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ANTIGENIC RELATEDNESS OF ALPHAHERPESVIRUSES ISOLATED FROM FREE-LIVING RODENTS

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Summary. — Complement fixation and virus neutralization tests confirmed that alphaherpesviruses isolated from free-living *Apodemus flavicollis* and *Clethrionomys glareolus* rodents form an antigenically identical or very close group. Immunofluorescence showed that antigen assembly and distribution within the infected cell resembles that of members of the *Alphaherpesvirinae* subfamily. Radioimmunoassay revealed close antigenic relatedness between five rodent herpesvirus isolates. Moreover it suggested a possible relatedness of these viruses to some virus species isolated from humans and animals.

Key words: *rodent herpesviruses*; *antigenic relatedness*; *complement fixation test*; *virus neutralization test*; *immunofluorescence*; *radioimmunoassay*; *immunodiffusion*

Introduction

Based on electron microscopic studies on the structure of virions of 5 isolates from free-living rodents (Čiampor et al., 1981) as well as their growth characteristics, these viruses were preliminarily classified as members of the *Alphaherpesvirinae* subfamily (Blaškovič *et al.*, 1980; Svobodová *et al.*, 1982). Because the species *Apodemus flavicollis* and *Clethrionomys glareolus* are not taxonomixally closely related it was useful to prove whether any antigenic differences exist among these virus isolates.

Materials and Methods

Viruses and growth conditions for their propagation. Viruses isolated from two species of free-living rodents described previously (Blaškovič *et al.*, 1980; Čiampor *et al.*, 1981) were used. Further strains of viruses used were described by Svobodová *et al.* (1982) who also presented data on cell cultures and growth media as well as growth characteristics of the five isolates. These characteristics were compared with the growth of pseudorabies and bovine rhinotracheitis viruses in primary foetal calf kidney cells (Somogyiová, 1962), herpes simplex virus type 1, and guinea pig-like herpesvirus (Hsiung and Kaplow, 1969). Mouse cytomegalovirus, strain Smith, was obtained from the American Type Culture Collection and was grown in mouse embryo cells (MEC).

Purification of viral antigens for serology and immunization procedure. Alphaherpesviruses from free-living rodents were inoculated into cultures of a continuous cell line of rabbit embryo fibroblasts (REF) in Roux bottles at a multiplicity of infection (MOI) of 2 $TCID_{50}$ per cell. Extracellular virus obtained 5-6 days p.i. with a titre of 10^5—10^7 $TCID_{50}$ per ml medium was purified by a three-step procedure.

The first step comprosed two cycles of differential centrifugation. First, the virus-containing medium (1000—1500 ml) was clarified in a Janetzky K 23 centrifuge for 20 min at 3000 rev/min and then centrifuged in a R 30 rotor in a Spinco L 50 centrifuge for 60 min at 30 000 rev/min. The pellet was resuspended in 0.005 mol/l Tris buffer, pH 7.2 (this buffer was used throughout all purification procedures) and consequently sonicated in a Raytheon sonic oscillator 9 kc for 60 sec.

The second step of purification comprised centrifugation through a discontinuous Ficoll gradient prepared from equal volumes of 20, 10, and 5% (w/v) of Ficoll dissolved in 0.005 mol/l Tris buffer at pH 7.2. From 0.7—1 ml of sonicated viral suspension was applied on the gradient. After centrifugation (Spinco L 50 centrifuge, SW50 rotor, 30 000 rev/min), the viral material was transferred to another tube, diluted with Tris-HCL buffer and sedimented in a R 50 rotor of a Spinco L 50 centrifuge at 48 000 rev/min for 60 min.

In the third step of the purification procedure the resuspended and sonicated virus was layered on a 30% sucrose cushion and centrifuged (SW50 rotor at 30 000 rev/min for 90 min). After removal of sucrose, the pellet was resuspended in Tris-HCl buffer and sonicated again. Protein content in the purified material was determined according to Lowry *et al.* (1951).

The purity of the five rodent herpesvirus preparations was checked by electron microscopy. Enveloped, unenveloped and empty virions were present. The purified material contained from 1.6—10.8 mg/ml of protein.

Immunization. Two 2—3 months old rabbits (Dobrá Voda breed) weighing about 2 kg were each given five equal doses of antigen at intervals of 2—3 weeks. Before immunization, control blood samples were taken from each rabbit. The immunization procedure lasted for 90 days with a total antigen load of 650—1 400 μg of protein. The antigen was applied intramuscularly (i.m.) with complete Freund's adjuvant (Difco Labs., Detroit, USA) added to the first and the last dose. The remaining three doses were applied with incomplete adjuvant (Arlacel and Bayol 1 : 10). During the immunization period blood samples were taken for virus neutralizing (VN) antibody titre assay. After the three months' period, the rabbits were bled. The final VN titres varied from 32—256.

