Prevention of 334C Murine Virus-induced Leukemia by Transmission of Maternal Immunity to Offspring[1]

Rita F. Buffett

Department of Medical Viral Oncology, Roswell Park Memorial Institute, Buffalo, New York 14203, and Graduate Faculty in Microbiology, State University of New York at Buffalo (Roswell Park Division), Buffalo, New York 14214

SUMMARY

Mice were protected against development of virus-induced leukemia late in life when they were suckled on female mice immunized as adults with 334C murine leukemia virus, a member of the Friend-Moloney-Rauscher subgroup of murine leukemia viruses. Young adult, random-bred Ha/ICR Swiss females were immunized with one to three injections of virus filtrate from organs of leukemic mice at weekly intervals and were mated during or after immunization. Offspring were challenged at birth by injection with 334C virus and then suckled on immunized females until weaned. The incidence of leukemia was reduced to an average of 10% in offspring from immunized females, compared to an average of 72% in offspring from nonimmunized females. The capability of virus-immunized females to protect their young extended over a period of 5 to 6 months.

Neonatal mice also were protected against development of leukemia when they were suckled on virus-immunized females either before or after infection by vertically transmitted 334C virus in reciprocal foster nursing experiments. Offspring were suckled on virus-immunized mothers for 2, 8, and 14 days before being transferred to virus-infected females. Leukemia developed in 36, 15, and 14% of offspring, as compared with 71, 38, and 7% in control litters (offspring from nonimmunized mothers suckled on virus-infected females). When offspring were suckled on virus-infected mothers for 2, 8, and 14 days before being transferred for suckling to virus-immunized females, leukemia developed in 6, 33, and 81% as compared with 83, 68, and 72% in control litters (virus-infected offspring suckled on normal females).

The results of these experiments define a critical period, early in life, during which the course of virus infection can be altered and the incidence of leukemia in adult life greatly influenced. Thus, this murine system provides a model for exploration of the application of combined immunotherapy and antiviral chemotherapy to the prevention of virus-induced leukemia.

INTRODUCTION

Transmission of maternal immunity resulting in passive protection of offspring against the induction of leukemias and tumors later in life, following infection at birth with oncogenic viruses, has been reported in several animal systems (8-11, 14-16) including the 334C murine leukemia virus system in our laboratory (2, 6).

In the 334C virus system, as in other murine leukemia virus systems, the susceptibility of mice to leukemia development following infection with exogenous 334C virus is age dependent (2, 5). Very young mice are highly susceptible; 80 to 90% of mice given injections at birth develop leukemia in 6 to 8 months. However, adults are highly resistant; 10 to 20% of those given injections develop leukemia by 8 to 12 months of age.

The age at which mice are exposed to this virus also determines whether females are capable of transmitting virus or immunity to their offspring via the milk. Female mice, given injections of 334C virus CFF[2] at birth, transmit virus during lactation to their offspring, producing incidences of leukemia comparable to those in mice that have received injections of virus at birth (3). Vertical transmission of virus then continues through many generations (4). However, when female mice are given injections, or *immunized*, with 334C virus CFF as young adults, their offspring are protected, via the milk, against virus challenge by either injection or vertical transmission.

Using this murine system in which *either* virus or immunity can be transmitted via the milk, would it be possible (by manipulating events during this critical period early in life) to prevent leukemia development after infection of neonates had been accomplished? Reciprocal foster nursing experiments reported here show that it is indeed possible and that our murine system thus provides a promising model for exploration of immunotherapy and antiviral chemotherapy for the *in vivo* inactivation of virus and prevention of leukemia development.

MATERIALS AND METHODS

Animals. Random-bred Ha/ICR Swiss mice, obtained from the Roswell Park Memorial Institute breeding colony,

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[2] The abbreviations used are: CFF, cell-free filtrate(s); NKLS, normal kidney-liver-spleen; ID_{50}, dose of challenge virus producing death with leukemia in 50% of mice given injections of 10% 334C leukemia virus CFF.

were used. Litters produced in our laboratory were weaned at approximately 4 weeks of age, caged separately by sex, and maintained on Teklad mouse chow (Rockland Laboratory, Monmouth, Ill.) and tap water *ad libitum*. Intercurrent infections were treated with Achromycin (Lederle Laboratories, Pearl River, N. Y.), 50 mg/500 ml of drinking water, for 1 to 2 weeks.

