Proc. Nat. Acad. Sci. USA
Vol. 69, No. 5, pp. 1069–1072, May 1972

Activation of Leukemia Viruses by Graft-Versus-Host and Mixed Lymphocyte Reactions *In Vitro*

(mice/mitomycin C/phytohemagglutinin)

M. S. HIRSCH*, S. M. PHILLIPS†, C. SOLNIK‡, P. H. BLACK*,
R. S. SCHWARTZ‡, AND C. B. CARPENTER†

* Departments of Medicine, Harvard Medical School, Massachusetts General Hospital and † Peter Bent Brigham Hospital, Boston, Mass. 02114 and 02115, respectively; ‡ Department of Medicine, New England Medical Center Hospitals, Boston, Massachusetts 02111

Communicated by Albert H. Coons, February 16, 1972

ABSTRACT **Spleen cells from BALB/c or CAF_1 mice released little or no detectable leukemia virus when cultured 2–7 days *in vitro*. In contrast, spleen cells of CAF_1 mice previously inoculated with parental BALB/c spleen cells released leukemia viruses in 10 of 11 cases studied. Cultures of a mixture of spleen cells from normal BALB/c and CAF_1 mice also contained leukemia viruses. Phytohemagglutinin induced the transformation of lymphocytes in cultures of CAF_1 or BALB/c spleen cells, but this transformation did not activate leukemia viruses. It is concluded that mixed lymphocyte cultures *in vitro*, just as graft-versus-host reactions *in vivo*, can activate leukemia viruses that are normally present in a repressed form. This activation is not solely a function of lymphocyte transformation. The activated mouse leukemia virus may subsequently account for the observed high incidence of neoplasia in graft-versus-host disease.**

Many strains of mice carry C-type RNA leukemia viruses, which are detectable by various techniques (1, 2). In other strains, such viruses are not detectable under ordinary circumstances, but they can be activated *in vivo* by exposure to radiation or chemical carcinogens (2–6), or *in vitro* by aging, infection with other viruses, or exposure to chemicals such as 5-bromodeoxyuridine (BrdU) or 5-iododeoxyuridine (IdU) (7–10).

We have recently shown that murine leukemia viruses are also activated by the graft-versus-host reaction, an immunological response induced by transplantation of immunocompetent cells into recipients unable to react against them (11). The graft-versus-host reaction can be studied in F_1 hybrid recipients of parental immunocytes, because F_1 hybrids of highly inbred mice are unable to reject parental grafts, whereas lymphocytes in the inoculum of parental spleen cells respond with immunological specificity to antigens possessed by the hybrid recipient. We studied (BALB/c x A/J)F_1 hybrid mice, 6–8 weeks old (hereafter referred to as CAF_1) that were inoculated with parental BALB/c spleen cells. Leukemia viruses became readily detectable in the injected hosts within a week after inoculation, and a high incidence of reticulum-cell sarcomas developed at a relatively early age; 30% of the mice had lymphomas by 12 months and 80% had lymphomas by 24 months (11, 12). In contrast, CAF_1 mice that were not inoculated with parental cells remained virus-free at least until 12 months of age, and they had a low incidence of lympho–reticular neoplasms that occurred late in life (0% under 12 months of age, less than 5% between 12–24 months).

In the present investigations, we have examined whether leukemia viruses could also be detected when spleen cells of CAF_1 mice undergoing a graft-versus-host reaction were cultured *in vitro*. The possibility that leukemia viruses could be activated by the *in vitro* analogue of the graft-versus-host reaction—the mixed lymphocyte culture—was also studied. In addition, the influence of the lymphocyte mitogen phytohemagglutinin(PHA) on the activation of leukemia viruses in cultured mouse lymphocytes was studied.

