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Serological Definition of the Lentivirus Group of Retroviruses

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The major polypeptides of visna viruses and other lentiviruses have been isolated and shown to be closely related if not identical in radioimmunoassays. By this criterion the lentiviruses form a distinct group of retroviruses unrelated to spuma viruses, mammalian and avian retroviruses that cause tumors, and unclassified retroviruses of cattle and horses. Two sera obtained from goats immunized with Mason-Pfizer monkey virus or squirrel monkey virus reacted with visna p30. Additional data suggest that this reaction represents infection of goats with a lentivirus or a new retrovirus closely related to the lentiviruses.

Visna, maedi, and progressive pneumonia viruses (PPV) are the cause of slow infections of the central nervous system and respiratory systems of sheep, respectively. All three agents are retroviruses on morphogenetic and biochemical grounds (12) and are related to one another by these criteria, by shared antigens (29), and by nucleic acid homology (16). There as yet has not been a comprehensive study of the taxonomic position of this group of lentiviruses vis-à-vis other members of the family of retroviruses.

In the work presented here, we have employed two sensitive radioimmunoassays (RIAs) for p30, the major structural polypeptide and cross-reacting antigen of retroviruses (9, 24, 25), to analyze potential taxonomic relationships between lentiviruses and other retroviruses. We show that the lentiviruses form a closely related and distinct group of agents that are not related to the RNA viruses that cause tumors or latent infections in animals or to a variety of unclassified retroviruses.

MATERIALS AND METHODS

Viruses: propagation, assay, labeling and purification. Visna (strain 1514) and other lentiviruses were propagated and assayed in sheep choroid plexus (SCP) cells as described previously (14); virion proteins were labeled to specific activities of 2,000 to 3,000 cpm/μg by growing virus in methionine-free medium containing 5 μCi of [^{35}S]methionine (>400 Ci/mmol, New England Nuclear) per ml. Viruses were purified by isopycnic sedimentation (13). The source of virus, antigen, or antisera in the case of other retroviruses is identified by appropriate reference in the text. Polypeptides were analyzed by electrophoresis in gels of polyacrylamide in the presence of sodium dodecyl sulfate (SDS-PAGE) in the discontinuous buffer system described by Laemmli; conditions for maximum resolution of polypeptides of the visna virus have been delineated (13); in this investigation we employed slab rather than cylindrical gels (28).

Isolation of p30. The major structural polypeptide of the lentiviruses and other retroviruses, designated p30 (1), was isolated by two methods differing in the conditions for dissociation. Conditions chosen to minimize denaturation were as follows: 1 to 3 mg of purified virions with 10^6 cpm of ^{35}S-labeled virus was pelleted, resuspended at 1 to 3 mg/ml in a solution of 6 M urea-0.1% Brij-0.1% β-mercaptoethanol, and incubated at 37°C for 30 min. The lysate was introduced into a 110-ml 4 to 8 urea gradient containing 2% ampholines in the pH range 3 to 10 and subsequently subjected to isoelectric focusing at 600 V for 24 h at 6°C. The pH and radioactivity were measured in fractions from the gradient. A typical profile of a gradient is shown in Fig. 1A; analysis of regions of the gradient by SDS-PAGE indicated that p30 migrates near neutrality. Fractions in this region were pooled and refocused in a narrower pH gradient of 5 to 8 (Fig. 1B). Concentrates of material at a pI of 6.9 contained p30 as a single homogeneous species on analysis by SDS-PAGE.

The major polypeptide constituents of the lentiviruses also were isolated by dissociation and chromatographic separation in guanidine hydrochloride (GuHCl) (8, 20, 21, 23). Virus preparations purified by isopycnic banding were concentrated by pelleting; 3 to 5 mg of virus containing 10^6 cpm of ^{35}S-labeled virus was disrupted in 600 μl of 8 M GuHCl-2% β-mercaptoethanol in a buffer containing 50 mM Tris-hydrochloride (pH 8.5)-10 mM EDTA. After incubation at 37°C for 30 min, the density of the sample was increased with sucrose (10% final), and it was loaded on a column (1.5 by 80 cm) containing Bio-Gel A-5 in 6 M GuHCl-20 mM sodium phosphate buffer (pH 6.5)-10 mM dithiothreitol. Fractions of 1 ml were eluted at a rate of 1 ml/h at a pressure of 10 cm; the radioactivity in each fraction was determined in a 20-μl portion. A typical profile is shown in Fig. 2A for visna virus; virtually identical profiles were obtained with maedi virus and PPV (data not shown). The excluded material consisted of gP135 and aggregates