Virus Filtrates. 334C murine leukemia virus, isolated in our laboratory in 1960 (5), is serotypically identified in the Friend-Moloney-Rauscher subgroup of murine leukemia viruses (7). It is routinely recovered from leukemic mice that have received, at birth, s.c. injections of 0.1 ml of a 10% (w/v) CFF from leukemic tissues (spleen, liver, kidney, lymph nodes, and thymus).

For the experiments reported here, tissues were obtained from groups of leukemic mice, pooled, and homogenized without diluent. Homogenates were stored in small aliquots at $-80°$ for 1 to 10 weeks; when thawed, sterile phosphate-buffered NaCl solution was added to make a final 10% suspension of homogenate. The homogenates were centrifuged at $2000 \times g$ for 1 hr at $4°$, and the supernatant fluids were passed through 0.45-μm Nalge grid membranes (Sybron Corporation, Rochester, N. Y.).

Normal Tissue Filtrates. Kidney, liver, and spleen were collected from groups of normal mice at ages comparable to those of leukemic mice, pooled, and processed, following the protocol for preparation of virus filtrates.

Immunization. Young adult females (± 6 weeks of age) were immunized with either 334C leukemia virus or NKLS CFF. Animals were given i.p. injections 1, 2, or 3 times, at weekly intervals, of 0.5 ml of 10% CFF containing virus doses of approximately 500 ID_{50}/ml. Females that were immunized more than once received freshly prepared CFF from stored aliquots of the same homogenate pool. Litters of newborn mice from nonimmunized females served as controls for infectivity (scored as leukemia incidence) of each CFF used to immunize adults. Over a period of 10 months, the incidences of leukemia in virus-treated control mice ranged from 73 to 83%; no leukemias occurred in litters given injections of NKLS CFF.

Virus Challenge. Litters from immunized females were challenged, by s.c. injection (0.1 ml), with virus doses of approximately 250 to 300 ID_{50}/ml in 10% CFF's from 3 different homogenate pools, over a period of 4 months. Quotas of 5 litters of 10 mice each per group were set. Newborn to 2-day-old babies were randomized prior to injection. Litters of comparable ages from nonimmunized females were given injections of each CFF used for virus challenge. A number of litters from immunized females that delivered before and after the quotas were filled remained as unchallenged controls; these litters were not randomized. Immunized mothers were discarded when litters were weaned. In reciprocal foster nursing experiments, litters from virus-immunized mothers were challenged with virus transmitted vertically via the milk from virus-infected females.

Reciprocal Foster Nursing. Litters from virus-infected and virus-immunized females (mated to normal males) were exchanged for foster nursing. Times of birth over a period of

72 hr were obtained by hourly inspections. Each virus-infected and virus-immunized female was kept with her foster daughters for the duration of the experiment. The parentage of each virus-infected and virus-immunized mother was recorded. To assure vertical transmission of virus, only those litters from mothers and/or grandmothers that developed leukemia were included.

RESULTS

Single *versus* Multiple Injections of Virus for Immunization of Adult Mice. Offspring from immunized adult females that had received 3 weekly injections of 334C virus CFF were completely protected against challenge with virus at birth (Table 1). Protection of offspring from females that had received only 1 or 2 injections was highly successful, also. Leukemias occurred in 1 unchallenged litter from a female that had been given 2 immunization injections; 5 of her 7 offspring developed leukemia between 194 and 290 days of age.

Duration of Immunity in Immunized Adults. The pooled data from experimental groups of offspring born to females over a period of 15 weeks following immunization are illustrated in Chart 1. Highly effective protection against development of leukemia occurred when offspring that were challenged with 334C virus in the neonatal period were suckled by 334C virus-immunized females. The capability of virus-immunized females to protect their litters was not altered by progressively longer intervals between immunization and mating (leukemia incidences varied randomly from 0 to 38%), nor was the degree of protection related to the dose of challenge virus. As noted in Table 1, leukemia developed in a small percentage of unchallenged offspring from virus-immunized mothers.

When the development of leukemia in *individual litters* (rather than in the total number of offspring) is considered (Table 2), about two-thirds of the passively immunized litters were *completely* protected. In the remaining litters, the incidence of leukemia was much lower than in individual NKLS-immunized and nonimmunized control litters challenged with virus. Unchallenged immunized litters also had a low incidence of leukemia.

Effects of Reciprocal Foster Nursing on the Incidence of Leukemia in Litters from Virus-infected and Virus-immunized Females. This study was based on the following observations: (*a*) mice given injections of 334C virus early in neonatal life transmit virus in high leukemia-producing titer (via the milk) to their offspring (3, 4); (*b*) susceptibility to leukemia development following injection with virus is age dependent (2, 5); and (*c*) the majority of mice immunized with this virus in adult life are capable of passively protecting their offspring (via the milk) against development of leukemia following neonatal injection of virus.

