Since the pathogenesis of acute rheumatic fever is largely unexplained, the significance of this increased adherence remains conjectural. Increased adherence may be necessary to prolong infection or to promote colonization with a critical mass of bacteria, factors that may be necessary for the induction of acute rheumatic fever. Since bacterial adherence is a cell surface phenomenon, the increased adherence of pharyngeal cells of certain individuals may reflect a relation between surface components of host cells and of streptococci, which in turn, may be important in the immunologic cross-reactivity which has been implicated in the pathogenesis of acute rheumatic fever. Certain similarities of cell surface components of patients with acute rheumatic fever, namely HLA (histocompatibility) and blood groups, have been investigated with equivocal results (*13*). Perhaps another cell surface component, yet to be identified, is important in this regard.

DANIEL S. SELINGER*
NEIL JULIE
WILLIAM P. REED
RALPH C. WILLIAMS, JR.
Medical Service, Veterans Administration Hospital, and *Bernalillo County Medical Center, University of New Mexico School of Medicine, Albuquerque 87108*

References and Notes

1. G. H. Stollerman, *Rheumatic Fever and Streptococcal Infection* (Grune & Stratton, New York, 1975).
2. A. L. Bisno, I. A. Pearce, H. P. Wall, M. D. Moody, G. H. Stollerman, *N. Engl. J. Med.* **283**, 561 (1970).
3. L. W. Wannamaker, *ibid.* **282**, 78 (1970).
4. C. H. Rammelcamp, Jr., F. W. Denny, L. W. Wannamaker, in *Rheumatic Fever*, L. Thomas, Ed. (Univ. of Minnesota Press, Minneapolis, 1952), p. 72; G. H. Stollerman, *Circulation* **43**, 915 (1971).
5. A. L. Bisno, I. A. Pearce, G. H. Stollerman, *J. Infect. Dis.* **136**, 278 (1977); A. L. Bisno, I. A. Pearce, H. P. Wall, M. D. Moody, G. H. Stollerman, *N. Engl. J. Med.* **283**, 561 (1970).
6. R. J. Gibbons, in *New Approaches for Inducing Natural Immunity to Pyogenic Organisms*, J. B. Robbins, R. E. Horton, R. M. Krause, Eds. (Publ. 74553, National Institutes of Health, Bethesda, Md., 1973), p. 115.
7. R. P. Ellen and R. J. Gibbons, *Infect. Immun.* **9**, 85 (1974); E. H. Beachey and I. Ofek, *J. Exp. Med.* **143**, 759 (1976).
8. B. Aly *et al.*, *Infect. Immun.* **17**, 546 (1977); J. E. Fowler, Jr., and T. A. Stamey, *J. Urol.* **117**, 472 (1977).
9. R. J. Gibbons and J. Van Houte, *Infect. Immun.* **3**, 567 (1971).
10. Provided by C. Rammelcamp, Case Western Reserve University.
11. Provided by L. Wannamaker, University of Minnesota College of Medical Sciences.
12. R. J. Gibbons, in *Microbiology 1977*, D. Schlessinger, Ed. (American Society for Microbiology, Washington, D.C., 1977), p. 395.
13. L. E. Glynn and E. J. Holbrow, *Arthritis Rheum.* **4**, 203 (1961); D. A. Brewerton and E. A. Albert, in *HLA and Disease*, G. Dausset and A. Suejgaard, Eds. (William & Wilkins, Baltimore, 1977), p. 99.
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* Requests for reprints should be addressed to Research Service (151), Veterans Administration Hospital, 2100 Ridgecrest Drive SE, Albuquerque, N.M. 87108.

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Genomic Masking of Nondefective Recombinant Murine Leukemia Virus in Moloney Virus Stocks

Abstract. *HIX virus cloned from Moloney leukemia virus stocks is a nondefective, leukemogenic, and amphotropic murine oncornavirus with a recombinant-type major glycoprotein. Although Moloney leukemia virus stocks generally contain little or no free amphotropic virus, dilution analysis of several virus stocks and the examination of virus progeny from individual foci revealed that HIX virus is present and functionally coated with ecotropic Moloney virus envelopes. Because most mice have serum factors that inactivate recombinant viruses, masking may represent a general survival mechanism for HIX as well as other analogous recombinant viruses.*

