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Comparative immunological characterization of type-specific and conserved B-cell epitopes of pinniped, felid and canid herpesviruses

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Summary. Murine monoclonal antibodies (MAbs) were generated against phocid herpesviruses (PhHV 2557/Han88 and 7848/Han90) isolated from European harbour seals (*Phoca vitulina*), and against strains of both felid (FHV strain FVR 605) and canid herpesviruses (CHV isolate 5105/Han89). MAbs were characterized with respect to certain biological properties and used to outline antigenicity profiles of isolates of PhHV (n = 8), FHV (n = 7) and CHV (n = 3) in enzyme immunoassays employing fixed infected cells. A close antigenic relationship between herpesviruses derived from pinnipeds and terrestrial carnivores became evident: The majority of the MAbs was directed against epitopes which were expressed by at least two of the viral species tested. A number of MAbs detected epitopes which were conserved between all isolates of PhHV, FHV and CHV. A few MAbs recognized type-specific B-cell epitopes and facilitated the identification of single viral species. Moreover, the PhHV isolate 7848/Han90 was antigenically distinguishable both from seven other phocid herpesvirus isolates and from FHV or CHV. PhHV 7848/Han90 proved to be antigenically distinct from all other viruses tested when examined by cross neutralization utilizing various reconvalescent and hyperimmune sera. Although more data are needed to ensure that PhHV 7848/Han90 indeed is a new genuine seal herpesvirus, the preliminary clustering of two groups of phocid herpesvirus isolates, tentatively designated PhHV-1 (type isolate 2557/Han88) and PhHV-2 (represented by 7848/Han90), seems to be justified. By using selected MAbs an unambiguous identification and typing of herpesvirus isolates derived from marine mammals and terrestrial carnivores is significantly facilitated.

Introduction

Herpesvirus infection associated with pneumonia and focal hepatitis in hospitalized harbour seal pups (*Phoca vitulina*) have been initially described in

1985 [25]. The so-called phocid herpesviruses (PhHV) were considered α-herpesviruses [29] with respect to their physico-chemical composition, a short replicative cycle leading to rapid mass destruction of susceptible seal kidney cell cultures, and an antigenic relationship to both felid and canid herpesviruses (FHV, CHV) detected by cross neutralization assay [25].

Since 1985 further herpesviruses were isolated from a California sea lion (*Zalophus californianus*) [19] and, more frequently, from harbour seals during the 1988 European morbillivirus seal mass mortality [10, 26]. Serosurveys indicated that infections of pinnipeds by PhHV or other closely related herpesviruses occur frequently and worldwide, including Arctic and Antarctic seal species [13, 32, 34]. Firm data on the incidence and kind of clinical disease in free-ranging seal populations due to herpesvirus infections is lacking. An association with respiratory disease and abortion, however, has been suspected [13, 32].

In the course of a natural infection described by Osterhaus et al. [25] a mortality rate of about 50% was observed in hospitalized seal pups. Fatal, natural infections of adult seals associated with high titres of herpesvirus in central nervous tissues have been reported in cases of concomitant infections with virulent phocid distemper morbillivirus (PDV) [35]. In contrast, experimental infection of susceptible juvenile harbour seals by the PhHV isolate 2501/Han88 resulted in an almost subclinical infection [15]. Apparently, the symptomatology due to PhHV infections may be variable, depending on the seal's age, properties of the virus strain and concomitant infections among other factors.

Evidence for an immunological relationship between PhHV and felid as well as canid herpesviruses is based on data obtained from cross neutralization experiments [13, 25, 32]. Further attempts to characterize pinniped herpesviruses have not been reported.

Monoclonal antibodies (MAbs) proved to be excellent tools both for taxonomic and diagnostic purposes in various herpesvirus species [6, 7, 11]. In order to specify the antigenic relationships among recent herpesvirus isolates of aquatic mammals and terrestrial carnivores, monoclonal antibodies were raised against two isolates of PhHV and a strain of both FHV and CHV. Here we show that the newly established panel of MAbs is useful for an unambiguous typing of isolates. Furthermore, we will provide evidence for the existence of two types of phocid herpesviruses which can be differentiated with respect to biological, immunological and epidemiological characteristics.

Materials and methods

Viruses and cells

Isolates and strains of PhHV, FHV, CHV and SHV-1 used in this study are summarized in Table 1. The majority of these isolates has been described earlier [3, 4, 9, 10, 13, 25, 27, 33]. The PhHV-isolates 2010/Han89 and 7848/Han90 were isolated in primary seal kidney cultures. Isolate 2010/Han89 originated from lung tissue homogenates of a carcass of an

Table 1. Origin of herpesviruses

Virus	Host	Disease	Specimen	Ref.
PhHV				
PB84	*Ph. vitulina*	respiratory	kidney culture	[25]
PB-6-II/85	*Ph. vitulina*	respiratory	lung	[25]
2158/Han88	*Ph. vitulina*	respiratory	lung	[10]
2160/Han88	*Ph. vitulina*	respiratory	liver	[10]
2501/Han88	*Ph. vitulina*	respiratory	lung	[10]
2557/Han88	*Ph. vitulina*	respiratory	leucocytes	[10]
2010/Han90	*Ph. vitulina*	carcass	lung	this work
7848/Han90	*Ph. vitulina*	generalized	leucocytes	this work
FHV				
FVR 605	*Felis catus*	–	vaccine strain[a]	[27]
F2	*Felis catus*	–	vaccine strain[b]	[3]
FHV/L	*Panthera leo*	encephalitis	tonsil	[33]
6084/Han90	*Felis catus*	rhinotracheitis	pharyng. swab	[14]
6887/Han91	*Felis catus*	rhinotracheitis	pharyng. swab	[14]
299/Han91	*Felis catus*	conjunctivitis	conjunct. swab	[14]
10499/Han91	*Felis catus*	rhinotracheitis	pharyng. swab	[14]
CHV				
5105/Han89	*Canis familiaris*	respiratory	lung	[13]
11190/Han91	*Canis familiaris*[c]	generalized	kidney	this work
11361/Han91	*Canis familiaris*[c]	generalized	liver	this work
SHV-1				
48-1/Han68	*Sus domesticus*	respiratory	lung	[9]