In order to establish the length of time essential for "maximum" infection via the milk, as measured by incidence of leukemia later in life, virus-infected mothers were allowed to deliver and suckle their offspring for intervals of 6 hr to 14 days after birth before their babies were

Table 1

Passive protection of offspring from 334C virus-immunized mothers against development of leukemia; incidence and latency of leukemia deaths in litters challenged with 334C virus

Young adult females (± 6 weeks old) were immunized 1, 2, or 3 times, at weekly intervals; 1 week after the 3rd immunization, all were mated to normal males. Babies were challenged at birth to 2 days of age.

	Dose of challenge virus											
	250 ID_{50}/ml				25 ID_{50}/ml				Unchallenged controls			
			Latency (days)				Latency (days)				Latency (days)	
Experimental groups	Leukemia incidence	%	Av.	Range	Leukemia incidence	%	Av.	Range	Leukemia incidence	%	Av.	Range
Immunized												
1 time	3/58[a]	5	255	221–280	0/50	0			0/49	0		
2 times	2/69	3	288	202–253	0/36	0			5/40	12	255	194–290
3 times	0/51	0			0/31	0			0/53	0		
Nonimmunized	190/255	75	194	85–286	105/168	62	211	111–282				

[a] Number of mice with leukemia/total number of mice observed.

transferred to normal foster mothers for the remainder of the suckling period. Suckling for as short a time as 6 hr produced leukemia in 48% of the offspring, and maximal infection resulting in 82% incidence of leukemia was achieved by suckling for 12 hr. The reciprocal procedure was carried out to determine age susceptibility to virus infection via the milk. Litters from normal females were transferred to virus-infected foster mothers at ages ranging from 12 hr (the average age of litters treated by *injection* with virus CFF) to 14 days after birth. Normal 12- to 48-hr old babies were highly susceptible to infection by virus transmitted via the milk; leukemia occurred in 70% of the offspring. Resistance to leukemia development was already evident at 8 days of age (38% incidence of leukemia), and by 14 days of age the incidence of disease was reduced further (7%) when normal mice were foster nursed on virus-infected females. These findings are comparable to those following injection of virus at these ages (2).

To determine whether passively transferred immunity could prevent the development of leukemia in mice previously infected by sustained delivery of virus via the milk, reciprocal foster nursing experiments were done with litters from virus-infected and virus-immunized females. Litters were exchanged after they had nursed on their own mothers for 2, 8, and 14 days (Table 3).

A marked reduction in the incidence of leukemia was obtained when babies were transferred to virus-immunized females after suckling on their virus-infected mothers for 2 days. Leukemias occurred in 2 of 6 litters (1 each); the other 4 litters remained leukemia free. Most surprisingly, leukemia incidence in mice receiving virus continuously for 8 days before transfer to virus-immunized females was also markedly reduced. In this group, leukemia occurred in 3 of 4 litters (1 of 3 in 2 litters, 2 of 4 in the 3rd); 1 litter remained leukemia free. By 14 days of age, transfer from virus-infected to virus-immunized mothers had no protective effect.

The incidence of leukemia was greatly reduced, also, in litters that were allowed to suckle on their virus-immunized mothers for 8 days before being exposed to infection
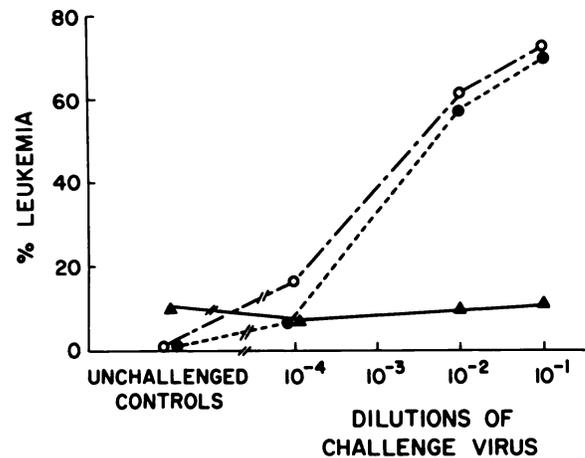

