Proc. Nat. Acad. Sci. USA
Vol. 71, No. 5, pp. 1957–1961, May 1974

Widespread Natural Occurrence of High Titers of Neutralizing Antibodies to a Specific Class of Endogenous Mouse Type-C Virus

(dominant genetic trait/RNA virus/cancer)

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Communicated by Robert J. Huebner, January 22, 1974

ABSTRACT **Mouse cells contain several biologically distinguishable endogenous type-C viruses. In the present studies, the immunologic response of different strains of mice to the natural expression of these viruses has been examined. High titers of neutralizing antibodies have been detected to a class of virus that lacks the ability to exogenously infect mouse cells. The expression of this virus, *in vivo* as well as *in vitro*, is shown to be vertically transmitted as a dominant genetic trait. The findings indicate the widespread occurrence of this endogenous virus and the ability of the mouse to immunologically respond to a class of type-C virus whose mode of transmission is similar to that of cellular genes.**

RNA type-C viruses that occur *in vivo* spontaneously (1) or after exposure to physical agents (2) are etiologically linked to leukemias of mice. Studies in tissue culture first suggested (3) and later demonstrated (4, 5) that these viruses exist in an unexpressed form in all cells. More recent genetic and biochemical evidence has shown that multiple biologically distinguishable type-C viruses are naturally integrated within the mouse-cell genome (6–8). These findings and the demonstration that at least one class of inducible mouse type-C virus causes lymphatic leukemia *in vivo* (10) have provided strong support for the hypothesis of Huebner and coworkers (11) that integrated RNA type-C viruses are etiologically involved in naturally occurring cancers.

Studies in tissue culture have shown that distinguishable endogenous type-C viruses are affected differently by intrinsic regulatory factors of the cell (7, 9, 12). However, little is yet known about virus regulation *in vivo*. In particular, the host immune system, if it were to recognize an endogenous virus as foreign, might influence both its expression and biologic activity. Thus, in the present report, the occurrence of natural antibodies to several classes of endogenous type-C viruses has been investigated. It has been possible to detect high titers of serum-neutralizing antibodies to one class of viruses, and at the same time to demonstrate widespread occurrence of this virus in many strains of mice. The ability of the animal to immunologically respond to a specific, naturally integrated type-C virus provides ready serological procedures for studying the natural history of this class of viruses in its natural host.

MATERIALS AND METHODS

Mice. C57BL/6N, NIH/N, BALB/cN, DBA/2N, CBA/HN, C3H/HEN, AL/N, NZB/BLN, NZW/BLN, A/HEN, and AKR/N mice were obtained from the colonies of the National Institutes of Health; C58/J and C57BL/10Sn mice were from the Jackson Laboratory, Bar Harbor, Me. F_1 hybrid mice were bred in our laboratory. Genetic crosses are designated with the female listed first.

Cell Culture. Cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum (Colorado Serum Co., Denver, Colo.), in 100 × 20-mm plastic petri dishes (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.). The cells used included clonal lines of continuous contact-inhibited mouse cells, BALB/3T3 (13) and NIH/3T3 (14), and a normal rat kidney (NRK) (15) line. Secondary cultures of individual embryos of NIH/N Swiss (NIH), NZB/BLN(NZB), BALB/cN (BALB/c), (NIH × NZB)F_1, and (NIH × BALB/c)F_1 genotypes were prepared by methods described (12).

