Jpn. J. Cancer Res. 80, 24–30, January 1989

Biochemical and Immunological Characterization of Murine Leukemia Viruses that Are Paralysis-inducing in Rats

Sei-Ichi Shibamura,[1] Kazushige Kai,[2,5] Toshitaka Akatsuka[3] and Takeshi Odaka[4]

[1]Research Institute of Dai-Ichi Seiyaku Co. Ltd., Kitakasai 1-16-13, Edogawa-ku, Tokyo 134, [2]Department of Veterinary Microbiology, Faculty of Agriculture, University of Yamaguchi, Yamaguchi-city 753, [3]Department of Enteroviruses, National Institute of Health, Murayama Annex, Gakuen 4-7-1, Musashimurayama, Tokyo 190-12 and [4]Department of Genetics, Institute of Medical Science, University of Tokyo, Shirokanedai 4-6-1, Minato-ku, Tokyo 108

The molecular size and pI of the viral structural proteins of four PVC viruses (PVC111, PVC211, PVC321 and PVC441) were compared by single or two-dimensional polyacrylamide gel electrophoresis. PVC111 had slightly larger p15E and gPr85 molecules (about 0.5 kilodalton) than did the other PVC viruses. On the other hand, the virion structural proteins p30, p15, p12E and p12 from all the viruses had the same molecular sizes and pIs. The gp70s and p10s from all the viruses showed the same molecular sizes. A monoclonal antibody to gp70 of PVC321 virus recognized the gp70s of all PVC viruses, but not the gp70s of four clones of the wild mouse ecotropic viruses, Friend murine leukemia viruses (F-MuLV), AKR ecotropic MuLV, dual-tropic F-MuLV or NZB endogenous xenotropic MuLV, revealing that these four PVC viruses are homologous with each other, but distinct from the known mouse retroviruses.

Key words: Paralysis — Murine leukemia virus — Structural proteins — Monoclonal antibody

We previously isolated four clones (PVC111, PVC211, PVC321, and PVC441) of MuLVs from a paralyzed rat which had been infected with rat-passaged Friend leukemia virus.[1)] These viruses, except for PVC111, induce hind leg paralysis in rats and differ from the other paralysis-inducing MuLVs in pathogenicity in the rat,[2)] in latency,[3)] in the N-, B-tropism,[4)] and in the distribution of viral antigens in the CNS tissues.[5)]

PVC211 and PVC321 induce paralysis in rats and kill them within 1 month, and PVC441 does so within 2 months, whereas PVC111 does neither within 4 months, though all of the PVC viruses induce neuronal degeneration in the CNS tissues of infected rats. We were interested in this variation of the 'latency' among PVC viruses, and in this study, tested whether the PVC viruses had mutually analogous genome structures or had heterogous genome structures by analyzing viral structural proteins biochemically, as in the studies that revealed the relationship between the N-, B-, NB-tropism of MuLV and the variances of p 30 proteins derived from various MuLVs,[6-8)] and immunologically, using a monoclonal antibody.

Abbreviations used in this paper: pI, isoelectric point; gPr85, *env* precursor protein; MuLV, murine leukemia virus; CNS, central nervous system; MEM, minimal essential medium; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; cpm, counts per minute; RLV, Rauscher leukemia virus; IgG, immunoglobulin G; Pr65, *gag* precursor protein.

MATERIALS AND METHODS

Viruses PVC viruses and F-MuLV were cloned in NRK cells as described previously.[1,9)] The ecotropic viruses of wild mice were provided by Drs. J. W. Hartley (National Institute of Allergy and Infections Disease, USA) and S. Rasheed (University of Southern California, USA). They were 1504E, 292E, 4070E and 4996E[4)] and were cloned in SC-1 cells.[10)] AKR ecotropic virus, NZB endogenous xenotropic virus, and dual-tropic F-MuLV were cloned in mink cells, and were provided by Dr. A. Ishimoto (Institute for Virus Research, Kyoto University). NRK and SC-1 cells infected with various viruses were maintained in Eagle's MEM (Nissui Co. Ltd., Tokyo) with 5% and 10% fetal calf serum, respectively. Virus-infected mink cells were maintained in RPMI1640 medium (Nissui) containing 5% fetal calf serum.

