Viral Induced Immunity to Syngeneic Rauscher Murine Leukemia Cells, in the Absence of Allogeneic Inhibition (34193)

MAX H. COHEN,[1,2] AND MARY ALEXANDER FINK[1]

Immunology Section, Viral Leukemia and Lymphoma Branch, Etiology, National Cancer Institute, Bethesda, Maryland 20014

It has been reported that mice immunized with formalin-treated murine leukemia viruses are resistant to challenge with the virus-transformed malignant cell (1, 2). Such immunity could be due to the formation of humoral antibody to the virus or virus associated antigens, to the formation of cell associated antibody to these antigens, or to the phenomenon of allogeneic inhibition. The latter, allogeneic inhibition, was suggested by Möeller (3) and by Klein (4) to explain the resistance of a host to its own tumor cells. The present study investigates the role of allogeneic inhibition in specifically immunized mice that are resistant to the transplantation of malignant ascites cells induced by a leukemia virus.

Materials and Methods. Mice. Standard NIH Balb/c mice of both sexes, 8–12 weeks old, were used.

Vaccines and immunizations. Formalin-treated Rauscher virus was prepared from a cell-free extract of spleens of leukemic BALB/c mice, as described elsewhere (5). In addition, live low virulence ("attenuated") virus harvested from the JLSV-5 tissue culture line as previously described (6) was obtained from Charles Pfizer and Co., under contract to NCI. Mice immunized with formalin-treated virus received a primary intraperitoneal injection of 0.25 ml of vaccine which had been either emulsified in an equal volume of complete Freund's adjuvant (Group 1), or mixed with an equal volume of saline (Group 2). Thirty, 40, and 50 days later each animal in Groups 1 and 2 received a subcutaneous booster injection of 0.1 ml of vaccine without adjuvant (5). Other mice received a single intraperitoneal injection of 0.05 ml of live attenuated virus either emulsified in an equal volume of Freund's adjuvant (Group 3), or mixed with an equal volume of saline (Group 4). Noninoculated mice of the same strain served as controls (Group 5).

Ascites cell challenge. Sixty-four days after the beginning of immunization, the immunized mice (Groups 1–4), and normal mice (Group 5), were challenged by interscapular subcutaneous inoculation with malignant ascites cells originally induced by Rauscher virus in a BALB/c mouse and maintained in this laboratory by serial passage of ascites cells every 6–10 days. These cells were in passages 80–100 during the course of these experiments. The standard challenge cell inoculum was 0.1 ml of a 10^{-6} dilution of freshly harvested ascites fluid. Hanks' buffered salt solution containing 2% normal mouse serum (HBSS-NMS) was the diluent used throughout these experiments. The 0.1-ml challenge inoculum consistently contained 30–40 ascites cells, and was the most dilute preparation consistently capable of inducing lethal tumors in at least 50% of normal control animals.

Transfer experiments. Two weeks after the final booster injection (day 64), 2 animals from each group were anesthetized with ether, and exsanguinated through the axillary artery. The bloods were pooled, the spleens were removed, and the spleen cells were teased into suspension in HBSS-NMS. After filtration through glass wool, the splenocytes

[1] Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Cancer Institute, Etiology Area, Bethesda, Maryland 20014.

[2] Present Address: Massachusetts General Hospital, Boston, Massachusetts.

were resuspended to a concentration of 5 × 10^7 viable spleen cells/ml. The ascites cell suspension, diluted 10^{-6} as described above, was mixed in equal volume with either the viable spleen cells or the serum, and incubated at 37° for 30 min.

Normal BALB/c mice were then inoculated subcutaneously in the interscapular region with 0.2 ml of the mixture of malignant cells and either serum or spleen cells.

X-Irradiation. Before incubation with malignant cells, some immune and normal spleen cell suspensions were subjected to 5000 R of x-irradiation, delivered at the NIH Radiation Therapy Unit, by a 250 kV machine operating without a filter at a distance of 25 cm over a 7.5 min exposure time.

