JOURNAL OF VIROLOGY, Feb. 1987, p. 383–387
0022-538X/87/020383-05$02.00/0

Vol. 61, No. 2

Abrogation of Resistance to Severe Mousepox in C57BL/6 Mice Infected with LP-BM5 Murine Leukemia Viruses

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Received 6 August 1986/Accepted 27 October 1986

Strain C57BL/6 (B6) mice infected with LP-BM5 murine leukemia virus (MuLV) develop a disease which combines abnormal lymphoproliferation with profound immunosuppression and has many features in common with human acquired immunodeficiency syndrome induced by HTLV-III/LAV retroviruses. To determine whether this LP-BM5 MuLV infection would affect the innate resistance of B6 mice to a naturally occurring, highly virulent murine pathogen, mice were exposed to ectromelia virus at various times after treatment with LP-BM5 viruses. At week 4 after infection with LP-BM5, mice challenged with ectromelia virus were unable to generate a humoral immune response to this virus, and between weeks 8 and 10 after infection, challenged mice lost the ability to generate an ectromelia virus-specific cytotoxic-T-cell response. Loss of the cellular immune responses to ectromelia virus was associated with an increased susceptibility to the lethal effects of the virus.

Retroviruses are widely distributed among the vertebrate species and have been studied intensively for insights into neoplasia; however, not all retroviruses are oncogenic for the host. For example, in certain strains of mice, murine leukemia viruses (MuLV) cause a number of nonneoplastic disorders such as spongiform encephalomyelopathy (8, 10), the early graying of coats (17), and deformed whiskers (23).

Recently a nonneoplastic, progressive, lymphoproliferative disease associated with polyclonal-B-cell activation, hypergammaglobulinemia, and profound immunosuppression (18, 21) has been observed in adult C57BL/6 (B6) mice infected with MuLV initially isolated by Laterjet and Duplan (15). A continuous cell line obtained from mice with this disease (9) produces a mixture of ecotropic and mink cell focus-inducing (MCF) viruses, termed LP-BM5 MuLV (18). The disease-inducing component in this mixture appears to be MCF virus that is totally dependent on the nonpathogenic helper ecotropic MuLV for spread within the host (R. A. Yetter, unpublished observations).

Although the syndrome induced by LP-BM5 MuLV has many histologic and immunologic features in common with human acquired immunodeficiency syndrome (AIDS), B6 mice infected with LP-BM5 MuLV appear to die with respiratory failure secondary to mediastinal lymph node enlargement, whereas AIDS patients succumb to a variety of opportunistic infections. Since laboratory mice live in a highly regulated environment, it was reasoned that the differences between the terminal stages of LP-BM5 virus-induced disease and AIDS might reflect a lack of exposure of LP-BM5-infected mice to normal murine pathogens. To test this hypothesis, adult B6 mice were infected with LP-BM5 MuLV and then challenged with ectromelia virus, an agent that causes an inapparent infection and no mortality when administered to immunocompetent B6 mice by footpad inoculation (19, 25).

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MATERIALS AND METHODS

Mice. C57BL/6J mice of both sexes were used at 4 to 6 weeks of age. Mice were purchased from the Jackson Laboratory, Bar Harbor, Maine.

Cell culture. BS-C-1 cells (a continuous African green monkey kidney cell line) were used exclusively for production of ectromelia virus stocks and for determination of virus infectivity (6).

MuLV LP-BM5. SC-1 cells were cocultivated with mitomycin C-treated lymph node cells from a B6 mouse infected with LP-BM5 MuLV containing a mixture of ecotropic and MCF MuLV. Twenty-four-hour culture supernatants of these cells contained 6.3×10^4 PFU of ecotropic virus per ml and 1.3×10^4 focus-forming units of MCF virus per ml. Throughout this communication, the designations LP-BM5 MuLV and LP-BM5 MuLV mixture refer to a combination of the ecotropic and MCF viruses.

Ecotropic virus was biologically cloned by two limiting dilutions in SC-1 cells. The cloned-virus preparation was shown to contain 5.0×10^3 PFU of ecotropic virus per ml (24) and to be free of MCF virus by both focus-forming and fluorescent antibody assays in mink lung cells (12).

