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SEROLOGICAL EVIDENCE OF HERPESVIRUS INFECTION IN CAPTIVE ASIAN ELEPHANTS (*ELEPHAS MAXIMUS*)

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ABSTRACT: In mid 1988 a 3-yr-old Asian elephant (*Elephas maximus*) from a circus in Switzerland died following generalized manifestation of a herpesvirus infection. In an effort to determine prevalence of infection with the herpesvirus, and due to lack of a corresponding virus isolate, it was decided to evaluate contact animals and elephants from a second herd for antibody to bovine herpesvirus 1 (BHV1) and bovine herpesvirus 2 (BHV2). Of 15 sera tested four displayed low neutralizing antibody titers to BHV2. None of the sera neutralized BHV1. However, as evidenced by protein A-mediated immunoprecipitation of metabolically radio-labeled virus-infected and mock-infected cell antigens, followed by separation of precipitation products in SDS-polyacrylamide gels, the 15 sera precipitated multiple antigens from both viruses. Similar results were obtained when using BHV4 antigens. The extent of reaction was most distinct with respect to BHV2 antigens, less prominent with BHV1 antigens, and least with BHV4 antigens. The respective protein patterns, although less marked, matched well with those obtained with bovine reference sera. Additional evaluation of sera from six elephants from two zoos in the Federal Republic of Germany gave essentially identical results. It was concluded that at least one herpesvirus, immunologically related to BHV2, may be widely distributed among captive Asian elephants, and that this virus apparently does not cause overt disease in the majority of animals.

Key words: Asian elephant, *Elephas maximus*, captive study, bovine herpesviruses, serology, neutralization, radio-immunoprecipitation, protein A, protein G, captive study.

INTRODUCTION

Elephants are classified (Schmidt, 1986) within the order Proboscidae. There are two genera in the single family Elephantidae, the Asian elephant (*Elephas maximus*) and the African elephant (*Loxodonta africana*).

To date, only a few viral infections have been recognized in wild and captive elephants. Among these are infections with the viruses of rabies, foot-and-mouth disease, rinderpest and encephalomyocarditis (Schmidt, 1986), infections with poxviruses (Pilaski et al., 1986; Pilaski and Rösen-Wolff, 1988), and cutaneous lesions induced by a papillomavirus (Sundberg et al., 1981). Previous reports indicated that African and Asian elephants were probably hosts for a hitherto unidentified herpesvirus. Thus, the early report of McCully et al. (1971) provided morphological evidence for the widespread occurrence of herpesvirus-related nodular lung alterations in African elephants. A recent report by Jacobson et al. (1986) showed that 27 members of the same species were heavily affected with proliferative cutaneous lesions attributed to a herpesvirus. Pilaski et al. (1987, 1988) described herpesvirus-associated nodular and warty skin afflictions in Asian elephants. Finally, a 3-yr-old Asian elephant from a circus in Switzerland died during the course of a herpesvirus-associated generalized illness of short duration. This diagnosis was based on postmortem examinations (P. Ossent and F. Guscetti, unpubl. data). Briefly, necropsy was characterized by extensive hemorrhages in multiple tissues. Microscopic findings were dominated by widespread necrotizing capillary damage and the presence of basophilic intranuclear inclusion bodies in numerous endothelial cells, and electron microscopy revealed herpesvirus particles in these inclusions. In an attempt to isolate the virus, triturated preparations of skeletal muscle (the only frozen tissue available) were inoculated into various cell cultures, i.e., primary bovine lung cells, chicken embryo fibroblasts as

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well as the bovine MDBK and the rabbit RK13 cell line. Virus isolation was not successful after three blind passages (A. E. Metzler, unpubl. data). This failure may have been due to temperature sensitivity of the virus, its inactivation by autolysis, inappropriate tissue samples, or inadequate host cells.

The above fatal case prompted a serological study to determine whether a herpesvirus was prevalent among contact animals and in elephants from other herds. Due to the lack of a corresponding elephant isolate, sera were evaluated for antibodies to the dermotropic bovine herpesvirus 2 (BHV2, bovine mammillitis virus). For comparative purposes the sera were also tested for activity against bovine herpesvirus 1 (BHV1, IBR/IPV virus) and bovine herpesvirus 4 (BHV4, 'Movar-type' herpesvirus).