[a] Dr. K. Danner, Behringwerke AG, Marburg/Lahn, F.R.G.
[b] Dr. A. Aubert, Virbac Laboratories, Carosse, France
[c] Newborn whelps

adult seal. Cultivation of peripheral mononuclear blood cells of an adult harbour seal showing severe respiratory disease yielded isolate 7848/Han90. Both isolates, like other phocid herpesviruses, replicated in a clone of CRFK cells [5], requiring no phase of adaptation. Canine herpesviruses were isolated in primary canine kidney cell cultures: 5105/Han89 originated from lung tissues of a dog with concomitant distemper virus infection; CHV 1119/Han91 and 11361/Han91 were isolated from kidney and liver tissues, respectively, of newborn pups of two different litters which died due to generalized disease. For further propagation of CHV the canine fibroblast cell line A 72[1] was employed [2]. Herpesvirus isolates of feline origin were recovered by use of a feline semicontinuous cell line (cat fetus cells[2]) or CRFK cells from oropharyngeal or conjunctival swabs of cats with upper respiratory disease or conjunctivitis [14]. The suid herpesvirus-1 isolate was also

[1] Kindly donated by Dr. S. Kölbl, Bundesanstalt für Virusseuchenbekämpfung, Vienna, Austria.

[2] Supplied by Dr. K. Danner, Behringwerke AG, Marburg/Lahn, F.R.G.

grown on CRFK cells. All viruses were subjected to at least three cycles of plaque purification before further use in this study.

For the preparation of concentrated virion suspensions large scale roller cultures of CRFK (PhHV, FHV) or A 72 cells (CHV) were harvested when viral cytopathic destruction was advanced, usually after three days of incubation. Following one freeze/thaw-cycle cellular debris was removed by centrifugation at 10 000 × **g** (15 min, 4 °C). Clarified supernatants were subjected to ultracentrifugation for 1 h at 85 000 × **g**. The pellets were resuspended in TNE-buffer (10 mM Tris-HCl, pH 7.4 100 mM NaCl, 1 mM EDTA) and centrifuged through a sucrose cushion (30% w/v in TNE) at 85 000 × **g** for 2 h. Virion-enriched pellets were resuspended in TNE-buffer and repelleted at 85.000 × **g** for 40 min. The pellets were reconstituted in 5 × volume of TNE-buffer, aliquoted in 0.2 ml portions and stored at −80 °C until use.

Monoclonal antibodies

Splenocytes obtained from Balb/c mice which had been hyperimmunized with concentrated virion suspensions emulsified in equal volumes of Freund's complete and incomplete adjuvant, respectively, were fused with cells of the X 63-AG 8.653 myeloma line [17] according to standard protocols. Hybridoma were grown in selective medium [22] to which as a source of cytokines supernatant of a fetal bovine splenocyte culture stimulated with Concanavalin A was added to 20% (v/v). The peroxidase-linked antibody assay (PLA) was used to identify hybridoma colonies secreting antigen-specific antibodies. Concentrates of MAbs were obtained by immune-affinity chromatography from cell culture supernatants of stabile producing hydridoma lines which had undergone at least two cycles of single-cell cloning by limiting dilution.

Peroxidase-linked antibody assay (PLA)

The PLA technique, an enzyme-linked immuno assay employing heat-fixed infected cells, was adopted from Harder et al. [12]. Antibody-containing solutions were applied to heat-fixed cultures in microtitre plates and incubated for 2 h at room temperature or overnight at 4 °C. For the initial screening of hybridoma colonies, supernatant was added in a single dilution of 1/2 in PBST. Bound antibodies were detected by using horseradish-peroxidase (HRPO)-labeled caprine-anti-murine-IgG, and 3-amino-9-ethylcarbazole serving as the chromogen. The highest dilution of a MAb solution readily enabling signal detection by light microscopy was recorded as its PLA-titre.

For photografic documentation of the intracellular herpesviral antigen accumulation, high contrast immunocytochemical staining was achieved by use of 3,3′-diaminobenzidine and subsequent gold/silver enhancement according to Lazar and Taub [20]. Hematoxylin was used for counterstaining [24].

