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Murine Xenotropic Type C Viruses

I. Distribution and Further Characterization of the Virus in NZB Mice

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The xenotropic mouse type C virus, originally detected in cultured embryo cells from New Zealand Black (NZB) mice, has been recovered from over 50 adult NZB animals and 15 NZB embryos. Its presence is best detected by measuring its ability to rescue a murine sarcoma virus (MSV) genome from a non-virus-producing MSV-transformed rat cell. The virus can serve as a helper for replication of MSV. It has a distinct type-specific coat and is a prototype for a third serotype of mouse type C viruses, NZB. The xenotropic virus may have an evolutionary role since it has a wide host range, including the ability to infect avian cells. It is produced spontaneously by all cells cultivated from NZB tissues and accounts for the high concentration of viral antigens associated with NZB tissues. The extent of virus production is similar in both male and female mice. All cell clones established from embryos also produce the virus. A variability in the intracellular regulation of virus replication is suggested since tissue cells from the same animal differ quantitatively in their ability to produce xenotropic viruses. Since enhanced spontaneous virus production is associated with cells from NZB mice, the virus may play a role in the autoimmune disease of this mouse strain.

The inherited disease of New Zealand Black (NZB) mice presents a spectrum of autoimmune phenomena which resembles human systemic lupus erythematosus (7, 8, 11, 17-19, 41). Type C particles have been observed in tissues from this mouse strain (12, 33, 43) and the biological activity of these viruses was demonstrated by co-cultivation techniques (30). Similar type C viruses have recently been recovered from many different strains of mice (3, 6, 26, 27, 27a, 32). They represent a separate class of murine endogenous viruses distinguished by their antigenic envelope coat and their inability to infect mouse cells. We call them xenotropic (X-tropic) because of their tropism for cells foreign to the host species (26). They differ from the standard mouse-tropic viruses which we call ecotropic (27) (oikos, Gr. home, one's environment) since the later infect and grow preferentially in cells from their own species.

X-tropic virus is expressed only by certain cells from most mouse strains, but in the NZB mouse it is produced by all embryo and adult cells, often at an unusually high titer. For this reason, we have considered that this virus may play a role in the NZB autoimmune disease. Our working hypothesis (27) is that a gene(s) in NZB mice is responsible for the enhanced expression of the endogenous X-tropic virus genome. The resulting production of the virus on cell surfaces acts like a carrier-hapten system and induces an immunologic reaction against the virus as well as normal host tissue.

In this paper we report the distribution and further characterization of the NZB isolate, NZB-MuLV (murine leukemia virus).

MATERIALS AND METHODS

Cells. NIH Swiss and BALB/c mouse embryo cells, Fischer rat embryo cells, feline, bovine, and hamster embryo cells were supplied by Microbiological Associates, Bethesda, Md. Hamster embryo cell monolayers were also prepared in this laboratory from 15-day-old embryos removed from pregnant Syrian Golden hamsters received from Simonson Laboratory, Gilroy, Calif. Mouse 3T3 cell lines (4, 23) were received from S. Aaronson, Bethesda. Cells from other animal mammalian species were provided by W. Nelson-Rees, Oakland. Avian cells were supplied by W. Nelson-Ress and D. Fugita, San Francisco, and S. Rasheed, Los Angeles. The NRK cell line, a continuous line of normal rat kidney cells established from an

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Osborne-Mendel rat (10), has been maintained in this laboratory. For the assays, a clone of these cells which was highly sensitive to the virus was selected and used. Cultures of human foreskin cells were provided by Miriam Debby, San Francisco. Each established foreskin line was used up to passage 12 when sensitivity to the NZB virus usually decreased. W138 cells at passage level 23 were obtained from Microbiological Associates. A cell line from a human wart was established in this laboratory.

NZB mouse embryo cell monolayers (NZB-ME) were cultivated from mice supplied by Norman Talal, San Francisco, and Robert Schwartz, Boston. An established line of NZB embryo cells, NZB-Q, was given to us by Marilyn Lander, Bethesda, in passage 89.

The non-virus-producing (NP) line, NRK-Harvey, was established from NRK cells originally transformed by the Harvey strain of murine sarcoma virus (H-MSV) (25). A clone of NP hamster cells transformed by Moloney (M)-MSV was isolated under agar from the original HT-1 line (20) which has been maintained in this laboratory. The non-virus-yielding BALB/c cells transformed by M-MSV, the MSV-8 line (2), were provided by S. Aaronson.