Viruses included clonal strains of two chemically induced endogenous viruses of BALB/c mouse cells, BALB:virus-1 and BALB:virus-2 (7, 9), and a strain of induced mouse leukemia virus (MuLV) from C58 embryo cells (C58-MuLV) (7, 12). Spontaneously replicating NZB-MuLV was obtained from tissue culture fluids of NZB embryo cells in culture (16). Rauscher (R)-MuLV and Kirsten (Ki)-MuLV (6) have been described. MuLV pseudotypes of the Kirsten strain of murine sarcoma virus (KiMSV) were obtained by infection of appropriate KiMSV-transformed nonproducer lines (17, 18) with MuLV by published methods (17). Since BALB:virus-2 and NZB-MuLV could be propagated in rat but not in mouse cells, all virus stocks were grown in rat-derived NRK cells to control for the specificity of the neutralization reactions. Rat type-C virus, known to be present in NRK cells and inducible by halogenated pyrimidines (38), was shown to be unaffected by any of the normal mouse sera used in these studies (data not shown).

Virus Assays. Neutralization tests were performed by a focus reduction method (7). Virion-associated RNA-directed DNA polymerase activity in tissue culture fluids was assayed as described (12).

Antisera. Mouse sera were obtained by carotid artery puncture. Standard rat antisera against Moloney-MSV (R-MuLV) and antisera against AKR-MuLV, which neutralize the Friend–Moloney–Rauscher (FMR) and Gross-type subgroups of MuLV, respectively (3), were generously provided by R. Wilsnack, Huntingdon Research Laboratories, through

Abbreviations: MuLV, murine leukemia virus; Ki-MuLV and R-MuLV, Kirsten and Rauscher strains of MuLV, respectively.

TABLE 1. *High titers of neutralizing activity against BALB:virus-2 in sera of normal BALB/c mice*

Serum		MuLV pseudotype of Ki-MSV* (% reduction of focus formation)			
Source	Final dilution	R-MuLV	Ki-MuLV	BALB:virus-1	BALB:virus-2
Rat					
anti-Rauscher	1:100	95	<10	15	20
anti-AKR	1:100	<10	95	95	95
BALB/c					
2 months	1:20	<10	<10	<10	97
	1:200	—	—	—	90
6 months	1:20	<10	<10	<10	98
	1:200	—	—	—	92
24 months	1:20	<10	<10	<10	100
	1:200	—	—	—	92

* Neutralization tests were performed by the focus-reduction method (7). Around 100 focus-forming units (FFU) of each MuLV pseudotype of KiMSV were incubated with the appropriate dilution of neutralizing antisera for 30 min at 37°. BALB:virus-2 pseudotypes were assayed on polybrene (2 μg/ml)-treated NRK cells, whereas each of the other pseudotypes was assayed on polybrene-treated NIH/3T3 cells. The number of MSV foci was scored at 7 days. Results are expressed as mean values of three separate experiments.

the auspices of the Resources and Logistics Segment, NCI. All sera were heat-inactivated at 56° for 15 min, and passed through a 0.45-nm Millipore filter before use in neutralization assays.

RESULTS

Natural Immunity to BALB:Virus-2 in Sera of BALB/c Mice. BALB/c mouse cells contain at least two biologically distinguishable endogenous type-C viruses (7, 9). The first, designated BALB:virus-1, grows inefficiently in BALB/c cells but replicates to high titer in cells of NIH mouse origin (9, 12). The other, BALB:virus-2, is not detectably infectious for either BALB/c or NIH cells (7), prototype strains that grow almost all other MuLV isolates. However, this virus replicates efficiently in cells of rat origin (7). To determine the natural host response to these viruses, sera from BALB/c mice were tested for virus-neutralizing activity. As shown in Table 1, pooled sera from BALB/c mice at 2, 6, and 24 months of age were each strikingly inhibitory to BALB:virus-2. At 1:200 dilution, each serum inhibited the infectivity of this virus by more than 90%. In contrast, the same sera did not detectably neutralize BALB:virus-1 or representative viruses of the Gross and Friend—Moloney–Rauscher (FMR) subgroups, Ki-MuLV and R-MuLV. The results of neutralization tests with standard Gross and FMR typing sera indicated that both BALB:virus-1 and 2 were effectively neutralized by antisera against Gross but not by antisera against FMR (Table 1). The neutralization patterns observed indicate that the inhibitory activity of normal BALB/c sera against BALB:virus-2 was attributable to natural antibodies directed against specific envelope antigen(s) of that virus.

