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Susceptibility of newborn mice with H-2^k backgrounds to lymphocytic choriomeningitis virus infection

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Summary. Many strains of mice, when injected at birth with an ordinarily lethal dose of lymphocytic choriomeningitis virus (LCMV), grow to adulthood despite maintaining a persistent virus infection and chronic virus-induced immune complex disease. Because the susceptibility to LCMV infection changed over several years of observation, a number of murine strains with different histocompatibility gene loci and genetic backgrounds were compared. Neonatal mice with H-2^b, H-2^d, and H-2^q backgrounds were relatively insensitive to the effects of LCMV infection compared to mice with H-2^k backgrounds, which had a high mortality rate in this situation. Expression of the H-2^k gene locus itself did not affect the rate of mortality. Use of recombinant mice indicated that susceptibility was linked to H-2^k backgrounds and not H-2^k gene loci. The low survival rate of newborn mice with H-2^k backgrounds infected with LCMV was not caused by cytotoxic natural killer cells, cytotoxic T lymphocytes, excessive amounts of virus in the organs, a unique distribution of virus or expression of viral antigens *in vivo* or unusual pathology in tissues.

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INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) infection of mice and cultured cells has been a useful probe for understanding basic immunobiological and immunopathological events. Traub (1936) first noted that LCMV persists in naturally infected mice, and Hotchin & Cintis (1958) later simulated this state in an experimental model by injecting LCMV into newborn mice. Since then, other investigators have used both natural and experimentally induced models of this infection in embryos, newborns, or adult mice to detail several important biological observations. For example, in studying the acute LCMV infection of adult animals Rowe (1954) first described virus-induced immunopathological disease, which has been subsequently confirmed by multiple investigators using a variety of immunological depletion and reconstitution experiments for LCMV (East, Parrott, & Seamer, 1964; Cole, Gilden, Monjan & Nathanson, 1971; Hotchin & Weigand, 1961; Oldstone & Dixon, 1970, 1971a, b; Hirsch, Murphy, Russe, & Hicklin, 1967; Lundstedt & Volkert, 1967; Gilden, Cole & Nathanson, 1972; Johnson, Monjan & Morse, 1977; Zinkernagel & Welsh, 1976) and other virus infections (reviewed by Oldstone & Dixon, 1976; Blanden, 1971). The initial evidence of the generation and cytotoxic activity of T lymphocytes against virus-infected targets was described using the LCMV model (Cole, Nathanson & Prendergast, 1972; Marker & Volkert,

1973) and this virus infection provided the initial observation for the H-2 restriction phenomena (Zinkernagel & Doherty, 1974), Others studying persistent virus infection used this model to define virus-induced immune complex disease (Oldstone & Dixon, 1967, 1969; Buchmeier & Oldstone, 1978) and provided the initial experimental evidence to support the concept that aberrations in the cell differentiation (luxury) function occurred during virus infection without impairment of the cells' vital function (Oldstone, Holmstoen & Welsh, 1977).

With an increasing number of investigators studying immunobiological and viral pathological phenomena derived from the LCMV–murine host system, changes from that ordinarily expected in the basic model become both of interest and importance. We have previously noted (Oldstone & Dixon, 1973) a change in the susceptibility of mouse strain C3H/HeJ (H-2^k) to LCMV infection. Before 1970, the majority of C3H/HeJ mice when inoculated as newborns, like mice of other strains, withstood a dose of LCMV that would be lethal to an adult mouse. However, since 1979, 99% of the over 200 C3H/HeJ mice inoculated in exactly the same way have died within 3–4 weeks. In contrast, newborn mice of other H-2^k strains (C3H/St, AKR, CBA) survivied just as before. They became persistently infected adults and carried virus as well as mounting anti-viral immune responses throughout their life (Oldstone & Dixon, 1973; Tishon & Oldstone, unpublished data).