Plaque reduction neutralization assay (PRNT)

MAb concentrates were diluted to a protein concentration of approximately 25–50 $\mu g\,ml^{-1}$ in DMEM to be tested for neutralizing capacities against 50–100 plaque forming units (pfu) of their homologous virus (PhHV 2557/Han88, PhHV 7848/Han90, FHV FVR 605 and CHV 5105/Han89). A polyclonal serum known to neutralize the respective virus in a microneutralization assay (see below) served as positive control. A neutralizing MAb raised against the H-protein of phocid distemper virus (PDV) represented the negative control [12]. Virus-MAb or – polyclonal serum mixtures were incubated overnight at 4 °C before inoculation in duplicate onto monolayers of susceptible (either CRFK or A 72) cells for

an adsorption period of 2 h. In a parallel series, virus-MAb mixtures were incubated in the presence of 4% (v/v, final) of ginuea pig complement. After incubation for 72 h plaques formed by PhHV 2557/Han88, FHV FVR 605, and CHV 5105/Han89 were macroscopically visible. In the case of PhHV 7848/Han90 the incubation period was prolonged to 6 days and a second overlay was added at day 4. Plaques were then immunocytochemically stained by the PLA-technique utilizing MAb 7848 2.4D7 or 2.6E5 as the first layer.

The percentage reduction of plaque formation by a certain MAb was calculated with respect to the number of plaques induced in the negative control.

Microneutralization assay (MNT)

Sera of naturally and experimentally herpesvirus-infected animals were assayed for their neutralizing capacities in the presence of 4% (v/v) guinea pig complement solution against PhHV, FHV, CHV and SHV-1. The test was essentially performed as described [13]. 50% end point titres were calculated according to Kaerber [16].

Results

Growth properties of phocid herpesviruses in vitro

All PhHV isolates replicated in primary seal kidney cells as well as in CRFK cells, but not in cells of canine origin. However, neither FHV nor CHV could be adapted to phocine cells by three blind passages (data not shown). Cytopathogenic effects induced by all PhHV isolates except 7848/Han90 were characterized by development of single rounded single cells gradually detaching from the vessel surface (Fig. 1). The onset of cytopathogenic effects induced by PhHV 7848/Han90 was delayed in comparison to other PhHV isolates and consisted mainly of syncytia formation. Under a semisolid overlay PhHV 2557/Han88, FHV and CHV strains generated macroscopically visible plaques of 1–3 mm in diameter within three days. Plaques induced by PhHV 7848/Han90, in contrast, did not exceed pin-point size even after prolonged incubation of six days.

Accumulation sites of viral antigens in CRFK cells infected by PhHV

A high-contrast peroxidase-linked immunocytochemical staining assay was used to demonstrate the accumulation of PhHV viral antigens in infected cells (Fig. 1). Using the panel of MAbs raised against the PhHV isolates 2557/Han88 and 7848/Han90 three different patterns were identified (Table 2):

The majority of the MAbs stained viral antigen which was evenly distributed throughout the cytoplasm (Fig. 1). Antigens detected by MAbs 7848 1.4C6 and 1.6C5 showed a restricted perinuclear localization surrounding the nucleus like a corona. In early stages of viral replication this antigen was found in the cytoplasm near the poles of the nucleus (Fig. 1.2). Some MAbs recognized viral antigen which was confined to the nucleus (Fig. 1.3, Fig. 1.4). While nuclei of cells apparantly in early stages of viral replication revealed a scattered staining pattern (Fig. 1.4), enlarged nuclei of cells located in the centre of viral plaques were homogenously filled with antigen (Fig. 1.3).