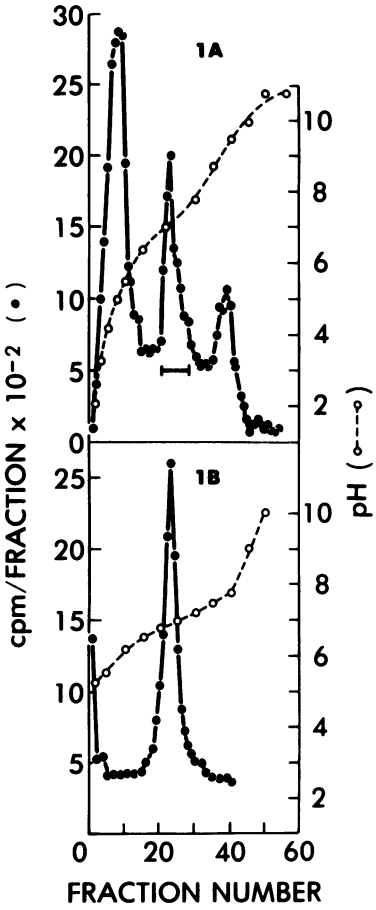

FIG. 1. *Purification of visna virus p30 by isoelectric focusing. Visna virus purified by isopycnic sedimentation was lysed with Brij and urea and focused in a urea gradient, pH 3 to 10 (A). Fractions in the region delimited by the bars were pooled and refocused in a pH 5 to 8 gradient (B).*

containing other virion polypeptides (for nomenclatures, cf. references 13 and 1). Fractions in peaks 1, 2, and 3 were pooled as shown, dialyzed against 140 mM NaCl and then 10 mM NH_4 acetate, and concentrated by lyophilization. Analysis by SDS-PAGE (Fig. 2B) shows that peak 1 contains p30, peak 2 contains p16, and peak 3 corresponds to p14.

Iodination. p30 was iodinated to specific activities generally of about 10^7 to 3×10^7 cpm/μg by oxidation with chloramine T (18). Reaction mixtures contained 10 μg of p30, 1 mCi of ^{125}I, 0.5 μg of chloramine T, 100 mM NaCl, and 10 mM sodium phosphate buffer, pH 7.5. After 10 min at room temperature, the reaction was terminated with 10 μg of sodium metabisulfite, and the ^{125}I-p30 was separated from unincorporated isotope by desalting on a column of Sephadex G15 equilibrated with reaction buffer containing 0.5% bovine serum albumin (BSA); material eluting in the void volume was pooled and stored at 4°C. The ^{125}I-p30 comigrated in PAGE with virion p30 as a single peak (Fig. 3), and >95% was precipitable by antibody to p30.

Immunization. Antisera to visna virus p30 and other isolated components were obtained by immunizing 1-kg guinea pigs in the footpads with virus (100 μg) or p30 (20 μg) emulsified in normal saline and complete Freund adjuvant (15). At 3 weeks the animals were bled by intracardiac puncture and boosted at this time and subsequently at monthly intervals by intramuscular injection of antigens (50 μg of virus; 10 μg of p30) in incomplete Freund adjuvant. Sera obtained 7 days later were tested for antibody against the respective antigens by immunodiffusion and by competition RIA.