MATERIALS AND METHODS

Cells and viruses

Madin Darby bovine kidney (MDBK) cells (Flow Laboratories AG, Binningerstrasse 116, CH-4123 Allschwil, Switzerland) were used throughout. These were subcultivated twice per week and used for the experiments as subconfluent monolayers. Growth medium was Eagle's minimal essential medium (Amimed AG, Austrasse 35, CH-4051 Basel, Switzerland) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (North American Biologicals, Miami, Florida 33169, USA). Serum was reduced to 0.2% in maintenance medium. For metabolic labeling with [^{35}S]methionine, maintenance medium was modified to contain 10% of the usual concentration of the corresponding amino acid. Cells were incubated at 37 C.

Sera were reacted with the following virus strains: Jura (Friedli and Metzler, 1987), TVA (Rweyemamu and Johnson, 1969) and LVR140 (Wellemans et al., 1986; Augsburger and Metzler, 1989) as representatives of BHV1, BHV2 and BHV4, respectively. After plaque purification these viruses were passed through a limited number (<10) of cell cultures. Virus stocks were obtained from cell cultures following inoculation with virus at a multiplicity of infection (ratio virus/cell) of 0.01 and harvesting at an advanced stage of virus-induced cytopathic effect. Cells and culture supernatants were frozen and thawed three times, clarified by centrifugation and stored at -60 C. Infectivity titrations were performed by inoculating series of tenfold virus dilutions in quadruplicate onto cell monolayers in 96-well microtiter plates (Falcon® 3072, Becton Dickinson AG, Immengasse 7, CH-4056 Basel, Switzerland). Plates were incubated for 4 to 7 days and examined for evidence of cytopathic effects. Fifty percent endpoints were calculated by the Kärber method (Kärber, 1931).

Serum samples

All wildlife samples originated from female Asian elephants. Initially, pericardial fluid was collected at necropsy from Lohimi which died in mid July 1988 following generalized herpesvirus infection. Two months later blood was obtained from 10 elephants from the same herd (Knie Brothers, Swiss National Circus Corporation, St. Wendelinstrasse 10, CH-8640 Rapperswil, Switzerland). One of these animals (Indi) recently had been introduced into the herd following its importation from Burma. An additional newly imported elephant (Mapalai from Burma) was bled upon arrival in December, and a second sample was obtained from Tima.

Blood was taken in September 1988 from three animals of the Zürich Zoo (Zoo Zürich, Zürichbergstrasse 221, CH-8044 Zürich, Switzerland). The herds from Zürich and Rapperswil had breeding contact from 1982 to 1983 through Claudia, Lohimi's dam.

In addition, we received lyophilized sera from six elephants of two herds in the Federal Republic of Germany (Pilaski et al., 1987, 1988). These samples, collected between 1986 and 1987, were included in some experiments.

Four bovine sera were used for reference purposes. One serum was from a lactating cow following experimental inoculation with BHV1 (Jura strain) and subsequent to viral reactivation with corticosteroids (Probst et al., 1985). Another serum originated from a naturally infected cow with a rising antibody titer to BHV2 following treatment with corticosteroids (Hofmann et al., 1986). The third serum, with antibody against BHV4, originated from a naturally infected bull and the fourth, a normal bovine serum, served as negative control (Metzler and Wyler, 1986).

Seroneutralization test (SNT)

Sera were tested for neutralizing antibody to BHV1, BHV2 and BHV4. Neutralization tests with BHV4 were performed knowing that bovine reference sera lack homologous virus neutralization (Wellemans et al., 1986). Serial twofold dilutions of heat-inactivated (30 min, 56 C) serum in maintenance medium were mixed with an equal volume of the appropriate virus ad-

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justed to 10^3 $TCID_{50}$ per ml. Mixtures were incubated for 5 hr at 37 C. Subsequently, 100 µl aliquots from each mixture were inoculated in quadruplicate onto preconfluent cell monolayers in microtiter plates. These were prepared by seeding 10,000 MDBK cells in 50 µl of growth medium and incubating for 24 hr. Serum controls were included in each test. Readings were made following an incubation period of 4 to 7 days, after completion of the cytopathic effect in virus control cultures. Antibody titers were expressed as 50% endpoint titers (Kärber, 1931). Sera were tested at least twice. Titers equal to or higher than 1:2 were considered positive (Darcel and Kozub, 1984; Meteyer et al., 1989).

