Physico-chemical and Serological Characterization of Five Rhabdoviruses Infecting Fish

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SUMMARY

Viruses isolated from fish with viral haemorrhagic septicaemia (VHS), infectious haematopoietic necrosis (IHN), spring viraemia of carp (SVC), swim-bladder inflammation (SBI) and pike fry disease (PFD) have been grown to high titre in fathead minnow cells. While our preparations of the IHN, SVC, SBI and PFD viruses showed typical rhabdovirus morphology with bullet-shaped particles and distinct surface projections, the VHS virus preparations had a less typical rhabdovirus morphology but were pleomorphic with a preponderance of flexuous rods. Using virus labelled with [3H]-uridine, it was shown that each virus contained RNA which sedimented at 38 to 40 S and was hydrolysed by very low concentrations of ribonuclease. The viruses of SVC, PFD and SBI had a polypeptide composition similar to that of vesicular stomatitis virus, the prototype rhabdovirus, but the IHN and VHS viruses gave a pattern similar to that of rabies virus. In serum neutralization tests the SVC and SBI viruses were indistinguishable. VHS virus showed no serological relationship with the other four viruses but there was a low level of cross-reaction between the PFD, IHN and SVC-SBI viruses.

INTRODUCTION

Bullet-shaped viruses have been isolated from fish with viral haemorrhagic septicaemia (VHS; Zwillicnberg, Jensen & Zwillicnberg, 1965; de Kinkelin & Scherrer, 1970), infectious haematopoietic necrosis (IHN; Amend, Yasutake & Mead, 1969; Amend & Chambers, 1970), spring viraemia of carp (SVC: Fijan et al. 1971) swim bladder inflammation of cyprinids (SBI; Bachmann & Ahne, 1973, 1974) and pike fry disease (PFD; de Kinkelin, Galimard & Bootsma, 1973). Two other bullet-shaped viruses isolated from cases of Chinook Salmon Disease (Pariosot, Yasutake & Klontz, 1965) and Sockeye Salmon Disease (Wingfield, Fryer & Pilcher, 1969) have been shown on the basis of pathology (Yasutake, 1970), morphology (Amend & Chambers, 1970; Darlington, Trafford & Wolf, 1972) and serology (McCain, Fryer & Pilcher, 1971) to be strains of IHN virus rather than distinct viruses. All these viruses are regarded as rhabdoviruses from their morphology. However, apart from the work of de Kinkelin and his colleagues (Lenoir, 1973; de Kinkelin, le Berre & Lenoir, 1974) which has shown that the SVC and PFD viruses have a polypeptide composition similar to that of vesicular stomatitis virus (VSV), little is known about the physico-chemical
characteristics of the viruses. One of the aims of the present work was to determine some of these properties and compare them with the properties of other rhabdoviruses.

Our preliminary experiments have shown that the five viruses contain single-stranded RNA sedimenting at 38 to 40S similar to that of VSV. Analysis by polyacrylamide gel electrophoresis indicates that the SVC, SBI and PFD viruses have polypeptide compositions similar to that of VSV. However, the polypeptide compositions of the VHS and IHN viruses were similar to each other and to that of rabies virus but differed from that of VSV. A second aim was to ascertain whether the viruses were serologically related. The evidence from serum neutralization tests indicates that the VHS, IHN, PFD and SVC viruses are distinct but the SVC and SBI viruses are indistinguishable.

**METHODS**

*Viruses.* The strains of virus used were VHS (strain F-I, provided by Dr P. E. Vestergaard-Jorgensen, State Veterinary Serum Laboratory, Aarhus, Denmark); IHN (isolated from rainbow trout, provided by Dr D. F. Amend, Western Fish Disease Laboratory, Seattle, Washington, U.S.A.); SVC (strain S/3o, provided by Dr N. Fijan, Veterinary Faculty, University of Zagreb, Yugoslavia); SBI (strain 10/3, provided by Dr W. Ahne, Institute of Zoology and Parasitology, University of Munich, West Germany; PFD (provided by Dr P. de Kinkelin, Laboratoire d’Ictyopathologie, Thiverval-Grignon, France). All the strains had been plaque cloned. The viruses were grown in monolayers of fathead minnow (FHM) cells (Gravell & Marlsberger, 1965) at 14 °C (VHS and IHN) or 20 °C (SVC, SBI and PFD) maintained in Eagle’s Minimal Essential Medium (MEM, Glasgow modification) buffered at pH 7.4 to 7.6 with 0.16 M-tris-HCl and supplemented with 2 % foetal calf serum and 10 % tryptose phosphate broth. Viruses containing [3H]-uridine were grown in the same medium supplemented with about 3 μc [3H]-uridine/ml. The cells were infected at a multiplicity of 0.01 to 0.1 to avoid interference effects and the viruses were harvested when gross c.p.e. were observed. This usually occurred after 40 to 48 h for SVC, SBI and PFD viruses, about 72 h for VHS virus and about 96 h for IHN virus. Titres of 10⁸ to 10⁹ p.f.u./ml were usually obtained with the SVC, SBI and PFD viruses and 10⁷ to 10⁸ p.f.u./ml with the VHS and IHN viruses.