Media. Cell lines and monolayers plated for type C virus propagation were maintained in Eagle minimal essential medium (Grand Island Biological, New York) with 10% unheated fetal bovine serum, 1% glutamine (2 mM), and antibiotics (250 μm of penicillin per ml; 250 μg of streptomycin per ml). Focus formation assays were performed on cells grown in McCoy 5A medium or Eagle minimal essential medium supplemented with 5% heated (56 C, 30 min) calf serum and 1% glutamine and antibiotics.

Virus. Preparations of NZB pseudotypes of M-MSV and H-MSV (M-MSV[NZB-MuLV], H-MSV[NZB-MuLV]) were made by co-cultivation of NZB-ME with the HT-1 and the NRK-Harvey cell lines, respectively. H-MSV and M-MSV were provided by Janet Hartley, Bethesda, and passaged in this laboratory. All virus stocks were stored at −70 C. Freezing and thawing of NZB viruses more than once usually reduced their titer 10-fold.

Concentrated virus preparations. One to 2 liters of supernatants from NZB embryo cells and NZB co-cultivation cultures were collected and centrifuged for 20 min at 5,000 rpm. The supernatants were centrifuged again at 52,000 × g for 2 h in a refrigerated Spinco International centrifuge. The pellets were resuspended in 10 ml of 0.1 M Tris-NaCl (pH 7.4) and used for virus assay and a source of antigen for rabbit hyperimmunization (26, 27). By this procedure, the titer of NZB pseudotype virus preparations rose from 10^4 focus-forming units (FFU)/ml to 10^6 FFU/ml.

Cultivation of animal tissues. Lymphoid tissues from NZB adult mice and NZB embryos were removed, teased apart, and plated in petri dishes with maintenance medium. Kidneys were removed from the capsule, teased apart, and trypsinized (0.25% trypsin, Grand Island Biological) for 1 to 2 h. Cells were then sedimented by low-speed centrifugation, plated in Falcon flasks, and grown to confluency. The NZB mice were obtained from five different sources: N. Talal, San Francisco; B. Mintz, Philadelphia; R. Mishell, Oakland; R. Schwartz, Boston; and S. Poiley, Bethesda.

Leukemia virus detection. Cell cultures were generally checked for the presence of MuLV by detection of the group-specific (gs), complement-fixing (CF) antigen. Ten percent extracts of animal tissues prepared by standard technique were also tested. The CF tests were performed under the supervision of P. Hill in R. Huebner's laboratory, Bethesda, using materials and methods described previously (15).

Cultures were also examined for the induction of plaques after co-cultivation with XC cells according to the procedure of Rowe et al. (38).

Co-cultivation assays. HT-1, NRK-Harvey, or MSV-8 cells at 10^4 cells/culture were grown together with 3.5×10^5 cells of the cultivated tissue being assayed for the presence of NZB-MuLV. Virus recovery was two to three times more efficient and reproducible when these assays were performed in 60-mm plastic petri dishes rather than in plastic flasks. Culture medium was changed every 2 to 3 days. Supernatants were removed on day 7, filtered (Millipore, 0.45 nm), and assayed for focus formation on mouse, rat, and human cells (20).

Focus formation assays. All cell monolayers were prepared and maintained as previously described (14, 25, 30, 36). Twenty-four hours after plating and just prior to virus inoculation, they were treated for 30 min with DEAE-dextran (DEAE-D) (25 μg/ml) to increase the efficiency of the assay (9). Cells not treated with DEAE-D were 2 to 3 logs less sensitive to infection by X-tropic viruses. Treatment of human cells with DEAE-D for more than 30 min decreased their sensitivity two- to threefold. Moreover, washing the human cells after DEAE-D treatment was necessary for the most efficient focus formation. For all focus formation assays, virus was adsorbed for 30 min at 37 C. Focus assay readings were taken on days 5 to 9. Virus titers were recorded as FFU per milliliter. For subsequent CF testing for gs antigen (15), the monolayers were changed to Eagle minimal essential medium with 10% fetal calf serum and maintained for 21 days.

RESULTS

Detection of NZB-MuLV: development of sensitive assays. (i) CF. CF testing was used to detect the MuLV gs antigen. The antigen was found in most NZB-ME cultures and was present in the NZB-Q line up to passage 98. Tissue extracts of thymus, liver, spleen, lymph nodes, skin, and embryos from NZB mice generally contained gs antigen. Those tissues which had no detectable gs antigen always demonstrated a very low production of NZB-MuLV as measured by virus rescue technique (see below).