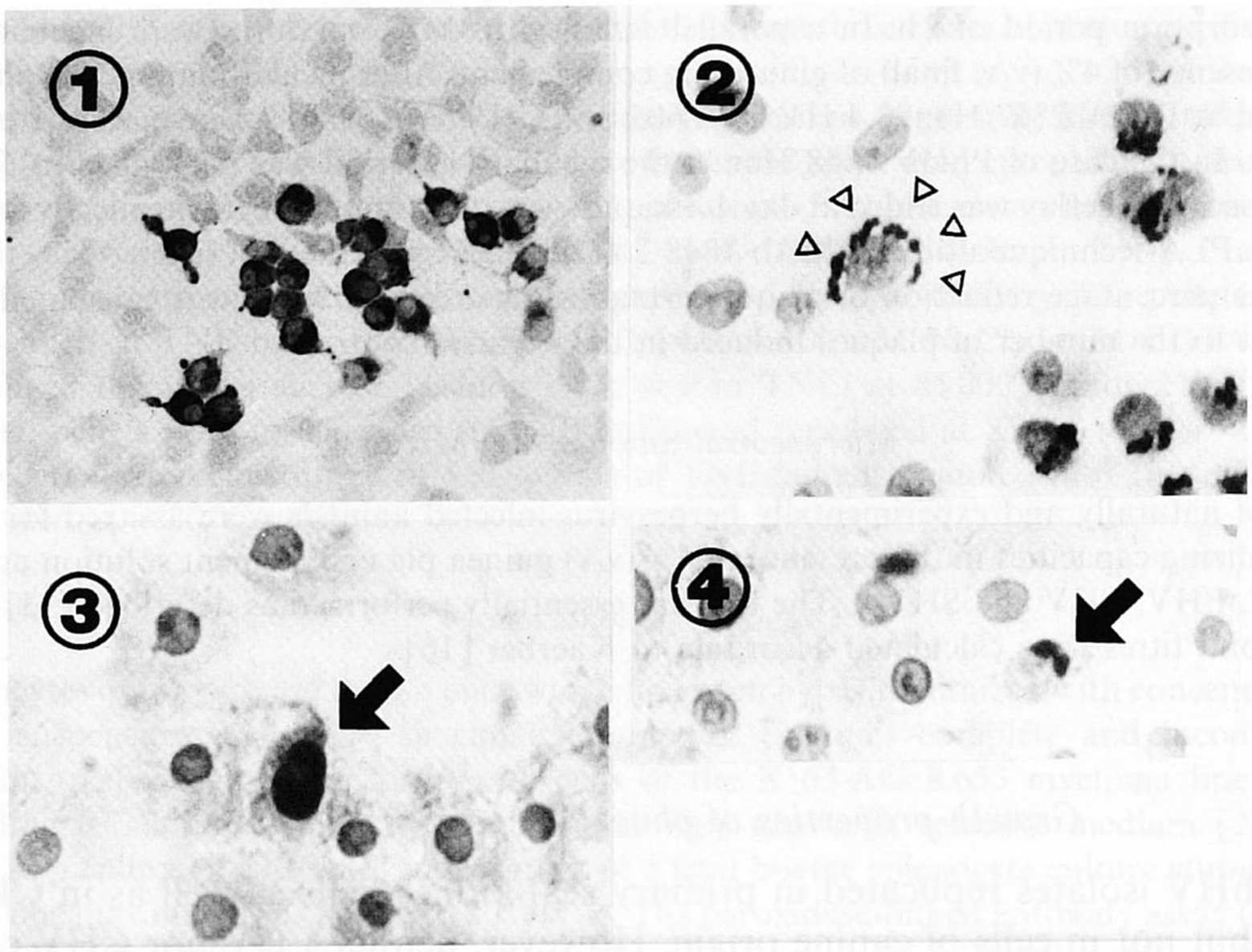

Fig. 1. Intracellular distribution of antigen in CRFK cells infected by phocid herpesvirus isolates identified by high contrast peroxidase-linked immunocytochemical staining utilizing different monoclonal antibodies *1* Plaque of CRFK cells infected by PhHV-1 2557/Han88 and pancytoplasmically stained using MAb PhHV-1 2557 3.1D3. *2* Perinuclear localization of antigen (open triangles) identified by MAb PhHV-2 7848 1.6C5 in CRFK cells infected by PhHV-2 7848/Han90. *3* Pannuclear distribution pattern of antigen (closed arrow) stained by MAb PhHV-2 7848 1.9A5 in CRFK cells infected by PhHV-2 7848/Han90. *4* Polar accumulation of antigen (closed arrow) in a nucleus of a CRFK cell infected by PhHV-2 7848/Han90 (MAb PhHV-2 7848 1.7C8)

Identification of conserved and type-specific epitopes of phocid, felid and canid herpesviruses

The reactivity and affinity of MAbs established against different herpesviruses was determined by use of an enzyme immunoassay employing fixed infected cells (peroxidase-linked antibody assay, PLA). As a measure of relative antibody affinity (AF-index) the ratio between the PLA-titre and the protein content (IgG) ml^{-1} of a given MAb concentrate was calculated.

As shown in Fig. 2 out of 20 MAbs raised against PhHV 2557/Han88 all but one reacted with CHV isolates whereas nine had also a high affinity to most FHV strains. Three MAbs, namely 2557 2.3F4, 2.3F7 and 2.9A12. also weakly reacted with pseudorabies virus (data not shown). The PhHV isolate 7848/Han90 was distinguishable both from seven other phocid herpesvirus isolates as well as from CHV and FHV (Fig. 2): Only four of the 20 MAbs produced against PhHV 2557/Han88 also detected PhHV 7848/Han90 antigen. In contrast, the majority of MAbs raised against the latter virus recognized

Table 2. Characterization of MAbs raised against phocid herpesvirus isolates 2557/Han88 and 7848/Han90, felid herpesvirus strain FVR 605 and canid herpesvirus isolate 5105/Han89

MAb designation	Antigen localization[a]	Neutralization[b]
PhHV 2557/Han88[c]		
1.1H3[d], 2.2F2[d]	cytoplasmic	100
3.4B3, 3.4G1, 3.4G6, 3.5F1, 3.6A12, 3.6E7, 3.7B9	cytoplasmic	60–90
2.3F4, 2.3F7, 2.5B4, 2.9A12, 3.1D3, 3.3G8, 3.3H5, 3.5B9, 3.7G3	cytoplasmic	⩽20
3.2C8, 3.3E4	nuclear	⩽20
PhHV7848/Han90[c]		
1.3E5, 2.4D7,. 2.6E5	cytoplasmic	⩽20
1.4C6, 1.6C5	perinuclear	⩽20
1.7C8, 1.8C3, 1.9A5	clear	⩽20
FHV FVR 605[c]		
1.4A1, 1.5H10, 1.8H6	cytoplasmic	⩽20
CHV 5105/Han89[c]		
2.3A10	cytoplasmic	70
1.2G7, 1.5A2, 2.9F6	cytoplasmic	⩽20

[a] Tested by peroxidase-linked antibody assay

[b] Percentage reduction of plaque forming units of the homologous virus (50 pfu) by 25–50 µg of antibody in the presence of 4% (v/v) of guinea pig complement

[c] Virus used to raise the MAbs

[d] MAbs did not require complement for neutralization

other PhHV isolates but displayed only limited reactivity with FHV and CHV. Most of the MAbs produced against FHV and CHV reacted with all PhHV isolates except PhHV 7848/Han90.

The monoclonal antibodies 2557 3.6E7; 7848 1.4C6, 1.6C5, 1.7C8, 2.4D7; FVR 2.8H6 and CHV 2.9F6 were found to bind antigen of other except their homologous virus species with very low affinity, if at all. Thus, these MAbs were used for identification of single viral species. In combination with cross reactive MAbs like 2557 3.3G8 and 3.1D3 an unambiguous antigenic identification and typing of herpesvirus isolates derived from terrestrial carnivores and pinnipeds using the PLA technique can easily be accomplished.