*Assay of infectivity.* Epithelioma papulosum carpio (EPC) cells were grown at 29 °C in MEM (Glasgow modification) containing 10 % foetal calf serum and 10 % tryptose phosphate broth. Confluent monolayers were obtained within 24 h, after which the medium was removed and the dishes inoculated in triplicate with 0.2 ml virus suspension. Virus was adsorbed for 1 h at 20 °C, after which the medium was removed and the cells overlayed with 2 ml of 0.5 % agarose in maintenance medium buffered at pH 7.4 to 7.6 with 0.25 M-tris-HCl. Dishes inoculated with the VHS and IHN viruses were stained after incubation at 14 °C for 3 days and 4 days respectively. Dishes inoculated with the SVC and SBI viruses were stained after 2 days at 20 °C and those of the PFD virus after 3 days at 20 °C. Each dish was covered with 2 ml of 0.01 % neutral red in MEM and incubated for 3 h in the dark at 20 °C to reveal the plaques.

*Production of antisera.* Pooled infected cell harvests of 30 to 50 ml were ultrasonically treated for 10 s and cell debris removed by centrifuging at 1000 g for 15 min. Virus was deposited from the supernatant fluid by centrifuging at 40000 g for 1 h, washed with phosphate buffered saline (PBS) and re-suspended in 3 ml PBS. Approx. 1 ml of the concentrated virus suspension was blended with an equal vol. of Freund’s complete adjuvant and 0.5 ml inoculated intramuscularly into each hind leg of a rabbit (New Zealand White). Three weeks
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later the rabbits were inoculated intravenously with 0.5 ml of virus in PBS followed by a second intravenous inoculation of 0.5 ml of virus 1 week later. After a further week, blood was collected and the serum separated. All sera were heat-inactivated at 56 °C for 30 min before use.

Neutralization tests. Virus stocks were diluted in maintenance medium to give 100 p.f.u./0.1 ml and 0.5 ml mixed with an equal vol. of serial 0.5 log dilutions of antisera ranging from 10^{-4} to 10^{-5}. Controls consisted of 0.5 ml virus dilution mixed with 0.5 ml of pre-immune serum diluted 10-fold in maintenance medium. Virus-serum mixtures were incubated at 20 °C for 1 h after which 0.2 ml volumes were inoculated on to triplicate Petri dishes of EPC cells and the residual plaque counts determined as described above.

Virus purification. The supernatant fluids were separated from cell debris by centrifuging at 2000 g for 5 min. The fluids were then centrifuged at 40000 g for 1 h and the pellets re-suspended in 2 ml 0.04 M-phosphate, pH 7.6. After removing debris, each concentrate was centrifuged for 2 h at 40000 g in 25 ml of a 15 to 45 % sucrose gradient. The viruses were clearly visible as opalescent bands. One ml fractions were collected from the bottom of the tube and 0.05 ml samples assayed for radio-activity.

Polyacrylamide gel electrophoresis of virus polypeptides. Mixtures of each virus and trace amounts of [35S]-methionine-labelled vesicular stomatitis virus, were disrupted according to the method described in detail by Cartwright, Talbot & Brown (1970). The amount of [35S]-methionine-labelled vesicular stomatitis virus used in each mixture was insufficient to stain with Coomassie blue. The mixture of polypeptides was separated by the method described in the same paper and stained with Coomassie blue. The position of the stained bands was traced with a densitometer and the gels were then frozen and sliced into 1 mm sections for the assay of radioactivity.

Electron microscopy. Pellets of the viruses obtained by centrifuging the virus harvests at 40000 g for 1 h were re-suspended in a few drops of water and applied to carbon formvar grids. After allowing a few minutes for adsorption, the samples were stained with 1 % phosphotungstic acid or 1 % sodium tungstate adjusted to pH 5.3 with formic acid and examined in a Siemens Elmiskop I.