Now we report a new change in mice bearing H-2^k backgrounds, such as C3H/St, AKR, C58, CBA, A/J which have become highly susceptible to neonatal infection with LCMV in comparison to mice with H-2^b, H-2^d and H-2^q backgrounds. We have examined this phenomenon in recombinant mice in which H-2^k background has been placed on H-2^b or H-2^d mice and H-2^b has been placed on an H-2^k background. These experiments provided evidence that the enhanced susceptibility of H-2^k mice was not controlled by the H-2^k locus but was associated with H-2^k background. Further, the inability of newborn H-2^k mice to survive inoculation with LCMV was not related to the generation of cytotoxic T or natural killer (NK) lymphocytes, enhanced production of infectious virus or changes in the location of viral replication in the infected animal.

MATERIALS AND METHODS

Mice

Adult mice of the test strains were obtained from Jackson Laboratories, Bar Harbor, Maine and from Scripps Clinic and Research Foundation, (SCRF) in La Jolla, California. The animals were bred at SCRF and newborn mice were inoculated with LCMV within the first 18 h of life. The H-2 locus and background of the 16 strains of mice used are recorded in Table 1.

Virus

The LCMV used was Armstrong strain. The cloning, handling, storage and use of virus as well as its biochemical markers have been recorded (Oldstone & Dixon, 1969; Buchmeier, Elder & Oldstone, 1978; Doyle & Oldstone, 1978). Mice less than 18 h of age were inoculated intracerebrally (i.c.) with 100 plaque-forming units (PFU) of LCMV stock. In some instances, newborn mice were inoculated with 1/10 of this dose of virus i.c. or intraperitoneally (i.p.). Infection of adult mice followed either i.c. or i.p. inoculation with 1000 PFU of LCMV.

Virus titration in various organs

Tissues collected from individual mice were placed in culture media and disrupted by Dounce homogenization. Medium consisted of 10% heat-inactivated foetal calf serum and 1% antibiotics and glutamine in MEM, pH 7·2. The resulting cell debris was removed by pelleting following centrifugation at 700 ***g***, and the supernatants were added to Vero cells and assayed for LCMV by using a plaque assay as previously described (Doyle & Oldstone, 1978).

Virus antigen localization in various organs

Tissues obtained from individual mice were snap-frozen in liquid nitrogen and 4 μm thin sections cut on a cryostat. Tissue sections on glass slides were fixed in 95% ethanol, ether:ethanol and stained with a monospecific antiserum to LCMV conjugated to fluorescein isothiocyanate. The details for fixing tissues, staining sections, the raising and specificity of the anti-viral antisera used have been recorded (Oldstone & Dixon, 1969; Buchmeier & Oldstone, 1978; Buchmeier *et al.*, 1978). The antisera used recognized all three structural LCMV polypeptides (Buchmeier & Oldstone, 1978).

Cytotoxic T lymphocyte and NK lymphocyte assay

The cytotoxic activity of lymphocytes from the spleens of LCMV-infected mice was determined using a quantitative ^{51}Cr- release assay. L-929 cells (H-2^k) were used as targets either uninfected or after exposure to LCMV at a multiplicity of infection (MOI) of 1. Varying ratios of effector to target cells were used. The

labelling of target cells with ^{51}Cr, the conditions of the assay, and the method of calculation have been described previously (Zinkernagel & Oldstone, 1976).

Histopathology

Multiple tissues taken were fixed in Bouin's solution and stained with haematoxylin–eosin and periodic acid Schiff.

RESULTS

Susceptibility of H-2^k mice to neonatal infection with LCMV

As shown in Table 1, inoculation of LCMV into newborn C3H/HeJ, C3H/St, C57BR/cdj, CBA/N, CBA/WEHI, AKR/Cum, B10/Br, C58/J, AKR/J, and C3H/Rv mice all of which have H-2^k locus yielded mortality rates ranging from 64·7 to 100%. In contrast, SWR/J, C57Bl/6, BALB/k and BALB/WEHI mice, with H-2^q, H-2^b, H-2^d backgrounds, respectively, had mortalities of only 23·0, 10·8, 13·8 and 15%. Mice homozygous and heterozygous for the nude gene but of CBA (H-2^k) background were relatively resistant. Based on smaller numbers of mice (6–12 per group) the susceptibility of H-2^k mice was similar when 100 PFU of virus was given i.p. or when 10 PFU was administered i.c. or i.p. Thus, neonates of the H-2^k type were more susceptible to LCMV than non-H-2^k neonates.