Radioimmunoprecipitation. Antisera were tested for antibody to radiolabeled antigen as follows. Serial twofold dilutions of serum in STE buffer (100 mM NaCl–10 mM Tris-hydrochloride (pH 7.4)–1 mM Na-EDTA) with 0.3% BSA were added to an equal volume of buffer containing 10,000 cpm of ^{125}I-p30 of visna virus. Two volumes of 5% normal guinea pig serum in STE-BSA was added, and the mixture was incubated at 37°C for 2 to 4 h. Antibody-antigen complexes were precipitated either by adding an equal volume of 80% saturated $(NH_4)_2SO_4$ (4) or an amount of anti-guinea pig immunoglobulin G previously shown to precipitate guinea pig immunoglobulin G maximally. Precipitates formed after incubation at 37 and 4°C overnight were collected by centrifugation (3,000 $\times$ *g* for 15 min), washed once in 40% saturated $(NH_4)_2SO_4$ or STE-BSA, and counted. Equivalent results were obtained with either method of precipitation. As Fig. 4 illustrates, high-titered antisera were obtained by immunizing guinea pigs with p30 obtained by isoelectric focusing, by GuHCl chromatography, or with disrupted virus. The extent of the reaction and slope of the curve in Fig. 4 tested with each antiserum against p30 were essentially coincident, suggesting that the antigenic determinants of p30 in virions and p30 purified as described were identical. Virus p30 in virions disrupted with a mild, nondenaturing detergent, Triton X-100 (22), and p30 prepared by chromatography in GuHCl also reacted identically in competition RIA (data not shown). Because of the interchangeability of antigens, we subsequently used p30 prepared by the method that gave the highest yield, chromatography in GuHCl.

Competition RIA. In competition RIA the serum dilution determined as described above to precipitate 50% of ^{125}I-p30 was incubated at 37°C for 2 h with the unlabeled antigen; ^{125}I-p30 was added, and the mixture was incubated at 37°C for an additional 2 h. Antibody-antigen complexes were precipitated and counted subsequently as in the radioimmunoprecipitation assay.

RESULTS

Lentivirus p30. The p30's of visna virus, PPV, and maedi virus were indistinguishable by competition RIA (Fig. 5). Moreover, antisera to PPV and Zwoegerziekte virus entirely precipitated visna ^{125}I-p30 (Fig. 1), and, in immunodiffusion, lines of identity were obtained with visna, maedi, or PPV p30 against visna anti-p30 or anti-PPV antisera (data not shown). These vi-