Tests for Naturally Occurring Antibodies to Other Endogenous Viruses. Sera from a number of strains of mice from which type-C viruses have been activated *in vitro* were tested for natural antibodies to different classes of inducible type-C

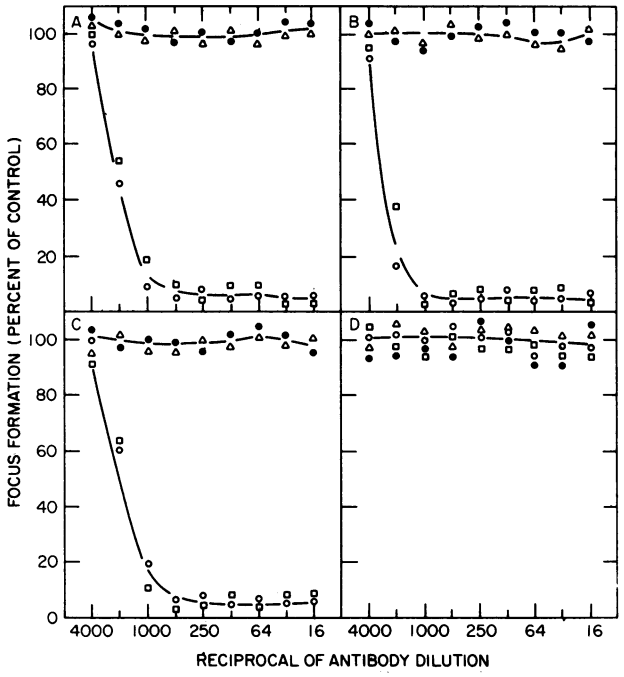

FIG. 1. Virus specificities of neutralizing antibodies in sera of different mouse strains. Neutralization tests were performed as described in the legend to Table 1. MuLV pseudotypes of KiMSV tested included: BALB:virus-1, △; BALB:virus-2, ○; C58-MuLV, ●; and NZB-MuLV, □. Sera from the following mouse strains were tested: (*A*) BALB/c; (*B*) NZB; (*C*) C58; and (*D*) NIH.

viruses. The results in Fig. 1*A* show that BALB/c sera that markedly neutralized BALB:virus-2 at 1:1000 had no detectable activity against BALB:virus-1 or C58-MuLV at the lowest dilution tested, 1:20. However, NZB-MuLV, spontaneously produced in culture by NZB embryo cells, was neutralized by BALB/c sera at an endpoint comparable to the titer against BALB:virus-2. Sera from NZB mice neutralized both NZB-MuLV and BALB:virus-2 at high titer but did not inhibit any of the other endogenous viruses tested (Fig. 1*B*). These results demonstrate the serologic relatedness of NZB-MuLV and BALB:virus-2 and the specificity of the immunologic reactivity of those strains to this serologic class of endogenous virus.

The reactivities of sera from the C58 mouse strain, which has a high leukemia incidence, are shown in Fig. 1*C*. These contained no detectable neutralizing activity for C58-MuLV or BALB:virus-1 but had very high neutralizing titers against both BALB:virus-2 and NZB-MuLV. These findings indicate that this high leukemic incidence strain contains an additional endogenous virus, serologically distinct from C58-MuLV or BALB:virus-1, and related to BALB:virus-2. Sera from the NIH mouse strain lacked detectable neutralizing activity against any of the endogenous viruses tested (Fig. 1*D*). It should be noted that in tissue culture, NIH embryo cells are not virus-inducible either spontaneously or after exposure to chemical activators under conditions where virus induction is readily demonstrable with cells of other strains (12, 19).

Natural Antibodies to a BALB:Virus-2-like Class of Endogenous Virus in Other Mouse Strains. The above results suggested a serologic test for the presence of naturally occurring viruses similar to BALB:virus-2 in other mouse strains.