Relationship of H-2^k locus or H-2^k background to lethal infection of newborn mice

The above results show that H-2^d and H-2^b newborn mice were far less susceptible to LCMV infection than were H-2^k mice. To determine whether H-2^k locus was directly responsible for the enhanced susceptibility of newborn mice, we used recombinant mice with the H-2^k background placed on H-2^d mice and mice with the H-2^b locus superimposed on an H-2^k background. As seen in Table 1, when the H-2^k locus was put on the H-2^d background, the BALB/k offspring were not susceptible to LCMV as newborns, more than 85% survived to adulthood. In fact, BALB/k and BALB/WEHI strains were equally low in mortality rates, even though the former possess the H-2^k gene locus and the latter is H-2^d. Both BALB/k and BALB/WEHI incorporate the BALB (H-2^d) background.

C57B1/6 newborn mice which contain H-2^b gene locus are resistant to LCMV infection (89% survival). However, when the H-2^b gene locus was placed on a C3H background to yield the C3H/SW mouse, the majority of offspring died by 3 weeks of age and the remainder by 4 weeks of age after viral inoculation.

Table 1. Susceptibility of murine strains to neonatal inoculation with LCMV

				4 weeks after LCMV inoculation at birth	
Strain	H-2 Locus	Background	Alive/total	Survival (%)	Mortality (%)
C3H/HeJ	kk	C3H (k)	0/16	0	100
C3H/St	kk	C3H (k)	0/26	0	100
C57BR/cdj	kk	C57BR (k)	0/48	0	100
CBA/N	kk	CBA (k)	0/22	0	100
CBA/WEHI	kk	CBA (k)	1/53	1·8	98·2
AKR/Cum	kk	AKR (k)	6/44	13·6	86·4
B10/BR	kk	B/10 (k)	8/30	26·6	73·4
C58/J	kk	C58 (k)	6/20	30·0	7·0
AKR/J	kk	AKR (k)	16/48	33·3	66·7
C3H/Rv	kk	C3H (k)	6/17	35·3	64·7
CBA/Nu/+	kk	CBAxSwiss (kxq)	20/36	55·5	44·5
SWR/J	qq	Swiss (q)	47/61	77·0	23·0
BALB/WEHI	dd	BALB (d)	85/100	85·0	15·0
C57Bl6/J	bb	C57Bl (b)	25/28	89·2	10·8
C3H/SW	bb	C3H (k)	0/72	0	100
BALB/k	kk	BALB (d)	25/29	86·2	13·8

Neonatal mice were inoculated i.c. with 100 PFU of LCMV within 18 h after birth. Litters were checked daily for survivors.

Cytotoxic lymphocytes do not play a role in the enhanced susceptibility of H-2^k mice to LCMV infection Two classes of cytotoxic lymphocytes are generated during acute LCMV infection (Welsh, 1978). Natural killer cells develop within 1–3 days, killing is not H-2 restricted between the effector cells and virus-infected target cells, and killing of both virus-infected and uninfected targets is equally efficient. In contrast, cytotoxic T lymphocytes are generated at days 5 through 8, show H-2 restriction and kill mainly virus-infected targets, not uninfected targets. Death from LCMV during acute infection of adult mice is associated predominatly with the generation and activity of cytotoxic T lymphocytes (Lundstedt & Volkert, 1967; Johnson *et al.*, 1977; Cole *et al.*, 1972; Zinkernagel & Doherty, 1973). Therefore, experiments were designed to determine whether the enhanced mortality of mice bearing H-2^k backgrounds was due to the generation of cytotoxic lymphocytes in LCMV-infected neonates. Spleen cells were taken from 17 day old mice that had been infected at birth with LCMV and were analysed *in vitro* on LCMV-infected and uninfected ^{51}Cr-labelled target cells. As the data in Table 2 show, these spleen cells did not kill significant numbers of either uninfected or LCMV-infected target cells. In contrast, Table 2 also shows that in assays run concurrently, spleen cells harvested from adult mice 7 days after initial exposure to LCMV significantly lysed ^{51}Cr-labelled virus-infected targets. In other experiments (data not shown), spleen cells harvested from neonatally infected C3H/St or BALB/k LCMV-infected mice 6–21 days after viral inoculation did not demonstrate cytotoxic activity against virus-infected or uninfected targets.