RESULTS

Morphology of the virus particles

The IHN, SVC, SBI and PFD virus particles had a rigid rod-like appearance. Surface projections were not immediately obvious, presumably because the specimens had been prepared by pelleting virus harvests and had not been purified by centrifuging in sucrose gradients, whereas the micrographs of VSV and rabies virus included in Fig. 1 for comparative purposes had been prepared with viruses purified in sucrose gradients. We did not use sucrose gradient purified preparations of the fish viruses for electron microscopy because these disintegrated when examined under the conditions we have used previously without difficulty for VSV and rabies virus. Nevertheless, it could be seen that the majority of the particles were about 120 nm long and 60 to 90 nm wide. Some larger particles, measuring about 180 × 80 nm, were found in the SVC virus preparations. The measurements of the shorter particles differ markedly from those found for VSV but are closer to those of the Flury LEP strain of rabies virus (Crick & Brown, 1970). The morphology of the VHS particles was distinct from that of the other four viruses in that the VHS virus particles were pleomorphic with a preponderance of flexuous rods which varied considerably in length. This morphology is different from that described for this virus by Zwillenberg et al. (1965) and de Kinkelin & Scherrer (1970), who found that it closely resembled VSV. A
Fig. 1. Electron micrographs of five fish rhabdoviruses, rabies virus and vesicular stomatitis virus.
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Fig. 2. Sucrose gradient sedimentation profiles of five [H]-uridine-labelled fish rhabdoviruses.

possible explanation for the difference between our observations and those reported by these workers is included in the Discussion.

Few short particles were seen in any of the preparations described here, presumably because the viruses had been grown under conditions of low input multiplicity to minimize interference effects. However, short particles can be obtained when the cells are infected at higher multiplicity, as with many other rhabdoviruses.

Purification of the virus particles

An opalescent band containing virus particles was obtained when each of the re-suspended pellets of [H]-uridine-labelled virus was centrifuged in a sucrose gradient. One ml fractions
were collected and counted for radioactivity. A peak of radioactivity coincident with the opalescent band was obtained with each virus (Fig. 2). A second small peak of radioactivity near the top of the tube was presumably due to interfering particles or ribonucleoprotein (RNP). The large peak at the top of the tube probably represented unincorporated [3H]-uridine.

**Sedimentation rate of virus RNA**

Purified preparations of the [3H]-uridine-labelled viruses were mixed with 0.1 M-acetate-0.1 % SDS, pH 5.0 and centrifuged in 5 to 25 % sucrose gradients prepared in the same buffer solution. The RNA from each virus sedimented as a fairly sharp peak at about 38
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Fig. 4. Densitometer tracings of SDS-polyacrylamide gel electropherograms of polypeptides of five fish rhabdoviruses. The number above each individual peak, which indicates the mol. wt. of each polypeptide, was calculated by reference to internal markers of [35S]-methionine-labelled VSV polypeptides (not shown).

Fig. 4 shows the electropherograms of the polypeptides of SVC, SBI, PFD, IHN, and VHS viruses. The numbers above each peak indicate the mol. wt. of the corresponding polypeptide.

Analysis of the virus polypeptides

Polyacrylamide gel electrophoresis of the polypeptides of the 5 viruses gave two distinct patterns of staining with Coomassie blue (Fig. 4). One pattern, similar to that obtained with VSV, was found for the SVC, SBI and PFD viruses and consisted of one large polypeptide, present in small amounts, and three major staining bands. The mol. wt. indicated on the tracings is as follows:

- SVC: mol. wt. > 150, 82, 44, 23
- SBI: mol. wt. > 150, 83, 52, 25
- PFD: mol. wt. > 150, 55, 28
- IHN: mol. wt. > 150, 55, 55, 33
- VHS: mol. wt. > 150, 55, 62, 33

Each RNA was hydrolysed to slowly sedimenting molecules by mixing with 0.01 μg ribonuclease/ml, indicating the single stranded nature of the molecules.

to 40S, relative to values of 28S and 18S for ribosomal RNA (Fig. 3). Each RNA was hydrolysed to slowly sedimenting molecules by mixing with 0.01 μg ribonuclease/ml, indicating the single stranded nature of the molecules.
Table 1. Cross neutralization tests with five rhabdoviruses