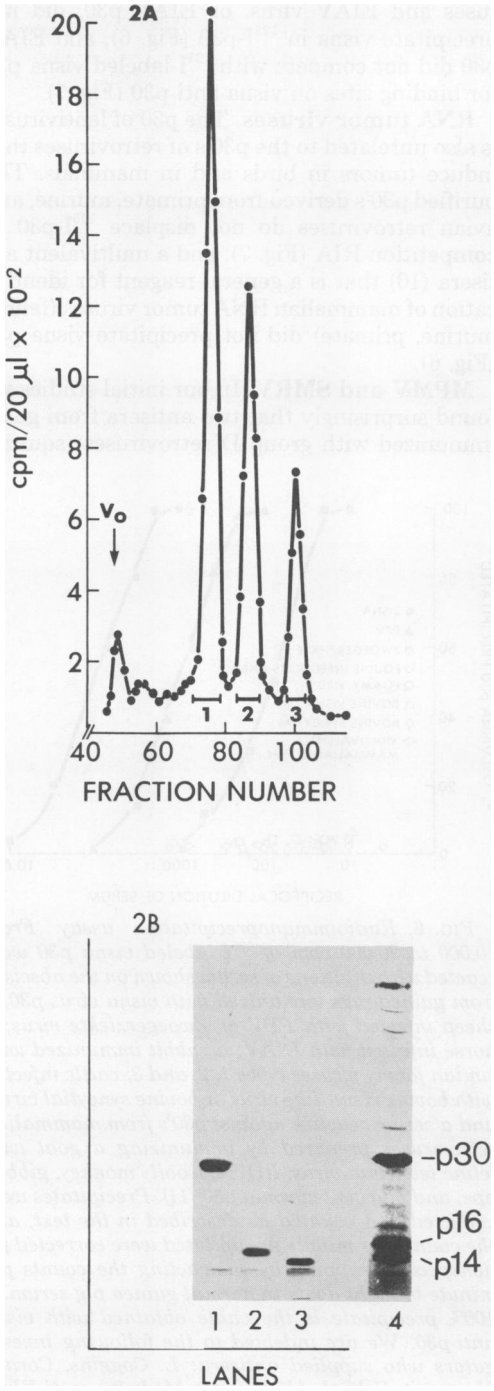

FIG. 2. *Purification of visna virus p30 by chromatography in GuHCl. Purified visna virus preparations were pelleted, disrupted in GuHCl, and chromatographed in GuHCl in a column containing Bio-Gel A-5-Sepharose. The profile of radioactivity of portions of each fraction is displayed in (A); the void volume (V_o) is marked by an arrow. Fractions in peaks designated 1, 2, and 3 were pooled as shown (⊢⊣), freed of GuHCl by dialysis, concentrated by lyophilization, and analyzed by SDS-PAGE in slab gels (B). Lanes 1 to 3 correspond to the respective peaks above in (A); lane 4 contained purified visna virus; the stained bands corresponding to p30, p16, and p14 are identified by symbols next to the band.*

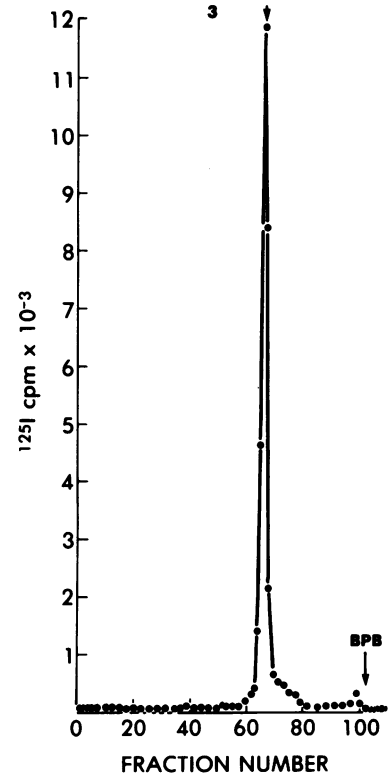

FIG. 3. *Iodinated visna p30. Visna p30 was isolated and iodinated as described in the text. A sample was analyzed by SDS-PAGE in a cylindrical gel. At the conclusion of electrophoresis, the gel was frozen, 1-mm segments were cut with razor blades, and the radioactivity in each fraction was measured in a well-type gamma counter (Nuclear Chicago). Arrows mark the position of visna p30 in a stained gel subjected to electrophoresis in parallel and the position of the bromophenol blue tracking dye (BPB).*

ruses thus have as a group determinants on p30 that are very closely related if not identical by immunological criteria.

Bovine viruses, spuma viruses, and EIAV. There are a number of parallels between lentiviruses, equine infectious anemia viruses (EIAV), unclassified retroviruses of cattle, and retroviruses associated with latent infection (2–4, 11, 22). We therefore looked for evidence of interrelationships in shared antigenic determinants. Antisera against bovine or spuma vi-

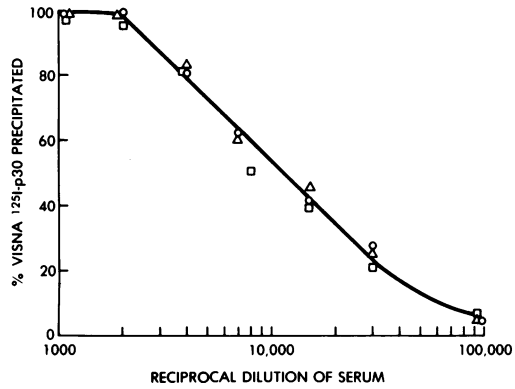

FIG. 4. *Immune precipitation of ^{125}I-labeled visna p30. Serial dilutions of guinea pig anti-visna p30 derived by immunization with p30 purified by isoelectric focusing (○) or GuHCl chromatography (△) or purified visna virus disrupted by freeze-thawing (□) were reacted with ^{125}I-labeled visna p30 purified in GuHCl chromatography and iodinated as described in the text. Precipitates obtained with anti-guinea pig immunoglobulin G were collected by centrifugation and counted. The immunoprecipitate is expressed as the percent counts per minute in the pellet after one washing divided by total immunoprecipitable counts per minute corrected for nonspecific trapping (counts per minute in pellet with serum from an unimmunized guinea pig).*

