VIROLOGY 179, 931–935 (1990)

Pathogenicity of BALB/c-Derived N-Tropic Murine Leukemia Viruses

L. PEDERSEN,* P. G. STRAUSS,† J. SCHMIDT,† A. LUZ,‡ V. ERFLE,†
P. JØRGENSEN,* N. O. KJELDGAARD,* AND F. S. Pedersen*,[1]

*Department of Molecular Biology and Plant Physiology, University of Aarhus, DK-8000 Århus C, Denmark; †GSF-Abteilung für Molekulare Zellpathologie; and ‡GSF-Institut für Pathologie, D-8042 Neuherberg, Federal Republic of Germany

Received May 15, 1990; accepted August 20, 1990

N-tropic murine leukemia viruses have been observed in connection with radiation-induced osteosarcomagenesis in BALB/c mice. We have investigated the bone disease-inducing potential of molecularly cloned, BALB/c-derived N-tropic viruses in the random-bred NMRI mouse strain. The germ-line virus and an exogenous virus isolate were found to induce high incidences of osteopetrosis and lymphomas and a lower incidence of osteomas. Two viruses derived from somatically acquired proviruses of independent radiation-induced osteosarcomas induced lower incidences of osteopetrosis and lymphomas. Nucleotide sequence analysis of the long terminal repeat regions and RNase T1 fingerprint analysis revealed only few differences between the isolates. The possible involvement of N-tropic murine leukemia viruses in radiation-induced osteosarcomagenesis in the BALB/c mouse strain is discussed.

Mice of the BALB/c strain harbor a single endogenous ecotropic provirus at the *emv*-1 locus (*1*). Ecotropic virus expression is only rarely detected in BALB/c mice before 6 months of age (*2*), most likely due to *Fv-1*$^{b/b}$ suppression of replication of the N-tropic virus (*3*). In contrast, N-tropic ecotropic murine leukemia viruses (MLVs) were activated during the early latency period of radiation-induced osteosarcomas (*4, 5*). Somatically acquired ecotropic proviruses were observed in 17 of 29 radiation-induced osteosarcomas of the BALB/c mouse (*6*). Two of the somatically acquired proviruses have been molecularly cloned and were found to encode N-tropic viruses ((*6*), this work). These observations suggest a possible role of N-tropic MLVs in radiation-induced osteosarcomagenesis in the BALB/c mouse. To address this issue, we have investigated the pathogenicity of BALB/c-derived N-tropic viruses in the outbred NMRI mouse strain, which is susceptible to replication of N-tropic viruses. The viruses used in this study included the molecular clone p7D (*7*) of the germ-line provirus and the molecular clone, pN20-7 (*8, 9*), representing an exogenous isolate from a normal BALB/c mouse, as well as two molecularly cloned, somatically acquired proviruses ((*6*), this work) from the radiation-induced osteosarcomas OTS-25 and OTS-72 (*6*). We have characterized these viruses, which exhibited various degrees of bone pathogenic potential in NMRI mice, by partial nucleotide sequence analysis and RNase T1 fingerprint analysis.

[1] To whom requests for reprints should be addressed.

Two independent clones (λOTS-25 and λOTS-25-2) of the single somatically acquired provirus in OTS-25 and a single clone (λOTS-72) of one of the three somatically acquired proviruses in OTS-72 were available after cloning as *Eco*RI restriction fragments in λgtWES.λB ((*6*), data not shown). DNA from λOTS-25, λOTS-25-2, λOTS-72, p7D, or pN20-7 was transfected (*10*) into NIH 3T3 cells to obtain infectious viruses, the production of which was monitored by reverse transcriptase assay (*11*). All viruses were N-tropic ((6–9), our own observations). The pN20-7 virus has previously been reported to be XC-positive (*9*); the other viruses were found to be negative in the XC-plaque assay (*12*) when tested 12 weeks post-transfection.