The gs antigen was not detected by CF in rat and human cells infected with NZB-MuLV or its pseudotype sarcoma virus unless they had

been passed several times or contained significant focus formation (>500 foci/dish). This CF technique therefore was not useful for X-tropic virus detection since substantial virus replication was required before the presence of gs antigen was recognized.

(ii) XC plaque assay. XC plaque formation, which has been a sensitive method for quantitating ecotropic MuLV, is also not applicable for detection of NZB-MuLV. Co-cultivation of NZB cells with XC cells or overlaying XC cells on co-cultivation cultures producing up to 8,000 FFU of NZB pseudotype sarcoma virus per ml yielded no syncytial formation. Moreover, NRK and human lines producing 5 to 6 logs of infectious NZB-MuLV or the NZB pseudotype virus did not form syncytia with XC cells.

(iii) Virus genome rescue. Since these standard techniques for demonstrating ecotropic MuLV were not satisfactory for detection of X-tropic virus, experiments were undertaken using virus genome rescue assays. Huebner et al. (20) first observed that non-virus-yielding MSV-transformed hamster cells (HT-1), when co-cultivated with MuLV-producing monolayer cells, yielded a replicating MSV. This virus was a pseudotype MSV since it contained the genome of the original MSV which transformed the NP cell, but had the envelope coat antigen and the host range of the MuLV which helped in its replication. Similar genome rescue experiments using NZB cells and the HT-1 line demonstrated the presence of the NZB virus which infected rat and human cells but not mouse or hamster cells (30; J. A. Levy, Abstr. Annu. Meet. Am. Soc. Microbiol., 1973, V169, p. 222). On the other hand, co-cultivation tests conducted with the NP mouse cell line (MSV-8) yielded no pseudotype sarcoma virus even after 12 days in culture.

Therefore, attempts were made to increase the sensitivity of the genome rescue assay by using NP MSV-transformed rat and human cells.

(iii) Virus genome rescue: selection of a sensitive NP cell line. When the NRK-Harvey cell line, a NP H-MSV-transformed rat cell, was co-cultivated with NZB cells 10 to 100 times more pseudotype virus was recovered than when co-cultivation assays were conducted using the HT-1 line (Table 1). Pseudotype virus production was assayed by focus formation on NRK cells. One-cell clones of the NRK-Harvey line were established as individual cell lines. One of these clones, B-4, was four times more efficient for detection of X-tropic virus in the rescue experiments than the parental line and has subsequently been used in all assays. Three NP MSV-transformed human cell lines received from P. Peebles and J. Rhim, Bethesda (35, 37), were also used successfully in co-cultivation assays. They were generally as sensitive for X-tropic virus detection as the parental NRK-Harvey line.

TABLE 1. *Representative yields of NZB pseudotype virus from co-cultivation cultures containing NZB cells and non-virus-producing MSV-transformed cells*[a]

Cell cultures	Co-cultivated	
	With NRK-Harvey	With HT-1
NZB kidney	>800	0
NZB kidney	650	3
NZB-Q line	50	3
Clone 4	3	0
Clone 28	10	1
Clone 30	40	5
Clone 2	75	0
Clone 22	125	2
Clone 12	400	1
Clone 35	>1,000	1

[a] Cell cultures derived from NZB kidneys and embryos were co-cultivated with non-virus-producing MSV-transformed rat (NRK-Harvey) or hamster (HT-1) cell lines. Figures represent the average number of foci formed in duplicate plates of NRK cells infected with 0.5 ml of filtered, 7-day supernatants from the co-cultivated cultures.

(iii) Virus genome rescue: conditions and kinetics of the assay. To ascertain the best conditions for X-tropic virus detection by genome rescue assays, the kinetics of virus production was measured using different input cell concentrations of the NRK-Harvey line and an NZB embryo cell clone. The kinetics of NZB pseudotype virus production was determined by assaying filtered fluids removed from alternate co-cultivation cultures from day 1 to day 12. In this manner the supernatant was sampled every 48 h. The results (Fig. 1) demonstrated that the optimum input of cells in a 60-mm petri dish was 3×10^5 to 4×10^5 monolayer cells and 10^4 NRK-Harvey cells. With these input concentrations, the pseudotype virus was recovered by day 3 and peak virus production (10^3 FFU/ml) was reached between days 6 and 7. The titer then remained relatively constant for 4 to 6 days. This efficient pseudotype virus recovery occurred when the monolayer cells were confluent and were 80 to 90% covered with NRK-Har-