Moloney murine leukemia virus (M-MuLV) passed in mouse cells can contain at least three kinds of nondefective murine oncornaviruses: typical ecotropic M-MuLV, xenotropic murine oncornavirus (MuX), and an amphotropic recombinant virus (HIX) that shares M-MuLV and MuX information (*1*). HIX virus breeds true in both mouse and nonmurine cells, and contains antigenic determinants of the group-specific antigen (gag) region, which are type-specific for M-MuLV (*1, 2*). The major glycoprotein (gp71) is neither eco-, xeno, or wild mouse amphotropic type as analyzed by interference properties, specific neutralization, and peptide maps. Partial relationships of HIX gp71 to both MuX and M-MuLV were observed by the above procedures (*1–3*). HIX virus was cloned by multiple cycles of focal and limiting dilution isolation, was grown in feline embryo fibroblast (FEF) cells, and was inoculated into newborn mice and cats. In several strains of mice, lymphomas were observed 2 to 3 months after inoculation. On the basis of the clinical syndrome and histopathology, these lymphomas were indistinguishable from the original M-MuLV–induced disease (*4, 5*). Extracts of leukemic organs contained high titers of virus with amphotropic properties. Further analysis of envelope properties and the genetic content of many individual infectious units of tumor-derived virus revealed that the only detectable virus in tumors was HIX (*6*).

Because leukemogenic HIX was isolated from M-MuLV stocks, we reexamined several questions. (i) Do M-MuLV–induced lymphomas contain HIX virus? Previously M-MuLV stocks were found to contain little or no detectable amphotropic virus (*1, 2, 6, 7*). (ii) Can HIX virus arise regularly by de novo recombination? (iii) Is the recombinant virus the actual leukemogenic agent in M-MuLV stocks? A similar virus has been isolated from lymphomas of AKR mice, and appeared to be a recombinant of AKR-MuLV and MuX; it was postulated to be the actual leukemogenic virus of AKR mice (*8*). Because this recombinant virus (MCF) could not be isolated in free form from AKR-MuLV stocks, the answers to the above questions could contribute to our understanding of virus participation in murine leukemia. Our examination of several lymphoma- and tissue culture–derived stocks of M-MuLV revealed that HIX virus was present in uncloned virus populations, but in a masked form. Single infectious units of HIX could be coated with ecotropic envelopes, giving the impression on assays that no virus with xenotropic properties existed in such stocks.

Several stocks of M-MuLV from NIH Swiss and BALB/c mouse lymphomas induced by the IC isolate (termed IC) of M-MuLV were reexamined for the presence of virus that could be detected in nonmurine cells (*4*). Assays for murine ecotropic and xenotropic viruses were performed in mouse or cat sarcoma-positive leukemia-negative (S+L−) cells (*9, 10*). Previously BALB/c lymphoma–derived M-MuLV (ICXB) had no detectable HIX-type virus. Because HIX virus was very susceptible to virus-inactivating factor found in normal mouse serums (MSF), it appeared possible that recombinant virus was already inactivated (*1, 11, 12*). The NIH Swiss strain of mice was unusual in that no MSF was detectable in the serum (*12*). Virus from extracts of NIH Swiss mouse tumors induced by the IC isolate of M-MuLV (ICXN) more than 8 years ago was also reexamined (*4*). The ICXN isolate was used to generate lymphomas in BALB/c mice. Both tumor-derived virus stocks had high titers of ecotropic M-MuLV (Table 1). ICXN had obvious xenotropic

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virus that produced typical HIX-type syncytial foci in cat 81 S+L− cells, but the ICXB stocks had no detectable virus in cat 81 cells (*5, 10*). However, the original HIX virus was isolated from 3T3FL mouse cells inoculated initially with ICXB virus (*1*). Accordingly, two stocks of virus from 3T3FL cells infected with ICXB several years previously were also examined. ICB-3T3-75 was typical in that very little, if any, xenotropic virus was present, whereas a more recent (ICB-3T3-77) stock was atypical in that it contained a relatively high quantity of xenotropic-type virus. Both 3T3FL-derived stocks had high titers of ecotropic M-MuLV. Presence of recombinant virus was first assessed by the examination of individual foci in the mouse S+L− clone FG-10 (*9*). No individual focus of ICXB or ICB-3T3-75 viruses contained any virus progeny capable of growth in S+L− cat 81 cells. In contrast ICXN and ICXB-77 stock did give rise to single foci whose progeny had xenotropic properties. Titers of virus based only on analysis of single foci suggested that 15.5 and 11.2 percent of virus in ICXN and ICB-3T3-77 stocks, respectively, had xenotropic properties.