Radiolabeling NRK cells infected with each PVC virus were grown subconfluently in 100 mm plastic dishes (Lux Corp., USA). After the removal of the culture media, 200 μCi/ml of ^{3}H-amino acid mixture (Amersham Japan, Tokyo) in Hanks' balanced salt solution supplemented with 10% dialyzed fetal calf serum was added to the cultures. The cultures were incubated with frequent rocking for 8 h. Then, 4 ml of normal culture medium was added to the cultures. After 16 h of incubation, the culture media were harvested and centrifuged at 3,000 rpm for 10 min. The supernatant fractions were overlaid on linear sucrose gradients of 15–

60%. The gradients were centrifuged at 25,000 rpm for 16 h in a Beckmann SW28.1 rotor at 4°C. The gradients were fractionated and the radioactivity of every fraction was counted. The fractions containing virions were collected, dialyzed against distilled water and lyophilized. These samples were used for analyses of virion structural proteins.

For analysis of cellular virus-related proteins, virus-infected cells were labeled with ^{3}H-leucine (Amersham; 200 μCi/ml) in leucine-free MEM (Nissui) with 10% dialyzed fetal calf serum, for 7–8 h. The labeled cells were harvested with a rubber policeman, washed three times with PBS and lysed with a buffer composed of 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS in PBS. About 3×10^6 cpm of lysate was subjected to immunoprecipitation performed in the same manner as described previously.[9]

Antibodies Monospecific goat antisera to gp70 of RLV were provided by the Division of Cancer Cause and Prevention, NCI, Bethesda, USA. Rabbit antiserum to RLV was prepared with purified RLV virions. Rat antisera to PVC321 virus were obtained from adult Fischer (F344) rats injected four times weekly with the spleen cells of 2–3 paralyzed F344 rats which had been infected with PVC321 virus.

Monoclonal antibodies Spleen cells from a rat immunized three times as above with the spleen cells of paralyzed rats were fused with the mouse myeloma cells, NS-1 according to the method of Oi and Herzenberg[11] for obtaining somatic cell hybrids.[12] Hybrids were selected in HAT medium and the culture supernatants were tested for anti-PVC321 reactivity with a peroxidase-conjugated rabbit antibody to rat IgG (Cappel, USA), on microtiter plates in which the NRK cells infected with PVC321 had been grown confluently, and fixed with 10% formalin in PBS for 10 min. After two cloning cycles of the hybrids, the culture media were harvested from the hybridoma cultures, concentrated about 30-fold by precipitation with ammonium sulfate, and dialyzed against PBS. Immunoprecipitation with these monoclonal antibodies was carried out with the second antibody (rabbit serum) to rat IgG.

Electrophoresis SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously[9] with the following molecular weight markers: β-galactosidase (130 kilodaltons (K)), phosphorylase *a* (94K), bovine serum albumin (67K), ovalbumin (45K), chymotrypsinogen A (25K) and cytochrome *c* (12.5K). Two-dimensional gel electrophoresis was carried out according to the method of O'Farrell *et al.*[13] The non-equilibrium pH gradient gel electrophoreses (NEPHGE) were performed with 2% ampholite (LKB, Sweden) of pH 3.5–10, for 5,000 V·h.

