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Interspecies radioimmunoassay for the major structural proteins of primate type-D retroviruses

(squirrel monkey retrovirus/Mason-Pfizer monkey virus/viral proteins/endogenous primate viruses/RNA tumor viruses)

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ABSTRACT **A competition radioimmunoassay has been developed in which type-D retroviruses from three primate species compete. The assay utilizes the major structural protein (36,000 daltons) of the endogenous squirrel monkey retrovirus and antisera directed against the major structural protein (27,000 daltons) of the Mason-Pfizer monkey virus isolated from rhesus monkeys. Purified preparations of both viruses grown in heterologous cells, as well as extracts of heterologous cells infected with squirrel monkey retrovirus or Mason-Pfizer monkey virus, compete completely in the assay. Addition of an endogenous virus of the langur monkey also results in complete blocking. No blocking in the assay is observed with type-C baboon viruses, woolly monkey virus, and gibbon virus. Various other type-C and type-B viruses also showed no reactivity. An interspecies assay has thus been developed that recognizes the type-D retroviruses from both Old World monkey (rhesus and langur) and New World monkey (squirrel) species.**

The various retrovirus isolates (1-4) from a rhesus carcinoma, rhesus lactating mammary gland, and rhesus placenta all share a common morphology, distinct from mammalian type-B and type-C retroviruses, and are now referred to as type-D retroviruses (5). The prototype virus of this group, the Mason-Pfizer monkey virus (MPMV), is transmitted in the rhesus (*Macaca mulatta*) population by a non-germ-line mechanism (6, 7). A virus morphologically similar to MPMV has recently been isolated from a spectacled langur (*Presbytis obscuris*) and has been shown to be an endogenous virus of that species (8). Both the rhesus and langur are Old World monkeys.

The squirrel monkey (*Saimiri sciureus*) is a New World species. Heberling *et al.* (9) reported that a virus morphologically similar to MPMV could be isolated from squirrel monkey tissues by the cocultivation of those tissues with cells of another species. We have recently shown that the squirrel monkey retrovirus (SMRV) is an endogenous virus of squirrel monkeys (10). Morphologically similar viruses from squirrel monkeys have also been isolated by Todaro *et al.* (8). To date, little if any nucleic acid sequence homology has been detected between the RNA genomes of SMRV and MPMV (10), whereas some homology is observed between the RNA genomes of the Old World monkey isolates, MPMV and the langur virus (8).

We report here that the major structural proteins of MPMV (27,000 daltons, p27) and SMRV (36,000 daltons, p36) share common antigenic determinants. We have developed a competition radioimmunoassay using radioactively labeled p36 of SMRV and antisera prepared against the p27 of MPMV. In this assay, complete blocking is observed upon the addition of the retroviruses isolated from rhesus, langur, or squirrel monkeys. No blocking is observed when mammalian type-C or type-B viruses are added. This interspecies assay thus recognizes both Old World and New World monkey type-D retrovirus isolates.

MATERIALS AND METHODS

Viruses. Squirrel monkey retrovirus (SMRV) was isolated from supernatant fluids from mixed cultures of squirrel monkey (*Saimiri sciureus*) lung cells and fetal canine thymus cells, Cf2Th (11). These cells were kindly supplied by R. Heberling and S. Kalter, Southwest Research Foundation, San Antonio, TX. After several months in culture, all cells were shown to be canine (10). Two additional SMRV isolates, one from a squirrel monkey kidney and the other from a spleen (and both grown in canine thymus cells), were kindly supplied by G. Todaro, National Cancer Institute. Mason-Pfizer monkey virus (MPMV) was grown in suspension cultures of the normal human lymphocyte cell line NC-37 (J. L. Smith Memorial Laboratories, Pfizer, Inc. Maywood, NJ), or in transformed Aleutian mink lung cells (12) kindly supplied by E. Scolnick, National Cancer Institute. SMRV and MPMV were purified from culture supernatant fluids by equilibrium density gradient centrifugation and concentrated as previously described (13).