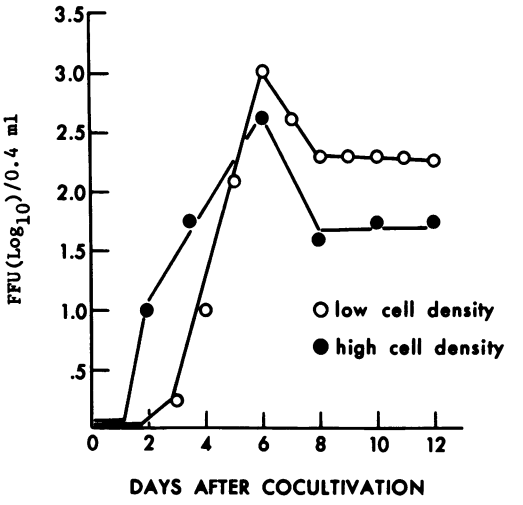

FIG. 1. *Recovery of NZB pseudotype sarcoma virus by co-cultivation of NZB cells with the NRK-Harvey cell line. The non-virus-producing, MSV-transformed NRK-Harvey cell line and a clone of NZB mouse embryo cells were co-cultivated at high and low cell input densities. The low density cultures began with 350,000 NZB cells and 10,000 NRK-Harvey cells. The high density cultures began with 800,000 cells from both cell lines. Fluids were collected daily from alternate plates so every point represents a culture supernatant after 48 h. Titer of pseudotype sarcoma virus recovered is expressed as FFU (log_{10}) as determined in NRK cells.*

vey cells. Cultures begun with a high input of cells released NZB pseudotype sarcoma virus 1 day earlier than cultures containing less input cells (Fig. 1), but the high cell density cultures yielded less virus and did not maintain efficient pseudotype virus production. The rapid decrease in pseudotype virus recovery in the high density cultures was apparently due to the complete displacement of the NZB monolayer cells by the NRK-Harvey cells. Addition of fresh NZB cells to these cultures resulted in a new burst of pseudotype virus production with often a 10-fold increase.

Distribution of NZB-MuLV in NZB mice. (i) Whole tissues. All tissues from over 50 NZB adult mice and 15 NZB embryos, cultivated as fibroblasts or epithelial-like cell cultures (e.g., kidney cells), showed the presence of a biologically active X-tropic MuLV (Table 2). The highest rescue efficiency (~1,000 to 10,000 FFU/ml) was observed with NZB fibroblasts which originated most often from lymphoid organs (e.g., spleen, thymus), but this quantity was also obtained with some cultures established from NZB kidneys and embryos. The amount of NZB pseudotype sarcoma virus recovered from co-cultivation assays of different tissues even from the same mouse often varied between 10- to 100-fold.

Female NZB mice have a more severe autoimmune disease than male NZB mice (19, 41); therefore, studies were directed at determining whether the variation in virus production by some NZB mice might be regulated by the X chromosome. Ten female and ten male mice of the same age were examined for expression of X-tropic virus. The results showed no correlation of virus recovery with sex of the animal (Table 3). The ability of tissues from male and female mice to rescue the H-MSV genome and produce pseudotype viruses varied widely, and even cultures established from separate organs from the same animal had different degrees of virus expression. This kind of variability paralleled the results of virus recovery from tissues from randomly selected NZB mice.

Although a Moloney-type MuLV has been isolated from one colony of NZB (24), no ecotropic virus has been recovered from any adult tissues taken from five different colonies of NZB mice nor from the cultivated NZB embryos.

(ii) Cloned embryo cells. To determine whether every NZB embryo cell contains NZB-MuLV, cloning of NZB primary cells was attempted immediately after trypsinization of an individual embryo. Cells were diluted and plated in wells of Microtest II dishes. Twenty-four hours later the wells were examined for

TABLE 2. *Demonstration of X-tropic NZB-MuLV in tissues cultivated from NZB mice*[a]

Tissues	No. positive/ no. assayed
Embryo	
Complete	12/12
Cell clones	42/42
Liver	1/1
Kidney	1/1
Adult	
Kidney	50/50
Thymus	9/9
Spleen	11/11
Liver	2/2
Skin	1/1

[a] Tissues were removed from NZB mice, washed, minced, and plated. Kidney tissues were trypsinized and plated. Fibroblast monolayers were co-cultivated with non-virus-yielding MSV-transformed rat cells (NRK-Harvey line). The presence of NZB-MuLV was indicated by the production by these co-cultivated cultures of an NZB pseudotype sarcoma virus as detected by focus formation in NRK cells.