Chart 1. Incidence of leukemia in offspring suckled by 334C virus-immunized (▲), NKLS-immunized (●), and normal (○) mice. Adult females were immunized with 3 weekly injections of 334C virus or NKLS CFF and subsequently mated at weekly or biweekly intervals to normal males over a period of 15 weeks. Neonates were given s.c. injections, at birth to 2 days of age, of challenge virus from 3 different homogenate pools. ID_{50} virus doses at dilutions of challenge virus were: 250 to 300/ml at 10^{-1}, 25 to 30/ml at 10^{-2}, and 0.25 to 0.30/ml at 10^{-4}. Numbers of mice observed in each group were: ▲, 10^{-1} = 383, 10^{-2} = 483, 10^{-4} = 609, unchallenged = 541; ●, 10^{-1} = 101, 10^{-2} = 96, 10^{-4} = 112, unchallenged = 140; ○, 10^{-1} = 580, 10^{-2} = 472, 10^{-4} = 506, unchallenged = 203.

following transfer to virus-infected females (approximately one-half that in normal babies exposed to virus transmitted via the milk from virus-infected females when 8 days of age). A more surprising finding was a comparable reduction in the incidence of leukemia in litters that had suckled on their immunized mothers for as short a time as 2 days before being subjected to sustained delivery of virus via the milk for most of the suckling period.

DISCUSSION

Protection against development of leukemia late in life, following neonatal infection with 334C murine leukemia

virus, was transmitted to offspring from virus-immunized females via the lactation fluid. The protection of the young was marked, although not complete, and extended over a period of 5 to 6 months following immunization of females destined to become mothers. These data confirm and extend observations made by others (8-11, 14-16); they define a critical period early in neonatal life when virus infection-inhibition may influence the incidence of disease in adult life.

We do not yet know the identity of the protective factor, but we presume it to be antibody. The extended duration of immunity in the immunized females is consistent with this assumption. In addition, when 1st-generation offspring from immunized females were mated, they did not transmit protection against challenge with virus at birth to their offspring (R. F. Buffett, unpublished data).

Table 2

Percentage of litters from virus-immunized females that were not completely protected against neonatal challenge with 334C virus; comparison with litters from NKLS-immunized and nonimmunized females

The degree of partial protection of litters may be assessed on the basis of incidences of leukemia in individual litters. Data are pooled from groups that received 250- to 300- and 25- to 30-ID_{50}/ml doses of challenge virus (see Chart 1).

Litters from mothers	334C virus challenge				No challenge			
	Litters with leukemia (%)	No. of individual litters with incidence of leukemia of			Litters with leukemia (%)	No. of individual litters with incidence of leukemia of		
		≤25%	≤50%	>50%		≤25%	≤50%	>50%
334C-immunized	32 (34/107)[a]	22	5	7	31 (22/70)	12	2	8
NKLS-immunized	96 (24/25)		5	19[b]	11 (2/19)	2		
Nonimmunized	97 (145/150)	12	24	109[b]				

[a] Numbers in parentheses, number of litters with leukemia/total number of litters observed.

[b] The percentage of litters with >50% incidence of leukemia in individual litters was higher in those challenged with the higher doses of virus than in those challenged with the lower doses; *i.e.*, 12 of 13 (92%) of NKLS-immunized litters and 73 of 87 (84%) of nonimmunized litters challenged with 250 to 300 ID_{50} doses as compared with 7 of 11 (64%) of NKLS-immunized litters and 41 of 63 (65%) of nonimmunized litters challenged with 25 to 30 ID_{50} doses. Conversely, the percentage of litters with <50% incidence in inidivual litters was higher in those challenged with the lower doses of virus than in those challenged with the higher doses; *i.e.*, 4 of 11 (36%) of NKLS-immunized litters and 21 of 60 (35%) of nonimmunized litters challenged with 25 to 30 ID_{50} doses as compared with 1 of 13 (8%) of NKLS-immunized litters and 15 of 85 (18%) of nonimmunized litters challenged with 250 to 300 ID_{50} doses. However, this was not the case with litters from virus-immunized mothers, in which percentages of leukemias in litters challenged with both doses of virus were identical.

Table 3

Effect of foster nursing on the induction of leukemia by vertically transmitted 334C murine leukemia virus; reciprocal foster nursing by virus-immunized and virus-infected females

Virus-infected females (grandmothers), obtained from litters that had been given injections of virus (±400 ID_{50} doses/ml in 10% CFF) at birth, were mated to normal males to produce the 1st generation of vertically virus-infected mothers of 2nd generation vertically virus-infected litters for this experiment. Young adult females (mothers), obtained from litters of previously virus-immunized females (grandmothers), were immunized and mated to normal males to produce the virus-immunized litters for this experiment. All immunized females received 3 immunization injections of freshly prepared virus CFF (250- to 300-ID_{50} doses/ml), starting at ±6 weeks of age, at weekly intervals, and were mated 1 week later.