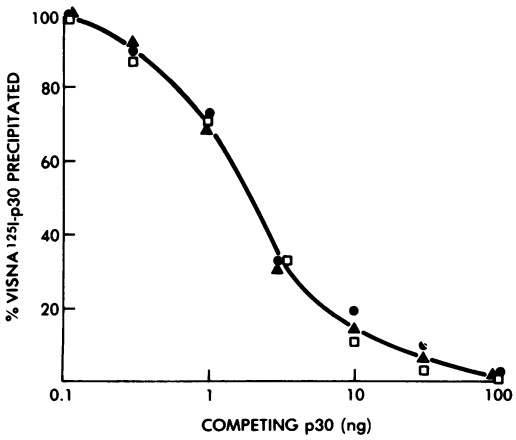

FIG. 5. *Competition RIA, lentivirus p30's. Increasing amounts of unlabeled p30 from visna virus (●), maedi virus (□), or PPV (▲) were added to a dilution of guinea pig anti-visna p30 serum that precipitated 50% of visna ^{125}I-p30. Subsequently, visna ^{125}I-p30 was added, and antibody-antigen complexes were precipitated as described in the text (radioimmunoprecipitation assay). The degree of competition is expressed as a decrease in the percent visna ^{125}I-p30 precipitated relative to the counts per minute in the precipitate obtained without competing unlabeled p30 (100%).*

ruses and EIAV virus, or EIAV p30, did not precipitate visna in ^{125}I-p30 (Fig. 6), and EIAV p30 did not compete with ^{125}I-labeled visna p30 for binding sites on visna anti-p30 (Fig. 7).

RNA tumor viruses. The p30 of lentiviruses is also unrelated to the p30's of retroviruses that induce tumors in birds and in mammals. The purified p30's derived from primate, murine, and avian retroviruses do not displace ^{125}I-p30 in competition RIA (Fig. 7); and a multivalent antisera (10) that is a general reagent for identification of mammalian RNA tumor viruses (feline, murine, primate) did not precipitate visna p30 (Fig. 6).

MPMV and SMRV. In our initial studies we found surprisingly that two antisera from goats immunized with group D retroviruses (squirrel

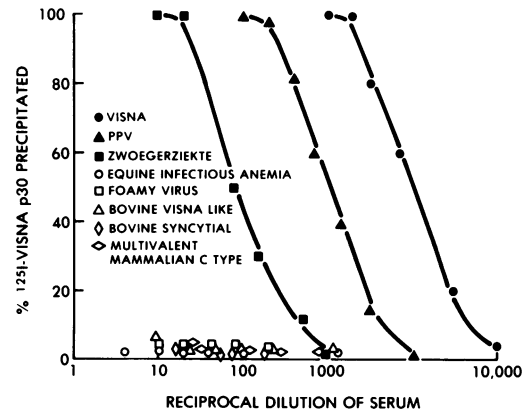

FIG. 6. *Radioimmunoprecipitation assay. From 10,000 to 20,000 cpm of ^{125}I-labeled visna p30 were reacted with dilutions of serum shown on the abscissa from guinea pigs immunized with visna virus p30; a sheep infected with PPV or Zwoegerziekte virus; a horse infected with EIAV; a rabbit immunized with simian foamy viruses types 1, 2, and 3; cattle infected with bovine visna-like virus or bovine syncytial virus; and a serum reactive against p30's from mammalian retroviruses prepared by immunizing a goat with feline leukemia virus, RD114, woolly monkey, gibbon ape, and murine leukemia p30 (11). Precipitates were collected and counted as described in the text, and the counts per minute precipitated were corrected for nonspecific trapping by subtracting the counts per minute brought down in normal guinea pig serum. A 100% precipitate is the value obtained with visna anti-p30. We are indebted to the following investigators who supplied antisera: L. Coggins, Cornell University School of Veterinary Medicine, anti-EIAV (5); J. Coe, W. Hadlow, Rocky Mountain National Laboratory, anti-PPV (19); G. deBoer, Central Veterinary Institute, The Netherlands, anti-Zwoegerziekte (3); J. von der Maatin, U.S. Department of Agriculture, Ames, Iowa, anti-bovine syncytial virus (2); W. Park, National Institutes of Health, Bethesda, Md., antifoamy viruses.*