TABLE 3. *Expression of X-tropic virus by cultured cells from male and female NZB mice*[a]

Pseudotype virus recovered (FFU/0.4 ml)	Male			Female		
	S	T	K	S	T	K
1–24	1			1		
25–99	2		4	2	1	4
100–500		2	3	3		2
>500		3	3	2	7	2

[a] Spleen (S), thymus (T), and kidney (K) cultures from male and female NZB mice 3 months old were co-cultivated with the NRK-Harvey cells (22). Seven-day filtered supernatants from these cultures were assayed for focus-forming virus on NRK cells. The figures given represent the number of cultures of each organ yielding pseudotype sarcoma virus particles in the range listed.

viable cells. From several wells containing originally one to three viable cells, 14 cell cultures were established in 35-mm plastic dishes. NRK-Harvey cells (10^3) were then added to each culture and the 7-day supernatants were collected and tested for the presence of NZB pseudotype virus. Twenty-eight one-cell clones from the NZB-Q line were also established by plating cell dilutions in Terasaki plates by the procedure described above. These clones were tested for their ability to rescue the MSV genome from both HT-1 and NRK-Harvey cells. As illustrated in Table 4, both the primary embryo cell lines and the NZB-Q clones showed a rescue capability which yielded NZB pseudotype sarcoma virus in a range varying from less than 10 to >500 FFU/ml. In all cases, the efficiency of virus rescue was better with the non-virus-producing rat cell line than with the HT-1 line (Table 1). Clone 35 of the NZB-Q line gave the best yields of NZB pseudotype sarcoma virus (>1,000 FFU/ml) and clone 4 gave the least (2 to 3 FFU/ml) (Table 1). The majority of the NZB clones after co-cultivation with NRK-Harvey cells produced 50 to 200 FFU of the sarcoma virus per ml. The ability of individual clones to rescue MSV has remained stable after five to six passages. This variability in virus rescue is similar to that noted with whole NZB embryos and tissues from adult mice.

Detection of NZB-MuLV production. Virus genome rescue experiments demonstrated that NZB-MuLV was associated with NZB cells. These assays, however, did not indicate whether X-tropic virus was spontaneously produced by NZB cells since cell fusion after co-cultivation may have been required for NZB-MuLV detection. Filtered supernatants from NZB cultures were therefore titered on the NRK-Harvey cells, as well as on human foreskin cells. These infected human cells, after three passages, were then co-cultivated with the NRK-Harvey cells and the production of pseudotype virus was subsequently measured. The human foreskin cells were used to enhance detection of infectious virus since they are most sensitive to NZB-MuLV infection (27, 27a, 28, see below).

These studies indicated that all cultures which can yield pseudotype virus after co-cultivation with NP cells are producing NZB-MuLV. In general, cultures from lymphoid organs of NZB mice produced the highest titers of infectious NZB-MuLV. Its presence in these cultures could be detected by direct inoculation of the supernatant fluids onto NRK-Harvey cells followed by focus assays on NRK or human cells. Supernatants from other tissue culture cells had to be inoculated first onto human cells where amplification of the virus occurred so that its detection was enhanced at least 10-fold. The largest quantity of NZB-MuLV obtained from supernatants of cultured NZB cells was about 100 to 400 infectious particles/ml as measured by titration on human cells with subsequent co-cultivation as described above.

With all the NZB cell cultures tested, the capacity for pseudotype virus production correlated with the amount of infectious X-tropic virus spontaneously produced by the cells. The extent of NZB-MuLV production could then be determined in a semi-quantitative manner by measuring the amount of pseudotype virus recovered after co-cultivation. Those cultures yielding over 500 FFU/ml after co-cultivation produced at least 100 infectious particles/0.4 ml as measured by titration in human cells with

TABLE 4. *Expression of X-tropic virus by clones of NZB cells*[a]

Pseudotype virus recovered (FFU)[b]	Primary embryo cells	NZB-Q cell clones[c]
1–24	3	4
25–99	6	10
100–500	4	11
>500	1	3

[a] The NZB cells were co-cultivated with NRK-Harvey cells as described in text. The figures given indicate the number of cell cultures whose supernatants, after co-cultivation, yielded NZB pseudotype virus in the titers listed.

[b] Number of focus-forming particles/0.4 ml as determined in NRK cells.

[c] NZB-Q is an established line of NZB-ME cells.

subsequent co-cultivation with NRK-Harvey cells and focus formation assays as described above. The NZB-MuLV in these supernatants could also be detected by pseudotype virus formation after inoculating the undiluted supernatants directly on the NRK-Harvey line. NZB-MuLV produced by other cells was only detected by inoculating the supernatants first onto human cells.