In general, isolates of PhHV (expect 7848/Han90) and CHV displayed homogenous reactivity patterns to the MAb panel. However, among felid herpesviruses, a diffuse intraspecies antigeneic heterogeneity was detectable with some MAbs: MAb 5105 1.2G7, for example, moderately reacted with FHV/L, FHV 6084, and 6887 but not with other FHV isolates including the vaccine strains. A general and concise clustering, e.g. into vaccine strains and isolates obtained from cats showing signs of rhinotracheitis or conjunctivitis, was not possible.

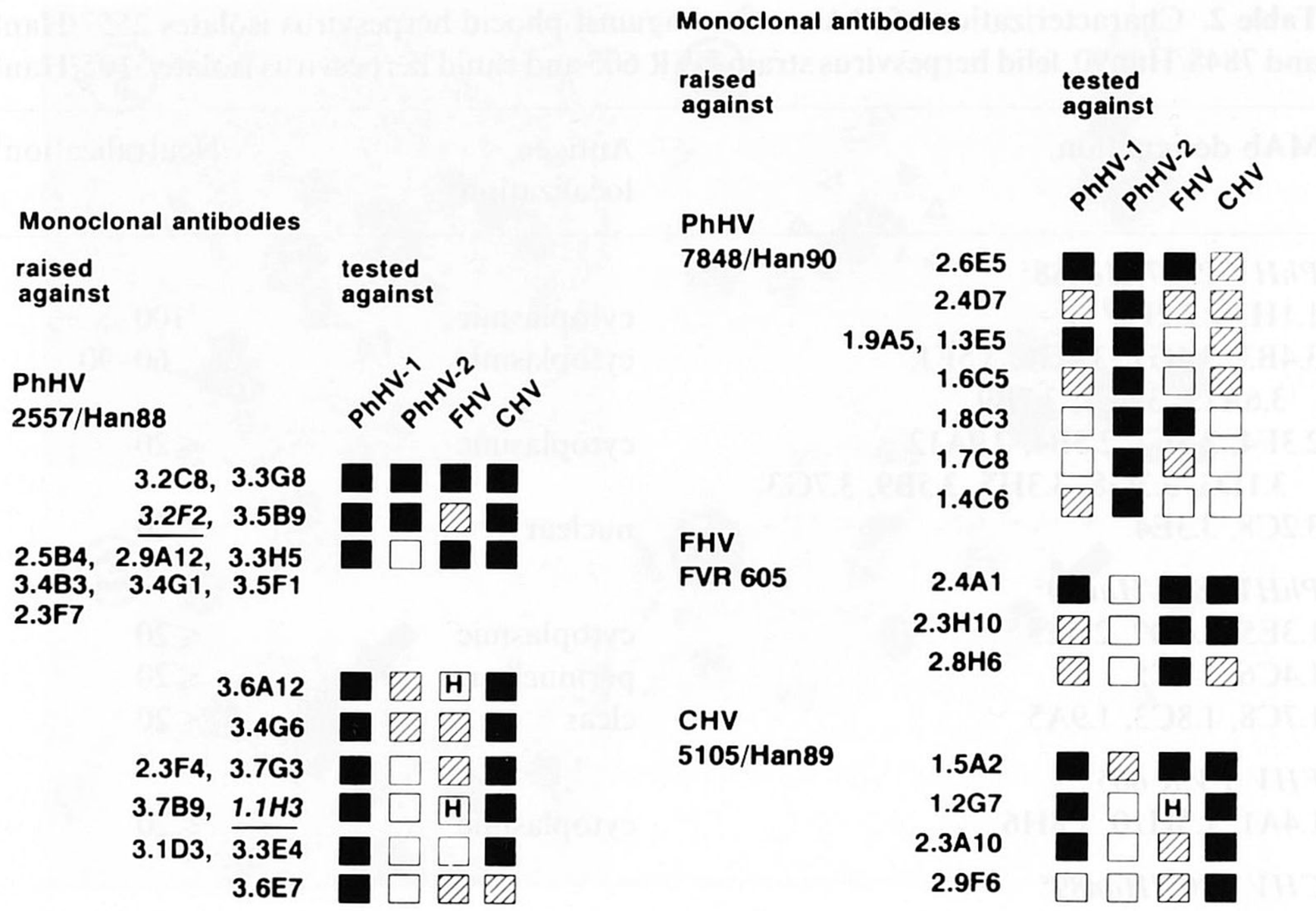

Fig. 2. Reactivity of monoclonal antibodies (MAbs) against phocid (*PhHV*), felid (*FHV*) and canid (*CHV*) herpesviruses analyzed by peroxidase-linked antibody assay. Reactivity gradations are based on affinity values (see Materials and methods for calculation). ■ strong, ▨ weak, [H] heterogenous, □ not detectable. With respect to the reactivity patterns the herpesvirus isolates and strains tested could be clustered as follows: *PhHV-1* (n = 7): PB 84, PB 6-II/85, 2158/Han88, 2160/Han88, 2501/Han88, 2557/Han88, 2010/Han90; *PhHV*-2 (n = 1): 7848/Han90; *FHV* (n = 7): FVR 605, F2, FHV/L, 6084/Han90, 6887/Han91, 299/Han91, 10499/Han91; *CHV* (n = 3): 5105/Han89, 11190/Han91, 11361/Han91. Underlined letters indicate single MAbs which were shown to completely neutralize their homologous virus independently from complement supplementation

Neutralization of viral infectivity by monoclonal antibodies

A number of MAbs generated against PhHV 2557/Han88, FHV and CHV partially (60–90%) reduced the formation of plaques of their homologous virus when assayed in a comparatively high protein concentration of 25–50 µg ml^{-1} (Table 2). Only two MAbs generated against PhHV 2557/Han88 (1.1H3 and 3.2 F2) completely neutralized viral infectivity even without complement supplementation and in higher dilutions. However, these MAbs were uncapable of cross neutralizing other herpesvirus species (data not shown).