Further assessment for the presence of virus with xenotropic properties was carried out by making serial dilutions of the four virus stocks in mouse S+L− cells and determining the $TCID_{50}$ (tissue culture infective dose for 50 percent of the cells) dilution that could give rise to focus-inducing units (FIU) detectable in the cat 81 cell assay. All four virus stocks contained such virus; however, ICXB and ICB-3T3-75 had only several hundred FIU for 81 cells, whereas ICXN and ICB-3T3-77 had higher ($\geq 10^4$ FIU per milliliter titers. Accordingly, most of the virus (90 to 99 percent) with xenotropic potential was not detected by direct assays.

Dilution passage of ICB virus in mouse S+L− cells was also repeated in half-log dilutions both in the absence and in the presence of a concentration of MSF that could inactivate ≥ 99.9 percent of xenotropic or recombinant virus. In both cases, the emergent recombinant virus (MCF) titer was from 200 to 400 $TCID_{50}$ per milliliter. Accordingly, recombinant virus genomes were inaccessible to the action of MSF.

To determine whether xenotropic-type virus seen in individual foci and in low dilutions of passaged M-MuLV's was truly recombinant type, further passage in mouse and cat cells was combined with treatment with MSF (Table 2). This method permitted us to distinguish between genomic masking and the presence of genetic information greater than that of a single virus genome. For comparison, individual foci of HIX tumor-derived pure HIX virus and ecotropic M-MuLV were also examined. On the basis of similar titers in mouse and cat S+L− cells, all progeny of the total number (81) of individual HIX foci had an amphotropic host range and were completely

Table 1. Examination of M-MuLV stocks by direct titration, blind dilution, and individual focus analyses.

M-MuLV stocks	Direct assays (FIU/ml)*			Ratio of foci (progeny to number examined)†		Titer of xenotropic genomes†		Masking (%)	Recombinant in stock (%)
	Ecotropic	Xenotropic	Ratio	Ecotropic	Xenotropic	Single focus‡	Virus dilution§		
Lymphoma extract from									
ICXN: NIH Swiss mouse	3.8×10^5	5.0×10^2	$10^{-2.9}$	32/32	5/32	5.9×10^4	3.3×10^4	99	12.1
ICXB: BALB/2 mouse	1.1×10^6	≤ 2.5	$10^{-5.6}$	41/41	0/41	0	2×10^4	≥ 99	0.02
3T3FL cells inoculated with									
ICXB, ICB-3T3-75	1.1×10^6	10	$10^{-5.0}$	32/32	0/32	0	2×10^2‖	≥ 99	0.02
ICXB, ICB-3T3-77	1.25×10^6	5.5×10^3	$10^{-2.3}$	44/44	5/44	1.4×10^5	3.3×10^4	90	6.9
M-MuLV 1869 isolate in SC-1 cells	1.8×10^6	0		33/33	0/33	0	N.D.¶	N.D.¶	N.D.¶

*Each virus stock was assayed on mouse S+L− cells (clone FG-10) and cat S+L− cells (clone 81) under standard conditions (*9, 10*). HIX syncytial-type foci were obvious in ICXN and ICB-3T3-77 virus stocks. †FG-10 (10^3 cells per miniwell) were plated in flat-bottomed well plates (Falcon Microtest 96). On the next day virus was added at an effective efficiency of plating of 0.2 to 0.4 FIU per well. The observed numbers and distribution of foci followed Poisson statistics. Virus progeny of each well was passed to both fresh FG-10 and cat 81 cells to determine the host range. ‡Focus-inducing units (per milliliter) were calculated from the ratio of xenotropic to ecotropic individual foci relative to total number of FIU for FG-10 cells in that virus stock. §Half-log dilutions (from 1:1 to 1:10,000) of each virus stock were inoculated in individual dishes of FG-10 cells under standard conditions. Virus was harvested 4 to 7 days after infection and used to infect both FG-10 and cat 81 cells. The virus titer is expressed as $TCID_{50}$, a dilution at which half the infected dishes yielded xenotropic virus progency. ‖The parental virus stock, essentially identical to ICB-3T3-75 in ecotropic and xenotropic virus content, was used for blind dilution analysis. ¶None detected.

Table 2. Analysis of host range and mouse serum factor susceptibility of progeny virus derived from selected individual foci from various virus sources.