Avian myeloblastosis virus was obtained from the plasma of leukemic chickens (Life Sciences, Inc., St. Petersburg, FL). Feline leukemia virus (Rickard) was grown in feline thymus cells. Murine leukemia virus (Rauscher) was obtained from plasma of infected BALB/c mice (University Laboratories, Inc., Highland Park, NJ). Simian sarcoma virus 1 was grown in NC-37 cells. Baboon virus (BKCT) was obtained from a cocultivation of baboon kidney cells with canine thymus cells. RD-114 virus was grown in RD cells (Pfizer Inc., Maywood, NJ). Murine mammary tumor virus was grown in C3H murine mammary tumor cells (Frederick Cancer Research Center, Frederick, MD). The above seven viruses were obtained via the Virus Cancer Program Office of Resources and Logistics, National Cancer Institute. The following viruses were kindly supplied by G. Todaro and L. Fedele, National Cancer Institute: gibbon brain virus, grown in bat lung cells; baboon (*Papio papio*) virus, grown in kidney cells; baboon (*Papio cynocephalus*) M7 virus, grown in canine thymus cells; spectacled langur virus, grown in bat lung cells; European wildcat virus, grown in bat lung cells; and *Mus cervicolor* virus (14), grown in murine NIH 3T3 cells.

Purification of Viruses. Amino acid-labeled SMRV was obtained from supernatant fluids of cells grown with ^{14}C-labeled mixed amino acids (50 μCi/ml) as previously described

Abbreviations: MPMV, Mason-Pfizer monkey virus; SMRV, squirrel monkey retrovirus; TNE, Tris/NaCl/EDTA buffer; $NaDodSO_4$, sodium dodecyl sulfate.

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(15). SMRV was concentrated by ammonium sulfate precipitation (50% saturation). The precipitate was pelleted at 4° at 5000 × *g* and suspended in TNE (0.01 M Tris·HCl, pH 7.2/0.1 M NaCl/0.001 M EDTA). The virus was purified by sedimentation through 30% (wt/vol) sucrose in TNE for 30 min at 50,000 rpm in a SW 50.1 rotor (Beckman) onto a 65% (wt/vol) sucrose in TNE pad. The virus was collected from the top of the 65% sucrose, diluted, and layered over a 15–65% sucrose gradient in TNE and centrifuged at 50,000 rpm for 60 min. The virus was collected at a density of 1.17 g/ml and pelleted at 50,000 rpm for 30 min.

Unlabeled SMRV and MPMV were concentrated from cell culture supernatant fluids by continuous flow centrifugation and further purified by two successive equilibrium density gradients as described above. Virus was then pelleted and protein concentrations were determined using the method of Lowry *et al.* (16).

Iodination. Purified SMRV (200 μg in 500 μl of 0.25 M sodium phosphate buffer at pH 7.2), disrupted in 0.5% nonionic detergent NP-40 at 37° for 15 min, was iodinated using a modification of the lactoperoxidase method of Witte *et al.* (17). The reaction was catalyzed by 1 μg of lactoperoxidase in the presence of 20 μM potassium iodide and 1 mCi of $Na^{125}I$ (Amersham/Searle). Unbound ^{125}I was removed by gel filtration. The labeled sample was dialyzed until the trichloroacetic acid precipitability was greater than 90% of the total radioactivity.

Purification of SMRV 36,000-Dalton Protein (p36). The ^{125}I-labeled SMRV was added to 0.01 M sodium phosphate buffer at pH 7.2, 1% sodium dodecyl sulfate ($NaDodSO_4$), 1 M urea, 1% (vol/vol) mercaptoethanol and 10% (vol/vol) glycerol, and immersed in boiling water for 1 min. Bromocresol green marker dye was added, and the samples were subjected to electrophoresis through 10% acrylamide gels (0.5 × 10 cm) at 3 mA per gel as described by Maizel (18). Electrophoresis was stopped when the marker dye reached the bottom of the gel (about 16 hr). Gels were then frozen and sliced in 1-mm sections. The radioactivity in the slices was measured in a γ counter, and the ^{125}I label in the 36,000-dalton region was eluted from gel slices by overnight incubation at 37° in TNE containing 0.1% Triton X-100 and 0.2% bovine serum albumin.