MATERIALS AND METHODS

Mice. BALB/c, A/J, C_{57}B1/6, DBA/2, CAF_1, and (C_{57}B1/6 x DBA/2)F_1 (hereafter referred to as BDF_1) mice were obtained from Jackson Laboratories, Bar Harbor, Maine. CD(R)-1 Swiss mice were obtained from Charles River Farms, Boston, Mass. 6- to 8-week-old CAF_1 mice received 4 weekly intraperitoneal injections of 50×10^6 viable spleen cells prepared as described (13) from 6-week-old male BALB/c donors. BDF_1 mice were injected with spleen cells from DBA/2 donors in an identical manner. Uninoculated F_1 mice of the same ages were used as controls. Mice were studied 4–6 weeks after the last injection of cells.

Lymphocyte Cultures. Lymphocyte suspensions were prepared as described (14) by mincing of spleens, gently pressing the fragments through a 100-gauge stainless steel mesh, allowing the cells to settle, and then passing them through a fine glass-bead column. The cells were washed and suspended in various densities ($1.5–5.0 \times 10^6$/ml), depending on the nature of the experiment, in Roswell Park Memorial Institute media 1640 (15) containing 10% heat-inactivated (56°C, 30 min) fresh normal human serum, 250 units of penicillin per ml, 100 μg of streptomycin per ml, and 5 mM HEPES buffer (pH 5.4). Cells were treated with 25 μg of mitomycin C per ml for 30 min at 37°, and then washed three times with Roswell Park Memorial Institute media 1640 with HEPES buffer (16).

Stimulation was assessed by addition of 1 μl of PHA/ml to cultures of lymphocytes. All cultures were assessed for cell proliferation by the addition of 1 μCi of [^{3}H]thymidine (New England Nuclear 6.7 Ci/mmol) 4 hr before termination of culture. The cells were harvested on glass filter discs, washed

Abbreviations: PHA, phytohemagglutinin; $TCID_{50}$, 50% tissue culture infectious doses.

with Hank's medium (pH 7.2), and precipitated with 10% Cl_3CCOOH and absolute alcohol. The glass-filter discs were placed in vials, and radioactivity was assessed by standard liquid scintillation techniques. Cultures with PHA were assayed after 2 days in culture, and mixed lymphocyte cultures were assayed after 3–7 days in culture.

Leukemia Virus Assays. Lymphocyte cultures were tested for leukemia virus by the mixed-culture cytopathogenicity assay reported by Klement *et al.* (17) and described more fully in (11). XC cells were provided by Dr. W. P. Rowe. Spleen-cell suspensions from allogenic and normal BDF_1 mice were also tested for virus by this technique. Experimental and control cells were examined under a light microscope at a magnification of ×100, and multinucleate cells (more than four nuclei per cell) were counted per 100 fields per dish. A given sample (dilution) was considered positive for virus if the number of multinucleate cells per dish was four times the mean of the number of multinucleate cells in the control.

The same samples were also tested for leukemia viruses by a focus assay on mouse cells transformed by murine sarcoma virus, but not releasing infectious virus (sarcoma positive-leukemia negative cells, provided by Dr. R. Bassin) (18). Cells were plated in 60-mm petri dishes (Falcon Plastics) at a density of 10^5 cells per dish and were infected the following day. Lytic lesions, easily seen by light microscopy, appeared 4–5 days after inoculation. Positive and negative controls, as in the mixed-culture cytopathogenicity assay, were included in each assay. After several weeks evidence was obtained indicating that the sarcoma-positive, leukemia-negative cells were contaminated with mycoplasma, and this assay was discontinued. Before that, there was concordance between the results from the mixed-culture cytopathogenicity assays and those from the cultures of sarcoma-positive, leukemia-negative cells.

RESULTS

Activation of leukemia virus during the graft-versus-host reaction

Cultures of spleen cells from uninoculated CAF_1 and BALB/c mice were found to be negative for leukemia viruses (Table 1) by the mixed-culture cytopathogenicity or transformed-cell assays, or both, in 15 of 16 experiments. However, cultures of spleen cells from CAF_1 mice previously inoculated with parental BALB/c cells were positive for leukemia virus by the same assays in 10 of 11 experiments; titers in these cultures ranged from $10^{1.2}$ to more than $10^{3.2}$ $TCID_{50}$ (50% tissue culture infectious doses)/ml.