Ectromelia virus strain 79. Ectromelia virus strain 79 was isolated during an outbreak of mousepox at the National Institutes of Health in 1979 and 1980 (1) and biologically cloned as described previously (6). Sucrose gradient-purified virus (11), which was divided into portions and stored at −70°C until use, was used in all experiments. All manipulations involving live virus were carried out in a class II hood at biosafety level 3.

Ectromelia virus plaque assay. Ectromelia virus infectivity was estimated by inoculations of BS-C-1 confluent monolayers (60-mm diameter) with 0.5 ml of serial 10-fold dilutions of virus in phosphate-buffered saline (without Ca^{2+} or Mg^{2+}) plus 0.1% bovine serum albumin (6).

Mouse inoculation. On day 1 in the experimental protocol, C57BL/6J male or female mice were inoculated by the

intraperitoneal route with 0.1 ml of either biologically cloned LP-BM5 ecotropic MuLV or the mixed virus preparation. These inocula resulted in comparable levels (10^4 PFU/10^7 cells) of ecotropic virus production in spleens at 3 weeks after infection as determined by the infectious-center assay. At various times after retrovirus injection, mice were challenged by footpad inoculation with a 0.05-ml suspension containing approximately 10^4 PFU of ectromelia virus, unless otherwise indicated.

Serology. Ectromelia virus neutralization assays were carried out as described previously (6). The highest dilution of plasma that yielded a 50% reduction in the number of indicator virus plaques was chosen as the assay endpoint. The neutralization titer was expressed as a reciprocal of this dilution.

Assays for CTL. Primary in vitro ectromelia virus-specific cytotoxic-T-lymphocyte (CTL) responses were measured in a ^{51}Cr-release assay. Target cells were prepared by labeling cultured EL-4 tumor cells with ^{51}Cr and then infecting them with either ectromelia virus or an antigenically cross-reacting vaccinia virus strain WR (20 PFU per cell, 5 to 6 h, 37°C). Single-cell suspensions (0.1 ml) of effector cells prepared from spleens were added in triplicate to infected or uninfected target cells (10^5 cells per 0.1 ml) at appropriate effector-to-target ratios (see below). After 4 h at 37°C, 0.1 ml of the culture supernatant was removed to measure the release of ^{51}Cr. Spontaneous release of ^{51}Cr was determined for target cells incubated with medium alone, and maximum ^{51}Cr release was determined by lysis of targets with distilled water. Data are presented as percent specific release, defined as 100 × (experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm).

RESULTS

Ectromelia virus-induced mortality in B6 mice infected with LP-BM5 MuLV. Ectromelia virus is a naturally-occurring murine pathogen that is probably transmitted in the wild through skin abrasions (7). In the laboratory, footpad inoculation closely approximates this route of infection. Strains of inbred mice vary considerably in their response to ectromelia virus challenge by this route (25). Some strains, such as A/J, rapidly succumb to a systemic infection associated with severe hepatic and splenic necrosis and high levels of virus (>10^7 PFU/g) in both types of tissue. Other strains, such as B6, experience no mortality and little if any morbidity, and they replicate virus at significantly lower levels (<10^5 PFU/g) in spleen and liver, when infected with high doses of virus by a peripheral route of inoculation (19, 25). Strain variations in response to this infection have been shown to be genetically determined (26) and in part may reflect differential capacities to generate virus-specific CTL responses (2, 3, 20).

Earlier studies of adult B6 mice infected with LP-BM5 MuLV demonstrated that they rapidly lost their ability to respond specifically to a variety of antigenic stimuli in vitro (18). To determine whether this MuLV strain would also affect the ability of mice to control an in vivo infection, B6 mice inoculated as adults with LP-BM5 virus mixture or with ecotropic virus alone were challenged with ectromelia virus and observed for morbidity and mortality (Fig. 1). The results of these studies demonstrated several points. (i) Ectromelia virus-induced mortality was observed only in mice infected with the LP-BM5 virus mixture; no deaths occurred in mice previously infected with LP-BM5 ecotropic virus alone. (ii) The frequency of ectromelia virus-induced deaths increased with the time after exposure to the LP-BM5 virus mixture. (iii) All deaths observed in these studies were caused by ectromelia virus infection. At necropsy, all animals were found to exhibit splenic and hepatic necrosis like that observed in susceptible A/J mice infected with ectromelia virus or had ectromelia virus infectivity titers of greater than 10^7 PFU/g in spleen and liver cells (data not shown). These results indicated that infection with the LP-BM5 virus mixture progressively impaired the ability of B6 mice to resist the lethal consequences of ectromelia virus infection.