Preparation of Cell Extracts. Approximately 2–3 g of cells was diluted with 10 ml of extraction buffer (0.05 M Tris·HCl, pH 8.0/0.4 M KCl/5 mM sodium acetate/0.5% Triton X-100) and disrupted with a Silverson homogenizer for 2 min. The extract was clarified by centrifugation at 3000 × *g* for 10 min. Sodium deoxycholate was added to the supernatant to a final concentration of 0.5%, and the volume was adjusted to 15 ml. After incubation for 5 min at 37°, the sample was extracted twice with 2 volumes of ether. The aqueous phase was concentrated using Aquacide II (Calbiochem) to approximately 5 ml after the ether was evaporated with nitrogen. The protein extracts were then dialyzed against 0.01 M Tris·HCl (pH 7.8), and the protein concentration was determined.

Radioimmunoassays. Radioimmunoassays were performed in TNE containing 0.1% Triton X-100 and 0.2% bovine serum albumin. Competing antigens in 100 μl were added to 20 μl of 3% NP-40 and incubated at 37° for 15 min. Ten microliters of appropriately diluted goat antisera to MPMV p27 (obtained from Huntington Laboratories via the Virus Cancer Program Office of Resources and Logistics, National Cancer Institute) was then added and incubated at 37° for 2 hr. Iodinated SMRV p36 (5000 cpm) in 20 μl was then added, followed by incubation at 37° for 2 hr. Finally, 50 μl of porcine anti-goat IgG was added, and the incubation was continued for an additional hr at 37° and then overnight at 4°. Following centrifugation at 10,000 × *g* for 1.5 min, 100 μl of the supernatant was removed and assayed for radioactivity. The remaining 100 μl of the total sample was also assayed for radioactivity. Percent precipitation was calculated by dividing the difference in cpm between the two 100-μl aliquots by the sum in cpm of the same aliquots and multiplying by 100. Percent bound was calculated by dividing percent precipitation in the presence of competing antigen by the percent precipitation in the absence of competing antigen and multiplying by 100. Binding of SMRV ^{125}I-labeled p36 (^{125}I-p36) in the presence of normal goat serum was less than 3%.

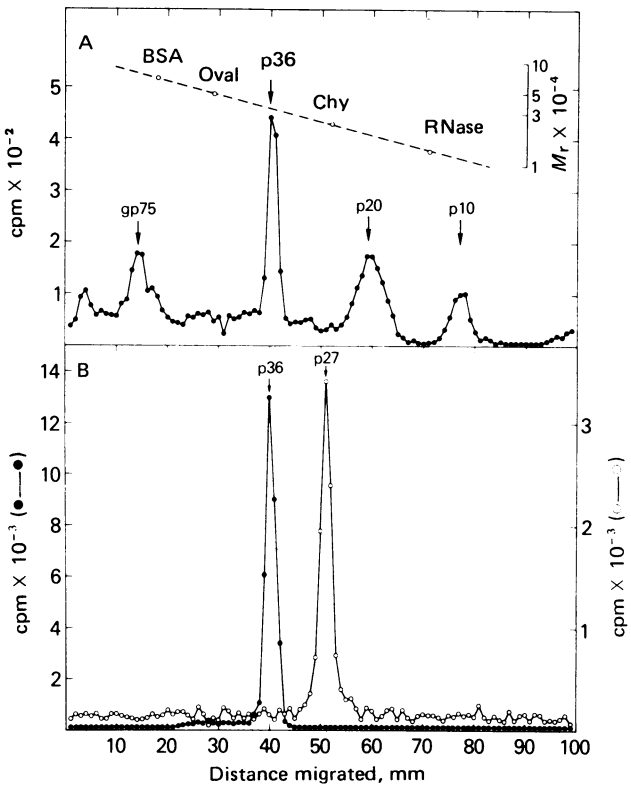

FIG. 1. (*A*) $NaDodSO_4$/polyacrylamide gel electrophoresis of SMRV ^{14}C-labeled polypeptides. SMRV-infected canine cells were grown with ^{14}C-labeled mixed amino acids. Virus was concentrated and purified by equilibrium density gradient analysis. Molecular weight (M_r) markers used were: bovine serum albumin (BSA); ovalbumin (Oval); chymotrypsin (Chy); ribonuclease (RNase). (*B*) $NaDodSO_4$/polyacrylamide gel electrophoresis of the ^{125}I-labeled major structural proteins of SMRV (p36) and MPMV (p27). Purified SMRV and MPMV preparations were disrupted with 0.5% NP-40 and iodinated by the lactoperoxidase method. The p36 of SMRV and the p27 of MPMV were eluted from gels and rerun on $NaDodSO_4$/polyacrylamide gels.