RESULTS

Analyses of virion structural proteins of PVC viruses PVC viruses were labeled with a mixture of ^{3}H-amino acids and purified by ultracentrifugation in a 15–60% linear sucrose gradient. Purified virions were analyzed by SDS-PAGE or by two-dimensional gel electrophoresis (Fig. 1). As shown in Fig. 1 (A), the virion structural proteins, gp70, p30, p15, p12E, p12, and p10 of various PVC viruses all showed the same mobilities. The p12E protein, when immunoprecipitated with anti gp70 serum, was revealed to be located between p15 and p12 *gag* proteins (data not shown). When these virions were subjected to two-dimensional NEPHGE-SDS-PAGE singly or mixed with PVC321 virions, they all gave similar electrophoretic patterns (Fig. 1: 1, 2, 3, 4, 1+3, 2+3, and 4+3), as illustrated in Fig. 1 (B). In NEPHGE, a major spot and a minor spot beside the major ones of p30 and p15 were observed in the neutral region, while p12E and p12 were located in a fairly acidic region, and p10 was so far into the basic region that it almost moved out of the gel, appearing as a trace in Fig. 1: 1+3. Thus, no difference in the molecular sizes or the pIs of major structural proteins was observed among the PVC viruses.

Analyses of viral-specific proteins in virus-infected cells The NRK cells infected with each of the PVC viruses were labeled with ^{3}H-leucine. The cell lysate of infected cells was immunoprecipitated with anti RLV rabbit serum (Fig. 2, A), and with anti gp70 serum (Fig. 2, B). From both panels, A and B, the precipitated 85K protein was identified as gPr85, the 70K protein as gp70, the 65K protein as Pr65, the 30K protein as p30, and the 19K protein as p15E. The gPr85 and p15E proteins of PVC111 virus (lane 1) moved slightly more slowly than those of PVC211 (lane 2), PVC321 (lane 3) and PVC441 (lane 4), while the Pr65s and p30s of all the PVC viruses showed the same mobilities (Fig. 2, A).

The immunoprecipitates with anti gp70 serum were further analyzed by two-dimensional gel-electrophoresis to determine the pIs of p15E proteins. As shown in Fig. 3, the precipitate of PVC111 virus and that of PVC211 virus showed similar patterns (1 and 2), even when both precipitates were coelectrophoresed (1+2), as summarized in panel A. Two spots of p15E of each PVC virus were found in the neutral region and were distinguished clearly from each other. When coelectrophoresed, the p15Es of PVC111 and PVC211 virus showed complete coincidence. Because the difference found in the molecular weights of p15Es (Fig. 2) was so small, the p15Es of PVC111 were not separable from those of PVC211 in the second dimension. Thus, the result shown in (1+2) seemed to be reasonable if the p15Es of both PVC111 and PVC211 had the same pI.