Further studies were undertaken to determine whether infection with LP-BM5 MuLV affected the ability of B6 mice to control replication of ectromelia virus in spleen and liver cells at a time (week 8 post-MuLV infection) when most mice survived the infection. Ectromelia virus titers in spleen and liver cells were measured 7, 13 and 17 days after the ectromelia virus challenge of mice inoculated with the LP-

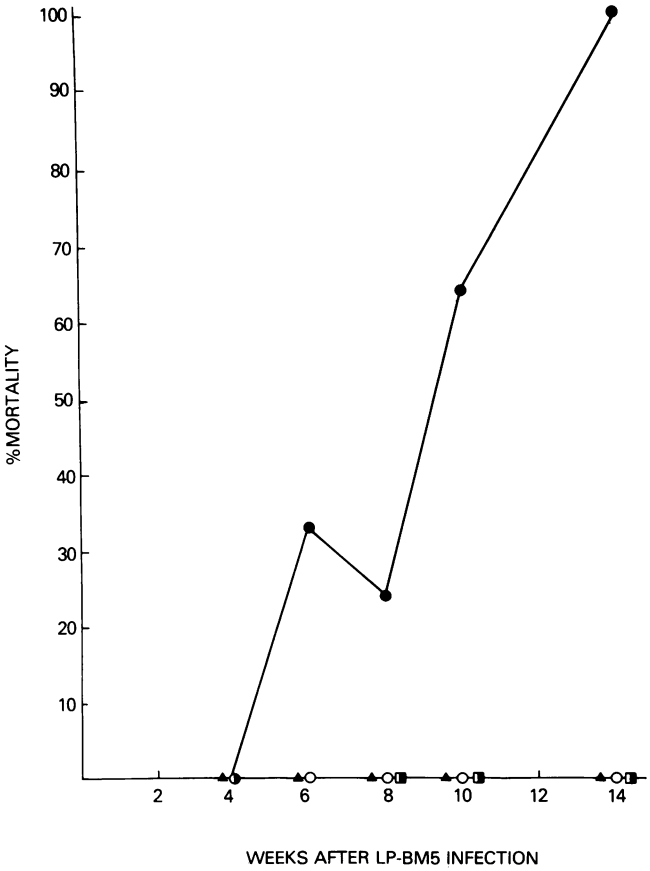

FIG. 1. Ectromelia virus-induced mortality in B6 mice. Adult B6 mice inoculated with the LP-BM5 virus mixture (circles), LP-BM5 ecotropic virus alone (squares), or saline (triangles) were challenged 4, 6, 8, 10 or 14 weeks later by footpad inoculation with 10^4 PFU of ectromelia virus (closed symbols) or saline (open symbols). Points indicated percent mortality for 4 to 17 mice during an 18-day observation period in three separate experiments. The numbers of LP-BM5 mixed-virus-infected mice challenged with ectromelia virus were 12 at week 4, 9 at week 6, 17 at week 8, 15 at week 10, and 4 at week 14. Mortality for ectromelia virus-challenged mice was significantly greater at weeks 6 and 10 ($P < 0.05$, Fisher exact test) in mice previously infected with a mixture of LP-BM5 MuLV than in mice previously infected with LP-BM5 ecotropic MuLV or mock infected with saline.

BM5 virus mixture or ecotropic virus alone (Table 1). At days 7 and 13 after challenge, ectromelia virus levels (on average) were higher in the spleens and livers of mice infected with the LP-BM5 virus mixture than in mice infected with the ecotropic virus alone. However, by day 17 after infection, no ectromelia virus infectivity was detected in the tissues of mice inoculated with either MuLV preparation. Thus, the impaired ability to control replication of ectromelia virus was evident even in stages of LP-BM5 infection preceding the time when the inoculation of ectromelia virus resulted in high mortality.