Antisera to SMRV were prepared in rabbits by inoculation with 200 μg of SMRV in complete Freund's adjuvant, followed by monthly intradermal inoculations with 200 μg of SMRV.

RESULTS

Polypeptide Composition of SMRV. SMRV, grown in canine cells, was labeled with ^{14}C-labeled mixed amino acids, and purified by equilibrium density gradient analysis as described in *Materials and Methods*. The virus, banding at a density of 1.17 g/ml, was pelleted and the polypeptides were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. As can be seen in Fig. 1*A*, the major virus-associated polypeptides have molecular weights of 75,000, 36,000, 20,000, and 10,000. The 75,000-dalton protein was shown to be a glycoprotein by

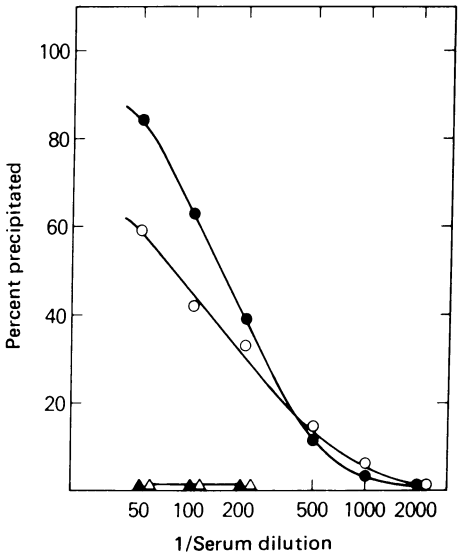

FIG. 2. Precipitation of SMRV ^{125}I-p36 by antisera to SMRV and MPMV. Various input dilutions of rabbit anti-SMRV (●) were incubated with ^{125}I-labeled SMRV p36; immune complexes were precipitated using goat anti-rabbit IgG. Serum obtained from the same rabbit prior to immunization was used as a control (▲). Various input dilutions of goat anti-MPMV p27 (○) were incubated with ^{125}I-labeled SMRV p36; immune complexes were precipitated with porcine anti-goat IgG. Serum obtained from the same goat prior to immunization was used as a control (△).

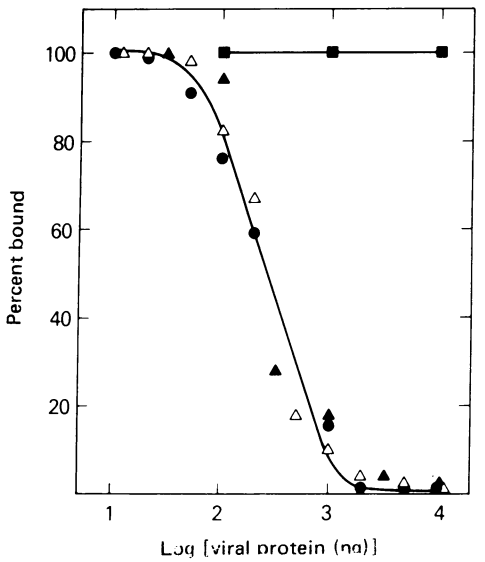

FIG. 3. Competitive radioimmunoassay using antisera to MPMV p27 and SMRV ^{125}I-p36. The assay used anti-MPMV p27 at a final dilution of 1:2000 and 5000 cpm ^{125}I-p36 of SMRV. The following purified virion preparations were used as competitors: MPMV grown in human NC-37 cells (△); MPMV grown in mink cells (▲); SMRV grown in canine cells (●); baboon virus grown in canine cells (■).

[^{3}H]glucosamine labeling and by periodic acid Schiff staining. This is in general agreement, with some differences, with another recent report (19).