Radio-immunoprecipitation (RIP)

Cell monolayers, consisting of 1.5×10^6 MDBK cells in 25 cm^2 plastic flasks (Corning Glass Works, Corning, New York 14831, USA) were inoculated with virus at a multiplicity of five to 10 or they were left as uninfected control. After an adsorption period of 3 hr at 37 C inocula were removed and monolayers washed with phosphate buffered saline (PBS, pH 7.3). Cultures were then replenished with labeling medium or maintenance medium. [^{3}H]glucosamine (50 µCi/ml; specific activity 40 Ci/mmol) (Hans Rahn and Co., Dept. Amersham, Dörflistrasse 120, CH-8050 Zürich, Switzerland) was added at 3 hr postinoculation (p.i.) and [^{35}S]methionine (5 µCi/ml; specific activity 1,426 Ci/mmol) (Hans Rahn and Co.) at 8 hr p.i. and cultures were incubated at 37 C. At 22 to 30 hr p.i. cultures were harvested by washing monolayers with PBS and dislodging adherent cells into the same buffer. Cells were partitioned into two aliquots (90 and 10%) and briefly centrifuged. The 10% aliquot was solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl, pH 6.8, 3% SDS, 1% 2-mercaptoethanol, 15% glycerol, and 0.01% bromphenol blue), denatured by heating for 3 min at 100 C (Augsburger and Metzler, 1989) and used to evaluate infected cell protein (ICP) profiles by SDS-PAGE. This provided a baseline for identifying immunoprecipitated polypeptides.

Cells from the 90% aliquot were resuspended in RIP buffer (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Triton-X100, 0.5% sodium deoxycholate). After incubation for 1 hr at 4 C, suspensions were centrifuged (90 min 100,000 *g*) and resulting supernatants used as antigens for RIP assay (Augsburger and Metzler, 1989). Polypeptides obtained from approximately 75,000 virus-infected and mock-infected cells, each contained in 250 µl RIP buffer, were mixed with 5 µl test serum. Mixtures were incubated over night at 4 C. This was followed by addition of 10 mg Protein A-Sepharose® CL-4B (Pharmacia AG, Lagerstrasse 14, CH-8600 Dübendorf, Switzerland) in RIP buffer or a corresponding amount of Protein G-Sepharose® 4 Fast Flow (Pharmacia AG) in the same buffer. Samples were incubated with occasional shaking for 1 hr at room temperature. Sepharose beads were washed five times with RIP buffer. Bound immune complexes were then solubilized and denatured by suspension in 65 µl of SDS-PAGE sample buffer and heating for 3 min in a water bath at 100 C. Resulting protein samples were briefly centrifuged at 10,000 *g* and analyzed by SDS-PAGE and fluorography.

In view of the lack of any agreed system of quantitative expression of RIP results the extent of virus-specific precipitation was deduced from fluorographs and scored as following: +++, strong reaction; ++, distinct reaction; +, weak reaction; ±, faint or questionable reaction, and −, no reaction.

SDS-PAGE

Discontinuous polyacrylamide slab gels (10% monomer concentration) were prepared according to Laemmli (1970) with bis-acrylamide as the cross-linker. Electrophoresis was performed at 25 mA per gel using 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3 as running buffer (Friedli and Metzler, 1987). To determine molecular weights (MW) of viral polypeptides, a mixture of radio-labeled reference proteins (Hans Rahn and Co., Code CFA.626) were separated in parallel channels. These proteins were myosin (200 kDa), phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (18.4 kDa). After electrophoresis gels were prepared for fluorography using En^{3}Hance (Du Pont de Nemours International SA, Biotechnology Systems Division, Pumpwerkstrasse 15, CH-8105 Regensdorf, Switzerland) and following instructions supplied by the manufacturer. Gels were dried on filter paper and placed at −60 C for 3 to 7 days in contact with Kodak XAR-5 films (Kodak SA, Postfach, CH-1001 Lausanne, Switzerland).