Table 2. Cytotoxic T-cell activity in spleens of mice neonatally inoculated with LCMV

Strain	Effector cells	Target cells	Effector:target ratio	% ^{51}Cr Release*
Adult C3H/St	Spleen lymphocytes†	L-929 Cells	75:1	0·0 ± 0·28
			37:1	7·9 ± 0·11
			18:1	4·2 ± 0·01
			9:1	5·0 ± 0·05
Neonate C3H/St	Spleen lymphocytes‡	L-929 Cells	23:1	0·9 ± 0·04
			2·3:1	4·1 ± 0·04
			0·23:1	8·9 ± 0·27
			0·023:1	6·9 ± 0·29
Adult C3H/St	Spleen lymphocytes	LCMV§ L-929 Cells	75:1	84·0 ± 0·42
			37:1	77·1 ± 1·18
			18:1	58·7 ± 0·60
			9:1	37·0 ± 0·60
Neonate C3H/St	Spleen lymphocytes	LCMV L-929 Cells	23:1	0·0 ± 0·08
			2·3:1	2·7 ± 0·01
			0·23:1	6·4 ± 0·7
			0·023:1	8·2 ± 0·15

* % ^{51}Cr release was calculated by

$$\frac{\text{c.p.m. sample} - \text{c.p.m. spontaneous release}}{\text{Total c.p.m.} - \text{c.p.m. spontaneous release}} \times 100$$

± one standard error.

† Three month old C3H/St adults were injected i.p. with 1000 PFU of LCMV. Seven days later their spleen cells were assayed for activity.

‡ Newborn C3H/St mice were injected i.c. with 100 PFU of LCMV; 17 days later their spleens were assayed for activity.

§ L-929 cells were infected with LCMV at an MOI of 13 days before testing.

The amount of infectious virus carried and the distribution of viral antigens in various organs of H-2^k neonatal mice infected with LCMV

Levels of virus were similar in many organs assayed from C3H/St (H-2^k) and BALB/WEHI (H-2^d) matched mice 6–16 days old. Table 3 shows amounts of infectious virus carried in 12–13 day old C3H/St and BALB/w mice. Similar results occurred when three other individual C3H/St and BALB/WEHI mice studied, i.e. average virus titres in 12–13 day old C3H/St:BALB/WEHI mice kidneys $1{\cdot}5\times10^7$: $5{\cdot}2\times10^{7{\cdot}5}$; liver $2{\cdot}4\times10^5$:$3{\cdot}3\times10^5$; brain $6{\cdot}5\times10^7$: $1{\cdot}4\times10^{7{\cdot}5}$; thymus $3{\cdot}4\times10^7$:$5{\cdot}0\times10^{7{\cdot}5}$. Mice from both strains had higher virus titres when studied during the second week of life than did 2 month old BALB/WEHI mice which survive the neonatal infection and carry virus in their tissues (Table 3). The plaques produced by cells from LCMV-infected neonates on Vero cells were clear, not turbid. Plaques formed by C3H/St cells were in general smaller (average 2 mm) than those from BALB/WEHI cells (average 4 mm). The yields of infectious virus did not change when plaquing was done at 34°, 37°, or 39·5° indicating that infectious virus harvested from organs of both C3H/St and BALB/WEHI neonates was not temperature sensitive. Similar results were obtained when stock LCMV was plaqued at these various temperatures (data not shown).

Brain, spleen, thymus, kidney and heart tissues of C3H/St and BALB/WEHI LCMV-infected neonates were studied for the distribution of viral antigens. In mice from each group examined at 6, 13 and 21 days after their neonatal LCMV inoculations, the distribution of antigen was similar in these various tissues despite the discordance of mortality among both strains of mice. All organs from 6 day old mice contained more viral antigens than did organs from 21 day old mice. In the brain, viral antigen was noted mainly in neurons, occasionally in glia and endothelial cells and infrequently in meningeal cells. In kidney and heart tissues, viral antigen was found in parenchyma cells, blood vessels and connective tissue cells. In the spleen, virus was detected in macrophages, fibrocytes and occasionally in lymphocytes, whereas in the thymus, viral antigen was found in lymphoid cells in both the cortical and medullary areas. Observations with these two strains of mice (BALB/WEHI, C3H/St) were similar to that previously reported for other mouse strains (Oldstone & Dixon, 1969).