monkey retrovirus [SMRV] and Mason-Pfizer monkey virus [MPMV]) precipitated ^{125}I-labeled visna p30 (Fig. 8A). On further investigation, we found that only these two antisera were reactive. Other goats immunized with p30's of these viruses, intact or disrupted viruses, or MPMV p10–12 did not react with visna p30, and a rabbit immunized with SMRV p30 did not precipitate visna p30. Moreover, in competition RIA there was no evidence that group D virus p30's share antigenic determinants with visna virus (Fig. 8B). Finally, in the one instance where a prebleed was available for testing, we found antibody to visna p30 (Fig. 8B) in the goat that was immunized with SMRV p30.

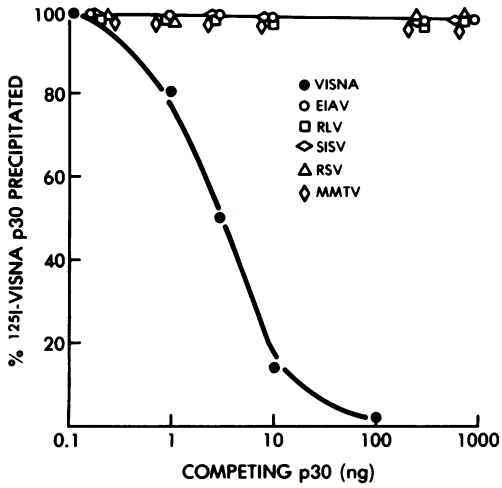

FIG. 7. *Competition RIA, unrelated viruses. A competition RIA was carried out with ^{125}I-labeled visna p30 and unlabeled p30's from visna virus, EIAV, Rauscher leukemia virus (RLV), simian sarcoma virus (SISV), Rous sarcoma virus (RSV), or murine mammary tumor virus (MMTV). We are indebted to T. Crawford, Washington State University, Pullman, Wash., for EIAV p30; J. Gruber, National Cancer Institute, Bethesda, Md., for RLV and SISV p30; and H. Oppermann, University of California, San Francisco, for RSV p30.*

DISCUSSION

In these studies we have employed two sensitive RIAs to arrive at an operational serological definition of retroviruses that cause slow and persistent infections in animals. The lentiviruses visna, maedi, PPV, and Zwoegerziekte show common antigenic determinants on p30, the major structural polypeptide. By this criterion, they form a distinct group apart from spuma viruses, EIAV, and bovine retroviruses that bear some superficial resemblance to lentiviruses in their ability to cause persistent infection (17, 26), in certain aspects of the pathological states that they induce (3), in their morphology (2, 11), and in their ability to cause cell fusion (17). RIAs are not only useful in examining possible interviral relationships, but also in tracing the often unexpected patterns of transmission of retroviruses in the distant past. We recently showed, for