Only certain tissue extracts, usually of lymphoid origin, demonstrated detectable amounts of infectious NZB-MuLV. This virus was recovered only after the extracts were inoculated first on human foreskin cells. Circulating NZB-MuLV was detected in sera from NZB mice using this same technique.

Host range of the NZB virus. Progeny virus production was not detected on petri dish cultures of NRK cells containing less than 100 foci induced by NZB pseudotype sarcoma virus (J A. Levy, Abstr. Annu. Meet. Am. Soc. Microbiol., 1973, V 169, p. 222). However, supernatants from NRK cultures with at least 500 foci contained small amounts of progeny NZB sarcoma virus (~10 FFU/ml) as detected by focus formation assay on fresh NRK monolayers. Since replication of MSV requires a leukemia virus (1, 14, 25, 31, 36) these observations indicated that the propagation of the NZB-MuLV in rat cells was limited. To find animal cells with the best sensitivity for X-tropic virus detection a variety of cells from different animal species was tested. The NZB pseudotype sarcoma virus provided an easy marker (i.e., focus formation) for measuring infection and replication of NZB-MuLV, since the host range and replication of MSV depends on its helper MuLV (1, 16, 20, 25, 31, 36).

As noted previously (27, 27a, 28), the most efficient focus formation and best progeny production were obtained in early passaged human cells, particularly those cultivated from warts and foreskins. Less efficient growth was seen in W138 cells. The NZB pseudotype virus was infectious and could be propagated in a large variety of other mammalian cells including guinea pig, rabbit, cat, cow, bear, lion, marmoset, horse, and mongoose. It was not infectious for hamster, pig, bat, or mouse cells (Table 5: see also reference 28; Table 1), including the established NIH and BALB/c 3T3 mouse lines. No gs antigen was found in hamster cells nor mouse cells inoculated with NZB cell culture supernatants or concentrated NZB pseudotype virus preparations. The NZB-MuLV grew efficiently in duck and ring-necked pheasant cells and moderately well in quail, parakeet, and squab cells. No infection of lymphomatous-free White Leghorn (C/O) chicken cells (including *chf*-negative embryos) was detected.

Human foreskin cells were two to 10 times more sensitive for detection of X-tropic pseudotype virus than rat cells and 10 to 100 times more efficient for progeny production (27, 27a, 28). After inoculation of NZB-MuLV onto human foreskin cells, virus production approached 10^3 infectious particles/ml as measured by titration of the culture fluid in human cells with subsequent co-cultivation and focus assays as described above. The NZB-MuLV preparation inoculated onto NRK cells generally yielded only 10 infectious particles/ml. One trans-

TABLE 5. *Host range of X-tropic NZB virus: sensitivity of various animal cell cultures to infection with NZB-MuLV*[a]

Good	Moderate to low	Resistant
Human	Dog	Mouse
Rat	Deer	NIH Swiss embryo
Guinea pig	Racoon	BALB/c mouse embryo
Rabbit	Gazelle	C57/Leaden embryo
Chimpanzee	Rhesus monkey	Wild mouse kidney, San Francisco
Marmoset		*Mus molossinus* kidney
Cat	Quail	Chinese hamster
Cow	Parakeet (Australian)	Syrian Golden hamster
Water mongoose	Squab	Pig
Horse		Bat
Bear		African green monkey
Lion		
		Chicken (C/O), lymphomatous-free
Duck (Pekin)		Chicken (C/O), *chf*-negative
Duck (Moscovy)		
Ring-necked pheasant		

[a] Sensitivity of the cells was measured by degree of focus formation and progeny production after infection with a standard quantity of NZB pseudotype sarcoma virus.

formed human foreskin cell line in its seventh passage produced $10^{6.3}$ FFU/ml of NZB pseudotype virus and $10^{6.0}$ infectious NZB-MuLV as detected by titration in human cells.

Since human foreskin cells were very sensitive to NZB-MuLV, attempts were made to derive an NP MSV-transformed human foreskin cell for genome rescue assays. Although one-hit kinetics of focus formation was observed in these human cells, propagation of individual foci has not yet been possible.