Differential neutralization by sera

A complement-enhanced microneutralization assay was used to investigate whether PhHV 7848/Han90 can also be distinguished from other PhHV isolates by polyclonal antibodies derived from various naturally and experimentally herpesvirus-infected species.

Table 3. Cross neutralization of phocid herpesviruses and antigenically related herpesviruses of terrestrial carnivores

Serum	PhHV-1 2557/Han88	PhHV-2 7848/Han90	FHV FVR 605	CHV 5105/Han89	SHV-1 48/Han68
Weddell seals					
5255/91	65[a]	⩽5	15	53	⩽5
5257/91	40	⩽5	⩽5	8	⩽5
5258/91	52	⩽5	⩽5	53	10
5259/91	40	⩽5	⩽5	53	⩽5
5272/91	52	⩽5	⩽5	28	⩽5
Harbour seals					
3128/88	220	40	80	46	7
3394/88	80	⩽5	28	130	⩽5
3400/88	80	⩽5	92	113	⩽5
7848/90	450	46	46	180	⩽5
8549/92	48	⩽5	⩽5	⩽5	⩽5
11135/92	450	⩽5	130	130	⩽5
11142/92	370	⩽5	46	23	⩽5
11164/92	192	26	23	32	⩽5
11166/92	256	⩽5	160	32	⩽5
11173/92	450	105	23	56	⩽5
Reference sera					
725C	13	⩽5	⩽5	40	⩽5
αFVR605S	14	⩽5	3100	⩽5	⩽5
AK-F	⩽5	⩽5	⩽5	⩽5	92

[a] Reciprocals of ND_{50}

Sera nos. 5255 to 5272 were collected from Antarctic Weddell seals (*Leptonychaetes weddellii*) in 1990 [13]. Nos. 3128 to 11173 originated from free-ranging harbour seals (*Phoca vitulina*) of the German wadden sea 1988–1992. Serum 725C resulted from a dog experimentally infected by CHV 5101/Han89 (Harder 1990, unpubl.). The anti-FHV serum (αFVR605S) was raised in a sheep against FVR 605 (Dr. K. Danner, Behringwerke AG, Marburg, F.R.G.). A porcine field serum (AK-F) served as a reference for SHV-1-specific antibodies

For use in this study pinniped sera were selected which displayed high neutralizing titres to the PhHV isolate 2557/Han88. The majority of these sera also strongly neutralized CHV (Table 3). In addition, sera sampled from European harbour seals cross neutralized FHV whereas this was not achieved with sera of Antarctic Weddell seals. All sera, however, enabled the differentiation of the phocid herpesvirus isolate 7848/Han90 which appeared to be more distantly related to PhHV 2557/Han88 than FHV but not as distant as SHV-1.

Discussion

Previous studies based on cross neutralization experiments employing reconvalescent seal sera have demonstrated an antigenic relationship between genuine

herpesviruses isolated from cats, dogs and European harbour seals (*Phoca vitulina*) [13, 25, 32]. More detailed comparisons of FHV and CHV led to the identification of some glycoproteins sharing a number of conserved epitopes [21, 31].

By using a new panel of monoclonal antibodies raised against two PhHV isolates we confirm and extend these data. Eight phocid herpesvirus isolates obtained during 1984–1990 from different harbour seals with different clinical syndromes were taken under study and compared to vaccine strains and recent field isolates of felid and canid herpesviruses. Seven PhHV isolates displayed a homogenous pattern of reactivity to 35 MAbs. Isolates of seemingly different pathogenicity (e.g. PhHV PB 84 and 2501/Han88) could not be distinguished. The isolate 7848/Han90, in contrast, differed in a number of epitopes defined by MAbs from other PhHV isolates as well as from FHV and CHV. Multiple cross neutralization experiments employing reconvalescent sera of different seal species as well as reference sera to FHV or CHV and a serum from a SHV-1 infected pig gave further evidence that PhHV 7848/Han90 is immunologically distinct, though distantly related, from all other viruses tested here. In addition, some in vitro properties, e.g. comparatively slow growth with predominant formation of syncytia, are not shared with other PhHV isolates. 7848/Han90 was isolated from leucocytes, but not from other tissues of an adult harbour seal which succumbed to a respiratory disease. The isolation of herpesviruses from seals sharing the properties of 7848/Han90 had not yet been reported. Definite proof that 7848/Han90 indeed represents a new genuine seal herpesvirus thereby ruling out the possibility of a contamination, e.g. originating from fetal calf serum, must come from restriction enzyme analysis and sequence data of the genome. Until then the differentiation of two distinct groups of phocid herpesviruses and their tentative designation as PhHV-1 (represented by PhHV 2557/Han88) and PhHV-2 (represented by PhHV 7848/Han90) may be justified.

Some diffuse and heterogenous antigenic differences were also observed among vaccine strains of FHV and herpesvirus isolates recovered from diseased cats. However, a correlation of loss or presence of single antibody binding sites and clinical entities was not feasible.

