Type C Virus Expression in Lymphoma-Paralysis-Prone Wild Mice[1,2]

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ABSTRACT—Wild mice trapped near Lake Casitas (LC) in southern California showed a high prevalence of infectious type C virus in the liver, spleen, and thymus within the first few weeks of life. By young adulthood about 80% of LC mice (including their genital tissues) were infected. Virus isolates from these mice cause lymphoma and lower limb paralysis under both natural and experimental conditions. Mice destined to develop paralysis showed higher levels of serum gs antigen early in life, whereas mice destined to develop lymphoma or remain free of these diseases could not be distinguished by this test. The individual variation in virus expression suggested that differences in virus type or in the immune or other host defense mechanisms greatly influenced susceptibility or resistance to indigenous type C virus-caused disease in LC wild mice.—J Natl Cancer Inst 57: 585–590, 1976.

Wild mice (*Mus musculus*) from a trapping area in southern California near Lake Casitas (LC) show, in comparison to most other wild mice, a high level of indigenous type C virus gs antigen expression; with increased age, they are prone to spontaneous lymphoma, other cancers, and a neurogenic hind leg paralysis (1, 2). We have presented evidence that the indigenous type C virus is the essential determinant of both lymphomatous and paralytic diseases (2, 3).

In this paper, we indicate that LC mice generally have infectious virus early in life, and that the serum gs antigen level is elevated especially in individual mice destined to develop paralytic disease. These findings help to explain the pathogenesis of type C virus-related diseases in wild mice.

MATERIALS AND METHODS

Live wild mice from the LC trapping site (1) were brought to the laboratory, weighed, and bled by retro-orbital puncture. They were shown to be completely free of antibodies by hemagglutination inhibition and CF tests to a battery of 16 murine virus antigens.[8] Newly trapped nonpregnant LC mice were either promptly killed for collection of tissues or individually housed in Mason jars and allowed to live out their life-spans under laboratory observation (4). Some LC mice, pregnant when trapped, were promptly killed to obtain fetal and maternal tissues; others delivered their progeny, which were individually housed in the laboratory and killed at various predetermined postnatal intervals from 1 to 42 days. Necropsies of all sick, paralyzed, or suspect tumor-bearing mice under long-term laboratory observation were made for microscopic and virologic study.

Established procedures were used for histopathology, EM, and CF tests (5). The routine EM screening procedure consisted of a thorough scan at 15,000× of three grids from each of two blocks of tissue. To be positive, more than one typical budding or extracellular type C particle had to be seen. For CF testing, individual whole embryos and separately pooled tissues (viscera, carcass,

liver, spleen, thymus, muscle) from 3–4 fetal and postnatal littermates up to 21 days of age were collected; at 42 days of age tissues from individual mice were mostly tested by CF. Some fetal and newborn mice were rinsed with ether to prevent possible virus contamination from the maternal genital tract. Primary tissues and tissue culture cell packs were sonically disrupted, and 10% (wt/vol) aqueous extracts were tested by CF at 1:2 and 1:4 antigen dilutions. In mice under 15 g, individual spleen and thymus extracts were, however, approximately 5% (wt/vol) strength because of the small size of these organs. MuSV rat serum pools #30, 322, 3490, and 4918 were used during this study. By comparison with guinea pig antisera to purified MuLV gs (p30) antigen (6), they were sensitive and specific at the dilution used (1:20) for murine gs (p30) antigen.

For virus isolation, 10% tissue extracts frozen at −70° C for 1–6 months were thawed; 0.1 ml was assayed undiluted and, in most instances, also at a 10^{-1} dilution for induction of gs antigen detected by CF on secondary NIH Swiss embryo cells after 21 days (COMUL) (7). The same MuSV rat sera were used. Some of the cultures that were COMUL-negative at 21 days were passaged and retested after 42 days. Since little difference was noted in gs antigen or infectious virus detection whether undiluted or 10^{-1}-diluted extracts were used and whether or not fetuses and newborns were rinsed with ether before dissection, these data are combined in the "Results" section.