The pathogenicity of these viruses was investigated in mice of the outbred NMRI strain. Newborn animals were injected with cell-free supernatant from MLV-producing NIH 3T3 cells, which had been cultivated for 7 weeks post-transfection. The results are shown in Table 1. Although few animals were included in the present study the results demonstrate a clear pathogenic effect of some of the viruses and reveal pathogenic differences between the isolates, in particular with respect to the induction of bone lesions. Viruses derived from the germ-line provirus p7D and the exogenous isolate pN20-7 induced osteopetrosis and malignant lymphomas with high incidences and osteomas with a lower incidence. The viruses derived from the λOTS-25, λOTS-25-2, and λOTS-72 clones induced osteopetrosis, except for λOTS-25, and malignant lymphomas, but with lower incidences and longer latency periods

0042-6822/90 $3.00

TABLE 1

DISEASE PATTERN INDUCED BY BALB/c-DERIVED MLVs IN NMRI MICE

Virus strain[a]	Malignant lymphoma[b] Animals	Mean latent period (days)[c]	Osteopetrosis[b] animals	Osteoma[b] animals
λOTS-25 (5×10^5 iu/ml)	6/9 (67%)	455 ± 138	0/9 (0%)	0/9 (0%)
λOTS-25-2 (5×10^5 iu/ml)	6/10 (60%)	496 ± 106	2/10 (20%)	0/10 (0%)
λOTS-72 (9×10^3 iu/ml)	4/8 (50%)	417 ± 87	2/8 (25%)	0/8 (0%)
p7D (4×10^5 iu/ml)	9/10 (90%)	366 ± 112	5/10 (50%)	1/10 (10%)
pN20-7 (3×10^5 iu/ml)	8/9 (89%)	333 ± 95	8/9 (89%)	3/9 (33%)
Control[d]	3/9 (33%)	258 ± 115	0/9 (0%)	0/9 (0%)
Historical controls[e]				
Control	7/39 (18%)	363 ± 165	0/39 (0%)	0/39 (0%)
Noninfected	4/150 (3%)	365 ± 157	0/150 (0%)	0/150 (0%)

[a] Newborn mice were intraperitoneally injected with 0.1 ml cell-free supernatant from MLV-producing NIH 3T3 cells. The number of infectious units per milliliter (iu/ml) was determined by infectious center assay on NIH 3T3 cells (*26*).

[b] The mice were kept under observation 5 days a week until they showed overt signs of illness or until the end of the observation period of 601 days. Moribund animals were killed and autopsied. All carcasses were examined by X-ray analysis. The criteria for gross appearance of malignant lymphomas have been described (*17*) and include enlargement of either peripheral lymph nodes, thymus, mesenterial lymph node, spleen, left lobe of liver, or abundant effusions in the thoracic or abdominal cavity. Osteopetrosis was diagnosed when loss of spongious structure in vertebrae and/or loss of the bone marrow cavity in other bones resulting from a thickening of spongious and cortical bone was clearly detectable in the X-ray photograph (*16*). The criterion for diagnosis of osteoma was the presence of dense bone tumors with a diameter of at least 1 mm in the X-ray photograph (19). All diagnoses were confirmed by histological examination.

[c] The mean latent period and SEM were calculated from animals sacrificed with malignant lymphoma, which was the life-limiting factor in these experiments. The bone lesions were diagnosed in the postmortem X-ray analysis. Hence, the mean latent periods of the bone lesions would refer to the time of detection rather than to the time of their appearance and are therefore not given.

[d] Mock-treated animals were injected with 0.1 ml complete medium.

[e] Pooled data of experiments carried out previously ((*5, 17*), unpublished results).

than the p7D- and pN20-7-derived viruses. Though a fairly high incidence of spontaneous malignant lymphomas (33%) was observed in the mock-treated NMRI mice, no bone lesions were detected in the present controls or in the historical controls compiled from previous experiments (Table 1). Our data therefore indicate a direct role of the MLVs in the observed induction of bone lesions in the NMRI mice.