Freeze–thaw of spleen cell suspensions. In some cases spleen cells were subjected to 3 cycles of freezing in dry ice, and thawing in a 37° waterbath. Cells were then washed once in buffer, resuspended in the original volume, and incubated with challenge inoculum in the usual manner.

Phytohemagglutinin. In some experiments Difco Phytohemagglutinin P (purified) was used to agglutinate spleen cells to target cells during incubation.

Complement. Complement was obtained as lyophilized guinea pig serum from Baltimore Biological Laboratories, Baltimore, Maryland. After reconstitution, the serum was absorbed three times with equal volumes of washed normal BALB/c mouse liver and spleen cell homogenate. Each absorption of the raw guinea pig serum was performed at 4° for 30 min to remove trace levels of nonspecific toxicity against the mouse tumor cells.

Adsorption of immune serum onto normal spleen cells. In some experiments, a suspension of normal spleen cells, prepared as described above, was incubated with an equal volume of immune serum for 45 min. The spleen cell suspension was then centrifuged at 300 *g* for 4 min, and washed twice in a total of 5 vol of diluent, resuspended to original volume, and incubated with an equal volume of challenge inoculum. A 0.2-ml portion of this mixture was administered subcutaneously to each test animal, in the usual manner.

Adsorption of inactivated immune serum onto target cells. When indicated, the standard challenge cell inoculum was preincubated at room temperature for 45 min with an equal volume of inactivated immune serum. Serum and malignant cells were gently agitated periodically during incubation, and then centrifuged at 500 *g* for 5 min. The serum was then discarded and the target cells were resuspended in 1 ml of diluent and incubated in the usual way with an equal volume of an immune spleen cell suspension. After incubation, 0.2 ml of the mixture was administered subcutaneously to each test animal.

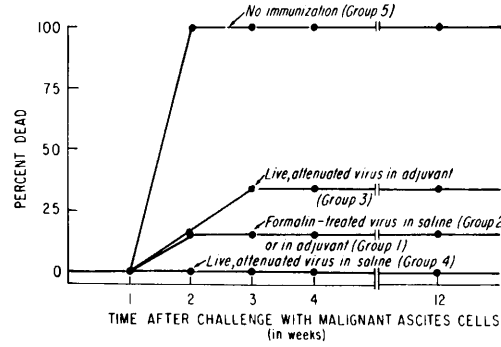

FIG. 1. Percentage of Balb/c mice immunized with formalin-treated or attenuated leukemia virus, and dying after challenge with virus-transformed malignant ascites cells.

Results. Considering all animals immunized with attenuated virus or with formalin treated vaccine, the death rate 3 months after challenge with syngeneic malignant cells was 17% (4/24) as compared to nonimmunized mice which were all dead within 2 weeks after challenge (Fig. 1). The death rate of mice immunized with formalin-treated vaccine in adjuvant (Group 1) was 1/6. In Group 2 (formalin-treated vaccine in saline) the death rate was also 1/6. In Group 3 (live, attenuated virus in adjuvant) it was 2/6, and Group 4 (live, attenuated virus in saline) it was 0/6. The death rate was 5/5 in nonimmunized controls (Group 5). All animals dying in these groups, and in the transfer studies described below, at autopsy showed a metastasizing lymphosarcoma, originating at the inoculation site.

Other immunized mice, instead of being challenged on day 64, were sacrificed to allow

TABLE I. Ability of Serum and Spleen Cells from Immune Mice to Prevent the Transplantability of Malignant Ascites Cells after *in Vitro* Incubation.

Method of immunization of serum and cells donor	No. dead/no. challenged of normal mice receiving ascites cells and:	
	Serum	Spleen cells
Formalinized vaccine in		
Adjuvant (Group 1)	2/7	4/7
Saline (Group 2)	3/5	7/7
Live, attenuated virus in		
Adjuvant (Group 3)	2/7	5/5
Saline (Group 4)	3/7	1/6
None (Group 5)	6/7	7/7

separate evaluation of their serums and spleen cells. The results presented in Table I show that the serums and spleen cells were most effective in the transfer tests when taken from mice immunized with killed vaccine in adjuvant or from mice immunized with live virus in saline. The complement dependence of the serum reaction was indicated by the experiments reported in Table II, using serum from mice immunized with live virus. Other immune spleen cell suspensions, which were obtained from mice immunized with live virus in saline, were subjected to 5000 R of X-irradiation. The data in Table III show that this irradiation eliminated the cytotoxic effect of the immune spleen cells. These data also show that 3 cycles of freezing and thawing eliminate the cytotoxicity of the spleen cells against the malignant ascites cells.