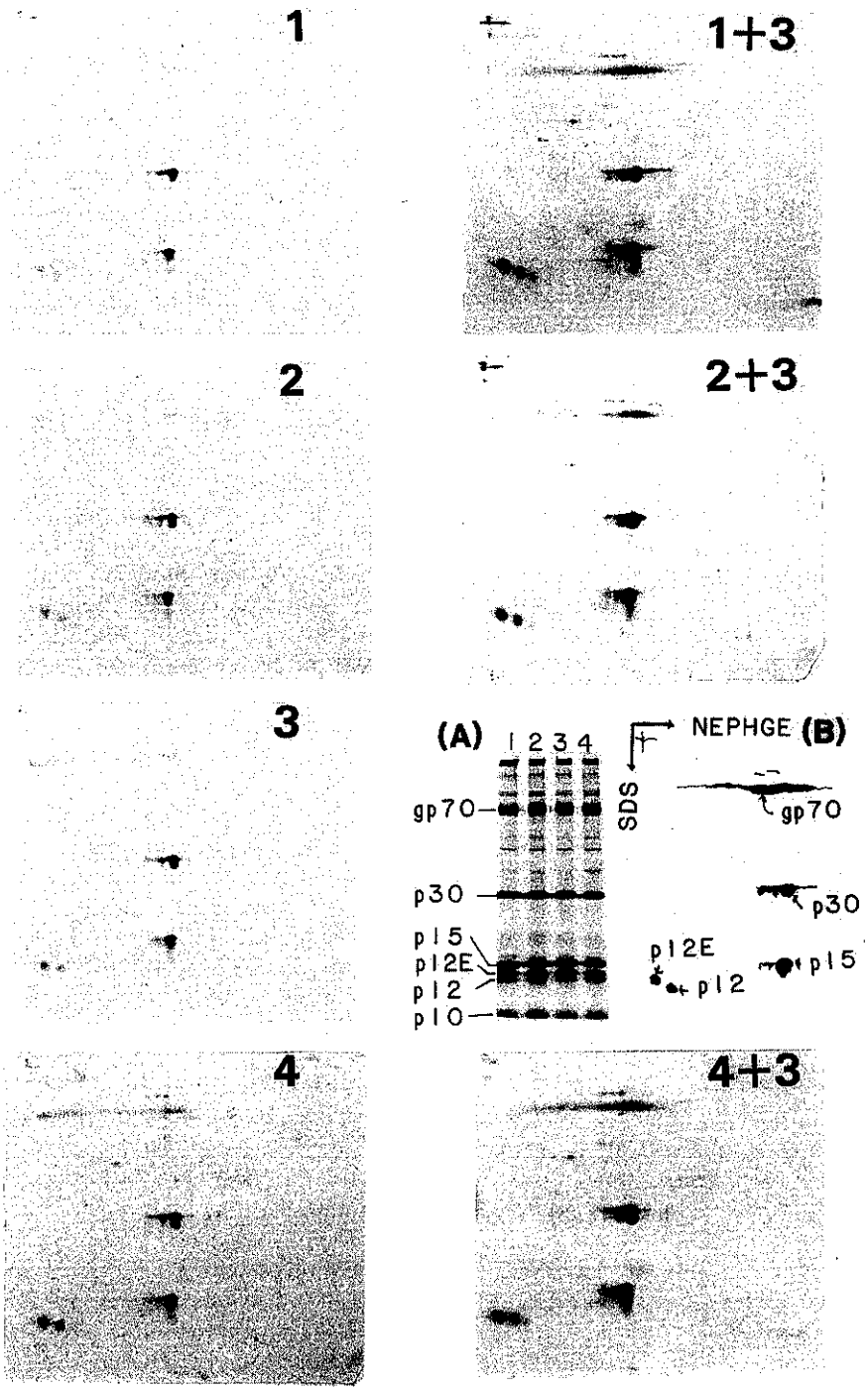

Fig. 1. Analyses of virion structural proteins of PVC viruses. ^{3}H-amino acid-labeled virions of about 10^4 cpm were subjected to SDS-PAGE (A), or to two-dimensional electrophoreses. The results were obtained with PVC111 (lane 1 and 1), PVC211 (lane 2, 2), PVC321 (lane 3, 3) and PVC441 (lane 4, 4) virions. (+) indicates the mixing of samples. The results are summarized in illustration (B).

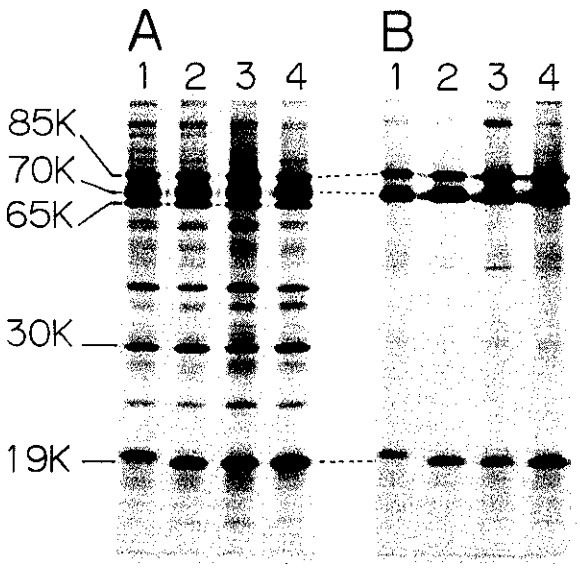

Fig. 2. SDS-PAGE of the immunoprecipitates from the lysates of ^{3}H-leucine-labeled NRK cells infected with PVC111 (lane 1), PVC211 (lane 2), PVC321 (lane 3) and PVC441 (lane 4), with antisera to RLV (A) and gp70 (B).