TABLE 1. *Leukemia viruses recoverable from CAF_1 and BALB/c spleen cells*

Cells	No.	No. with leukemia virus
CAF_1 normal-spleen suspensions*	15	0
CAF_1 normal-cultured spleen cells†	13	1
BALB/c normal-spleen suspensions*	6	0
BALB/c normal-cultured spleen cells†	3	0
Allogeneic CAF_1-spleen suspensions*‡	23	22
Allogeneic CAF_1-cultured spleen cells†	11	10
CAF_1 × BALB/c MLC	11	7

* 20% Suspensions of spleen cells (or extracts thereof) cultured directly onto mouse embryo cells, followed in 14 days by mixed-culture cell assays (reported in ref. 11).

† Spleen cells cultured *in vitro* for 3 days before subculture on mouse embryo cells.

‡ Allogeneic spleen cell suspension are spleen cells prepared from F_1 hybrid mice that were undergoing the graft-versus-host reaction after receiving parental spleen cells.

In contrast to the results observed with CAF_1 cells, leukemia viruses were not frequently recovered from BDF_1 cells, irrespective of treatment (Table 2). Virus was detectable in only one of 10 control spleen-cell preparations from BDF_1 mice, and in 4 of 16 allogeneic spleen-cell preparations. Virus titers in the positive BDF_1 cultures did not exceed $10^{1.2}$ $TCID_{50}$/ml.

Activation of leukemia viruses in mixed-lymphocyte cultures

3- to 7-day cultures of mixtures of BALB/c and CAF_1 spleen cells were positive for leukemia viruses by the mixed-culture or transformed-cell assays, or both, in 7 of 11 experiments (Tables 1 and 3). In three cultures that did not produce virus, lymphocyte stimulation was suboptimal, with isotope incorporation ratios only 2.5 times those of the controls, whereas the seven positive cultures were stimulated a minimum of five times the controls. CAF_1 and BALB/c spleen cells cultured separately for the same periods were negative for viruses.

Relationship of blastogenesis to activation of virus

Lymphocytes of CAF_1, BALB/c, or A/J mice cultured in the presence of PHA underwent marked blastogenesis but did not release virus after treatment with PHA (Table 3). Similarly, titers of leukemia virus from cultured spleen cells of CAF_1 mice undergoing a graft-versus-host reaction were not altered by treatment with PHA ($10^{1.2}$ $TCID_{50}$/ml with or without PHA). The proliferative response of spleen cells from CAF_1 mice with the graft-versus-host reaction to PHA was significantly less than that of normal CAF_1 spleen cells. Spontaneous blastogenesis in these cultures during the first day was 2.8 times that in the controls, but fell below that in the controls by the third day (19).

Both mixed-lymphocyte cultures that were positive for leukemia virus and PHA cultures that were negative for leukemia virus showed proliferative responses, as measured by incorporation of isotopes (Table 3). Treatment of responding BALB/c cells with mitomycin C prevented both blastogenesis and release of virus.

DISCUSSION

The results confirm our earlier finding of the activation of leukemia virus by the graft-versus-host reaction (11), and

TABLE 2. *Leukemia viruses recoverable from BDF_1 spleen cells*

Cells	No.	No. with leukemia virus
BDF_1 normal-spleen suspensions*	8	1
BDF_1 normal-cultured spleen cells†	2	0
BDF_1 allogeneic-spleen suspensions*‡	11	2
BDF_1 allogeneic-cultured spleen cells†	5	2

*, †, ‡ are as in footnotes to Table 1.

TABLE 3. *Effects of blastogenesis on activation of leukemia virus*

Cells	No. with leukemia virus/Total no. cultures	With PHA cpm (range)*	With PHA Virus titer†	Without PHA cpm (range)*	Without PHA Virus titer†
Normal spleen‡	0/8	29,500 (19,100–56,100)	0	980 (640–1240)	0
Allogeneic CAF_1	2/2	9700 (7750–15,800)	1.2	1040 (420–1970)	1.2
CAF_1 x BALB/c MLC	7/11	—	—	—	—
Virus positive	7/7	—	—	10,600 (5630–17,500)	2.2
Virus negative	0/4	—	—	3270 (1420–8450)	0
CAF_1 x BALB/c–mitomycin C	0/2	—	—	1400 (650–1860)	0

* Average cpm computed from mean incorporations of tritiated thymidine in individual replicate cultures; standard errors of replicate cultures were less than 10%. Cells for blastogenic and viral studies were harvested after 2 days in PHA and after 3 or 6 days in mixed-lymphocyte cultures (MLC).