The results presented in Table IV show that immune serum adsorbed onto the surface of normal spleen cells was not able to confer immunological capability upon these spleen cells, as indicated by their subsequent inability to influence the growth potential of the malignant target cells. The data there also indicate that the immune serum did not inhibit the immune spleen cell—malignant cell interaction.

TABLE III. Ability of X-Irradiation[a] or Freezing and Thawing[b] to Eliminate Cytotoxicity of Immune Spleen Cells against Malignant Target Cells.

Spleen cell donors	Treatment of spleen cells before incubation with malignant cells	No. dead/no. challenged
Normal	None	7/7
Immune	None	0/7
Normal	5000 R	5/7
Immune	5000 R	5/7
Immune	Freeze–thaw (3 cycles)	5/7

[a] Dose: 5000 R.

[b] Freeze (—70°) and thaw (37°) three times.

In other experiments, phytohemagglutinin (PHA) was used during the incubation period to agglutinate normal nonimmune cells to malignant cells, with the result that PHA was either without effect on the tumor cell–normal cell mixture, or was nonspecifically toxic to the tumor cell.

Discussion. It has recently been demonstrated that altered murine leukemia virus can be used as an immunizing agent in the

TABLE II. Complement Dependence of the Reaction between Immune Serum[a] and Malignant Cells.

Serum donor	Treatment of serum prior to its incubation with malignant cell challenge	No. dead/no. challenged
Normal	None	6/7
Immune	None	1/7
	56° for 45 min (''inactivated'')	5/7
	''Inactivated'' with added complement[b]	1/7
Normal	''Inactivated'' with added complement[b]	5/7

[a] Immunized with live, attenuated virus in saline (see text).

[b] Supplied as lyophilized guinea pig serum from Baltimore Biological Laboratories, and diluted 1:6 by test serum.

TABLE IV. Inability of Immune Serum to Confer Immunological Capability on Normal Spleen Cells, or to Competitively Inhibit the Action of Immune Spleen Cells.

First incubation		Second incubation	No. dead/no. challenged
Normal spleen cell suspension and immune serum (equal volume)	Wash →	Normal spleen cells ("coated with immune serum") and challenge cell inoculum	7/7
Challenge cell inoculum and inactivated immune serum	Wash →	Challenge cell inoculum ("coated with inactivated immune serum") and immune spleen cells	0/7

host of origin (1, 5, 6). These observations argue against the hypothesis that specific tolerance to the virus (if such tolerance exists) prevents active immunization against viral challenge. Indeed, the presence of tolerance in other murine virus systems (e.g., lymphocytic choriomeningitis) has also been recently questioned (7). The present studies indicate that mice specifically immunized against Rauscher leukemia virus have antibody and immunocytes capable of causing lethal damage to the virus transformed malignant cell.

Hellström (8) while studying the growth of parental tumors in F_1 hybrid recipients, confirmed a previous observation (9) that tumor growth was sometimes markedly inhibited in F_1 hybrid hosts. He suggested the term "allogeneic inhibition" to refer to the nonimmunological restriction of tumor growth due to contact with foreign histocompatibility antigens. Nonimmune allogeneic cells which were aggregated to target cells *in vitro* by phytohemagglutinin or by heterologous serum, similarly inhibited tumor cells. Since the inhibiting cells were from unimmunized animals and often of nonlymphoid origin, the mechanism was presumably nonimmunologic. These observations led Möeller (3) and Klein (4) to the hypothesis that there may be a similar nonimmunological component in the resistance of a host to syngeneic or autochthonous tumor cells. According to this proposal, tumor specific "new cell antigens" would create a mild histoincompatibility between neoplastic cells and the host's normal cells, with the subsequent destruction of the neoplastic cells by allogeneic inhibition.