Fibroblast cultures initiated from minced whole embryos with or without initial ether rinse were fed Eagle's minimum essential medium with 10% heated fetal calf

serum, 2 mM glutamine, 100 U penicillin/ml, and 100 µg streptomycin/ml. Cultures were grown in 75-cm² Falcon flasks incubated at 37° C in 5% CO₂ and subcultured once weekly with 0.1% trypsin in 0.02% Versene in Hanks' balanced salt solution. Supernatants (0.2 ml) were harvested in primary or secondary subpassage and assayed undiluted for infectious virus by COMUL on secondary NIH Swiss embryo cells.

Sera were analyzed for MuLV gs (p30) antigen in Dr. Frank Dixon's laboratory (Scripps Clinic and Research Foundation) by a double antibody RIA that is a modification of the methods described by Oroszlan et al. (8) and Parks and Scolnick (9). The Scripps' leukemia virus p30 was labeled with ¹²⁵I (10); guinea pig antisera to MuLV p30 from Dr. R. Gilden (Flow Laboratories, Rockville, Md.) was used, and saturated ammonium sulfate was the precipitating reagent for antigen–antibody complexes. The specificity of the RIA for MuLV p30 antigen was demonstrated by the fact that this antigen was completely precipitable by antibody prepared against MuLV p30 but not by antibody against fetal calf serum or MuLV gp69/71. Specificity of the p30 RIA was further supported by the fact that lysates of murine cell lines shown to be nonproducers of type C virus by other methods were negative by RIA for p30 antigen.

RESULTS

Type C Virus Expression in Different Weight Groups of Newly Trapped LC Wild Mice

Virus expression as measured by liver and spleen gs antigen assay by CF and virus isolation from frozen spleen extracts in newly trapped, apparently healthy, LC mice according to weight is shown in table 1. An equally high prevalence (70–80%) and titer (≥1:4) of gs antigen were detected in the extracts of liver in each age group. Due to the small size of the spleens (<60 mg) in the mice having lower weights, the extracts of this organ were only 5%, which is probably reflected in the lower amount of antigen detected in this group. In the mice having heavier weights, the prevalence and amount of gs antigen closely approximated that in the corresponding liver. In about 10% of skeletal muscle extracts, gs antigen was detected by CF. Virus was recovered in vitro from nearly 80% of spleen extracts in each weight group.

Type C Virus Expression in Young LC Mice of Known Age

Tissues from laboratory-born LC mice of known age that were the offspring of mothers caught in the wild were tested for gs antigen by CF, for type C particles by EM, and for infectious virus by COMUL (table 2). Infectious virus and gs antigen were almost completely undetected in fetal tissues. By EM, however, extracellular and budding type C particles were seen in association with hematopoietic cells in 4 of 24 fetal livers. In newborn and 3-day-old mice, although gs antigen and type C particles were almost entirely undetected, virus could be isolated from 3 of 10 pooled liver and spleen extracts. The first appreciable occurrence (13%) of gs antigen

TABLE 1.—*Type C virus expression in different weight groups of newly trapped healthy LC wild mice*[a]

Weight,[b] g	Tissue[c]	gs antigen titer by CF[d]		Virus isolation[e]
		1:2	≥1:4	
5.0–9.9	Liver	14/20[d]	14/20	
	Spleen	5/20	2/20	8/9
	Muscle	0/18	–	
10.0–14.9	Liver	15/18	15/18	
	Spleen	12/18	4/18	7/10
	Muscle	2/18	1/18	
15.0–19.9	Liver	16/22	14/22	
	Spleen	18/22	13/22	5/7
	Muscle	2/22	1/22	
Over 20.0	Liver	14/20	14/20	
	Spleen	16/20	15/20	7/8
	Muscle	0/20		

[a] Values are number positive/No. tested.

[b] Mice in these weight categories were considered successively older age groups. Mice weighing 5.0–9.9 g at trapping were estimated to be about 3 months old.

[c] Values are for individual mice (tissue not pooled).

[d] 10% (wt/vol) extracts were tested in CF vs. MuSV rat serum pools #30, 322, 3490, and 4918. Spleen extracts from mice weighing 5.0–9.9 g were only 5%.