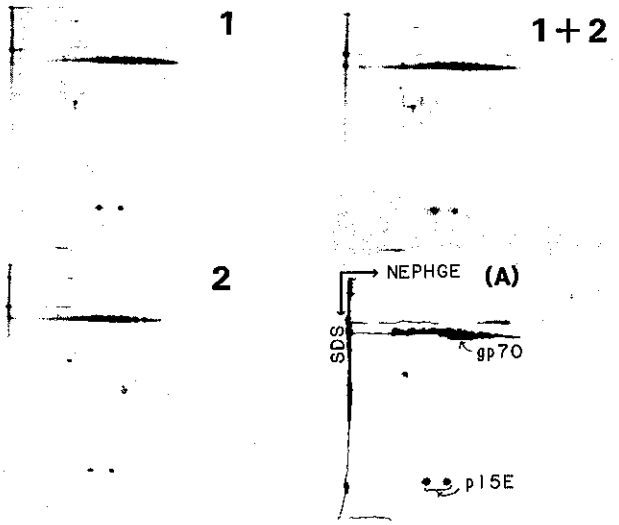

Fig. 3. Two-dimensional analyses of the immunoprecipitates with the antiserum to gp70 from the lysates of ^{3}H-leucine-labeled NRK cells infected with PVC111 (1) and PVC211 (2). (+) indicates the mixing of samples. The results are summarized in (A).

Similarly the precipitates of PVC321 and PVC441 virus were electrophoresed singly or together with that of PVC211, and all of the p15Es showed complete coincidence in mobility (data not shown).

Immunological characterization of PVC viruses Two hybridoma clones, of the 20 clones obtained by fusing mouse myeloma NS-1 cells with the spleen cells of a rat immunized with PVC321 virus, appeared to be producing monoclonal antibody which reacted with viral structural protein. One monoclonal antibody precipitated only p15Es of PVC viruses, dual-tropic F-MuLV, and NZB endogenous xenotropic virus, but did not coprecipitate gp70 or gPr85 (data not shown). Thus, this monoclonal antibody has broad specificity. The other monoclonal antibody precipitated gp70, gPr85 and p15E of PVC321, and so must have recognized gp70 of PVC321.

With this monoclonal antibody to gp70, the ^{3}H-leucine-labeled cell lysates of NRK cells infected with each of the PVC viruses or F-MuLV were immunoprecipitated (Fig. 4 A). The gPr85, gp70 and p15E proteins were immunoprecipitated from the lysates of the PVC group, but not from that of F-MuLV. When the supernatant fractions after the first immunoprecipitation were immunoprecipitated with rat anti PVC321 serum, the gPr85, gp70, Pr65, p30 and p15E proteins were precipitated from all of the lysates (Fig. 4 B). Because of the low titer of the monoclonal antibody, fairly large amounts of gPr85, gp70 and p15E were left unabsorbed by the first precipitation. Thus, the monoclonal antibody recognized the gp70s of PVC viruses but not the gp70 of F-MuLV. Also, the monoclonal antibody did not recognize the gp70s of AKR ecotropic MuLV, xenotropic MuLV, or dual-tropic F-MuLV (data not shown). This indicated specific recognition of the gp70s of PVC viruses by the monoclonal antibody.

Since the ecotropic viruses of wild mice induce paralysis in mice and the viral gene related to paralysis induction is suggested to be *env* gene,[14, 15] it was of interest to test whether the monoclonal antibody to gp70 recognizes gp70s of wild mouse ecotropic viruses. SC-1 cells infected with 1504E, 292E, 4070E or 4996E viruses were labeled with ^{3}H-leucine, and the cell lysates were immunoprecipitated with the monoclonal antibody. As shown in Fig. 5, the monoclonal antibody did not precipitate the gp70 of wild mouse viruses (panel A) as normal rat serum did (panel B), while the anti PVC321 rat serum precipitated gPr85, gp70, Pr65, p30 and p15E proteins (panel C). Of the wild mouse viruses, 4996E virus is not paralysis-inducing,[4] and interestingly, had large gp70 and p15E (panel C, lane 4).