Effects of LP-BM5 infection on immune responses to ectromelia virus. Mice infected with the LP-BM5 virus mixture or ecotropic virus alone were challenged with ectromelia virus at various times after infection. Virus-neutralizing antibody titers in serum were determined 21 days later. No circulating antibody to ectromelia virus was present in the sera of mice infected 4 or 6 weeks earlier with the mixture of LP-BM5 viruses, whereas sera from mice infected previously with LP-BM5 ecotropic virus or from mock-infected mice contained neutralizing antibody (Table 2). The detection of a low level of neutralizing antibody at week 8 in mice infected with the LP-BM5 virus mixture may have resulted from the polyclonal-B-cell activation and production of cross-reactive antibodies. Although a humoral immune response to ectromelia virus was absent at week 4 after LP-BM5 inoculation, no ectromelia virus-induced mortality was observed in the experiments. This indicated that neutralizing antibody does not contribute significantly to recovery from a primary ectromelia virus infection.

TABLE 1. Persistence of ectromelia virus infectivity in spleen and liver cells of challenged C57BL/6 mice infected 8 weeks previously with LP-BM5 MuLV[a]

LP-BM5 MuLV genotype	Days post-ectromelia virus challenge	Mouse	Log_{10} ectromelia virus infectivity (PFU/g) in:	
			Spleen	Liver
Mixture	7	1	6.4[b]	4.2[c]
	7	2	7.9	5.4
	7	3	8.0	8.4
	13	4	<3.2	<2.7
	13	5	5.7	4.3
	13	6	5.9	<2.7
	17	7	<3.2	<2.7
	17	8	<3.2	<2.7
	17	9	<3.2	<2.7
Ecotropic	7	10	4.3[b]	<2.7[c]
	7	11	4.7	<2.7
	7	12	3.7	<2.7
	13	13	<3.2	<2.7
	13	14	<3.2	<2.7
	13	15	<3.2	<2.7
	17	16	<3.2	<2.7
	17	17	<3.2	<2.7
	17	18	<3.2	<2.7

[a] One of six and none of six mice died between 1 and 18 days of ectromelia virus challenge when mice were infected previously with LP-BM5 virus mixture and ecotropic virus, respectively.

[b] In mixed infections the geometric mean $2.8 \times 10^7 \times\div 3.4$ PFU/g of virus titers at day 7 postchallenge, from the spleens of three animals, was significantly greater than the corresponding mean of ecotropic controls ($1.7 \times 10^4 \times\div 1.9$ PFU/g); $P < 0.01$ (Student t test).

[c] In mixed infections the geometric mean $9.6 \times 10^6 \times\div 18$ PFU/g of virus titers at day 7 postchallenge, from the livers of three animals, was significantly greater than the corresponding mean of ecotropic controls (4.8×10^2 PFU/g); $P < 0.05$ (Student t test).

TABLE 2. Effect of prior infection of C57BL/6 Mice with the LP-BM5 virus mixture on in vivo antibody response to ectromelia virus challenge

Week[a] of ectromelia virus inoculation	LP-BM5 genotypes	Reciprocal of anti-ectromelia virus-neutralizing antibody titer at day 21 postinfection[b]
4	Mixture	<40 (12)[c]
6	Mixture	<40 (5)
8	Mixture	$51 \times\div 1.2$[d] (12; 40–254)
10	Mixture	<40 (6)
10	Ecotropic	$376 \times\div 1.4$ (8; 109–1420)
10	Mock	$343 \times\div 1.3$ (4; 186–502)
14[e]	Ecotropic	$3395 \times\div 1.1$ (2; 3192–3615)
14	Mock	$1029 \times\div 1.2$ (4; 558–1506)

[a] Week after infection with LP-BM5 MuLV.

[b] Fourfold differences in titer are significant.

[c] Numbers in parentheses indicate the number of mice tested and range of antibody titers where appropriate.

[d] Geometric mean and relative standard error; uninfected control B6 mice routinely gave a neutralizing antibody titer of <40.

[e] The 14-week data sets were from a second experiment in which no challenged mice inoculated with the LP-BM5 mixture survived challenge (see Fig. 1).