Purified SMRV, disrupted with 0.5% NP-40 and iodinated using lactoperoxidase, gave polypeptide profiles similar to that of the ^{14}C-labeled SMRV polypeptides when analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. When the major polypeptide, p36, was eluted from the gels and rerun via NaDodSO$_4$/polyacrylamide gel electrophoresis, the polypeptide was shown to be greater than 90% pure (Fig. 1*B*). The major internal protein of MPMV, p27, was similarly prepared and also shown to be greater than 90% pure (Fig. 1*B*). These iodinated polypeptides were used in the radioimmunoassays described below.

Titration of Antisera. Antisera prepared in rabbits against disrupted SMRV were tested for their ability to bind iodinated p36 isolated from SMRV by NaDodSO$_4$/polyacrylamide gel electrophoresis. Various dilutions (1:50–1:10,000) were incubated with SMRV ^{125}I-p36; the immune complexes were precipitated using goat anti-rabbit IgG. As can be seen in Fig. 2, greater than 80% of the SMRV ^{125}I-p36 was precipitated by the anti-SMRV sera, while none of the labeled p36 was precipitated by serum obtained from the same rabbit prior to inoculation with SMRV.

The antiserum prepared in goats against MPMV p27 was also tested for its ability to bind the SMRV ^{125}I-p36. Up to 60% of SMRV ^{125}I-p36 was bound by the anti-MPMV p27 (Fig. 2). None of the labeled SMRV p36 was precipitated, however, when the preimmune serum from the same goat was used. The goat anti-MPMV p27 showed one line of precipitation to disrupted MPMV in Ouchterlony tests, did not react with fetal calf serum proteins, and has been extensively characterized previously (20).

Interspecies Competitive Radioimmunoassay. Goat antisera to MPMV p27 was used at an input dilution of $^1/_{100}$ to bind and precipitate SMRV ^{125}I-p36. This binding could be completely blocked by the input of detergent-disrupted SMRV or MPMV as competitors (Fig. 3). Inputs of 250 ng of purified SMRV or MPMV gave 50% blocking, while complete blocking could be obtained using 2000 ng. The blocking by SMRV and MPMV could not be due to a primate-specific protein because the MPMV used was grown in human, rhesus, and mink cells, and the SMRV was grown in canine cells (Fig. 3).

The p27 of MPMV was purified by the same technique de-

Table 1. Specificity of the type-D retrovirus interspecies assay

Competitor	% blocking at 10^4 ng*	Ref.
Primate viruses		
MPMV	100	1
SMRV, lung isolate	100	9
spleen isolate	100	8
kidney isolate	100	8
Langur virus	100	8
Baboon virus, *Papio cynocephalus*, M7	<5	21
Papio cynocephalus, BKCT	<5	22
Papio papio	<5	22
Woolly monkey virus	<5	23
Gibbon virus	<5	24, 25
Nonprimate viruses		
Murine mammary tumor virus (C3H)	<5	26
Mus cervicolor virus (M432)	<5	14
Murine leukemia virus (Rauscher)	<5	27
Feline leukemia virus (Rickard)	<5	28
RD-114	<5	29
European wildcat virus	<5	30
Avian myeloblastosis virus	<5	31
Fetal calf serum	<5	

* The competitive radioimmunoassay utilized anti-MPMV p27 and SMRV ^{125}I-p36 as described in *Materials and Methods*.

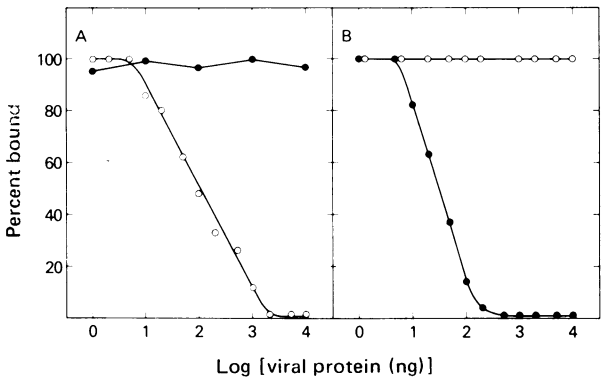

FIG. 4. Homologous competitive radioimmunoassays for MPMV p27 and SMRV p36. (*A*) Competitive radioimmunoassay for MPMV p27 using goat anti-p27 (1:20,000 final dilution in the assay) and MPMV ^{125}I-p27 as described (20). (*B*) Competitive radioimmunoassay for SMRV p36 using rabbit anti-SMRV (1:4000 final dilution in the assay) and SMRV ^{125}I-p36 as described in the *text*. Purified preparations of MPMV (○) and SMRV (●) were used as competitors.

