwartz, A. Muftuoglu, L. Beldotti, and R. S. Schwartz. 1968. Chronic allogeneic disease. I. Development of glomerulonephritis. J. Exp. Med. 128:653.
11. Moloney, J. B. 1960. Biological studies on a lymphoid-leukemia virus extracted from Sarcoma 37. I. Origin and introductory investigations. J. Natl. Cancer Inst. 24:933.
12. Dunn, T. B. 1954. Normal and pathologic anatomy of the reticular tissue in laboratory mice. With a classification and discussion of neoplasms. J. Natl. Cancer Inst. 14:1281.
13. Rowe, W. P., and T. Pincus. 1972. Quantitative studies of naturally occurring murine leukemia virus infection of AKR mice. J. Exp. Med. 135:429.
14. Hartley, J. W., W. P. Rowe, W. I. Capps, and R. J. Huebner. 1965. Complement fixation and tissue culture assays for mouse leukemia viruses. Proc. Natl. Acad. Sci. U.S.A. 53:931.
15. Mollenhauer, H. H. 1964. Plastic embedding mixtures for use in electron microscopy. Stain Technol. 39:111.
16. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17:208.
17. Dalton, A. J. 1972. Further analysis of the detailed structure of type B and C particles. *J. Natl. Cancer Inst.* **48**:1095.
18. Dalton, A. J. 1972. RNA tumor viruses. Terminology and ultrastructural aspects of virion morphology and replication. *J. Natl. Cancer Inst.* **49**:323.
19. Hirsch, M. S., P. H. Black, G. S. Tracy, S. Leibowitz, and R. S. Schwartz. 1970. Leukemia virus activation in chronic allogeneic disease. *Proc. Natl. Acad. Sci. U.S.A.* **67**:1194.
20. Armstrong, M. Y. K., F. L. Black, and F. F. Richards. 1972. Tumor induction by cell-free extracts derived from mice with graft-versus-host disease. *Nat. New Biol.* **236**:153.
21. Hartley, J. W., W. P. Rowe, W. I. Capps, and R. J. Huebner. 1969. Isolation of naturally occurring viruses of the murine leukemia virus group in tissue culture. *J. Virol.* **3**:126.
22. Pincus, T., J. W. Hartley, and W. P. Rowe. 1971. A major genetic locus affecting resistance to infection with murine leukemia viruses. I. Tissue culture studies of naturally occurring viruses. *J. Exp. Med.* **133**:1219.
23. Rowe, W. P., and J. W. Hartley. 1972. Studies of genetic transmission of murine leukemia virus by AKR mice. II. Crosses with Fe-P strains of mice. *J. Exp. Med.* **136**:1286.
24. Stephenson, J. R., and S. A. Aaronson. 1972. Genetic factors influencing C-type RNA virus induction. *J. Exp. Med.* **136**:175.
25. Murphy, E. D. 1966. Characteristic tumors. In Biology of the Laboratory Mouse. E. L. Green, editor. McGraw-Hill Book Company, New York. 521.
26. Bloom, B. R., L. Jimenez, and P. I. Marcus. 1970. A plaque assay for enumerating antigen-sensitive cells in delayed-type hypersensitivity. *J. Exp. Med.* **132**:16.
27. Hirsch, M. S., S. M. Phillips, C. Solnick, P. H. Black, R. S. Schwartz, and C. B. Carpenter. 1972. Activation of leukemia viruses by graft-versus-host and mixed lymphocyte reactions *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* **69**:1069.
28. Culliton, B. J. 1972. Cancer virus theories: focus of research debate. *Science (Wash. D.C.)* **177**:44.
29. Huebner, R. J., and G. J. Todaro. 1969. Oncogenes of RNA tumor viruses as determinants of cancer. *Proc. Natl. Acad. Sci. U.S.A.* **64**:1087.
30. Lowy, D. R., W. P. Rowe, N. Teich, and J. W. Hartley. 1971. Murine leukemia virus: high-frequency activation *in vitro* by 5-iododeoxyuridine and 5-bromo-deoxyuridine. *Science (Wash. D.C.)* **174**:155.
31. Aaronson, S. A., G. J. Todaro, and E. M. Scolnick. 1971. Induction of murine C-type viruses from clonal lines of virus-free BALB/3T3 cells. *Science (Wash. D.C.)* **174**:157.
32. O'Connor, G. T. 1970. Persistent immunologic stimulation as a factor in oncogenesis, with specific reference to Burkitt's tumor. *Am. J. Med.* **48**:279.
33. Gerber, P., J. Whang-Peng, and J. H. Monroe. 1969. Transformation and chromosome changes induced by Epstein-Barr virus in normal human leukocyte cultures. *Proc. Natl. Acad. Sci. U.S.A.* **63**:740.
34. Probert, M., and M. A. Epstein. 1972. Morphological transformation *in vitro* of human fibroblasts by Epstein-Barr virus: preliminary observations. *Science (Wash. D.C.)* **175**:202.