Previous reports on the pathogenicity of N-tropic viruses from normal BALB/c mice have been contradictory. One isolate from a normal animal was reported to induce high incidences of malignant lymphomas, osteopetrosis, and osteomas in NMRI mice (*5*), and viruses isolated from BALB/c embryo cells were found to induce myeloid metaplasia in NIH Swiss mice (*13*). However, the viral isolate N-CI-35, from which the clone pN20-7 was derived, and the isolate N-CI-5 were reported to be nonpathogenic in outbred NIH Swiss and CD^R-1 mice, respectively (*14*). This discrepancy with our findings may relate to different susceptibilities of the mouse strains used or to genetic differences between the viruses studied.

A partial structural analysis of the viral genomes including nucleotide sequence determination of the LTR regions and RNase T1 fingerprint analysis was carried out as a first step towards elucidating the genetic basis of the pathogenicity.

The nucleotide sequence of the LTR of λOTS-72 was determined by dideoxy sequencing with synthetic oligonucleotide primers after subcloning in bacteriophage M13 vectors as described (*6*), and found to be identical to the published sequence of the LTRs of p7D (*7*) and λOTS-25 (*6*). Sequence analysis of the pN20-7 plasmid revealed two base differences relative to the LTR sequence of the other BALB/c viruses (*7*), in positions 64 and 375 in U_3 (*7*), where the pN20-7 sequence has A instead of G residues. The nucleotide sequence of the LTR region of the pN20-7 clone showed two base differences relative to the published nucleotide sequence (*9*) of this clone, in positions 421 and 565 (*9*), where we found A and T residues instead of G and C residues, respectively. The presence of an adenine residue in position 421 in pN20-7 was confirmed by the absence of oligonucleotide 34 in the fingerprint of pN20-7-derived viruses (Figs. 1B, 1D).

For RNase T1 fingerprint analysis viral 70 S RNAs were harvested from NIH 3T3 producer cell lines and subjected to RNase T1 digestion, radioactive labeling,