Proteins induced by BHV1 and BHV4 were described previously (Metzler et al., 1985, 1986; Friedli and Metzler, 1987; Augsburger and Metzler, 1989). Based on preliminary evaluations the TVA strain of BHV2 was observed to generate at least 31 infected cell proteins, ranging in apparent MW from 280 to 31 kDa.

RESULTS

Results of SNT and RIP assays are summarized in Table 1. Of 15 elephant sera tested, four displayed low neutralizing an-

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TABLE 1. Results from seroneutralization test (SNT) and radio-immunoprecipitation (RIP) assay of elephant and bovine reference sera with the bovine herpesviruses 1 (BHV1), 2 (BHV2) and 4 (BHV4).

Animal origin	Number/Name	Age in years	Antibody titer					
			BHV1		BHV2		BHV4	
			SNT	RIP	SNT	RIP	SNT[a]	RIP
Knie's	1/Indi[b]	2	−[c]	+[d]	−	+	−	+
Circus	2/Mapalai[b]	3	−	+	2	+	−	+
	3/Lohimi[b]	3	−	±	−	±	−	+
	4/Ceylon	17	−	+	−	+	−	+
	5/Dehli	20	−	+	2	+	−	±
	6/Tima	23	−	+	2	+	−	±
	7/Siri	25	−	+	−	+	−	+
	8/Sumatra	25	−	+	−	+	−	+
	9/Claudia	26	−	+	−	+	−	+
	10/Patma	26	−	+	−	+	−	+
	11/Miniak	38	−	+	−	+	−	+
	12/Java	47	−	+	−	+	−	+
Zürich Zoo	13/Ceyla	13	t	++	t	++	t	+
	14/Zella	20	−	++	2	++	−	±
	15/Chhukha	21	−	+	−	+	−	+
Reference sera	16/Bovine antiserum to BHV1		512	+++	−	++	−	−
	17/Bovine antiserum to BHV2		−	++	16	+++	−	−
	18/Bovine antiserum to BHV4		−	−	−	−	−	+++
	19/Bovine normal serum		−	−	−	−	−	−

[a] Negative throughout. However, SNT not applicable since reference sera to BHV4 are known to lack homologous virus neutralization (Wellemans et al., 1986).

[b] Indi and Mapalai were imported from Burma shortly before blood collection. Lohimi died of a generalized herpesvirus infection.

[c] Antibody titers are expressed as the reciprocal of the highest serum dilution protecting 50% of the inoculated cultures. A − denotes an antibody titer <2.

[d] Antibody activities are expressed as relative amount of precipitated viral antigens: +++, strong reaction; ++, distinct reaction; +, weak reaction; ±, faint or questionable reaction; −, no reaction.

[e] Serum toxic for indicator cells.

tibody titers to BHV2. In contrast, BHV1 (and BHV4) remained unaffected. Due to toxicity, serum from Ceyla could not be evaluated by SNT.

When assayed by protein A-mediated RIP, and using [^{35}S]methionine-labeled antigens, the elephant sera not only displayed a reaction to multiple BHV2 polypeptides but also to antigens of BHV1 and BHV4. SDS-PAGE profiles of immunoprecipitation products obtained with BHV2 and BHV1 antigens are illustrated in Figure 1. The elephant sera recognized a limited number of BHV2 antigens as compared to the respective reference serum. A slight but distinct virus-directed reaction was consistently demonstrated with serum from Lohimi. Serum from Tima, showing the most prominent reaction among the three elephants shown, clearly precipitated three BHV2 polypeptides and additional faint bands, ranging in apparent MW from 152 (the major capsid protein) to 40 kDa. The same antigens, yet significantly more pronounced, were found in the precipitation product obtained with the BHV2 reference serum. Precipitation of radio-labeled viral antigens could be abolished by prior reaction of sera with non-radio-labeled homologous antigens.