[e] COMUL was done at 21 days on secondary NIH Swiss embryo cells.

detectable by CF in liver and spleen at low titer (1:2) was at 3 days of age. By 7–10 days of age, the frequency of low-titered gs antigen was 23% in liver and spleen and 48% in thymus. At 2–3 weeks of age, gs antigen was found by CF in higher titer (≥1:4) in liver, spleen, and thymus in 60–67% of LC mice and at 42 days in 100% of individual liver and pooled liver, spleen, and thymus extracts. In LC mice 6 weeks of age and older, the titer of gs antigen in liver and spleen often reached end points of 1:8–1:32. From 14 to 42 days of age, virus was recovered from 34 of 52 (65%) of the total assays done on liver, spleen, thymus, and muscle extracts; during this period type C particles were found by EM in 4 of 9 spleens and 6 of 10 thymuses. After 14 days of age a strong positive correlation was observed between the three assays when done on the same tissues. However, virus was sometimes recovered from skeletal muscle despite the absence of gs antigen detectable by CF in the corresponding tissue extract.

Evidence for a Litter Effect on Type C Virus Expression

Liver and spleen pools from each of 5 littermates from 8 newly trapped pregnant LC wild mice were tested by CF at 2 weeks of age for gs antigen and infectious virus (table 3). A considerable litter-to-litter variation was noted in the prevalence of detectable gs antigen and infectious virus in liver and spleen. In the different litters, antigen was found in 0, 1, 2, 3, 4, or all 5 littermates. Virus was isolated from the corresponding gs-positive tissues and occasionally from gs-negative tissues. In at least one litter (#4), virus expression appeared significantly less than in the other litters. In several litters (#1, #3 and #5), virus expression seemed exceptionally high in that CF assays revealed that vir-

TABLE 2.—*Type C virus expression in healthy LC wild mice of known age, born in captivity*[a]

Age	Tissue	gs antigen titer by CF[b]		Type C particles by EM	Virus isolation[c]
		1:2	≥1:4		
Fetal	Pooled viscera[d]	0/18		4/20(liver)	1/18
	Pooled carcass[d]	0/5			0/5
	Individual whole embryo	1/8	0/8		0/8
Newborn	Pooled liver and spleen[e]	1/40 (2.5%)	1/40	0/4(spleen)	1/4
	Pooled thymus[e]	0/16			
	Pooled muscle[e]	0/6			
3 days	Pooled liver and spleen[e]	4/30 (13%)	0/30	0/4(spleen)	2/6
	Pooled thymus[e]	1/2	0/12	0/1	
	Pooled muscle[e]	0/20			
7-10 days	Pooled liver and spleen[e]	11/48 (23%)	1/48	1/4(spleen)	1/16
	Pooled thymus[e]	11/23	3/23	4/4	
	Pooled muscle[e]	0/31			
14 days	Pooled liver and spleen[f]	24/40 (60%)	20/40		17/23
	Pooled thymus[e]	8/8	6/8		1/2
	Pooled muscle[f]	4/5	2/15		1/3
21 days	Pooled spleen[e]	14/21 (67%)	12/21	2/4	2/3
	Pooled liver[e]	15/21	12/21		
	Pooled thymus[e]	17/20	15/20	4/5	1/4
	Pooled muscle[e]	0/20			
42 days	Individual liver[g]	26/26 (100%)	24/26		
	Pooled liver[h]	3/3	2/3		1/1
	Individual spleen[g]	4/17	0/17	2/5	3/4
	Pooled spleen[h]	3/3	2/3		1/1
	Individual thymus[g]	6/17	0/17	2/4	
	Pooled thymus[h]	5/5	5/5		3/3
	Individual muscle[g]	1/26	0/26		4/7
	Pooled muscle[h]	0/3			0/1

[a] Values are number positive/No. tested.

[b] Same as footnote [d] in table 1. Individual spleen and thymus extracts from 42-day-old mice were only 5%.

[c] Same as footnote [e] in table 1, except some of the COMUL's negative at 21 days were redone after 42 days.