The ability of LP-BM5-infected mice to mount an ectromelia virus-specific CTL response was determined at two different times: week 8 after LP-BM5 infection, when most mice were resistant to the lethal effects of ectromelia virus, and week 10 after infection when most mice were susceptible (Fig. 1). When assayed at day 7 after ectromelia virus challenge, mice infected 8 weeks earlier with the LP-BM5 virus mixture had CTL levels that were approximately half those of mice infected with ecotropic virus alone (Fig. 2A, B). By comparison, no ectromelia virus-specific CTL responses were obtained from the spleen cells of mice inoculated 10 weeks earlier with the LP-BM5 virus mixture and tested for CTL activity on day 5 (Fig. 2C, D), 7 (Fig. 2E, F), or 10 (Fig. 2G, H) after challenge with ectromelia virus.

In this experiment, 6 of 12 mice inoculated with the LP-BM5 virus mixture, in parallel with the mice used in the week 10 CTL assays, died after infection with ectromelia virus. All mice had high virus titers in the spleen and liver (Table 3). Five additional mice sacrificed at day 9 after challenge exhibited extensive ectromelia virus-induced splenic and hepatic necrosis. The six surviving mice were sacrificed at day 24 after ectromelia virus challenge for histopathologic studies and assays for ectromelia virus in spleen and liver tissues. All six mice had histologic changes in the lymph nodes and spleen consistent with advanced LP-BM5-induced disease, but no infectious ectromelia virus was detected in spleen or liver tissue. The mice were known to have been infected, as they previously had shown primary lesions at the sites of inoculation.

DISCUSSION

The data presented here demonstrate that infection of adult B6 mice with the LP-BM5 virus mixture rendered this normally highly resistant strain susceptible to the lethal effects of ectromelia virus (Fig. 1). Mortality was observed in some mice challenged with ectromelia virus as soon as week 6 after inoculation with the LP-BM5 virus mixture, and when challenged 14 weeks after LP-BM5 virus inoculation, all mice died with severe mousepox. This effect can most likely be ascribed to the activity of the MCF virus present in the

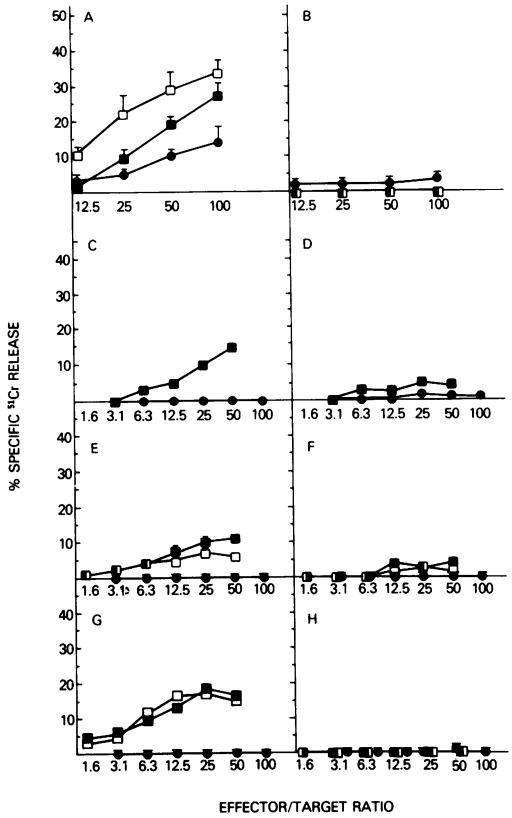

FIG. 2. Primary in vivo ectromelia virus-specific CTL responses. Adult B6 mice inoculated with the LP-BM5 virus mixture (●), LP-BM5 ecotropic virus alone (■), or saline (□) were challenged 8 (A and B) or 10 weeks later (C through H) by foodpad inoculation with ~5 × 10^2 PFU of ectromelia virus. Splenic CTL responses to ectromelia virus-infected, ^{51}Cr-labeled EL-4 cells (A,C,E,G) or uninfected, labeled cells (B,D,F,H) were determined on day 5 (C,D), 7 (A,B,E,F), or 10 after challenge (G,H). Points indicate percent specific lysis for individual mice (no standard error bars) or the mean values for two individual mice (with bars indicating one standard error of the mean). Spontaneous ^{51}Cr release varied from 19 to 48% of maximum release. Nonimmune spleen cells from uninfected control mice gave no specific lysis (0%) at all of the indicated effector/target ratios.