The elephant sera reacted similarly with respect to BHV1 antigens. Protein profiles showed slight variation in the number of viral antigens found with precipitation

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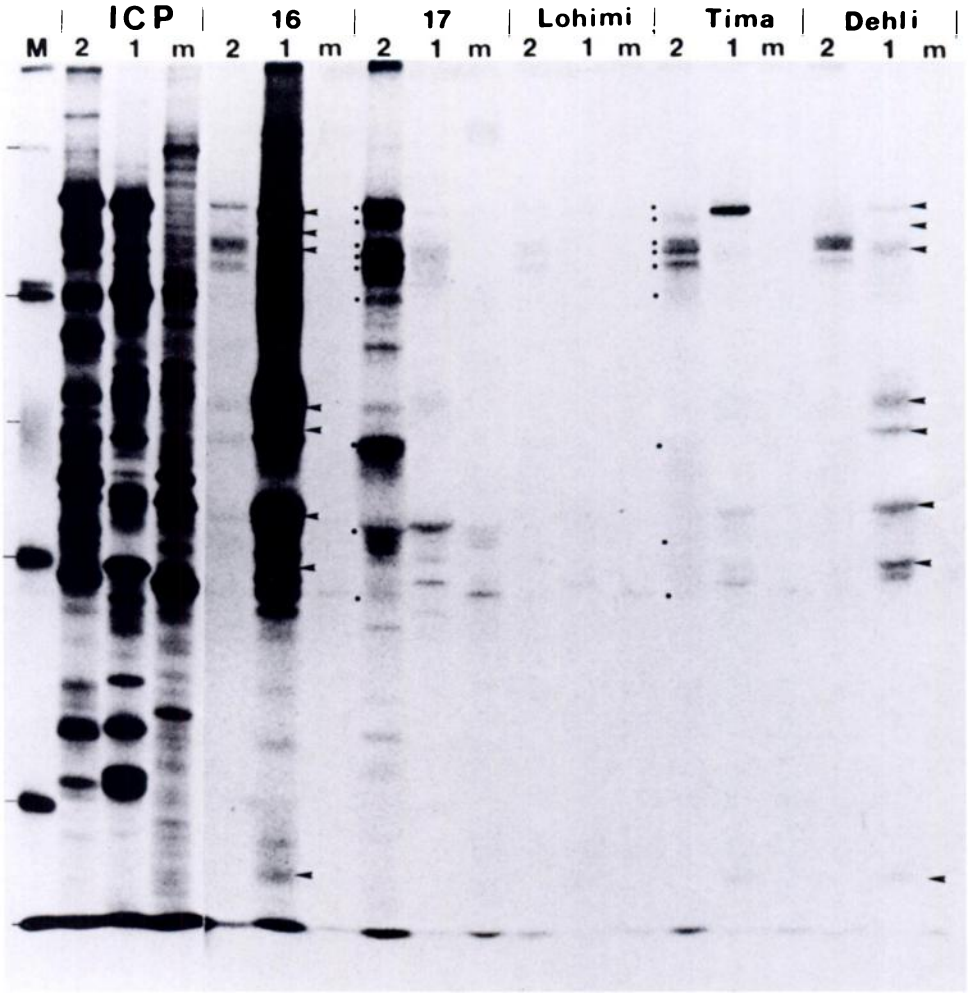


FIGURE. 1. SDS-PAGE profiles of [^{35}S]methionine-labeled, protein A-mediated immunoprecipitation products generated by bovine reference sera to BHV1 (16) and BHV2 (17), and by three elephant sera (Lohimi, Tima, and Dehli), using antigens from BHV2- (lanes 2), BHV1- (lanes 1) and mock-infected (lanes m) cells. Immune complexes were harvested with protein A-sepharose. Infected and mock-infected cell proteins (ICP) are shown on the left of the figure. M denotes molecular weight marker proteins (200, 92.5, 69, 46, 30, and 18.4 kDa). Arrowheads mark BHV1 proteins and dots BHV2 proteins most consistently precipitated by elephant sera.