TABLE 2. *Neutralization of endogenous type-C viruses by sera from different strains of mice*

Sera from:*	No. of sera tested	Neutralizing antibody titer against† BALB: virus-1	BALB: virus-2	C58-MuLV
NIH	10	<20	<20	<20
C57BL/6N	3	<20	500–2000	<20
C3H/HEN	3	<20	500–2000	<20
AKR/N	3	<20	500–2000	<20
AL/N	3	<20	500–1000	<20
DBA/2N	3	<20	500–1000	<20
NZW/BLN	3	<20	500–1000	<20
CBA/HN	3	<20	500–1000	<20
A/HEN	3	<20	500–1000	<20
C57BL/10Sn	3	<20	500–1000	<20

* Sera were from mice at 2–3 months of age.

† Neutralization tests were performed as described in the legend to Table 1. Results are presented as the reciprocal of the highest serum dilution giving 67% or greater reduction in the number of MSV foci when tested against approximately 100 FFU of the appropriate MuLV pseudotype of KiMSV.

While the lack of BALB:virus-2-neutralizing activity in sera of a specific strain would not necessarily indicate that the viral genetic information was absent, the detection of such activity would provide strong evidence for the natural occurrence and *in vivo* expression of this virus class. Individual sera from adult mice of a number of different strains were, thus, tested for neutralizing activity against BALB:virus-2. As shown in Table 2, sera from only one strain, NIH, lacked detectable neutralizing antibodies to BALB:virus-2. Sera from every other strain tested contained high titers of antibodies to BALB:virus-2. The specificity of their reactivity was indicated by the absence of detectable neutralizing antibodies to BALB:virus-1 or C58-MuLV. These results provide evidence for the existence and natural expression of an endogenous virus serologically related to BALB:virus-2 in a large number of mouse strains.

Neutralizing Antibodies to Inducible Viruses by Immunization of a Heterologous Species. One possible explanation for the presence of naturally occurring high titers of neutralizing antibodies against one but not other classes of endogenous type-C virus in sera of different mouse strains could be differences in the immunogenicities of the viruses. To examine this possibility, the immunologic response of a heterologous species to purified virus preparations was examined. The magnitude of the immune response in rabbits immunized against BALB:virus-2 was very similar to that obtained against other induced viruses tested, including BALB:virus-1 and C58-MuLV (data not shown). These findings suggest that the differences in the natural immune response of mice to different endogenous viruses are due to factors other than differences in the immunogenicities of the viruses, themselves.

Genetic Factors Involved in Expression of the Class of Endogenous Viruses Resembling BALB:Virus-2. The serologic relatedness of BALB:virus-2 and NZB-MuLV demonstrated above led us to compare in more detail the expression *in vitro* and *in vivo* of these two viruses. As shown in Table 3, NZB embryo cells in culture spontaneously produced readily detectable amounts of type-C virus. The virion-associated RNA-directed DNA polymerase activity in tissue culture fluids of five separate embryo cultures and 10 different clonal lines derived from one of these embryos ranged from 20 to 50 pmol of [^{3}H]dTTP per ml per 10^6 cells under conditions where supernatants of virus-negative cells incorporated less than 0.2 pmol/ml. The viruses released were each related to BALB:virus-2, both in serologic characteristics and host range (7). The level of spontaneous virus production by BALB/c and NIH cells was below the sensitivity of the polymerase assay. Thus, each released at least 100-fold less virus than NZB embryo cells. Chemical induction of BALB/c cells resulted in transient type-C virus production at one-tenth to one-half the level spontaneously released by NZB cells. Other studies have shown that BALB/c cells spontaneously release BALB:virus-2 at a very low frequency (20). The serologic status *in vivo* of each of these three mouse strains correlated with their virologic status *in vitro*. High titers of neutralizing antibodies against BALB:virus-2 and NZB-MuLV were present in NZB and BALB/c sera but not in the sera of NIH mice.