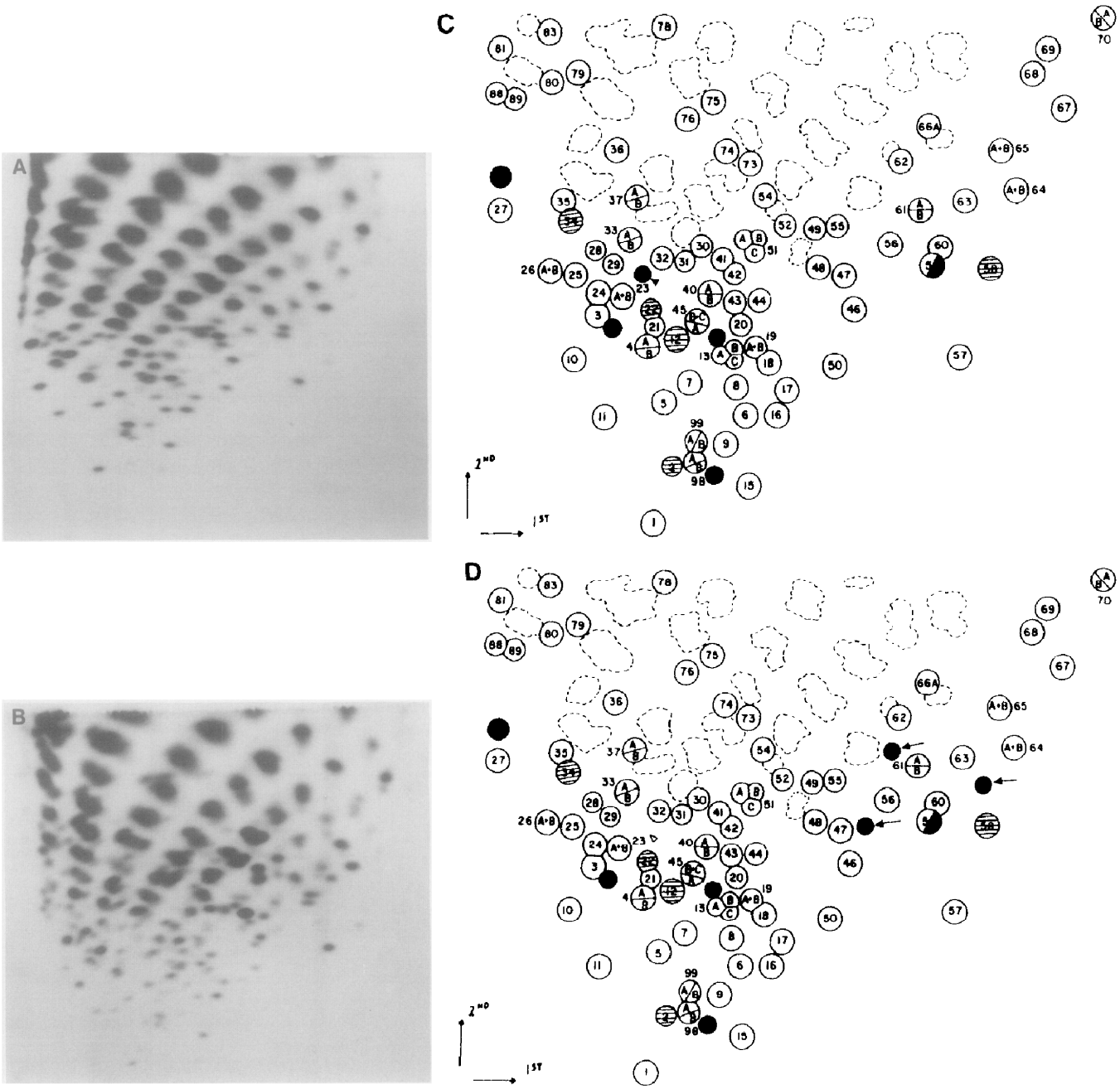

FIG. 1. RNase T1 fingerprint analysis of BALB/c-derived viruses. (A) The fingerprint pattern of viral RNAs derived from p7D, λOTS-25, λOTS-25-2, and λOTS-72; autoradiogram shown is from λOTS-25. (B) The fingerprint pattern of pN20-7 viral RNA. (C) and (D) Schematic comparisons of the fingerprint patterns of (A) and (B), respectively, with the fingerprint pattern of Akv MLV using the numbering system of Pedersen and Haseltine (*15*). The positions of 95 unique oligonucleotides have been assigned to the nucleotide map of the Akv MLV (*27*). Open circles represent oligonucleotides shared by Akv and the BALB/c-derived viruses, closed circles represent oligonucleotides unique to BALB/c-derived viruses, and crosshatched circles represent oligonucleotides unique to Akv MLV. The closed arrowhead in (C) indicates the position of the oligonucleotide associated with XC-negativity absent in (D) as marked by the open arrowhead. The arrows in (D) indicate RNase T1-resistant oligonucleotides not present in (A).

The schematic diagrams are based on the analysis of multiple experiments.

and two-dimensional gel electrophoresis as described (*15*). No difference was found between the fingerprints of viruses derived from the λOTS-25 (Figs. 1A, 1C), λOTS-25-2, λOTS-72, and p7D clones (data not shown), and only minor differences were observed between the fingerprints of these viruses and that of the exogenous isolate pN20-7 (Figs. 1B, 1D). Three unique oligonucleotides were observed in the fingerprint of

pN20-7. One oligonucleotide, indicated by an arrowhead in Fig. 1C, was common to p7D and the somatically acquired proviruses but absent in pN20-7 (Figs. 1B, 1D). According to the migration rules for oligonucleotides in the two-dimensional electrophoresis system (*15*), the composition of this oligonucleotide corresponds to that of the oligonucleotide predicted from the sequence associated with the determinant of XC-negativity at the gp70–p15E proteolytic cleavage site (*7*). Our results, including additional nucleotide sequence analysis of the relevant regions of the molecular clones, allow us to conclude that the oligonucleotide marked by an arrowhead in Fig. 1C represents the allelic determinant of XC-negativity.