DISCUSSION

Among PVC viruses, obvious genetic differences were observed only in the molecular size of p15E among the proteins tested. The *gag* proteins p15, p12, and p30, and

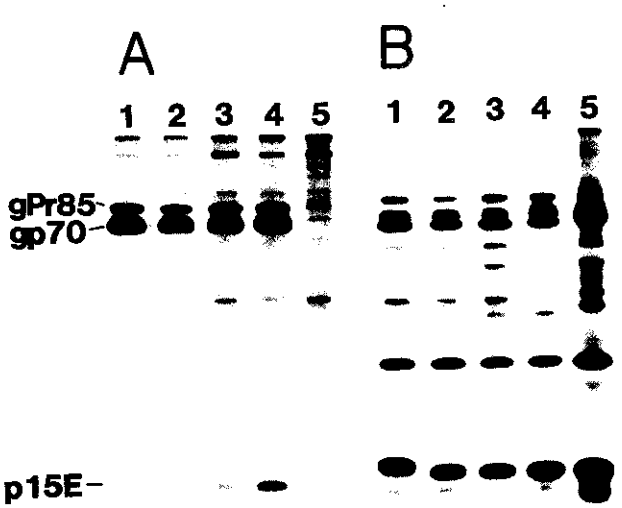

Fig. 4. (A): SDS-PAGE of the immunoprecipitates with the monoclonal antibody to gp70 of PVC321 virus from the lysates of ^{3}H-leucine-labeled NRK cells infected with PVC111 (lane 1), PVC211 (lane 2), PVC321 (lane 3), PVC441 (lane 4) and F-MuLV (lane 5). (B): The supernatants resulting from the first immunoprecipitation were further immunoprecipitated with rat anti-PVC321 serum and analyzed as in (A). The numbering of the lanes is the same as in (A).

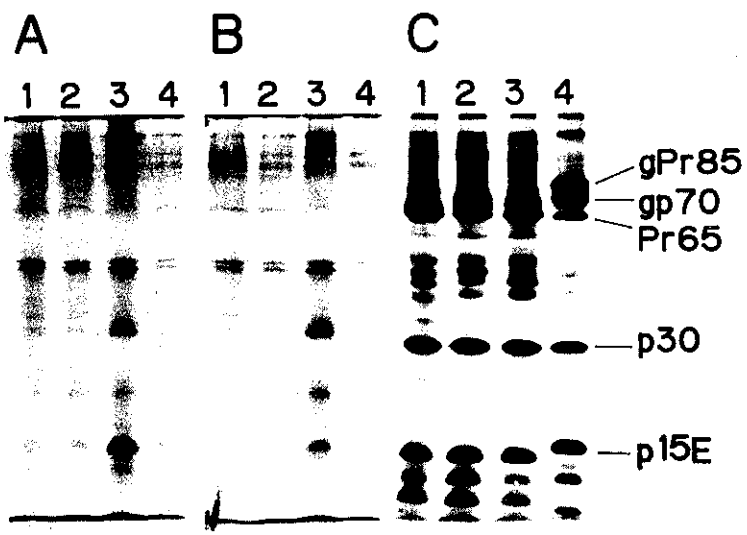

Fig. 5. SDS-PAGE of the immunoprecipitates with monoclonal antibody to gp70 of PVC321 virus (A), normal rat serum (B) and anti PVC321 rat serum (C) from the lysates of ^{3}H-leucine-labeled SC-1 cells infected with 1504E (lane 1), 292E (lane 2), 4070E (lane 3) and 4996E (lane 4).

the *env* protein p12E derived from various PVC viruses coincided in both molecular size and pI (Fig. 1). Since the Pr65s of all PVC viruses showed the same mobility in SDS-PAGE, the p10s of these viruses may also be the same, at least in molecular size (Fig. 2). The gp70s of the PVC viruses coincided in molecular size (Fig. 1) and in immunological reactivity to the monoclonal antibody to gp70 of PVC321 (Fig. 4). Thus, the PVC viruses have very similar genome structures. The p15E of PVC111 was larger (about 0.5K) in molecular size than those of the other viruses (Fig. 2). Since this protein had the same pI as those of the other PVC viruses (Fig. 3), several additional neutral amino acids may be linked covalently to the common structure of p15Es of the others. The large p15E might cause poor growth of PVC111 virus in rats, especially in CNS tissue,[1] as a result of unsatisfactory processing from p15E to p12E.