Kinetics of focus formation in NZB-ME cells: helper role of NZB-MuLV. Since MuLV is necessary for focus formation in mouse embryo cells (1, 14, 25, 31, 36), the ability of the NZB-MuLV present in NZB-ME cells to "help" in focus formation by MSV was examined. A known helper-dependent pool of MSV (36) was titered on secondary NZB-ME. The dose response curve obtained varied and did not show one-hit kinetics; the virus preparation titered only $10^{3.5}$ FFU/ml. One-hit kinetics and a titer of $10^{4.3}$ FFU/ml were observed, however, after the inoculation of additional M-MuLV. These results illustrated that ecotropic MuLV can infect NZB cells and provide a helper function despite the presence of X-tropic MuLV. The experiment also indicated that the expression of X-tropic virus did not enhance the independent propagation of MSV-transformed NZB-ME cells. NZB-ME, like other mouse embryo cells, appear unable to replicate after transformation by MSV and focus formation only occurs with infection of nearby cells by progeny MSV (1, 14, 25, 31, 36). Endogenous X-tropic virus could not enhance this formation of foci in NZB-ME since any progeny MSV produced with its help carried the envelope coat of NZB-MuLV and was unable to infect neighboring mouse cells.

To demonstrate that infection and replication of MSV occurred in some NZB-ME without formation of foci, the following experiment was performed. H-MSV was titered on NZB embryo cells and, 7 days after virus inoculation, two of four cell monolayers at each virus dilution were overlaid with NRK cells. All cultures were then read 1 week later for focus formation. The rat cells were used since they are sensitive to X-tropic virus, and focus formation in these cells occurs by cell multiplication without requiring infection of neighboring cells by progeny MSV (25, 36). The results showed that the pool of H-MSV titered more than 1 log higher ($10^{4.9}$ FFU/ml) with this overlay technique than it titered in the NZB-ME alone (Table 6), and the kinetics of focus formation were one-hit. In most supernatants from cultures containing transformed NRK cells either X-tropic pseudotype sarcoma virus alone or both X-tropic sarcoma virus and parental type H-MSV were recovered. These observations indicated that H-MSV did infect NZB-ME and replicated with the help of either H-MuLV or NZB-MuLV. Focus formation was not efficient in these mouse cells since NZB-MuLV served as helper for a significant portion of the H-MSV particles, and the resulting progeny MSV carried the NZB coat. These latter NZB pseudotype viruses were only detectable by focus formation in the rat cells.

TABLE 6. *MSV infection of NZB cells*[a]

Dilution of H-MSV added	FFU/0.4 ml[b]	
	NZB	NZB/NRK
10^{-2}	50	TNC
$10^{-3.0}$	0	88
$10^{-3.5}$	0	33
$10^{-4.0}$	0	8

[a] Quadruplicate plates of NZB embryo cells were inoculated with dilutions of H-MSV. After 7 days, 5×10^5 NRK cells were added to two of the NZB monolayers. All cultures were read for focus formation 1 week later.

[b] Figures given represent the average number of foci formed (FFU) in duplicate plates of NZB-ME cells cultured alone, or NZB-ME cells overlaid with NRK cells (NZB/NRK). TNC, too numerous to count.

When H-MSV was titered directly on an established clone of the NZB-Q cell line (C1S2), the kinetics of focus formation were one-hit. Since the NZB-Q cells, unlike NZB-ME, are able to replicate independently after they are transformed, progeny production was not necessary in this case for focus formation. This same phenomena explains the one-hit kinetics observed with MSV in rat cells (25, 36) and established 3T3 mouse lines (1, 2, 5) whose MSV-transformed cells are able to replicate independently.

The helper role of NZB-MuLV was also observed when the C1S2 line was infected with an end titration of H-MSV. Cells were then infected only by H-MSV, not H-MuLV. Lines were established from nine different propagating foci. In all cases, the cells did not release any virus which was infectious for mouse cells, but did produce a virus which transformed rat and human cells. The different clones, moreover, varied in the range of virus production by up to 1 log (Table 7). The virus recovered was not neutralized by antisera to H-MSV but was neutralized by rabbit antisera prepared against the NZB virus. Certain of these lines, yielding

TABLE 7. *Production of pseudotype virus by various clones of NZB-ME cells transformed with H-MSV*[a]

Clone	FFU (log_{10})/0.4 ml
13A	3.6
1B	3.0
1C	3.0
13C	3.0
1A	2.2
13B	2.1
11A	2.0
11B	1.9
10A	1.7

[a] A clone of NZB-ME cells (Cl S2) was infected with an end titration of H-MSV. Individual foci were isolated and cloned. Their production of NZB pseudotype sarcoma virus was measured by the focus-forming activity (FFU) of filtered supernatants in NRK cells.

up to 8,000 FFU/ml, provide an excellent source of NZB pseudotype sarcoma virus.