The complement fixation test (CFT) was performed by a micromethod in plastic plates (Microtitration plate, Behringwerke, FRG) according to a standard method (Lennette and Schmidt, 1969). Complement titration was performed in test tubes.

The virus neutralization test (VNT) was performed with different dilutions of hyperimmune sera to which 100 CPD_{50}/ml of the appropriate virus was added. The virus-serum mixtures were incubated in a water bath at 37 °C for 90 min and then inoculated in 24—48 hr old REF, MEC and FCKC cell cultures according to the growth requirements of the respective virus.

The VN titre was established on days 7—10 when a complete CPE could be observed as compared with control cell cultures. The titre was estimated according to Reed and Muench.

Fluorescent antibody technique. Coverslip REF cultures were infected at MOI of 10. Rodent herpesvirus No. 60 was used as a prototype of the five isolates. Coverslip cell cultures were taken at 24 and 48 hr p.i., fixed with acetone, treated with immune rabbit serum with a titre of 64 and then stained with labelled swine anti-rabbit conjugate (SwARa, Sevac, Prague).

Radioimmunoassay (RIA) procedure. Purified antigen of rodent herpesvirus isolate No. 68, purified HSV type 1 and 2, and nucleocapsids of HSV typd 1 were firmly fixed to polystyrene plate wells. Rabbit immune sera against five strains of rodent herpesviruses, HSV type 1 and 2, HSV type 1 envelope antigen and pseudorabies virus, human immune cytomegalovirus serum and 12 rabbit normal sera (controls) were used. The binding of antibodies with firmly fixed antigens was detected using ^{125}I-labelled protein A. Radioactivity was measured in a Packard Model PGD PRIAS automatic gamma counter.

Gel double diffusion test (immunodiffusion, ID) was performed according to Styk *et al.* (1971) and Styk and Russ (1978) in 1% agarose (Loba, Austria) gels. Purified viral antigens from rodent herpesviruses were treated with 1% sodium deoxycholate for 20 min at room temperature. All rabbit immune sera against herpesvirus isolates Nos. 60, 68, 72, 76 and 78 were absorbed with REF cells resuspended in Basal Eagle's Medium with 5% bovine serum and subsequently saturated with inactivated calf serum.

Table 1. Complement fixation test with rodent herpesvirus isolate No. 60 and five rabbit homologous and heterologous immune sera

Rabbit immune serum against isolate No.	Antibody titre*
60	64
68	64
72	32
76	64
78	32

* Serum dilution reciprocals

Results

Complement fixation tests

As antigen, herpesvirus isolate No. 60 and rabbit immune sera against virus isolates Nos. 60, 68, 72, 76 and 78 absorbed with bovine protein powder and REF cells were used. Normal rabbit serum was used as control. The results are given in Table 1. The CFT was repeated three times with identical results. All five isolates form according to the CFT homogeneous group. Control rabbit did not react with the antigens.

Virus neutralization tests

Rabbit immune sera against 5 rodent herpesvirus isolates (Nos. 60, 68, 72, 76 and 78) were tested with homologous viruses and, in addition, with HSV type 1 and 2, pseudorabies virus, bovine infectious rhinotracheitis (IBR) virus and mouse cytomegalovirus (MCV) in cell cultures as indicated in Table 2. The VNT clearly showed that the five herpesvirus isolates reacted

Table 2. Virus neutralization tests with five rabbit hyperimmune sera against rodent herpesvirus isolates with homologous and heterologous viruses

Viruses	Hyperimmune rabbit sera against rodent herpesvirus isolates Nos.					Cell culture in which VNT was performed
	60	68	72	76	78	
60	64*	64	128	128	256	REF
68	64	32	256	256	256	
72	64	128	256	256	256	
76	128	128	256	128	128	
78	64	128	256	128	128	
HSV type 1	0	0	0	0	0	
HSV type 2	0	0	0	0	0	
Pseudorabies	0	0	0	0	0	
IBR	0	0	n.d.	n.d.	n.d.	FCKC
MCV	0	0	0	0	0	MEC

Control serum: no antibodies detected
* Serum dilution reciprocals (0 = no antibody detected)
n.d. — not done

with homologous and heterologous immune sera; however, they did not react with heterologous viruses from man or different animal species. No serological reaction was detected with MCV.