Wilson and Billingham raised theoretical objections to the phenomenon of allogeneic inhibition (10). These objections were based on observations made in nonsyngeneic systems, before allogeneic inhibition was described. In testing the resistance of mice to the transplantation of a virus-induced malignant ascites cell, the present report presents several lines of evidence to indicate that allogeneic inhibition is also of little importance in syngeneic and autochthonous systems. Irradiation of immune spleen cells eliminated their cytotoxic effect in our experiments, although lethal irradiation does not abrogate cytotoxicity mediated through allogeneic inhibition (11). In addition, disruption of splenocytes by freezing and thawing eliminated the cytotoxic cell-to-cell interaction in our studies, in contrast to the observation that physical disruption of allogeneic cells does not eliminate their cytotoxic potential (12) when mediated through allogeneic inhibition. Phytohemagglutinin was used to agglutinate normal nonimmune cells to antigenically distinct malignant cells, a procedure which has resulted in target cell death in systems demonstrating allogeneic inhibition (3, 13, 11). However, specific killing of target cells did not occur in our system. The PHA was either without effect on the tumor cell–normal cell mixture, or it was nonspecifically toxic to the tumor cell. This provided a further indication that simple cell-to-cell adherence is not sufficient to cause death of virus-induced target cells in this syngeneic system.

Finally, we attempted to test directly the hypothesis (3) that allogeneic inhibition may be the basis for the ordinary *immune* elimination of neoplastic cells in the autochthonous host. According to this theory, anti-

bodies on the surface of an immune lymphoid cell may recognize the neoplastic cell and destroy it by holding it in apposition to an antigenically normal (lymphoid) cell surface. However, immune serum adsorbed onto normal spleen cells was not able to confer immunological capability upon these spleen cells.

Summary. BALB/c mice were immunized with Rauscher murine leukemia virus. Both formalin-treated virulent virus, and a live, attenuated strain of the virus were used as antigens. Immune serums and immune spleen cells harvested 64 days later caused lethal damage to the virus transformed malignant cell. The effectiveness of immune serum was removed by heat inactivation and was restored by the addition of guinea pig complement. Either X-irradiation or freezing and thawing eliminated the competence of the immunocytes. Normal spleen cells agglutinated to target cells by phytohemagglutinin were not capable of causing lethal damage to the malignant cells. Although allogeneic inhibition has been suggested as the possible mechanism underlying tumor immunity and surveillance against neoplasia in syngeneic and autochthonous systems, it does not appear to play a significant role in the immunity to transplantation of malignant cells that follows immunization with killed or attenuated murine leukemia virus.

1. Friend, C., J. Exptl. Med. **109**, 217 (1959).

2. Fink, M. A., *in* Viruses Inducing Cancer (W. Burdette, ed.), p. 47. Univ. of Utah Press, Salt Lake City, Utah (1966).

3. Möeller, E. and Möeller, G., Cancer **20**, 871 (1967).

4. Klein, G., *in* Viruses Inducing Cancer (W. Burdette, ed.), p. 345. Univ. of Utah Press, Salt Lake City, Utah (1966).

5. Fink, M. A. and Rauscher, F. J., J. Natl. Cancer Inst. **32**, 1075 (1964).

6. Wright, B. S. and Lesfargues, J. C., Natl. Cancer Inst. Monograph **22**, 685 (1966).

7. Oldstone, M. B. A. and Dixon, F. J., J. Exptl. Med. **129**, 483 (1969).

8. Hellström, K. E., Nature **199**, 614 (1963).

9. Snell, G. D., J. Natl. Cancer Inst. **21**, 843 (1958).

10. Wilson, D. B. and Billingham, R. E., Advan. Immunol. **7**, 189 (1968).

11. Möeller, G. and Möeller, E., Ann. Med. Exptl. Biol. Fenniae (Helsinki) **44**, 181 (1966).

12. Hellström, K. E. and Hellström, I., Federation Proc. **27**, 39 (1968).

Received May 26, 1969. P.S.E.B.M., 1969, Vol. 132.