Virus source	FG-10 foci examined (No.)*	Fraction of single foci giving rise to FIU in indicated assay system:				
		Focus progeny into FG-10 cells		Focus progeny into 81 cells		
		Untreated	Treated with MSF	Untreated	Harvested from cat 81 cells inoculated into	
					FG-10 cells	Cat 81 cells
HIX-induced mouse tumors†	81	81/81	0/81	81/81	5/5 tested	NT
ICXN‡	5	5/5	1/5	5/5	5/5	5/5
ICB-3T3-77‡	4	4/4	4/4	4/4	4/4	4/4
M-MuLV without xenotropic virus§	65	65	20/20	0		

*All foci examined were picked from individual wells with single foci in well microtiter plates (Falcon 96) where the number and distribution of foci were compatible with a Poisson distribution. †Represents virus from extracts of tumors induced by HIX in either BALB/c or NIH Swiss mice and plated directly into FG-10 miniwells. ‡In ICXN and ICB-3T3-77 stocks only those foci were tested with MSF which yielded progeny capable also of infecting cat 81 cells. The contents of a miniwell with an individual focus were brought to 1.0 ml, of which 0.5 ml was put onto cat 81 cells; 0.25 ml was put onto FG-10 cells directly, and the remaining 0.25 ml was made up to 1:25 final concentration of STU normal mouse serum and kept at room temperature for 1 hour (*9*). Progeny per focus ranged from 20 to 500 FIU. Susceptibility to MSF was considered positive if ≥ 99 percent of virus was inactivated. §M-MuLV 1869 cloned stock and ICXB foci were randomly selected for host range and MSF analyses.

susceptible to MSF. In contrast, all progeny of the 65 ecotropic M-MuLV foci was infectious only for mouse cells, and none was susceptible to MSF. Progeny of each of five selected individual foci from ICXN (5/32) (Table 1) were amphotropic on passage, and in four of five they were completely inactivated by MSF. This indicated that the single infectious unit could consist of pure recombinant virus coated with ecotropic M-MuLV. Ecotropic M-MuLV that gave rise to de novo recombinants could not have been present because, otherwise, at least some of the focus progeny would not have been susceptible to MSF. The remaining focus could have consisted of both M-MuLV and HIX; after growth in cat 81 cells, virus progeny was still amphotropic and also susceptible to MSF. It was surprising to find relatively high titers of virus with xenotropic potential in ICB-3T3-77 virus stocks because most 3T3FL-derived M-MuLV stocks over the past several years had typically very little, if any, such virus. The original HIX virus isolate came from such a typical M-MuLV preparation after long-term infection of 3T3FL cells with ICXB virus (*1*). From the above dilution sequence of ICXB in mouse cells, the amphotropic virus isolated was also typical HIX virus. Virus isolated from those ICB-3T3-77 foci that contained progeny virus capable of growth in cat 81 cells was analyzed. Virus from all four foci was amphotropic, but foci in cat 81 cells were minute and atypical and continued to be low in titer despite passaging. In addition, the original focus progeny was not significantly inactivated by MSF. On the basis of Poisson counting statistics, the foci were derived from single infectious units. Other characteristics of this atypical virus remain to be determined.

The above were compared to the progeny of 65 foci from several M-MuLV stocks which did not yield any single foci with xeno- or amphotropic virus progeny. In all cases, virus remained ecotropic and was not susceptible to MSF. This suggested that de novo generation of recombinant virus is rare or does not occur in tissue culture. Virus from nine individual ecotropic M-MuLV foci was grown to high titer and passed in 3T3FL cells three consecutive times with high and low multiplicity of infection. No xeno- or amphotropic virus was detected. The same nine M-MuLV virus stocks were also passed in rat NRK cells several times; again no recombinant viruses were evident. Further analysis was done with cloned 1869 M-MuLV passed in SC-1 cells (*13*). This virus had no detectable virus with xeno- or amphotropic host range, either by the isolation of progeny virus of single foci or by dilution studies (Table 1). Attempts were made to generate recombinant virus by infection of mouse and rat NRK cells at multiplicities of infection ranging from 1 to 0.001. In all instances no xeno- or amphotropic virus was detected.