scribed to purify the SMRV p36 (Fig. 1*B*). Purified MPMV p27 and SMRV p36 both completely blocked the interspecies radioimmunoassay.

No blocking in the assay was observed following the addition of: three different baboon type-C virus isolates (Fig. 3 and Table 1), simian sarcoma virus, gibbon virus; and a variety of other type-C and type-B retroviruses (Table 1).

Homologous Radioimmunoassays. The specificity of the interspecies radioimmunoassay was further tested for possible artifacts by examining the addition of SMRV and MPMV in the homologous assays specific for MPMV p27 and SMRV p36. Goat anti-MPMV p27 was used at an input dilution of $^1/_{1000}$ to bind iodinated MPMV p27 in the radioimmunoassay for MPMV p27. As shown in Fig. 4*A*, binding was completely blocked by purified Mason-Pfizer virions at an input of 2 μg (50% point, 0.1 μg). No blocking was observed when SMRV was used as a competitor at inputs up to 10 μg (Fig. 4*A*). In the homologous SMRV assay, rabbit anti-SMRV was used at an input dilution of $^1/_{200}$ to bind the iodinated SMRV p36. This binding could be blocked completely by 0.5 μg of SMRV (50% point, 0.03 μg) (Fig. 4*B*). No blocking was observed when MPMV was used as a competitor at inputs up to 10 μg. These data are evidence that: (*a*) the affinity of binding is higher in the homologous assay than in the interspecies assay, and (*b*) the blocking observed by SMRV and MPMV in the interspecies aassay is not due to an artifact such as the presence of proteases in competitor preparations.

Cell Extracts. Extracts were prepared from canine thymus cells producing SMRV and human NC-37 cells producing MPMV as well as from the corresponding uninfected cells.

The extract of canine thymus cells producing SMRV completely blocked the binding in the interspecies radioimmunoassay between anti-MPMV p27 and SMRV ^{125}I-p36 at 500 μg of input. No blocking of the radioimmunoassay was observed with the protein extract from uninfected canine thymus cells (Table 2). The protein extract from NC-37 cells producing MPMV completely blocked the interspecies radioimmunoassay at an input of 100 μg; no significant blocking of the assay was observed with protein extracts from uninfected NC-37 cells at inputs up to 300 μg (Table 2).

Table 2. Detection of type-D retrovirus antigens in heterologous cell extracts

Cells	% blocking* 30 μg†	100 μg†	300 μg†
NC-37	<5	<5	<5
NC-37 infected with MPMV	80	100	100
Canine thymus	<5	<5	<5
Canine thymus infected with SMRV	20	55	90

* The competitive radioimmunoassay utilized anti-MPMV p27 and SMRV ^{125}I-p36.

† Input of protein from cell extracts.

Table 3. Properties shared by all the type-D retrovirus isolates

1. Intracytoplasmic A particles in the infected cell
2. Budding with nucleoid complete (in majority of particles)
3. Intermediate layer in mature particle
4. Absence of large spikes with knobs (distinguishing from murine mammary tumor virus)
5. Mg^{2+} preference for viral DNA polymerase
6. Molecular weight of viral DNA polymerase of 80,000–90,000
7. Some crossreactivity in the MPMV-baboon virus gp70 assay
8. Complete crossreactivity in the MPMV-SMRV p36 interspecies assays

See the following references for details: 1, 8, 9, 10, 19, 32, 33.

Other Type-D Isolates. Viruses morphologically similar to SMRV (9) have recently been isolated (8) from squirrel monkey spleen and kidney. The addition of these isolates to the interspecies assay also resulted in complete inhibition of binding (Fig. 5 and Table 1).