products of individual sera. Serum from Dehli, showing the strongest reaction among the three animals depicted in Figure 1, precipitated at least eight BHV1 antigens, ranging in apparent MW from 148 (major capsid protein) to 20 kDa. With BHV1 reference serum the same proteins were resolved, again with higher efficiency. The newly imported elephants Indi and Mapalai and the three animals from the Zürich Zoo displayed essentially the same type of antibody activity to BHV1 and BHV2 antigens as the adult elephants from Knie's Circus (Fig. 2). A second serum sample from Tima, obtained 3 mo after the first, did not reveal any change in antibody activities. When assayed with [^{3}H]glucosamine-labeled antigens the elephant sera failed to precipitate viral glycoproteins.

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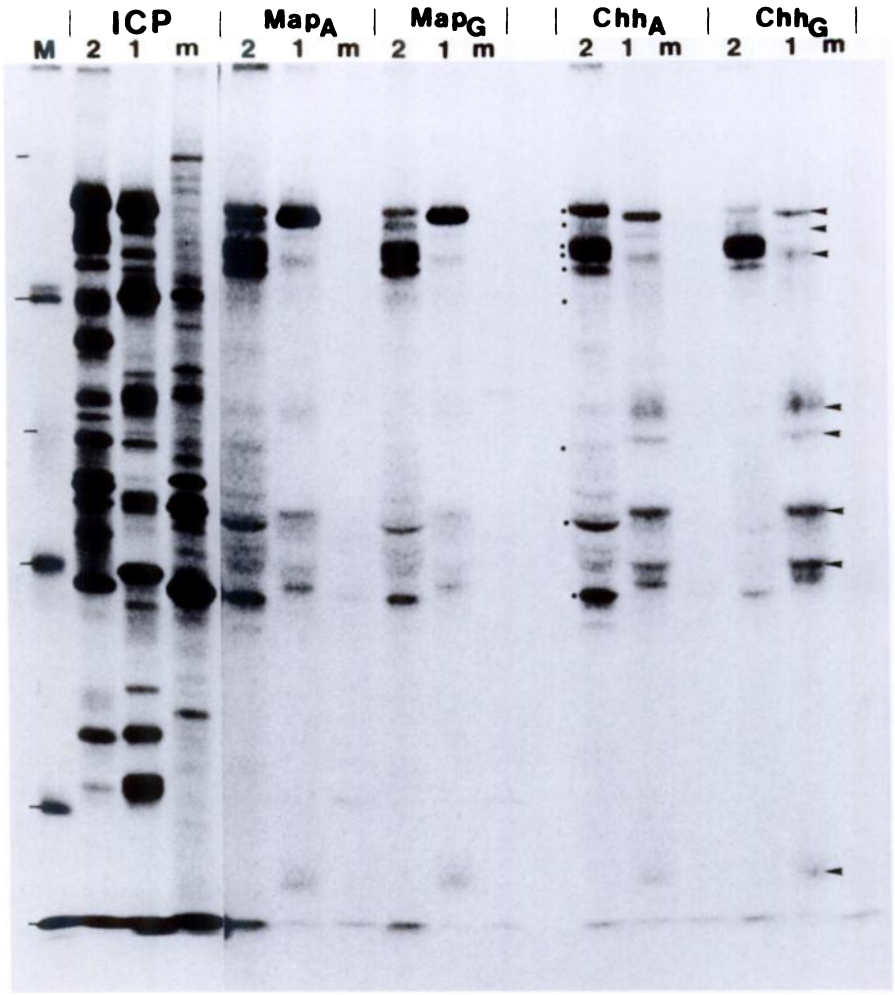


FIGURE 2. SDS-PAGE profiles of [^{35}S]methionine-labeled immunoprecipitation products generated by serum from the elephants Mapalai and Chhukha, using antigens from BHV2- (lanes 2), BHV1- (lanes 1), and mock-infected (lanes m) cells. Immune complexes were either harvested with protein A- (Map$_A$ and Chh$_A$) or with protein G-sepharose (Map$_G$ and Chh$_G$). M denotes molecular weight marker proteins; ICP denotes virus-infected and mock-infected cell proteins. Arrowheads mark BHV1 proteins and dots BHV2 proteins most consistently precipitated by elephant sera.