Litters born to	Nursed by mother for (days)	Transferred to foster mother	Incidence of leukemia (%)	Latency (days)	
				Av.	Range
Infected females[a]	2	Immunized females[b]	6 (2/32)[c]	149	100-198
	8		33 (4/12)	224	176-287
	14 ± 35		81 (29/36)	199	76-297
			79 (66/84)	183	68-231
Immunized females[b]	2	Infected females[a]	36 (8/22)	266	216-301
	8		15 (2/13)	277	254-299
	14 ± 35		14 (4/28)	235	200-296
			0 (0/61)		

[a] Of a total of 17 virus-infected mothers, 14 developed leukemia; 15 of 17 of their mothers (grandmothers) also developed leukemia, including the mothers of the 3 which remained leukemia free.

[b] Of a total of 17 virus-immunized mothers, only 2 developed leukemia. The litters from these mothers, reciprocally foster nursed by virus-infected females, one after 8 days and the other after 14 days, remained free of leukemia. Leukemias occurrred in 1 of 3 and 3 of 5 of the offspring in the litters from virus-infected mothers, reciprocally foster nursed by these immunized females. Of the 12 grandmothers, 2 developed leukemia; 1 was the mother of one of the leukemic mothers above (8-day group, 1 of 3 leukemic offspring); the daughter of the other remained leukemia free and had only 1 of 6 leukemic offspring (2-day group). None of the grandmothers or mothers of control litters developed leukemia.

[c] Numbers in parentheses, number of mice with leukemia/total number of mice observed.

The possible role of *prenatal* transfer of immunity in the protection of offspring in these experiments is not known at this time. Although small amounts of maternal antibody have been detected in the sera of unsuckled newborn mice, most of the immunity transferred to neonates is derived from colostrum and milk during postnatal suckling (1). Ioachim (10) has reported prenatal transfer of protection against induction of leukemia by Gross virus in rats; this was augmented by postnatal transfer of immunity during suckling. Experiments are under way to explore the question of prenatal *versus* postnatal transfer of immunity in our murine system by identification of the classes of immunoglobulins (*e.g.*, IgA and IgM) in milk and sera from immunized females and their pre- and postnatal offspring. Similarly, the possible role of passive transfer of cytotoxic antibody in our experiments is being determined.

The dramatic reduction in leukemia incidence in neonates that received passively transferred maternal immunity *after* virus infection, at a time when infection was maximal, implies the highly efficient capability of antibody in the milk to alter the course of infection early in life and of pathological events later in life. Neonatal mice absorb antibody from the gut for about 16 days, when absorption ceases abruptly (1). The reduction in the incidence of leukemia in mice receiving virus for 8 days, before being exposed to maternally transmitted antibody for an equivalent period, suggests new ways to evaluate antiviral agents as adjunctive therapy directed against virus that may escape the immunotherapy blockade.

For reasons not yet determined, immunization was unsuccessful in a small percentage of animals. A low incidence of leukemia (approximately 10%) occurred in virus-challenged and unchallenged offspring suckled on virus-immunized mothers. Presumably, vertical transmission of virus occurred in 31% of unchallenged litters, but the leukemia-inducing potential (possibly because of low concentration of virus) was considerably less than in litters receiving vertically transmitted virus from virus-infected mothers. One would expect complete (or nearly complete) protection of virus-challenged litters that received antibody in the lactation fluid from immunized mothers. We did observe complete protection; 68% of virus-challenged litters remained leukemia free. In the remaining 32% of virus-challenged litters, where offspring may have received vertically transmitted virus *in addition to* injected virus, a high incidence of leukemia would be expected. However, we *did not* observe this; the incidence of leukemia was less than 50% in the majority of individual litters and was not virus-dose related.

The active immune response of the offspring may play a role, since limited immunological response in mice exposed to virus during the early postnatal period is possible (12, 13). In the majority of instances, passive immunity may be effective enough to eliminate virus infection completely or nearly completely, thereby preventing the development of a state of tolerance to the virus. Any persisting virus may then be eliminated as the immunocompetence of the suckling animal develops. However, in some instances the neonates may become and remain immunologically crippled (or tolerant) and therefore susceptible to leukemia development later in life. Also, the degree of immunocompetence in the immunized adult may determine whether or not virus may be vertically transmitted.

ACKNOWLEDGMENTS

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