A virus similar to MPMV has recently been isolated from a spectacled langur (*Presbytis obscurus*) (8). When purified langur virus was used as competitor in the type-D interspecies assay, complete blocking was observed (Fig. 5). This demonstrates that the langur virus also shares the interspecies determinants that are found associated with the major structural proteins of MPMV and SMRV.

DISCUSSION

The assay described here distinguishes type-D virus isolates (of rhesus, langur, and squirrel monkeys) from known mammalian type-B and type-C retroviruses. To date there have been six non-human primate species from which retroviruses have been isolated; these include the baboon, woolly monkey, and gibbon,

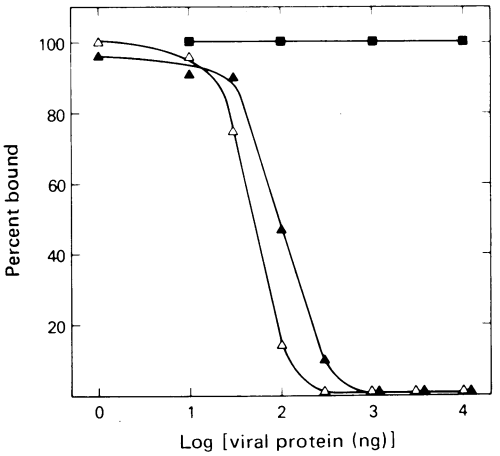

FIG. 5. Interspecies competitive radioimmunoassay for type-D retroviruses. The assay used antisera to MPMV p27 and ^{125}I-p36 of SMRV. The following purified virus preparations were used as competitors: langur virus grown in bat cells (△); a squirrel monkey kidney isolate grown in canine cells (▲); simian sarcoma virus grown in human NC-37 cells (■).

from which type-C viruses have been isolated, and the three species described here, from which type-D viruses have been isolated.

Table 3 contains a list of the various morphological, biochemical, and immunological properties shared by all the type-D isolates. Whereas some type-B and type-C viruses also share some of these properties, only the type-D viruses share all of them. For example, recent studies (8, 32) have shown that the following viruses compete in an assay utilizing antisera to MPMV and the gp70 of the baboon virus: MPMV, baboon virus, langur virus, SMRV, and RD-114. That assay, therefore, appears to be detecting determinants shared by some type-C and type-D viruses. It should be pointed out that three different baboon isolates were used in the assay described here and none showed blocking (Table 1).

The interspecies assay described here is most likely directed towards virus-coded determinants, because virus preparations grown in heterologous cells were used; furthermore, extracts of these same heterologous cells, when uninfected, showed no competition in the assay. The affinity of binding in the homologous reaction with antisera to MPMV and MPMV p27 is, as expected, stronger than it is with SMRV p36. This is evidenced by the fact that SMRV does not compete in the homologous MPMV p27 assay (Fig. 4*A*). The analogous situation is also true for the homologous SMRV assay, in which MPMV does not compete (Fig. 4*B*). It should be pointed out that the interspecies assay described here utilizes purified p36 (Fig. 1*B*) obtained from $NaDodSO_4$ gels. The antigenic nature of the ^{125}I-p36 used in the assay appears to be extremely similar to that of p36 in its native form in the virion; SMRV p36, purified by ion-exchange chromatography and gel filtration in the presence of 6 M guanidine hydrochloride, and purified virion preparations block completely (Fig. 3) and with the same slope as purified p36 preparations from $NaDodSO_4$ gels.

The lack of appreciable nucleic acid homology between the MPMV and SMRV genomes (10) is in concord with their hosts' distant geographic habitat and diverse evolution. The detection of common antigenic determinants between the major structural proteins of MPMV and SMRV indicates the differences in sensitivities of the immunologic versus the hybridization assays. It should be pointed out that little or no detectable nucleic acid sequence homology is observed between type-C leukemia viruses of various species, whereas various interspecies determinants exist (34–36). One of several possible explanations for the complete blocking observed by both MPMV and SMRV in the assay described here is the presence of highly conserved antigenic determinants shared by a wide range of diverged primates that contain type-D retroviruses.

(Following submission of this manuscript we became aware of an independent study providing similar evidence from another laboratory [see accompanying article (37)].)

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