As judged by the above analyses of single foci and dilution study of virus, leukemic extracts as well as M-MuLV derived from tissue culture contained HIX recombinant virus, which was masked so that a single genome of HIX was functionally enveloped by the ecotropic M-MuLV coat. This is not phenotypic mixing because (i) such HIX genomes would have had gp71 of both HIX and M-MuLV and, accordingly, should have been detected by direct assay on cat 81 cells; and (ii) the addition of MSF and ICXB stocks did not reduce the titer of emergent recombinant virus as seen on dilution passage. Although phenotypic mixing between eco- and xenotropic MuLV has been described, the possibility of recombinant virus or masking has not been rigidly excluded (*14*). Because HIX virus is not defective and can code for its own major glycoprotein, it should not be considered a "pseudotype." The persistence of masking of HIX virus in M-MuLV stocks in tissue culture is interesting; perhaps M-MuLV confers some replicative advantage because we have previously seen a spontaneous loss of pure HIX virus on passage in mouse cells (*1*). The difference in the quantity of HIX virus in NIH Swiss and BALB/c mouse tumor extracts could have been due to the absence of active MSF in the former and a high titer of MSF in the latter strain. No free amphotropic virus was seen in ICXB stocks, although tumor extracts that contained ICXB virus did not have free MSF when tested with pure HIX virus. The presence of MSF, which readily inactivated HIX, could be the mechanism that favored genomic masking of HIX virus to ensure survival. Such evolutionary pressure could have led to HIX, which readily accepted the M-MuLV coat even in tissue culture where MSF was absent. Genomic masking has been described in several picornavirus systems in cultures subjected to artificially induced survival pressure (*15*).

The presence of recombinant, nondefective, and leukemogenic HIX virus in M-MuLV stocks has a counterpart in the MCF virus (*8*). The latter is related to AKR-MuLV as seen by neutralization with type-specific antiserums to AKR-MuLV and by other criteria (*3, 8*). So far, no HIX isolate examined from any source has ever been susceptible to antiserum to the gp71 of AKR-MuLV (*5*). Evidently MCF cannot be found as free virus in leukemic plasma but must be isolated in culture by cocultivation (*8*). Our findings suggest that analogous genomic masking might be operative as a general survival mechanism. Recent studies of virus from AKR lymphomas also revealed masked MCF virus. The fact that HIX virus was quite leukemogenic in BALB/c mice and found as free virus in lymphomas was initially puzzling because BALB/c mice have MSF (*5, 11, 12*). This puzzle was solved in part because newborn BALB/c mice did not have MSF and because BALB/c mice with HIX-induced lymphomas did not have demonstrable MSF (*5*). Our experiments have also been partially confirmed by direct inoculation of M-MuLV into rat cells; however, the 10- to 100-fold lower susceptibility of rat cells to M-MuLV or HIX required higher inputs of viral genomes per cell. Whether the detectable recombinational event in the gp71 molecule is by itself causative of leukemogenic potential or whether additional changes in nucleotide sequence also play a role remains to be determined.

PETER J. FISCHINGER
CHARLOTTE S. BLEVINS
NANCY M. DUNLOP
Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20014

References and Notes

1. P. J. Fischinger, S. Nomura, D. P. Bolognesi, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 5150 (1975).
2. P. J. Fischinger, J. J. Elder, J. H. Ihle, D. P. Bolognesi, R. A. Lerner, in preparation.
3. J. J. Elder, F. C. Jensen, M. L. Bryant, R. A. Lerner, *Nature (London)* **267**, 23 (1977); J. J. Elder *et al.*, *Bibl. Haematol.*, in press.
4. P. J. Fischinger, C. O. Moore, T. E. O'Connor, *J. Natl. Cancer Inst.* **42**, 605 (1969).
5. P. J. Fischinger, J. N. Ihle, F. Noronha, D. P. Bolognesi, *Med. Microbiol. Immunol.* **164**, 119 (1977).
6. P. J. Fischinger, N. M. Dunlop, C. S. Blevins, *J. Virol.* **26**, 532 (1978).
7. P. J. Fischinger and S. Nomura, *Virology* **65**, 304 (1975).
8. J. W. Hartley, N. K. Wolford, L. J. Old, W. P. Rowe, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 785 (1977).
9. R. H. Bassin, N. Tuttle, P. J. Fischinger, *Nature (London)* **229**, 564 (1971).
10. P. J. Fischinger, C. S. Blevins, S. Nomura, *J. Virol.* **14**, 177 (1974).
11. P. J. Fischinger, J. N. Ihle, D. P. Bolognesi, W. Schafer, *Virology* **71**, 346 (1976).
12. J. A. Levy, J. N. Ihle, O. Oleszko, R. D. Barnes, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 5071 (1975).
13. J. W. Hartley, W. P. Rowe, R. J. Huebner, *J. Virol.* **5**, 221 (1970). This limiting dilution purified M-MuLV was obtained from J. Hartley through S. Nomura and passaged in our laboratory only through mouse SC-1 cells.
14. J. Levy, *Virology* **77**, 797 (1977).
15. J. J. Holland and C. E. Cords, *Proc. Natl. Acad. Sci. U.S.A.* **51**, 1082 (19 → E. Wecker and G. Lederhilger, *ibid.* **52**, 705 (1964).
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