Table 3. Titres of infectious LCMV in various organs of neonatally inoculated mice

	Strains and ages		
	C3H/St (Day 13)*	BALB/WEHI (Day 12)†	BALB/WEHI (2 months)‡
Organs	Virus titre (PFU/g)§		
Kidney	$1{\cdot}0\times10^8$	$1{\cdot}4\times10^8$	$1{\cdot}6\times10^6$
Spleen	$1{\cdot}1\times10^6$	$3{\cdot}5\times10^6$	$9{\cdot}6\times10^6$
Liver	$3{\cdot}6\times10^5$	$2{\cdot}6\times10^5$	$<10^4$
Heart	$5{\cdot}0\times10^7$	$2{\cdot}5\times10^6$	$<10^4$
Brain	$6{\cdot}0\times10^7$	$1{\cdot}6\times10^7$	$2{\cdot}0\times10^5$
Thymus	$4{\cdot}0\times10^7$	$1{\cdot}7\times10^8$	$1{\cdot}7\times10^8$
Serum	$5{\cdot}0\times10^5$ PFU/ml	$4{\cdot}0\times10^6$ PFU/ml	$4{\cdot}0\times10^3$ PFU/ml

* C3H/St mice injected i.c. with 100 PFU of LCMV at birth were killed 13 days later and their organs assayed for LCMV.

† Same as in * except BALB/WEHI mice were killed 12 days after birth.

‡ Same as in * except BALB/WEHI mice were killed 2 months after birth.

§ Organs were weighed and then disrupted by Dounce homogenization in medium. The cell debris was pelleted and the supernatant fluids assayed for LCMV using a plaque assay on Vero cells. Results are given in PFU per gram of tissue.

Clinical and histopathological studies

Mice with H-2^k backgrounds infected at birth with LCMV failed to grow normally compared to concurrently inoculated H-2^d, H-2^b, and H-2^q mice. Values for 10–13 day old C3H/St mice (H-2^k) and BALB/WEHI mice (H-2^d) appear in Table 4 and represent the

Table 4. Weights of various organs from neonatally inoculated mice

	Organ weight (g) Day 10–13*	
Organ	BALB/WEHI	C3H/St
Kidney	0·05128 ± 0·005	0·02978 ± 0·003
Spleen	0·06211 ± 0·011	0·01820 ± 0·003
Liver†	0·08496 ± 0·008	0·07943 ± 0·005
Heart	0·05534 ± 0·012	0·03219 ± 0·006
Brain	0·30023 ± 0·013	0·22512 ± 0·021
Thymus	0·03951 ± 0·006	0·01603 ± 0·003

* Mice were injected i.c. with 100 PFU of LCMV at birth. Numbers represent the mean ± standard error.

† One lobe of liver weighed.

results of four animals of each strain. Kidneys, spleens, hearts, brains, and thymi of LCMV-infected BALB/WEHI mice weighed significantly more ($P = < 0·01$) than those from infected C3H/St (H-2^k) mice. Similarly, kidneys, spleens, livers, hearts, brains, and thymi of 19–21 day old BALB/WEHI infected mice weighed significantly more ($P = 0·01$) than age-matched infected C3H/St mice.

Histopathological studies of brain, liver, spleen, thymus, kidney and heart revealed no major differences between C3H/St and BALB/WEHI LCMV-infected mice killed at 6, 13 or 21 days after their neonatal injection with LCMV. Neither inflammation nor tissue necrosis was apparent when tissues from four mice of each group were studied at these various time points.