[d] Viscera and carcass pools from several fetal littermates were collected from 9 pregnant mice of approximately 16-20 days' gestation. Individual whole embryos were from 4 separate litters of approximately 8-15 days' gestation. The single fetus whose extract yielded infectious virus was not prerinsed with ether.

[e] Each assay represents tissues pooled from 3-6 littermates, each littermate pool from a different mother. The single newborn yielding infectious virus was prerinsed with ether.

[f] Tissues were pooled from 5 mice in each of 8 litters from different mothers.

[g] Individual tissues were from nonlittermates and were different mice than those used for tissue pools.

[h] Pooled tissues were from nonlittermates.

TABLE 3.—*Type C virus expression at age 2 weeks in individual littermates from different newly trapped pregnant LC wild mice*[a]

Litter No.	gs antigen titer by CF		Virus isolation
	1:2	≥1:4	
1	4/5	3/5	5/5
2	2/5	0/5	4/5
3	5/5	5/5	5/5
4	0/5		1/5
5	5/5	4/5	ND[b]
6	3/5	0/5	ND[b]
7	4/5	1/5	ND[b]
8	1/5	0/5	2/3

[a] Values are number positive/No. tested; pooled liver and spleen were used.

[b] ND = not done.

tually each mouse had high-titered (≥1:4) gs antigen and infectious virus in liver and spleen.

Type C Virus Expression in Genital Tissues of Newly Trapped Adult LC Wild Mice

Genital tissues from random samples of newly trapped healthy LC mice (including pregnant mice whose fetal tissues were included in table 2) were exam-

ined for gs antigen by CF, for type C particles by EM, and for infectious virus by COMUL (table 4). In 36 of 52 extracts (70%) of ovary and uterus, and in 5 of 5 (100%) epididymis extracts, gs antigen was detected; it was detected in only 1 of 26 placenta extracts. Numerous type C particles, including budding and extracellular virions, were seen in ovary and uterus specimens but not in placenta or testis. Virus was isolated from 23 of 38 (61%) extracts of ovary, uterus, and placenta, from 12 of 12 (100%) extracts of amniotic fluid, and from 9 of 10 (90%) extracts of testis and epididymis. Virus was recovered from 10 placenta and testis extracts that were negative by CF or EM.

Type C Virus Expression in Cultures of Whole Embryos

Fibroblast cultures established from 18 whole embryos (12-19 days estimated gestation) from 12 newly trapped LC mice were examined in primary or secondary subpassage for infectious virus by COMUL on secondary NIH Swiss embryo cells. In primary culture, virus was isolated from 1 of 4 embryos rinsed in ether and 3 of 6 embryos not rinsed in ether; in secondary subculture of different embryos, virus was isolated from 1 of 2 em-

TABLE 4.—*Type C virus expression in genital tissues of newly trapped adult LC wild mice* [a]

Tissue [b]	gs antigen titer by CF [c] ≥1:2	Type C particles by EM	Virus isolation [d]
Ovary	15/26	4/7	9/13
Uterus	21/26	10/15	7/12
Placenta	1/26	0/8	7/13
Amniotic fluid	ND [e]	ND [e]	12/12
Testis	ND [e]	0/4	5/5
Epididymis	5/5	ND [e]	4/5

[a] Values are number positive/No. tested.

[b] Tissues were obtained from 26 pregnant females and 5 adult males. Data on fetal tissues from 13 of these 26 pregnant mice are shown in table 2. Of these 13 mice, virus was isolated from genital tract tissues in 10. The amniotic fluid samples were taken from 6 littermates in each of 2 litters from different mothers.

[c] Same as footnote [d] in table 1.

[d] Same as footnote [e] in table 1.

[e] ND = not done.

bryos rinsed in ether and 4 of 6 embryos not rinsed in ether.