† Average virus titers in $TCID_{50}$/ml.

‡ This group includes individual cultures of CAF_1, BALB/c, and A/J cells.

extend the *in vivo* findings to their probable *in vitro* counterpart, the mixed-lymphocyte reaction. The mechanism(s) of virus activation by these immunological reactions remain to be fully elucidated; however, activation does not appear to be solely the result of the transformation of lymphocytes, because PHA induced marked blast transformation in CAF_1 lymphocytes, yet activation of virus did not occur. Furthermore, PHA did not increase the titer of recoverable leukemia virus in lymphocytes from mice undergoing graft-versus-host reaction.

Not only is the extent of lymphocyte transformation in the mixed-lymphocyte cultures less than that induced by PHA (20), but some qualitative difference between the two systems must also exist, since leukemia virus was recoverable only from mixed-lymphocyte cultures. Since it is estimated that less than 2% of an initial lymphoid population can respond to an antigenic stimulation, whereas an excess of 80% can respond to PHA (20), the metabolic demands and products of metabolism may vary between the two systems as well. Perhaps the balance of these products known to directly stimulate or inhibit lymphocyte metabolism (21, 22) may affect the subsequent release or detection of virus. It is conceivable that this type of immunologic mechanism shared by the mixed-leukocyte cultures and the graft-versus-host reaction is unique, because other work (in preparation) has shown that *in vivo* challenge of CAF_1 mice with allogenic spleen cells from $C_{57}BL_6$ mice does not activate leukemia viruses; in these experiments each of four recipients received four doses of 50×10^6 $C_{57}BL_6$ spleen cells as described above, and spleens from these animals were uniformally negative for leukemia viruses 1 week after the fourth injection of cells. In addition, multiple injections of six CAF_1 mice with sheep erythrocytes, and six CAF_1 mice with Freunds adjuvant failed to activate leukemia viruses. A nonimmunological mechanism that could activate leukemia viruses during the mixed-lymphocyte culture seems remote because of the failure to find virus release upon *in vitro* cocultivation of CAF_1 and BALB/c spleen cells that were treated with mitomycin C. Under these conditions no immunologic response is expected, because F_1 lymphocytes are minimally responsive to parental cells, and the parental cells cannot react to F_1 antigens because of their inactivation by mitomycin C. The lack of immunological reactivity in cultures of this type was demonstrated by the isotope incorporation studies, which failed to show stimulation.

The cell of origin of the activated leukemia virus is unknown; responding cells, target cells, or third party cells are all possible virus producers. Preliminary results in our laboratories using iododeoxyuridine (IdU) as an activator of leukemia viruses (9, 10) demonstrate that both BALB/c and CAF_1 spleen cells possess the genetic information necessary to support the replication of leukemia viruses.

Is the immunologically-activated leukemia virus the cause of the lymphomas that develop in CAF_1 mice inoculated with BALB/c cells? Treatments that prevent cellular proliferation and virus activation, e.g., treatment of donor cells with mitomycin C, also prevent the graft-versus-host reaction and development of lymphoma (12). In addition, tumor cells from CAF_1 mice with the graft-versus-host reaction induced rat-type lymphosarcomas when inoculated into newborn Wistar–Furth rats, a strain highly susceptible to murine viruses (R. S. Schwartz, unpublished results); mature C-type particles were seen by electron microscopy in these rat tumors. It is of interest that activation of leukemia viruses was not detected frequently in a parent–hybrid combination in which lymphomas do not develop. For example, none of 81 $(C57B1/6 \times DBA/2)F_1$ mice developed a lymphoma after the administration of DBA/2 spleen cells (R. S. Schwartz, in preparation), and in this combination leukemia virus was not detected frequently either *in vivo* or *in vitro*. This result shows that not all graft-versus-host reactions or mixed-lymphocyte cultures will be associated with the activation of leukemia viruses, and again demonstrates that lymphocyte transformation, although necessary for viral activation, is not the sole requirement for this phenomenon. Further evidence that the viruses activated during the graft-versus-host reactions are relevant to the development of neoplasms in this condition was recently provided by the demonstration