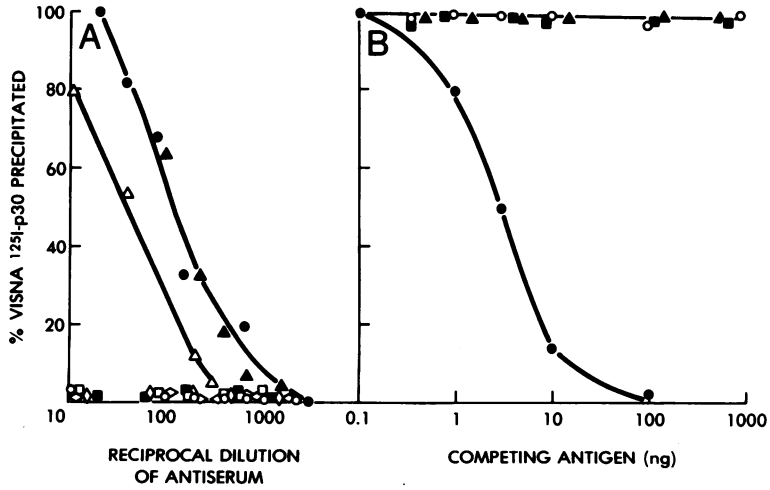

FIG. 8. *(A) Radioimmunoprecipitation assay with antisera to primate viruses. Dilutions of serum from a goat immunized with MPMV (●) p25 and a goat immunized with SMRV (▲) p37 were reacted with visna ^{125}I-p30. Immunoprecipitates were collected and counted as described in the text. Precipitate obtained with the goat serum obtained before immunizing with SMRV p37 (△); reaction of visna ^{125}I-p30 serum from a rabbit immunized with SMRV p37 (○); reaction of visna ^{125}I-p30 with sera from another goat immunized with MPMV p25 (□), goats immunized with MPMV disrupted with Tween-ether (◆) or GuHCl (◇), or intact virus (■). (B) Competition RIA. ^{125}I-labeled visna p30, anti-visna p30 competed with unlabeled p30 from visna virus (●), MPMV p25 (▲), MPMV p10–12 (○), and disrupted SMRV (■).*

example, that the p30 of reticuloendothelial virus, a horizontally transmitted avian virus, has interspecies determinants in common with mammalian C-type viruses (H. P. Charman, R. V. Gilden, and S. Oroszalan, J. Exp. Med., in press). Visna virus is the prototype of retroviruses that spread horizontally in natural infections and in tissue culture (12), but we found no evidence that the antigenic determinants of the lentivirus p30 are shared with mammalian or avian retroviruses that cause tumors or primate group D viruses. This result is in keeping with the distinct differences in the primary structure of visna p30 (S. Oroszlan and R. V. Gilden, *In* H. Fraenkel-Conrat and R. R. Wagner, *Comprehensive Virology*, in press) and the lack of sequence homology between the genome of visna virus and the Src sequences of RNA tumor viruses (27). The singular reactions with goat antisera to SMRV and MPMV, respectively, are most easily explained by infection of these animals by one of the lentiviruses or a virus closely related to them, such as the goat leucoencephalitis agent (6). This interpretation is supported by the presence of antibody to visna p30 in the prebleed, the failure of antisera directed against group D virus or p30 to react with visna p30, and the failure of these antigens to compete with visna p30 in RIA.