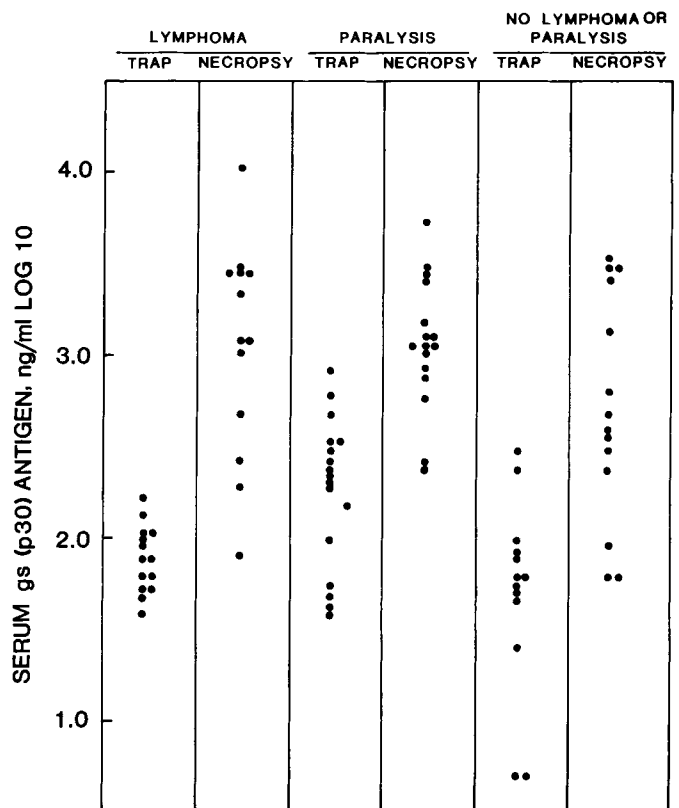## Serum gs (p30) Antigen Titer in Relation to Disease

Serum p30 antigen titers (ng/ml) were determined by RIA on the same individual 45 LC mice at trapping and 1–2 years later at death (text-fig. 1). The mice weighed 10–20 g at trapping and were assumed to be young adults at that time. Of these mice, 16 ultimately developed paralysis, 15 developed lymphoma, and 14 died from other causes. The average observation time in the laboratory was 10 months for the paralyzed mice, 15 months for the lymphomatous mice, and 24 months for mice remaining free of these diseases. Considerable individual variation was noted in p30 levels (text-fig. 1). However, mice destined to develop paralysis generally had a higher p30 titer at trapping than did those later developing lymphoma or remaining free of paralysis and lymphoma. At trapping, 11 of 16 (69%) mice eventually developing paralysis had serum p30 titers greater than 100 ng/ml, and only 4 of 15 (27%) developing lymphoma and 2 of 14 (14%) remaining free of these diseases had these serum p30 titers. When the paralysis group was compared with the combined lymphoma and "normal" groups by the Mann-Whitney U test ([11]), this difference was statistically significant ($P=0.004$). Mice dying with lymphoma showed a slightly, but not statistically significant, higher geometric mean titer of serum p30 at trapping than did mice that did not develop lymphoma. At death, markedly elevated serum p30 titers (>200 ng/ml) were found in almost all mice regardless of diagnosis, but mice dying with lymphoma or paralysis had slightly higher (not statistically significant) geometric mean titers (305 ng/ml) than did those dying from other causes (275 ng/ml).

DISCUSSION

These findings confirm and extend our earlier observations that a markedly elevated indigenous type C virus activity is present in LC wild mice. In addition to a high prevalence and amount of spleen gs antigen and type C

particles in adult LC mice ([1]), we now show that gs antigen and infectious virus are first detectable in liver and spleen in the perinatal period and can be found thereafter in a more widespread tissue distribution, including the genital tissues. The positive correlation between detection of gs antigen at higher titer (≥1:4), type C particles, and isolatable virus in about 80% of LC mice after several weeks of age and the rapidly increasing incidence of high-titered gs antigen with age indicates that, by 1–2 months of postnatal life, most LC mice are extensively replicating infectious virus. Viremia probably accounts for the occasional virus isolations from skeletal muscles, placenta, and other gs antigen-negative tissues as well as for the elevated serum p30 levels. In inbred mouse strains, serum p30 levels greater than 100 μg/ml were invariably associated with recovery of infectious virus from spleen cultures ([12]). Furthermore, in recent studies ([13], [14]), we demonstrated that about 80% of adult LC mice have free infectious virus and high levels of particulate reverse transcriptase activity in their sera.