that cell-free extracts of the spleens of CAF_1 mice that had been injected with BALB/c spleen cells induced lymphomas when injected into newborn CAF_1 mice (23).

Our results indicate that certain immunological reactions can activate infectious leukemia viruses both *in vivo* and *in vitro*. Although the full spectrum of immunological reactions capable of activating these viruses has not yet been tested, present information indicates strongly that lymphocyte–lymphocyte interactions, which are characteristic of graft-versus-host reaction and mixed-lymphocyte culture, are most liable to this event. Since no human oncogenic virus has yet been definitively isolated, one can only speculate on activation of leukemia virus in man. However, the high incidence of lymphoma and leukemia in patients with certain autoimmune diseases (24, 25) might be explained on the basis of our findings. Moreover, the increased incidence of neoplasms in recipients of renal allografts (26)—commonly attributed to immunosuppression—may be due to activation of leukemia viruses during the overt or subtle transplantation reactions in these patients. Finally, the recent report of Fialkow *et al.* (27), in which donor bone marrow cells became leukemic after grafting into a previously irradiated leukemic recipient, also suggest activation of leukemia viruses as a possible mechanism.

We thank Drs. John Merrill and Max Proffitt for their help and support, and Mrs. Gay Tracy, Miss Ruth Speer, and Miss Lorraine Beldotti for expert technical assistance. Supported by Public Health Service awards 5 T01-AI 00301, CA 100-18, CA 12464-01 and NIH NCI-72-2012.