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LITERATURE CITED

1. **August, J. T., D. P. Bolognesi, E. Fleissner, R. V. Gilden, and R. C. Nowinski.** 1974. A proposed nomenclature for the virion proteins of oncogenic RNA viruses. Virology **60**:595.
2. **Boothe, A. D., and J. van der Maaten.** 1974. Ultrastructural studies of a visna-like syncytia-producing virus from cattle with lymphocytosis. J. Virol. **13**:197-204.
3. **Charman, H. P., S. Bladen, R. V. Gilden, and L. Coggins.** 1976. Equine infectious anemia virus: evidence favoring classification as a retravirus. J. Virol. **19**:1073-1079.
4. **Charman, H. P., M. H. White, R. Rahman, and R. V. Gilden.** 1976. Species and interspecies radioimmunoassays for rat type C virus p30: interviral comparisons and assay of human tumor extracts. J. Virol. **17**:51-59.
5. **Coggins, L., and N. L. Norcross.** 1970. Immunodiffusion reaction in equine infectious anemia. Cornell Vet. **60**: 330-335.
6. **Cork, L. D.** 1976. Differential diagnosis of leucoencephalomyelinitis in goats. J. Am. Vet. Med. Assoc. **169**: 1303-1306.
7. **De Boer, G. F.** 1975. Zwoegerziekte virus, the causative agent for progressive interstitial pneumonia (maedi) and meningo-leucoencephalitis (visna) in sheep. Res. Vet. Sci. **18**:15-25.
8. **Fish, W. W., K. G. Mann, and C. Tanford.** 1969. The estimation of polypeptide chain molecular weights by gel filtration in 6M guanidine hydrochloride. J. Biol. Chem. **244**:4980-4996.
9. **Geering, G., T. Aoki, and L. J. Old.** 1973. Shared viral antigen of mammalian leukemia. Nature (London) **244**: 266-269.
10. **Gilden, R. V.** 1975. Interrelationships among RNA tumor viruses and host cells. Adv. Cancer Res. **22**:157.
11. **Gonda, M. A., H. P. Charman, J. L. Walker, and L. Coggins.** 1978. Scanning and transmission electron microscopic study of equine infectious anemia virus. Am. J. Vet. Res. **39**:731-740.
12. **Haase, A. T.** 1975. The slow infection caused by visna virus. Curr. Top. Microbiol. Immunol. **72**:101-156.
13. **Haase, A. T., and J. R. Baringer.** 1974. The structural polypeptides of RNA slow viruses. Virology **57**:238-250.
14. **Haase, A. T., and W. Levinson.** 1973. Inhibition of RNA slow viruses by thiosemicarbazones. Biochem. Biophys. Res. Commun. **51**:875-880.
15. **Haase, A. T., and H. G. Pereira.** 1972. Adenovirus immunoadsorbent: preparation of virus neutralizing antibody. J. Immunol. **108**:633-636.
16. **Harter, D. H., R. Axel, A. Burny, S. Gulati, J. Schlom, and S. Spiegelman.** 1973. The relationship of visna, maedi, and RNA tumor viruses as studied by molecular hybridization. Virology **52**:287-291.
17. **Hooks, J. J., and C. J. Gibbs, Jr.** 1975. The foamy viruses. Bacteriol. Rev. **39**:169-185.
18. **Hunter, W. M., and F. C. Greenwood.** 1962. Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature (London) **194**:495-496.
19. **Kennedy, R. C., E. M. Eklund, C. Lopez, and W. J. Hadlow.** 1968. Isolation of a virus from the lungs of Montana sheep affected with progressive pneumonia. Virology **35**:483-484.
20. **Lin, F. H.** 1978. Polyacrylamide gel electrophoresis of visna virus polypeptides isolated by agarose gel chromatography. J. Virol. **25**:207-214.
21. **Lin, F. H., and H. Thormar.** 1974. Substructures and polypeptides of Visna virus. J. Virol. **14**:782-790.
22. **Makino, S., J. A. Reynolds, and C. Tanford.** 1973. The binding of deoxycholate and Triton X-100 to proteins. J. Biol. Chem. **248**:4926-4932.
23. **Mehta, P. D., F. H. Lin, and H. Thormar.** 1976. Antigenic analysis of isolated polypeptides from Visna virus. Infect. Immun. **13**:1728-1732.
24. **Oroszlan, S., C. L. Fisher, T. B. Stanley, and R. V. Gilden.** 1971. Species-specific and interspecific antigenic determinants of feline C-type virus. Proc. Natl. Acad. Sci. U.S.A. **68**:901-904.
25. **Parks, W. P., and E. M. Scolnick.** 1972. Radioimmunoassay of mammalian type-C viral proteins: interspecies antigenic reactivities of the major internal polypeptide. Proc. Natl. Acad. Sci. U.S.A. **69**:1766-1770.
26. **Parks, W. P., and G. J. Todaro.** 1972. Biological properties of forming ("foamy") viruses. Virology **47**: 673-783.
27. **Stehelin, D., R. V. Guntaka, H. E. Varmus, and J. M. Bishop.** 1976. Purification of DNA complementary to nucleotide sequences required for neoplastic transformation of fibroblasts by avian sarcoma viruses. J. Mol. Biol. **101**:349-365.
28. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**: 237-248.
29. **Weiss, M. J., E. P. Zeelon, R. W. Sweet, D. H. Harter, and S. Spiegelman.** 1977. Immunological cross-reactions of the major internal protein component from "slow" viruses of sheep. Virology **76**:851-854.