The polymorphism of p30 was observed as one major spot and one minor spot in our case, unlike other cases in which several species of p30 are present in the virions,[16,17] and rather like the case of Katoh *et al.*[18] Since the polymorphism was demonstrated not to be due

to a technical artifact,[18] the minor p30 might be a product of processing of the *gag-pol* precursor protein, as proposed by Katoh *et al.*[18] In these previous studies, the virions used were all produced from mouse cells, which may produce endogenous MuLV, while our virions were produced from cells originating from rat tissue (NRK) which produces no comparable endogenous MuLV. Thus, it seems that the expression of endogenous MuLV may contribute to the polymorphism of p30.

The polymorphisms of p15E and p12E were also reported by Karshin *et al.*[16] They found two major species of p15E and p12E in the neutral region, while we found two species of p15E in the neutral region and one species of p12E in a fairly acidic region (more acidic than p12) (Fig. 1 and Fig. 3). It is unknown whether this apparent difference in the character of p12E was caused by the difference of virus strain or by the different first dimensions of gel electrophoresis performed (equilibrium or non-equilibrium pH gradient electrophoresis).

In this report, we could not obtain any critical evidence as to whether some gene product(s) of PVC viruses is related to paralysis induction. We tested whether the PVC viruses code a gene responsible for paralysis induction in their genome, like the *src* gene of Rous sarcoma virus, but not the usual *gag, pol,* and *env* genes, by immunizing F344 rats with homogenate of the spleens of F344 rats paralyzed with the PVC321 virus. However, no specific protein was found (data not shown). On the other hand, the monoclonal antibody to gp70 of PVC321 virus showed rigid specificity to the gp70s of PVC viruses but not to those of F-MuLV, AKR ecotropic MuLV, dual-tropic F-MuLV, NZB xenotropic MuLV, and ecotropic viruses of wild mice. This bore some relationship to evidence that the *env* gene of wild mouse ecotropic viruses,[14, 15] or the *ts* mutant of Moloney MuLV[19, 20] is responsible for paralysis induction. Our PVC viruses, except for PVC111, induced tremor in the hind limb, but rarely hind limb paralysis, in NFS mice, and this tremor lasted till the mice died of the leukemia that then developed (manuscript in preparation). These clinical findings apparently differ from those in mice infected with ecotropic viruses of wild mice or with the *ts* mutant of Moloney MuLV. Thus, there is probably no disparity between the fact that the monoclonal antibody to gp70 of PVC321 did not recognize the gp70s of wild mouse ecotropic viruses and the possibility that the *env* gene of PVC viruses may be responsible for the induction of a neurological disorder.

ACKNOWLEDGMENTS

We thank Drs. J. W. Hartley, S. Rasheed and A. Ishimoto for providing the cells infected with various MuLVs. This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

(Received September 9, 1988/Accepted November 15, 1988)