Isolates of the proposed PhHV-1 type displayed a pronounced cross relationship especially to CHV and to a lesser extent also to FHV. A similar situation was unraveled by cross neutralization experiments. Titres of neutralizing antibodies in sera derived from different pinniped species were generally highest against PhHV-1 (2557/Han88). The majority of sera, at least those derived from European harbour seals, significantly cross neutralized CHV 5105 and FHV FVR 605 in contrast to PhHV-2 (7848/Han90). The serum of the seal from which PhHV-2 7848/Han90 was isolated displayed a high titre to PhHV-2. As an unexpected finding the titre of this serum against PhHV-1 was even higher. For an explanation it may be assumpted that seal 7848 has previously been infected by PhHV-1; upon a recent primary infection with PhHV-2 (7848/Han90) a presumably latent PhHV-1 infection was reactivated leading to a boost or an anamnestic response of PhHV-1 specific antibodies while PhHV-2 specific antibodies yet were not fully developed. However, it can not be excluded

that infections with PhHV-2 are not inducing neutralizing antibodies as readily as PhHV-1. Serological follow-up studies in this case were not possible since the animal died within a few days.

The host restriction of many α-herpesviruses both in vivo and in vitro is variable [29]. Some viruses, e.g. CHV, appear to be strictly confined to a single host species while others such as pseudorabies virus are capable of crossing host species boundaries. Diseases induced by most α-herpesviruses in their natural hosts are commonly self-limiting. Aberrant host species, in contrast, are more prone to develop severe and often fatal clinical syndromes. Herpesviruses were isolated from the faeces of dogs showing signs of gastroenteritis. These isolates replicated in feline cell cultures and were indistinguishable from felid herpesvirus by cross neutralization [8], restriction enzyme analysis and Southern blotting [30]. The infection of dogs with a felid herpesvirus variant provoked a clinical syndrome which is generally not seen in FHV infections of the natural host [28]. Data whether PhHV is carrying any potential to cross the species boundaries and infects members of the *canidae* or *felidae* families are presently not available. It is tempting to speculate that the latter would not be unlikely since both proposed types of PhHV did not require an adaptation phase to replicate productively in feline cell cultures. In contrast, cells of canine origin seem to be non-permissive. A system enabling an unambiguous identification and differentiation of herpesviruses isolated from sea mammals and terrestrial carnivores would be desirable both for diagnostic and epidemiological reasons. For this purpose some of the newly established MAbs are suitable (PhHV-1:2557 3.6E7, PhHV-2: 7848 1.4C6, FHV:FVR 8H6, CHV:5105 2.9F6). Once an isolate is available it can be typed by PLA within a couple of hours avoiding the more time consuming, laborious and sophisticated restriction enzyme analysis.

The panel of herpesvirus-specific MAbs characterized here may also aid in the diagnosis of herpesvirus infections in cetaceans which have recently been described in a harbour porpoise (*Phocoena phocoena*) showing encephalitis [18] and in Beluga whales (*Delphinapterus leucas*) with dermatitis [1, 23].

In conclusion, using a large panel of MAbs we provided evidence that herpesviruses of harbour seals are antigenically related to, though distinct from felid and canid herpesvirus. Two groups of phocid herpesviruses, tentatively designated PhHV-1 and PhHV-2, were differentiated by cross neutralization experiments and epitope-typing using the newly established MAb panel. While only a few antigenic differences between PhHV-1, CHV and FHV were detected PhHV-2 appears to be more distantly related to PhHV-1 isolates. Further work is in progress to characterize the genomes and proteins specified by PhHV-1 and -2 in order to confirm their classification as separate genuine pinniped herpesviruses.