Immunofluorescence

Twenty-four hr p. i. REF monolayers on coverslips infected with rodent herpesvirus isolate No. 60 (MOI 10 $TCID_{50}$/cell) were stained by the indirect technique. Bright fluorescence was observed in great foci consisting of 30—40 cells and in smaller foci consisting of 5—10 cells. The fluorescent antigen either in form of granules or in form of diffuse fluorescence was localized on the nuclear membrane and in the cytoplasm of infected cells. From 70—80% of cells in the monolayer contained fluorescent antigen. Forty-eight hr p. i., great morphological changes were observed in the cells. However, pathologically changed cells revealed only traces of fluorescence.

Radioimmunoassay

Purified antigens of rodent herpesvirus isolate No. 68, human herpesvirus type 1 and 2, and nucleocapsid of HSV type 1 firmly fixed in wells of polystyrene plates were assayed with rabbit immune sera against all five rodent herpesvirus isolates, against HSV type 1 and 2, against envelope from HSV 1, against pseudorabies virus and with human immune serum against human cytomegalovirus. Twelve different sera from normal rabbits were included as controls. Under the conditions described in Materials and Methods, the rodent herpesvirus isolates, forming a close group, could no be definitely differentiated.

Moreover, nonspecific reactions with serum proteins and perhaps cell components were not completely eliminated from immune sera and therefore they probably influenced the values obtained. The reactivity of normal (control) rabbit sera with practically all antigens used in the test was remarkably high.

Immunodiffusion

Each immune serum formed one or several (2—3) precipitation lines with the corresponding antigen located in the central well. The lines were of different intensity, corresponding to the potency of the antigen and the quality fo the serum used.

Antigen from rodent herpesvirus isolate No. 76 reacted with all five immune sera located symetrically in peripheral wells with a coalescent line around the central well, thus indicating the presence of at least one common antigen in all samples tested. To a lower extent, the antigens No. 68 and 76 have a coalescent precipitation line with their sera and with sera Nos. 60 and 72 (see Figs. 1 and 2).

Discussion

The aim of the present work was to decide whether the rodent herpesvirus isolates Nos. 60, 68, 72, 76 and 78 are antigenically identical, related or different. For this purpose several tests on the antigen — antibody binding

capacity were used. We are aware of the difficulties which may occur in performing such tests because of different potency of the antigens used and the individual serological response of immunized rabbits.

The CFT confirmed that all five rodent herpesvirus isolates from an antigenically uniform group with a common CF antigen. We suggest that nucleoprotein of the viruses tested is responsible for this uniformity.

The VNT is generally considered as the most specific test. All five viruses were neutralized by any antiserum in titres from 32—256 and higher. The highest titre was not necessarily reached within the homologous virus strain. One may speculate whether these discrepancies in titre values are due to quantitative differences in the antigenic potency of the antigens and/or in the individual immunological response of different animals. Nevertheless, we assume that if differences between the rodent herpesvisus strains do occur, they are of minor importance. On the other hand, we revealed no antigenic relatedness in the VNT between the herpesvirus isolate and HSV type 1 and 2, pseudorabies and bovine rhinotracheitis viruses and what we feel to be most important, no relatedness with the mouse cytomegalovirus.

Immunofluorescence confirmed the antigen formation in cells infected with rodent herpesviruses and the feasibility to detect viral antigens by this method. Once again it was shown that the rodent herpesvirus isolate belong according to the pathohistology of infected cells to the subfamily *Alphaherpesvirinae*.

Radioimmunoassay offered partial results thus far. It confirmed a most probable antigenic relatedness of the five rodent isolates and moreover suggested a possible antigenic relatedness to herpesviruses of other host species. Further experiments are necessary to show whether this assumption is correct and why normal rabbit sera react with herpesvirus isolates No. 68 and also to some extent with herpesvirus type 1. Antigenic differences between the rodent herpesviruses could be revealed by competitive RIA.

The immunodiffusion test suggested possible identity of the five isolates as far as one common antigen is concerned. Despite the fact that the immune rabbit sera against all five herpesvirus isolates (obtained after immunization with partially purified virus grown in REF with 5% inactivated calf serum) were absorbed with REF cells and bovine serum the results were not clear Further evidence should be obtained to differentiate the possible nonspecific reactions from specific virus-antibody binding. Thus the question as to whether herpesviruses isolated in identical biotopes from rodent species with different phylogenetic history are identical or slightly different remains unanswered.

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Explanation of Figures (Plate XLVI):

Figs. 1—2. Immunodiffusion reaction of purified antigens from rodent herpesvirus isolates with immune sera against homologous and heterologous virus antigens.
1 — Antigen of herpesvirus isolates No. 68 (central well); antisera against the isolates Nos. 60, 68, 72, 76 and 78 as indicated in peripheral wells.
2 — Antigen of herpesvirus isolate No. 76 (central well) and antisera against herpesvirus isolates in peripheral wells located as in Fig. 1.