Virus appearance in primary and secondary subpassage LC embryo cultures and early in postnatal life could be explained by transplacental epigenetic virus spread or spontaneous activation of endogenous, genetically integrated (vertically transmitted) virus genomes with subsequent cell-to-cell spread. The opportunity for epigenetic spread of virus among LC mice is amply afforded from the male and female genital tracts, including placentas and amniotic fluid, as well as postna-

TEXT-FIGURE 1.—Serum p30 antigen levels in relation to disease, as determined by RIA in individual LC wild mice at trapping and later at necropsy.

tally in milk (15) and saliva (16). In this regard suscepti-ble laboratory mice have been readily infected with the LC virus by foster nursing on LC females (Gardner MB, Klement V: Unpublished observations). Virus isolation from embryo cultures shows that the recovery of infectious virus was about twice as common in embryos that were not prerinsed with ether than in prerinsed ones. This suggests that external virus contamination from the maternal reproductive tract probably does account for some virus isolations, but it is not the only explanation for infectious virus recovered from the cultured embryos. The litter variation in virus expression also indicates a maternal influence on virus expression but does not establish whether this is a positive effect from epigenetic virus transmission or a negative effect from passively transferred antivirus neutralizing antibody. However, the former possibility seems most likely, since LC mice appear to have an ineffective humoral immune reactivity to their indigenous type C virus (13).

The cumulative disease incidence curves in aging LC mice indicate that, after a hiatus of 5–6 months, only 4% of the surviving mice develop either lymphoma or paralysis during each subsequent month of laboratory observation (4). We showed that little difference exists in prevalence and titer of spleen CF gs antigen at necropsy in tumor-bearing or paralyzed LC mice as compared with age-matched non-tumor-bearing and non-paralyzed LC mice (4). A similar conclusion was reached in a comparison of spleen p30 and gp69/71 antigen titers by RIA in lymphomatous, paralyzed, and normal LC wild mice (August JT: Personal communication). Little difference was also found between lymphomatous, paralyzed, and age-matched LG mice free of these diseases in the serum p30 antigen titers by RIA at death. However, a comparison of serum p30 antigen titers at trapping suggests that LC mice with the highest titer viremia early in life are those most at risk to the paralytic disease. Possibly only in those LC mice has virus infection been established in the central nervous system at a critically early age. On the other hand, LC mice destined to develop lymphoma could not be distinguished at trapping by the serum p30 titer from LC mice remaining lymphoma-free for a similar time. A more accurate index of virus load early in life (e.g., serum or tail infectious virus titers at 3–6 weeks of age) might better show in LC mice as in AKR × BALB/c crosses (17) a positive correlation in individual mice between virus titer early in life and relative risk to lymphoma.

These findings indicate that, although virus is the essential determinant of lymphoma and paralysis, factors other than virus titer alone are obviously involved in the pathogenesis of these diseases. The type C virus population in these wild mice is complex, consisting of a mixture of mouse-tropic virus and a new class of type C virus with a broad (amphotropic) host range (18–20). Both classes of virus are N-tropic for mouse cells and thus would be detected by the virus isolation technique used in this study. Preliminary in vivo transmission experiments with limiting dilution-cloned or fluorescent focus-purified viruses suggest that the mouse-tropic strains can induce both the paralytic disease and lym-

phoma, whereas the amphotropic strains induce only lymphoma [(20); Parker JC, Hartley JW: Personal communication]. Therefore, the differences in serum p30 levels detected early in life could represent different ratios of these two classes of virus. Although both classes are apparently subject to $Fv-1^b$ gene restriction, it is not yet determined if other genetic loci known to control susceptibility or resistance to viral leukemogenesis in laboratory mice (21) are also operative against each class of virus in wild mice. It will be of interest to relate lymphoma-paralysis resistance or susceptibility in individual LC wild mice with such genetic markers and with various measures of humoral and cellular immunity that may regulate the expression of each type of virus.

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