DISCUSSION

The results described in this paper further characterize the virus of NZB mice. It is a prototype for a new class of endogenous murine type C viruses which differs from standard ecotropic (mouse-tropic) viruses. NZB-MuLV has the MuLV gs antigen, can rescue the MSV genome and help in MSV replication, and contains murine reverse transcriptase; it does not, however, form syncytia with XC cells nor infect mouse cells even when highly concentrated amounts of virus are used. This lack of infectivity for mouse cells is further reflected in the failure of NZB-MuLV to induce gs antigen in these cells, its inability to rescue MSV from MSV-transformed mouse cells, and the lack of focus formation in mouse cells by the pseudotype virus even after the addition of excess Rauscher leukemia virus (26). The NZB isolate has a distinctive envelope antigen (26, 27), and forms a third serotype subgroup of MuLV, NZB. This class of viruses must be inherited vertically through the germ cell, since infection of mouse cells is not possible. Its existence provides confirmation of the virogene hypothesis (21).

The host range of X-tropic type C virus varies more widely than that of ecotropic MuLV and its ability to cross class barriers with efficient propagation in avian cells is unique. The variation in the ability of X-tropic virus to infect and replicate in cells from different hosts has suggested the presence of at least two general mechanisms for regulating infection of host cells by this virus: one at the surface (or receptor) level and the other at the intracellular level. The former involves those events leading to the entry of viral RNA into the cell; the latter involves those processes required for viral replication. Together they determine the input (virus penetration or uncoating) and output (virus replication) responses by cells to virus infection (28).

The susceptibility of cells to these viruses cannot then be measured only by reverse transcriptase assays since input response may be more efficient than output response. Co-cultivation of NZB cells with NP-transformed hamster cells yields pseudotype virus, although hamster cells cannot be infected by high titered stocks of NZB viruses (27, 27a, 28). These results imply that the block in hamster cells is at the input or receptor level and once heterokaryon formation occurs (as observed with co-cultivation) (40) X-tropic virus can be produced. In mouse cells, on the other hand, both a surface and intracellular block must be occurring since neither concentrated pseudotype MSV in the presence of excess ecotropic MuLV, or co-cultivation techniques using non-virus-producing MSV-transformed mouse cells, has yielded an NZB pseudotype sarcoma virus. Results from other laboratories support this conclusion (13, 39).

The recovery of NZB virus from all cells cultivated from adult NZB mice as well as NZB embryos including clones of primary cells indicates spontaneous production is associated with the NZB genome. Its expression in NZB embryos as well as those of other mice (27a) suggests it may normally play a role in fetal development. The variability in production of NZB-MuLV and NZB pseudotype virus by the cultivated NZB cells (Tables 4–6), even from the same animal, appears to be related to an intracellular mechanism regulating virus production. This mechanism may be similar to that controlling the output responses by cells of other species. It is not related to the X chromosome since comparable virus production is observed in both male and female mice. The phenomenon may be associated with an accumulation of virus RNA within the cell as has been observed with Newcastle disease virus infection (22). We are presently trying to determine if this variability is correlated with the presence of type C virus inhibitor produced by NZB cells (28a, 29; Levy, Abstr. Annu. Meet. Am. Soc. Microbiol., 1973, V 169, p. 222).

Production of X-tropic virus endogenously by mouse cells and exogenously by heterologous cells including human cells is much lower (10^3

infectious particles/ml) than that of ecotropic virus in permissive cells ($\sim 10^6$ infectious particles/ml). This observation suggests a general restriction of X-tropic virus replication which may be inherent in the virus genome itself or secondary to responses within the host cell. Addition of MSV appears to overcome this restriction, particularly in heterologous cells; pseudotype sarcoma virus preparations in human and duck cells have accompanying X-tropic MuLV in titers as high as 10^6 infectious particles/ml. Besides MSV, perhaps other type C virus genomes can act as helpers for X-tropic virus replication.

Although ecotropic viruses have been reported to be associated with one colony of NZB mice (24, 34), we have recovered only X-tropic virus from tissues and extracts obtained from over 50 NZB mice and 15 embryos from five different colonies. Our tissue culture studies suggest that X-tropic virus production in vivo is occurring in most, if not all, NZB cells from embryo to adulthood. This X-tropic virus accounts for the large quantity of viral protein recently described in tissues from NZB mice (42). The expression of X-tropic virus in other strains of mice, on the other hand, is less than that observed in NZB mice (26, 27, 27a, 32). Such observations suggest that the genetic information for X-tropic virus production is completely turned on in mice containing the NZB genome and this unique characteristic of NZB cells may be a reason for the development of autoimmune disease by the host.

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