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References

1. Barr B, Dunn L, Daniel MD, Banford A (1989) Herpes-like dermatitis in a Beluga whale (*Delphinapterus leucas*). J Wildl Dis 25: 608–611
2. Binn LN, Marchwicki RH, Stephenson EH (1980) Establishment of a canine cell line: derivation, characterization, and viral spectrum. Am J Vet Res 41: 855–860
3. Bittle JL, Rubic WL (1975) Immunogenic and protective effects of the F-2 strain of feline viral rhinotracheitis virus. Am J Vet Res 36: 89–91
4. Borst GHH, Walvoort HC, Reijnders PFH, van der Kamp JS, Osterhaus ADME (1986) An outbreak of a herpesvirus infection in harbour seals (*Phoca vitulina*). J Wildl Dis 22: 1–6
5. Crandell RA, Fabricant CG, Nelson-Rees WA (1973) Development, characterization and viral suspceptibility of a feline (*Felis catus*) renal cell line (CRFK). In Vitro 9: 176–185
6. Cropper LM, Lees DN, Patt R, Sharp IR, Brown D (1992) Monoclonal antibodies for the identification of herpesvirus simiae (B virus). Arch Virol 123: 267–277
7. Dubuisson J, Thiry E, Bublot M, Sneyers M, Boulanger D, Guillaume J, Pastoret P-P (1989) Production and characterization of monoclonal antibodies to bovid herpesvirus-4. Vet Microbiol 19: 305–315
8. Evermann JF, McKeirnan AF, Ott RL, Reed LA (1982) Diarrheal condition in dogs associated with viruses antigenetically related to feline herpesvirus. Cornell Vet 72: 285–291
9. Frey H-R (1972) Serologische Untersuchungen über den Verbreitungsgrad der Aujeszky'schen Virusinfektion bei Schweinen in Norddeutschland. Dtsch Tierärztl Wochenschr 79: 465–470
10. Frey H-R, Liess B, Haas L, Lehmann H, Marschall HJ (1989) Herpesvirus in harbour seals (*Phoca vitulina*): isolation, partial characterization, and distribution. J Vet Med B 36: 699–708
11. Friedli K, Metzler AE (1987) Reactivity of monoclonal antibodies to proteins of a neurotropic bovine herpesvirus 1 (BHV-1) strain and to proteins of representative BHV-1 strains. Arch Virol 94: 109–122
12. Harder TC, Moennig V, Greiser-Wilke I, Barrett T, Liess B (1991a) Analysis of antigenic differences between sixteen phocine distemper virus isolates and other morbilliviruses. Arch Virol 118: 261–268
13. Harder TC, Plötz J, Liess B (1991b) Antibodies against European phocine herpesvirus isolates detected in sera of Antarctic seals. Polar Biol 11: 509–512
14. Harder TC, Findik A, Nolte I, Liess B (1993) Untersuchung zum Spektrum viraler Erreger im Katzenschnupfen-Komplex – Therapieversuche mit dem Herpesvirustatikum Aciclovir (Zovirax). Kleintier-Praxis 39: 93–106
15. Horvat B, Willhaus Th, Frey H-R, Liess B (1989) Herpesvirus in harbour seals (*Phoca vitulina*): transmission in homologous host. J Vet Med B 36: 715–718
16. Kaerber G (1931) Beitrag zur Behandlung kollektiver Reihenversuche. Arch Exp Pathol Pharmkol 162: 527–539
17. Kearney JF, Radbruch A, Liesgang B, Rajesky K (1979) A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J Immunol 123: 1548
18. Kennedy S, Lindstedt IJ, McAlisky MM, McConnell SA, McCullough SJ (1993)

Herpesviral encephalitis in a harbour porpoise (*Phocoena phocoena*) J Zoo Wildl Med 23: 374–379
19. Kennedy-Stoskopf S, Stoskopf M, Eckhaus AM, Strandberg JD (1986) Isolation of a retrovirus and a herpesvirus from a captive California sea lion. J Wildl Dis 22: 156–164
20. Lazar JG, Taub FE (1992) A highly sensitive method for detecting peroxidase in *in situ* hybridization or immunohistochemical assays. In: Kessler C (ed) Nonradioactive labeling and detection of biomolecules. Springer Laboratory, New York, pp 135–142
21. Limcumpao JA, Horimoto T, Xuan X, Takashi E, Mikami T (1990) Immunological relationship between feline herpesvirus type 1 (FHV-1) and canine herpesvirus (CHV) as revealed by polyvalent and monoclonal antibodies. Arch Virol 111: 165–176
22. Littlefields JW (1964) Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science 145: 709–711
23. Martineau D, Lagace A, Beland P, Higgins R, Armstrong D, Shugart R (1988) Pathology of stranded Beluga whales (*Delphinapterus leucas*) from the St. Lawrence Estuary, Quebec, Canada. J Comp Pathol 98: 287–311
24. Morris R (1990) Immunoperoxidase staining of gene products in cultured cells using monoclonal antibodies. Methods Mol Biol 7: 339–359
25. Osterhaus ADME, Yang H, Spijkers HEM, Groen J,Teppema JS, van Steenis G (1985) The isolation and partial characterization of a highly pathogenic herpesvirus from the harbour seal (*Phoca vitulina*). Arch Virol 86: 239–251
26. Osterhaus ADME (1988) Seal death. Nature 334: 301–302
27. Povey RC (1975) In vitro antiviral efficacy of ribavirin against feline calicivirus, feline viral rhinotracheitis virus, and canine parainfluenza virus. Am J Vet Res 39: 175–178
28. Povey RC (1979) A review of feline viral rhinotracheitis (feline herpesvirus-1 infection). Comp Immunol Microbiol Infect Dis 2: 373–387
29. Roizman B, Desrosiers RC, Fleckenstein B, Lopez C, Minson AC, Studdert MJ (1992) The family *herpesviridae*: an update. Arch Virol 123: 425–449
30. Rota PA, Maes RK, Evermann JF (1986) Biochemical and antigenic characterization of feline herpesvirus-1-like isolates from dogs. Arch Virol 89: 57–68
31. Rota PA, Maes RK (1990) Homology between feline herpesvirus-1 and canine herpesvirus. Arch Virol 115: 139–145
32. Stenvers O, Plötz J, Ludwig H (1992) Antarctic seals carry antibodies against seal herpesvirus. Arch Virol 123: 421–424
33. Truyen U, Stockhofe-Zurwieden N, Kaaden OR, Pohlenz J (1990) A case report: encephalitis in lions. Pathological and virological findings. Dtsch Tierärztl Wochenschr 97: 89–91
34. Vedder L, Zarnke R, Spijkers I, Osterhaus ADME (1987) Prevalence of virus neutralizing antibodies to seal herpesvirus (phocid herpesvirus 1) in different pinniped species. Abstracts of the 7th Biennial Conference on the Biology of Marine Mammals, 5-9 December 1987, Miami, U.S.A.
35. Zhang XM, Mölle G, Hoffmann W, Hentschke J, Ludwig H (1989) Viral infections as aetiological factors of the seal death: coinfections with herpes- and morbilliviruses, Abstracts of the International Workshop on Current Research in Seal Diseases, Hannover Vet. School, 21–22 February 1989, p 32

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