1. Hartley, J. W., Rowe, W. P., Capps, W. I. & Huebner, R. J. (1969) "Isolation of naturally occurring viruses of the murine leukemia virus group in tissue culture," *J. Virol.* **3**, 126–132.
2. Huebner, R. J., Kelloff, G. J., Sarma, P. S., Lane, W. T., Turner, H. C., Gilden, R. V., Oroszlan, S., Meier, H., Myers, D. D. & Peters, R. L. (1970) "Group-specific antigen expression during embryogenesis of the genome of the C-type RNA tumor virus: implications for ontogenesis and oncogenesis," *Proc. Nat. Acad. Sci. USA* **67**, 366–376.
3. Kaplan, H. S. (1967) "On the natural history of the murine leukemias," *Cancer Res.* **27**, 1325–1340.
4. Haran-Ghera, N. (1966) "Leukemogenic activity of centrifugates from irradiated mouse thymus and bone marrow," *Int. J. Cancer* **1**, 81–87.
5. Gross, L. & Feldman, D. G. (1968) "Electron microscopic studies of radiation-induced leukemia in mice: virus release following total-body x-ray irradiation," *Cancer Res.* **28**, 1677–1681.
6. Igel, H. J., Huebner, R. J., Turner, H. C., Kotin, P. & Falk, H. L. (1969) "Mouse leukemia virus activation by chemical carcinogens," *Science* **166**, 1624–1626.
7. Aaronson, S. A., Hartley, J. W. & Todaro, G. J. (1969) "Mouse leukemia virus: spontaneous release by mouse embryo cells after long term in vitro cultivation," *Proc. Nat. Acad. Sci. USA* **64**, 87–94.
8. Rowe, W. P., Hartley, J. W., Lander, M. R., Pugh, W. E. & Teich, N. (1971) "Non-infectious AKR mouse embryo cell lines in which each cell has the capacity to be activated to produce infectious murine leukemia virus," *Virology* **46**, 866–876.
9. Lowy, D. R., Rowe, W. P., Teich, N. & Hartley, J. W. (1971) "Murine leukemia virus: high frequency activation in vitro by 5-iodeoxyuridine and 5-bromodeoxyuridine," *Science* **174**, 155–156.
10. Aaronson, S. A., Todaro, G. J. & Scolnick, E. M. (1971) "Induction of murine C-type viruses from clonal lines of virus-free BALB/3T3 cells," *Science* **174**, 157–159.
11. Hirsch, M. S., Black, P. H., Tracy, G. S., Leibowitz, S. & Schwartz, R. S. (1970) "Leukemia virus activation in chronic allogeneic disease," *Proc. Nat. Acad. Sci. USA* **67**, 1914–1917.
12. Armstrong, M. Y. K., Gleichmann, E., Gleichmann, H., Beldotti, L., Andre-Schwartz, J. & Schwartz, R. S. (1970) "Chronic allogeneic disease. II Development of lymphomas," *J. Exp. Med.* **132**, 417–439.
13. Lewis, R. M., Armstrong, M. Y. K., Andre-Schwartz, J., Muftuoglu, A., Beldotti, L. & Schwartz, R. S. (1968) "Chronic allogeneic disease. I Development of glomerulonephritis," *J. Exp. Med.* **128**, 653–679.
14. Phillips, S. M., Martin, W. J., Shaw, A. R. & Wegmann, T. G. (1971) "Serum-mediated immunological non-reactivity between histoincompatible cells in tetraparental mice," *Nature* **234**, 146–148.
15. Adler, W. H., Takiguchi, T., Marsh, B. & Smith, R. T. (1970) "Cellular recognition by mouse lymphocytes *in vitro*," *J. Immunol.* **105**, 984–1000.
16. Bach, F. H. & Voynow, N. K. (1966) "One way stimulation in mixed leukocyte cultures," *Science* **153**, 545–547.
17. Klement, V., Rowe, W. P., Hartley, J. W. & Pugh, W. E. (1969) "Mixed culture cytopathogenicity: a new test for growth of murine leukemia viruses in tissue culture," *Proc. Nat. Acad. Sci. USA* **63**, 753–758.
18. Bassin, R. H., Tuttle, N. & Fischinger, P.J. (1971) "Rapid cell culture assay technique for murine leukaemia viruses," *Nature* **229**, 564–566.
19. Phillips, S. M., Hirsch, M. S., Carpenter, C. B., Gleichmann, H. & Schwartz, R. S., "*In vitro* relationship of mouse lymphocyte reactivity with leukemia virus activation: a mechanism in chronic gvh disease," *Fed. Proc.*, in press.
20. Wilson, D. B., Blyth, J. L. & Nowell, P. C. (1968) "Quantitative studies on the mixed lymphocyte interaction in rats. III. Kinetics of response," *J. Exp. Med.* **128**, 1157–1181.
21. Kasakura, S. & Lowenstein, L. (1965) "A factor stimulating DNA synthesis derived from the medium of leukocyte cultures," *Nature* **208**, 794–795.
22. Granger, G. & Kolb, W. P. (1968) "Lymphocyte *in vitro* cytotoxicity: mechanism of immune and non-immune small lymphocyte mediated target L cell destruction," *J. Immunol.* **101**, 111–120.
23. Armstrong, M. Y. K., Black, F. L. & Richards, F. F., "Tumor induction by cell-free extracts derived from mice with graft versus host disease," *Nature*, in press.
24. Ziff, M. (1971) "Viruses and connective tissue disease," *Ann. Intern. Med.* **75**, 951–958.
25. Cummings, N. A., Schall, G. L., Asofsky, R., Anderson, L. G. & Talal, N. (1971) "Sjogren's syndrome—newer aspects of research, diagnosis, and treatment," *Ann. Intern. Med.* **75**, 937–950.
26. Hirsch, M. S., Black, P. H. & Proffitt, M. R. (1971) "Immunosuppression and oncogenic virus infections," *Fed. Proc.* **30**, 1852–1857.
27. Fialkow, P. J., Thomas, E. D., Bryant, J. I. & Neiman, P. E. (1971) "Leukemic transformation of engrafted human marrow cells *in vivo*," *Lancet*, **i**, 251–255.