In summary, the structural analysis of the viruses showed no indications of recombinational events involving different classes of endogenous viruses, nor any evidence for clustering of mutational differences in specific regions of the genomes. The two somatically acquired proviruses from independent tumors were closely related to the germ-line provirus. They do not seem to represent viruses selected for increased pathogenicity; rather, the somatically acquired viruses appeared less pathogenic than their presumed parental provirus, represented by p7D. A possible role of N-tropic ecotropic MLVs in radiation-induced osteosarcomagenesis in BALB/c mice might therefore be played by the germ-line ecotropic provirus itself.

Other ecotropic MLVs such as Akv MLV ((*16*), unpublished results), OA MLVs (*17, 18*), or RFB osteoma virus ((*16, 19*), manuscript in preparation) were found to induce disease patterns similar to those observed with p7D and pN20-7 when injected into newborn NMRI mice. The N-tropic Akv MLV was derived from the clone λ623 representing the *emv*-11 locus of the AKR mice (*16*). The N-tropic OA MLVs were obtained from spontaneous osteomas of strain 101 mice (*17*) and represent noncloned viruses (*17*), biologically cloned viruses (*17*), and a molecularly cloned virus (*18*). The RFB osteoma virus (*20*) was originally reported to induce only osteomas when injected into mice of the CF1, NIH Swiss, and CBA strains (*20, 21*). We found, however, that the RFB osteoma virus was a potent inducer of a disease pattern similar to that induced by p7D- and pN20-7-derived viruses after injection of newborn NMRI mice ((*16, 19*), manuscript in preparation). Experiments performed with viruses derived from the molecularly cloned OA MLV (*22*) indicated that the target cells for MLVs in the skeleton are progenitor cells of the osteoblastic lineage and that virus infection can indeed have a direct effect on the phenotype of the skeletal cells, leading to an enhanced expression of markers of osteogenic differentiation in the case of OA MLV infection.

Virus-induced osteopetrosis has otherwise been described only in chicken (*16*), where it is commonly observed in animals injected with avian leukosis viruses (ALVs) (*23*). The major osteopetrotic potential of ALVs has in several cases been mapped to regions outside the LTR (*24, 25*). We are currently constructing recombinants between selected MLVs which exert significantly different pathogenic effects on the mouse skeleton in order to identify viral sequences involved in MLV-induced bone lesions.

ACKNOWLEDGMENTS

We thank P. V. Mathiasen for excellent technical assistance. We are indebted to Dr. P. Jolicoeur for the gift of pN20-7 and Dr. R. Risser for the gift of p7D. This research was supported by EURATOM BI-6-0080-D and B10-D-547-DK and by the Danish Cancer Society.