The RIP assay could have been limited due to low efficiency of immunoglobulin binding to protein A. Therefore, RIP procedures were effectuated with protein G in place of protein A (Fig. 2). In contrast to bovine sera, virtually identical results were obtained with elephant sera irrespective of the type of protein being used. Nevertheless, with most elephant sera protein A performance was slightly better than protein G. This is illustrated by the efficiency of precipitation of the major capsid protein of BHV1 and BHV2 with serum from Chhukha (Fig. 2, Chh., lanes 2 and 1).

The elephant sera consistently reacted with a set of two closely migrating polypeptides of BHV4 with apparent MW of 140 to 138 kDa (Fig. 3). A corresponding reaction was not observed with the normal bovine serum. Some elephant sera precipitated additional viral antigens of 124 and 83 kDa. The bovine reference serum to BHV4 recognized a widened antigen spec-

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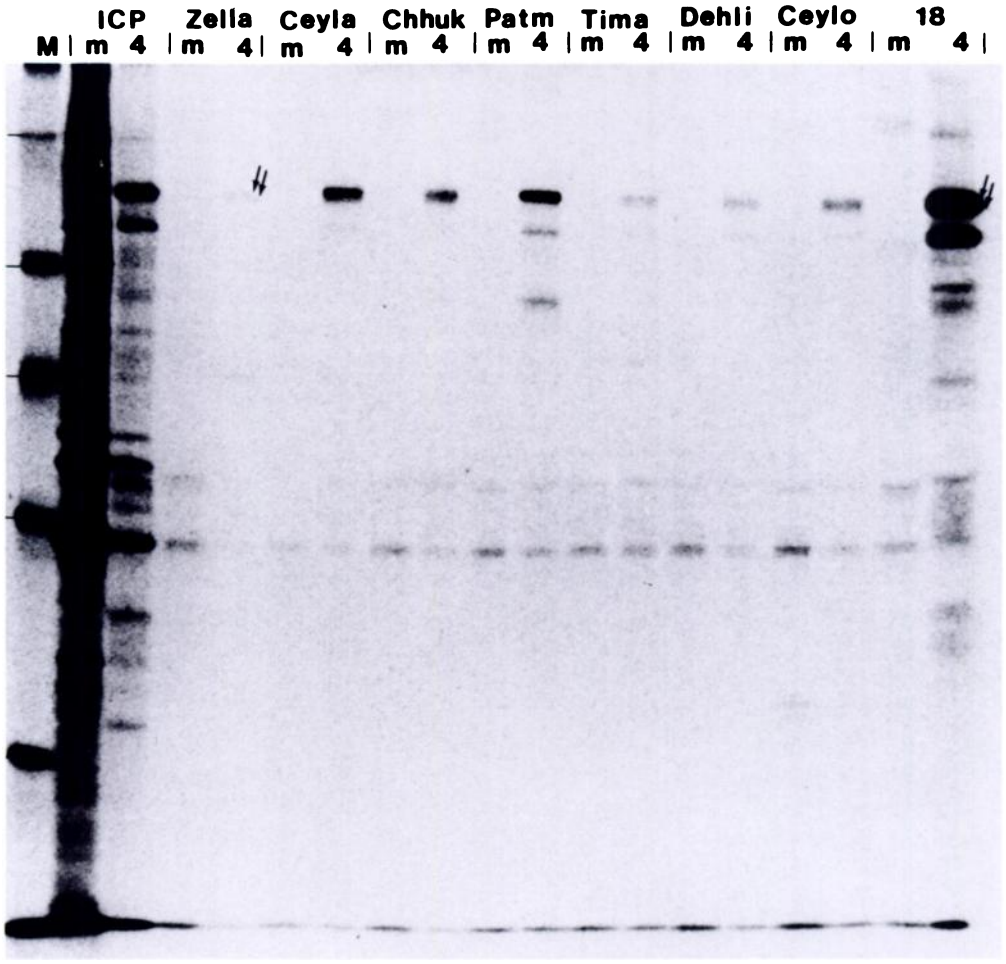