DISCUSSION

Mice infected at birth or *in utero* with LCMV usually survive their initial infection and subsequently maintain infectious virus in their tissues throughout life. Such mice mount immune responses and the interaction of anti-viral antibodies directed against all the viral polypeptides with infectious virus and viral antigens in the circulation results in the formation of virus–antibody immune complexes which become deposited in various tissues leading to immune complex disease (Oldstone & Dixon, 1969; Buchmeier & Oldstone, 1978; Oldstone, 1975). Several years ago (Oldstone & Dixon, 1973) it was reported that newborn C3H/HeJ mice inoculated with LCMV died when 2–4 weeks old. Although these mice were characterized by generalized stunted growth, examination of their tissues by immunopathological and histopathological techniques failed to demonstrate any abnormalities. Foster nursing did not prevent death. Other mice of related background (C3H) and H-2 type (H-2^k) such as AKR/J and C3H/St survived neonatal injection with LCMV and went on to become persistently infected adults.

In this report, we document a new change in susceptibility to LCMV infection of newborn mice with H-2^k backgrounds. These mice are now susceptible to LCMV and frequently do not survive past the fourth week of age. Thus, newborn mice with H-2^k backgrounds have a higher mortality and die earlier than mice with H-2^b, H-2^d, or H-2^q backgrounds after either i.c. or i.p. injection with LCMV. Using C3H/SW and BALB/k recombinant mice, we demonstrated clearly that susceptibility of newborn mice to LCMV is related to the H-2^k background, not to the H-2^k locus of the major histocompatibility gene complex. Thus BALB/k mice whose H-2 gene locus is kk but whose background is H-2^d readily survived LCMV infection whereas C3H/SW mice who possess the H-2^b gene locus on a C3H (H-2^k) background were susceptible to LCMV infection.

Tissue injury and death caused by virus infection are related either to a property of the virus *per se* or to the immune system reacting against the virus or their antigens on the cells' surfaces (Oldstone & Dixon, 1976). In association with both the acute and persistent LCMV infection tissue injury and death are products of the specific anti-viral immune response (Rowe, 1954; Oldstone & Dixon, 1976; Oldstone, 1975). During actue LCMV infection in adult mice, at least two kinds of killer lymphocytes are generated, and one of these, cytotoxic T lymphocytes, lyses virus infected targets *in vivo* and *in vitro* (Cole *et al.*, 1972; Lundstedt, 1969; Marker & Volkert, 1973; Zinkernagel & Doherty, 1973). For this reason, we studied susceptible and resistant mice after their infection at birth with LCMV for the presence of cytotoxic lymphocytes but neither (Table 2) cytotoxic T lympho-

cytes nor NK cells were found in these mice. In addition, the immunopathological and histopathological examination of tissues taken from these mice revealed no infiltrating lymphocytes or areas of tissue necrosis. Hence, there is little evidence at present that immune responses directed against LCMV cause the death of these neonatally infected mice with H-2^k backgrounds. LCMV can cause direct cytopathology as demonstrated by its ability to form plaques on certain cell lines. However the virus rapidly generates defective virus particles (Welsh & Oldstone, 1977; Welsh & Pfau, 1972) so that possible cytopathic effects are difficult to see in most assay systems. For these reasons we determined whether there was a difference in the amount of infectious virus generated, expression of viral antigen and distribution of viral antigen in mice with and without H-2^k backgrounds after infection at birth with LCMV. Using a sensitive *in vitro* plaque assay and a range of temperatures for culturing along with immunofluorescence as a means of detecting viral antigens, we found no differences in the quantity of infectious virus formed, morphology of virus plaques or expression of viral antigens between resistant and susceptible mice.

Thus, it is not clear why mice with H-2^k backgrounds following infection with LCMV at birth fail to grow normally and die within the first 28 days of life when compared to H-2^d, H-2^b, and H-2^q mice. Mice dying show neither immunopathological injury nor direct virus-associated damage. Other possible but unproved explanations is that a secondary agent or event activated by LCMV causes the death of H-2^k background mice or that a dysfunction of an endocrine system may occur in susceptible mice. LCMV could potentiate and activate other viruses (Oldstone & Dixon, 1973; Oldstone, Aoki, & Dixon, 1971) and LCMV might impair the function of unique cells that make neurotransmitters or hormones (Oldstone, *et al.*, 1977; Yoon, Onodera, & Notlans, 1978). Although the cause(s) of early death and stunted growth of mice with H-2^k backgrounds is undefined, the events described here provide an *in vivo* model for studying virus-induced inhibition of growth.

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