TABLE 3. *Effect of genetic crosses of BALB/c or NZB with NIH mice on endogenous virus expression in tissue culture and in vivo*

Strain	*In vitro:* Supernatant RNA-directed DNA polymerase activity (pmol/ml)* Spontaneous	IdU-activated	*In vivo:* Serum neutralizing antibody titer against† BALB: virus-1	BALB: virus-2	NZB-MuLV
Parental					
NIH	<0.2	<0.2	<20	<20	<20
BALB/c	<0.2	5–10	<20	1000	1000
NZB	20–50	N.T.	<20	1000	1000
F_1 hybrid					
(NIHxBALB/c)F_1	<0.2	5–10	<20	500	500
(NIHxNZB)F_1	5–20	N.T.	<20	1000	1000

* Supernatant fluids were assayed for poly(rA)·oligo(dT)-directed (dT) incorporation by methods described (12). Results are expressed as pmols of [^{3}H]dTTP incorporated per milliliter of tissue culture fluids per 10^6 cells. *N.T.* means not tested.

† Neutralization tests were performed as described in the legend to Table 1. The results represent the mean values from separate tests of sera from three animals in each group.

Previous studies have shown that the capacity of mouse cells to be chemically activated to produce type-C virus in tissue culture is inherited as a dominant genetic characteristic (12). The serologic assay for spontaneous expression *in vivo* of BALB:virus-2 or NZB-MuLV made it feasible to examine the expression of these viruses in genetic crosses of BALB/c or NZB strains with the noninducible NIH strain. As reported previously (9, 12) and confirmed by the results in Table 3, embryo cells of the (NIH × BALB/c)F_1 genotype were virus-inducible in culture. (NIH × NZB)F_1 embryo cells spontaneously produced virus, although the amount released was somewhat lower than that released by parental NZB cells. *In vivo*, sera of (NIH × BALB/c)F_1 and (NIH × NZB)F_1 hybrid mice were found to contain high titers of neutralizing anti-

bodies to BALB:virus-2 and NZB-MuLV. These results establish that the phenotypic properties, *in vitro* and *in vivo*, of both BALB:virus-2 and NZB-MuLV were transmitted as dominant genetic characteristics in F_1 hybrids of BALB/c or NZB strains with NIH mice.

DISCUSSION

Immunologic tolerance, a phenomenon whereby the immune system does not recognize components of the body as foreign, apparently develops during embryonic life (21). Burnet and Fenner (22) proposed that immunologic tolerance can also be induced to viruses by infection of the host *in utero*. More recent studies with several viruses, including LCM, LDH virus, and Aleutian mink disease (23–25), that establish chronic congenital infections indicate that these viruses do elicit an immunologic response by the host. RNA type-C viruses, in contrast to these nongenetically transmitted viruses, bear a unique relationship with the host in that they naturally exist within the cellular DNA. Endogenous viruses like BALB:virus-2 appear to be limited to the genetic mode of transmission due to their inability to productively infect cells of the host (7, 20). The present findings of high titers of neutralizing antibodies to this class of virus in normal mouse sera clearly indicate that the mouse is not tolerant to at least one virus whose mode of transmission is similar to that of cellular genes.

The mechanisms by which the host immune system recognizes this endogenous virus as foreign and the effects of the immune response on its biologic activity *in vivo* remain to be explained. The antibodies might be produced as a defense against, rather than as part of, an immune rejection mechanism against virus-expressing cells by functioning as blocking antibodies to cellular-mediated immunity (14). An alternative hypothesis would implicate the naturally occurring viral antibodies as components of the host defense against malignancy. Here, malignant transformation of a cell would directly or indirectly result in increased expression of specific viral-antigens on the cell surface and, thus, lead to antibody-mediated rejection of the tumor cell. In fact, in certain instances immunologic rejection of tumors due to their replication of highly antigenic type-C viruses has been demonstrated (26, 27). It should be noted that in the present studies, all viruses were grown in cells of a heterologous species rather than in their natural host, the mouse. This was due to the fact that endogenous viruses like BALB:virus-2 can not be propagated in mouse cells. Thus, the magnitude of the immune response to this class of virus in the natural situation *in vivo* or even *in vitro* has not been directly measured.