MuLV mixture, as no mortality was observed in ectromelia virus-challenged mice previously infected with the LP-BM5 ecotropic virus alone (Fig. 1; Tables 1 and 3; data not shown).

Earlier studies demonstrated that MCF viruses present in the LP-BM5 mixture rapidly induce profound suppression of a variety of T- and B-cell functions measured either in vitro or in vivo (18). In this report, in vivo antibody responses to challenge with ectromelia virus were found to be depressed as early as week 4 after infection with the LP-BM5 virus mixture (compare the week 4 mixed infection with the week 10 or 14 LP-BM5 ecotropic infection [Table 2]). Mice with no virus-specific antibody responses were still resistant to ectromelia virus-induced mortality, a result consistent with earlier studies indicating that antibody plays little if any role in recovery from mousepox (2, 3).

By comparison, the loss of virus-specific CTL responses in primary in vitro assays of spleen cells from ectromelia virus-infected mice was correlated with the extent of ectromelia virus-induced mortality (Fig. 1, 2). Virus-specific

TABLE 3. Mortality in C57BL/6 mice infected 10 weeks previously with LP-BM5 MuLV and challenged with ectromelia virus

LP-BM5 MuLV genotype	Mouse no.	Day of death	Ectromelia virus infectivity on day of death (PFU/g)[a]	
			Spleen	Liver
Mixture	1	10	9.8	9.3
	2	12	8.6	7.6
	3	12	8.6	7.8
	4	13	7.3	5.4
	5	15	5.8	<2.7
	6	17	<3.2	7.7
Ecotropic	7–14	NA[b]	NA	NA

[a] Six of twelve and none of eight mice previously infected with LP-BM5 virus mixture and ecotropic virus, respectively, died on challenge with ectromelia virus. At death, samples of liver and spleen tissues were removed for estimation of virus infectivity.
[b] Not applicable.

CTL activity was observed, at reduced levels, in mice challenged with ectromelia virus at week 8 after infection with the LP-BM5 virus mixture, a time at which most mice were still resistant to ectromelia virus-induced mortality. However, on challenge with ectromelia virus at week 10 after infection with the LP-BM5 virus mixture, ectromelia virus-specific CTL responses were no longer detectable, and ectromelia virus-induced mortality of 50% was observed (Table 3, Fig. 2). The mechanism(s) responsible for the clearance of ectromelia virus in mice surviving challenge at 10 weeks after infection with the LP-BM5 virus mixture is not known but may reflect the influx of CTL from sites not assayed (e.g., the lymph node) or the activity of Mac-1^+ cells present at increased frequency in the spleens of mice infected with the LP-BM5 virus mixture (18). A role for mononuclear phagocytes in the clearance of ectromelia virus from infected tissues was demonstrated previously (4, 5, 22), and ongoing studies of peritoneal macrophages from B6 mice exposed to LP-BM5 mixed-virus infection indicate that these cells function normally, at least through week 12 after infection (C. Nacy and R. Yetter, unpublished observations).

Taken together with the results from our earlier studies (18), these data extend the parallels between the effects of LP-BM5 infection on mice and the effects of HTLV-III/LAV infection on humans. Both diseases are associated with lymphadenopathy, polyclonal-B-cell activation, progressively severe immunosuppression, and enhanced susceptibility to the lethal effects of otherwise innocuous infections (13, 14, 16). Further studies of this retrovirus-induced murine AIDS may thus provide important insights into the host-virus interactions responsible for the development of AIDS.

ACKNOWLEDGMENTS

The support of the Division of Safety, Office of the Director, National Institutes of Health was essential to the operation of the BL4 containment laboratory. We thank D. Alling for expert statistical advice, C. Duarte for technical assistance, M. Rust for manuscript preparation, and B. Moss and J. Hartley for critical reviews of the manuscript.

R.E.Y. is a Special Fellow of the Leukemia Society of America.

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