REFERENCES

1. JENKINS, N. A., COPELAND, N. G., TAYLOR, B. A., and LEE, B. K., *J. Virol.* **43,** 26–36 (1982).
2. MCCUBREY, J., and RISSER, R., *J. Exp. Med.* **156,** 337–349 (1982).
3. STOYE, J., and COFFIN, J., *In* "RNA Tumor Viruses 2" (R. Weiss, N. Teich, H. Varmus, and J. Coffin, Eds.), 2nd ed., pp. 357–404. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1985.
4. SCHMIDT, J., ERFLE, V., and MÜLLER, W. A., *Radiat. Environ. Biophys.* **24,** 17–25 (1985).
5. SCHMIDT, J., LUZ, A., and ERFLE, V., *Leuk. Res.* **12,** 393–403 (1988).
6. STRAUSS, P. G., SCHMIDT, J., PEDERSEN, L., and ERFLE, V., *Int. J. Cancer* **41,** 616–621 (1988).
7. HOROWITZ, J. M., and RISSER, R., *J. Virol.* **56,** 798–806 (1985).
8. RASSART, E., DESGROSEILLERS, L., and JOLICOEUR, P., *J. Virol.* **39,** 162–171 (1981).
9. DESGROSEILLERS, L., RASSART, E., and JOLICOEUR, P., *Proc. Natl. Acad. Sci. USA* **80,** 4203–4207 (1983).
10. GORMAN, C., *In* "DNA cloning, Volume II" (D. M. Glover, Ed.), pp. 143–190, IRL Press, Oxford, 1985.
11. GALLAGHER, R. E., and GALLO, R. C., *Science* **187,** 350–353 (1975).
12. ROWE, W. P., PUGH, W. E., and HARTLEY, J. W., *Virology* **42,** 1136–1139 (1970).
13. GREENBERGER, J. S., STEPHENSON, J. R., MOLONEY, W. C., and AARONSON, S. A., *Cancer Res.* **35,** 245–252 (1975).
14. JOLICOEUR, P., ROSENBERG, N., COTELLESSA, A., and BALTIMORE, D., *J. Natl. Cancer Inst.* **60,** 1473–1476 (1978).
15. PEDERSEN, F. S., and HASELTINE, W. A., *J. Virol.* **33,** 349–365 (1980).
16. MURRAY, A. B., SCHMIDT, J., and LUZ, A., *In* "Monograph on Pathology of Laboratory Animals" (T. C. Jones, U. Mohr, and R. D. Hunt, Eds.). ILSI, Springer, New York, in press, 1990.
17. SCHMIDT, J., ERFLE, V., PEDERSEN, F. S., ROHMER, H., SCHETTERS, H., MARQUART, K., and LUZ, A., *J. Gen. Virol.* **65,** 2237–2248 (1984).
18. LEIB-MÖSCH, C., SCHMIDT, J., ETZERODT, M., PEDERSEN, F. S., HEHLMANN, R., and ERFLE, V., *Virology* **150,** 96–105 (1986).

19. LUZ, A., MURRAY, A. B., and SCHMIDT, J., *In* "Monograph on Pathology of Laboratory Animals" (T. C. Jones, U. Mohr, and R. D. Hunt, Eds.). ILSI, Springer, New York, in press, 1990.
20. FINKEL, M. P., REILLY, C. A., JR., BISKIS, B. O., and GRECO, I. L., *In* "Bone-Certain Aspects of Neoplasia" (C. H. G. Price and F. G. M. Ross, Eds.), pp. 353–366. Butterworths, London, 1973.
21. TEICH, N., WYKE, J., MAK, T., BERNSTEIN, A., and HARDY, W., *In* "RNA Tumor Viruses 1" (R. Weiss, N. Teich, H. Varmus, and J. Coffin, Eds.), 2nd ed., pp. 785–999. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1984.
22. SCHMIDT, J., CASSER-BETTE, M., MURRAY, A. B., LUZ, A., and ERFLE, V., *Am. J. Pathol.* **129,** 503–510 (1987).
23. SMITH, R. E., *Curr. Top. Microbiol. Immunol.* **101,** 75–94 (1982).
24. BROWN, D. W., BLAIS, B. P., and ROBINSON, H. L., *J. Virol.* **62,** 3431–3437 (1988).
25. ROBINSON, H. L., REINSCH, S. S., and SHANK, P. R., *J. Virol.* **59,** 45–49 (1986).
26. NEXØ, B. A., *Virology* **77,** 849–852 (1977).
27. ETZERODT, M., MIKKELSEN, T., PEDERSEN, F. S., KJELDGAARD, N. O., and JØRGENSEN, P., *Virology* **134,** 196–207 (1984).