There have been previous reports of naturally occurring, low titers of antibodies to antigens associated with Gross-type MuLV in some mouse strains (28, 29). Further, high titers of antibodies with specificities for MuLV envelope antigens have been detected in sera of several strains by radioimmunologic techniques (39). The present studies provide the first evidence of biologic activity of such antibodies and suggest that the immune responses detected in those studies may have been primarily directed against the BALB:virus-2 class of endogenous viruses but cross-reactive at lower level with Gross-type virus. Thus, there may be immunologic tolerance in the mouse to endogenous viruses, like BALB:virus-1 and C58-MuLV, analogous to the relative tolerance to one subviral component, the group-specific antigen (30), an antigen known to be expressed during embryonic development (31).

The detection of antibodies to BALB:virus-2 in sera of many strains of mice suggests the widespread natural occurrence and *in vivo* expression of this virus class. Since the strains examined are known to have large differences in their frequencies of spontaneous tumors, there does not appear to be a direct correlation between early expression of this virus and the occurrence of tumors in those strains. In contrast, a clear association has been demonstrated between strains with a high natural incidence of a specific tumor, lymphatic leukemia, and another class of endogenous virus, C58-MuLV. The C58 virus, induced from cells in tissue culture, is leukemogenic when inoculated into mice of a strain with low incidence of leukemia (10).

RNA type-C viruses have been implicated in the pathogenesis of a complex of diseases involving autoimmunity in mice (32, 33). In the NZB strain, which has a very high incidence of hemolytic anemia and renal glomerular disease, type-C viral antigens have been localized *in vivo* in the glomerular lesions (33). The present findings indicate that NZB cells have a genetically determined defect in regulatory processes that normally control expression of a specific class of endogenous virus that is naturally highly immunogenic in the NZB mouse and in many other mouse strains as well. In other strains, however, this virus is expressed at much lower levels. Thus, the defect in the cellular regulation of this virus may be a primary factor in the pathogenesis of autoimmune disease in the NZB strain.

While NIH Swiss mouse-embryo cells are nonvirus-inducible in culture (9, 12), recent evidence indicates that this strain does harbor a virus that is serologically related to NZB-MuLV; this virus can be isolated from adult NIH Swiss mice under certain conditions *in vivo* (34, 35) and from adult NIH Swiss tissues *in vitro* (19). The lack of detectable neutralizing antibodies in NIH Swiss mice to this virus class suggests that control of its spontaneous expression even in the adult may be very tightly restricted. It is clear from the present studies that whatever regulatory processes block expression of this virus in NIH cells, they are not dominant in genetic crosses with either the NZB strain, which normally generates large amounts of this virus, or the BALB/c strain, which spontaneously produces the virus at a much lower level. Because of the nonpermissiveness of mouse cells for endogenous viruses represented by BALB:virus-2 and NZB-MuLV, these viruses have recently been designated "xenotropic" (19). Xenotropic viruses are not affected by the known alleles of the Fv-1 gene (8), a gene that markedly influences the expression of other endogenous mouse type-C viruses (9, 12, 36, 37). It may be possible through genetic studies with the NZB and NIH strains to elucidate genetic factors that control the expression of this new class of viruses.

We thank Claire Y. Dunn and Roberta K. Reynolds for excellent technical assistance. This work was supported in part by Contract no. NCI-E-73-3212 of the Virus Cancer Program of the National Cancer Institute.

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