FIGURE 3. SDS-PAGE profiles of [^{35}S]methionine-labeled immunoprecipitation products generated by the elephant sera indicated on the top of the figure and by bovine reference serum to BHV4 (18), using antigens from mock- (lanes m) and BHV4-infected cells. Infected and mock-infected cell proteins (ICP) are shown on the left of the figure. Immune complexes were harvested with protein A-sepharose. M denotes molecular weight marker proteins. Arrows mark BHV4 proteins most consistently precipitated by elephant sera.

trum. The reaction was restricted to the homologous reference serum with [^{3}H]-glucosamine-labeled BHV4 antigens.

Virtually identical results were obtained with six elephant sera from Germany. A BHV2-neutralizing antibody titer of 1:8 was recorded in one case.

DISCUSSION

This study showed that 21 (100%) captive Asian elephants possessed serum antibodies with specificity for three bovine herpesviruses. Since the sera originated from animals representing different herds in Switzerland, Germany and Burma, it was concluded that infection with a hitherto unidentified herpesvirus(es) may be widespread among captive Asian elephants. This is not surprising, since most mammalian species harbor at least one herpesvirus (Roizman and Batterson, 1985). Moreover, almost 20 yr ago McCully et al. (1971) reported that lymphoid nodules with intranuclear inclusion bodies in epithelial lung cells of African elephants were as-

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sociated with a herpesvirus. Together with the findings of Pilaski et al. (1987, 1988) the present study also demonstrates that the Asian elephant may be a host for one or several herpesviruses. All herpesviruses studied to date persist in the host in some state of latency from which the virus can be reactivated (Roizman and Batterson, 1985). The same may be true in elephants. Viral reactivation and subsequent shedding are likely to contribute to maintenance of the virus within populations, even within small herds. At present it is not possible to name the factors which, as in the case of Lohimi, provoke systemic and eventually fatal disease. However, in view of the 100% prevalence of seropositive animals and the rarity of disease, it seems unlikely that the virus is a primary pathogen for elephants.

Neither BHV1, BHV2 nor BHV4 has been isolated from hosts outside the order Artiodactyla (Scott, 1989; Thiry et al., 1989; Wyler et al., 1989). Based on this it seems unlikely that the elephants were infected with these bovine viruses. Nevertheless, this anticipation remains to be confirmed. Antibody titers recorded in the elephant sera were admittedly low. This is probably because the serology was possibly conducted with heterologous viruses, thus detecting only cross-reacting antibodies. This seemed especially true with respect to reaction with heterologous viral glycoproteins. Considering the RIP results and the exclusive neutralization of BHV2 with some of the elephant sera, it is possible that a single virus, immunologically related to BHV2, was responsible for the generation of antibodies that cross-reacted with BHV1 and BHV4 antigens. This seems possible since a limited antigenic relationship of BHV2 with BHV1 and BHV4 has been documented (Wyler et al., 1989).

Bovine herpesvirus 2 also shares several genetic and antigenic characteristics with the human pathogen herpes simplex virus (HSV) (Scott, 1989). At least one glycoprotein of HSV (gC) and one glycoprotein of BHV2 (130 kDa) are known to induce cross-neutralizing antibodies. It cannot be ruled out that an infection of elephants with HSV by human carriers could lead to the generation of antibodies that neutralize BHV2. However, this possibility seems unlikely since HSV infections are restricted to humans and non-human primates.

The value of protein A as a successful laboratory reagent has been well documented. Protein A, however, does not bind IgG from all species or of all subclasses (Boyle and Reis, 1987). The recently recognized protein G is often a valuable alternative as was shown for bovine immunoglobulins (Boyle and Reis, 1987). We found protein G slightly less suited for RIP assays than protein A with elephant sera.

In summary, we found captive Asian elephants from various herds to display serum antibody with specificity for a herpesvirus of unknown identity. To substantiate the present findings, additional sera from captive as well as from free-living Asian and African elephant populations should be evaluated and further efforts should be made to isolate the herpesvirus.

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