REFERENCES

1) Kai, K. and Furuta, T. Isolation of paralysis-inducing murine leukemia viruses from Friend virus passaged in rats. *J. Virol.*, **50,** 970–973 (1984).
2) McCarter, J. A., Ball, J. K. and Frei, J. V. Lower limb paralysis induced in mice by a temperature-sensitive mutant of Moloney leukemia virus. *J. Natl. Cancer Inst.*, **59,** 179–183 (1977).
3) Officer, J. E., Tecson, N., Estes, J. D., Fontanilla, E., Rongey, R. W. and Gardner, M. B. Isolation of a neurotropic type C virus. *Science,* **181,** 945–947 (1973).
4) Gardner, M. B. Type C viruses of wild mice: characterization and natural history of amphotropic, ecotropic and xenotropic MuLV. *Curr. Top. Microbiol. Immunol.*, **79,** 215–259 (1978).
5) Oldstone, M. B. A., Lampert, P. W., Lee, S. and Dixson, F. J. Pathogenesis of the slow disease of the central nervous system associated with WM1504E virus. *Am. J. Pathol.*, **88,** 193–212 (1977).
6) Hopkins, N., Schindler, J. and Hynes, R. Six NB-tropic viruses derived from a B-tropic virus of BALB/c have altered p30. *J. Virol.*, **21,** 309–318 (1977).
7) Schindler, J., Hynes, R. and Hopkins, N. Evidence for recombination between N- and B-tropic murine leukemia viruses: analysis of three virion proteins by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. *J. Virol.*, **23,** 700–707 (1977).
8) Scindler, J., Gautsh, J. W., Lerner, R. A. and Hopkins, N. Biochemical analysis of the p30's of N-, B-, and NB-tropic murine leukemia viruses of BALB/c origin. *J. Virol.*, **39,** 703–712 (1981).
9) Kai, K., Yoshida, M. and Odaka, T. Strain difference in immune response of the rat to NRK cells infected with spleen focus-forming virus of the Friend leukaemia virus complex. *J. Gen. Virol.*, **64,** 2169–2176 (1983).
10) Hartley, J. W. and Rowe, W. P. Clonal cell lines from a feral mouse embryo which lack host range restrictions for murine leukemia viruses. *Virology,* **65,** 128–134 (1975).
11) Oi, V. T. and Herzenberg, L. A. Immunoglobulin-producing hybrid cell line. *In* "Selected Methods in Cellular Immunology," ed. S. M. Shiigi, pp. 351–372 (1980). W. H. Freeman and Co., San Francisco.
12) Koehler, G. and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature,* **256,** 495–497 (1975).

13) O'Farrell, P. Z., Goodman, H. M. and O'Farrell, P. H. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell,* **12,** 1133–1142 (1977).

14) Lai, M. M. C., Shimizu, S., Rasheed, S., Pal, B. K. and Gardner, M. B. Characterization of genome structure of amphotropic and ecotropic wild mouse retroviruses. *J. Virol.,* **41,** 605–614 (1982).

15) Rassart, E., Nelbach, L. and Jolicoeur, P. Cas-Br-E murine leukemia virus: sequencing of the paralytogenic region of its genome and derivation of specific probes to study its origin and the structure of its recombinant genomes in leukemic tissues. *J. Virol.,* **60,** 910–919 (1986).

16) Karshin, W. L., Arcement, L. J., Naso, R. B. and Arlinghaus, R. B. Common precursor for Rauscher leukemia virus gp69/71, p15(E), and p12(E). *J. Virol.,* **23,** 787–798 (1977).

17) Ledbetter, J. A. Two-dimensional analysis of murine leukemia virus *gag*-gene polyproteins. *Virology,* **95,** 85–98 (1979).

18) Katoh, I., Yoshinaka, Y. and Luftig, R. B. Murine leukaemia virus p30 heterogeneity as revealed by two-dimensional gel electrophoresis is not an artefact of the technique. *J. Gen. Virol.,* **65,** 733–741 (1984).

19) Wong, P. K. Y., Soong, M. M., McLeod, R., Gallick, G. and Yuen, P. H. A group of temperature-sensitive mutants of Moloney leukemia virus which is defective in cleavage of *env* precursor polypeptide in infected cells also induced hind-limb paralysis in newborn CFW/D mice. *Virology,* **125,** 513–518 (1983).

20) Yuen, P. H., Tzen, E., Knupp, C. and Wong, P. K. Y. The neurovirulent determinants of *ts*1, are localized in at least two functionally distinct regions of the genome. *J. Virol.,* **59,** 59–65 (1986).