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>SVC</th>
<th>SBI</th>
<th>PFD</th>
<th>IHN</th>
<th>VHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVC</td>
<td>6500</td>
<td>6200</td>
<td>20</td>
<td>20</td>
<td>&lt;10</td>
</tr>
<tr>
<td>SBI</td>
<td>980</td>
<td>980</td>
<td>68</td>
<td>50</td>
<td>&lt;10</td>
</tr>
<tr>
<td>PFD</td>
<td>33</td>
<td>28</td>
<td>200</td>
<td>27</td>
<td>&lt;10</td>
</tr>
<tr>
<td>IHN</td>
<td>25</td>
<td>25</td>
<td>27</td>
<td>1700</td>
<td>&lt;10</td>
</tr>
<tr>
<td>VHS</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>2300</td>
</tr>
</tbody>
</table>

individual densitometer tracings are similar to those found for VSV. A minor peak (44) in the SVC pattern, which was not found in our preparations of SBI and PFD, probably corresponds to the NS protein of VSV. The second pattern, which was obtained with the IHN and VHS viruses, more closely resembled that given by rabies virus (Sokol, Stancek & Koprowski, 1971) in containing two polypeptides with mol. wt. of about 35 and 40 × 10^3. In addition, however, the largest of the major staining bands of the IHN and VHS viruses appeared to consist of two polypeptides, whereas rabies virus contains only one staining band at this position.

Serological relationships of the five viruses

By plotting the percentage of plaques obtained at each dilution of antiserum (compared with the control) against the logarithm of antiserum dilution, neutralization curves with a typical sigmoid shape were obtained. The dilutions of antisera giving 50% reduction in plaque count were calculated from the graphs (Table 1). The SVC and SBI viruses could not be distinguished from each other in this test. The VHS virus did not show any serological relationships with the other four viruses but there was a low level of cross-reaction between the PFD, IHN and SVC-SBI viruses.

DISCUSSION

Four of the five viruses studied in this work, namely the IHN, SVC, SBI and PFD viruses showed typical rhabdovirus morphology but the size of the particles was closer to that found for the Flury strain of rabies virus (Crick & Brown, 1970) than it was to VSV, the prototype member of the rhabdovirus group. The particle size of the SVC, SBI and PFD viruses found in this work is similar to the values given by Fijan et al. (1971), Bachmann & Ahne (1973) and de Kinkelin et al. (1973). The micrographs of the different strains of IHN virus (Amend & Chambers, 1970; Darlington et al. 1972) suggest that these viruses also are shorter than VSV particles.

Our preparations of the VHS virus had a morphology which was quite distinct from that described by Zwillenberg et al. (1965) and de Kinkelin & Scherrer (1970). Whereas these workers found particles which closely resembled VSV in size and shape, our preparations contained pleomorphic particles with a preponderance of distorted or flexuous rod-shaped particles varying considerably in length. K. P. Olberding & J. W. Frost and A. I. Donaldson (separate personal communications) have also found that their preparations of VHS virus contained a significant proportion of distorted particles in addition to the more easily recognizable bullet-shaped particles typical of VSV. Donaldson also noticed that this proportion was larger when the virus was pelleted before examination, suggesting that VHS virus particles are fragile and distort readily when pelleted. This observation would explain why we have failed to obtain particles recognizable as typically bullet-shaped since in our
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study the virus was centrifuged prior to electron microscopy. Fixation prior to centrifuging may lead to improved preservation of structure.

Our preliminary examination of the physical and chemical characteristics of the fish rhabdoviruses shows that they all contain single-stranded RNA with a sedimentation coefficient of 38 to 40 S, similar to that for the RNAs of vesicular stomatitis virus and rabies virus. However, the viruses fall into two groups with regard to their polypeptide composition. The SVC, SBI and PFD viruses have a composition similar to that of VSV (Wagner et al. 1972). The mol. wt. of the three fish virus polypeptides are similar to those obtained for the polypeptides of SVC virus by Sokol et al. (1974) but differ from the much lower values obtained by Lenoir (1973). The IHN and VHS virus polypeptides gave patterns of staining which more closely resembled those produced by rabies virus (Sokol et al. 1971). In addition, the largest of the major staining bands of the IHN and VHS viruses appeared to contain two polypeptides instead of the single polypeptide described for rabies virus. It is not known whether the large major polypeptides of the fish rhabdoviruses are glycosylated as in VSV and rabies virus, because our attempts to incorporate glucosamine into the viruses were unsuccessful.

The serological data show that the VHS, IHN and PFD viruses are distinct from each other and from the SVC and SBI viruses. However, the SVC and SBI viruses were serologically indistinguishable. As spring viraemia of carp and swim bladder inflammation have generally been considered to be distinct diseases because of the differences in their pathogenesis, seasonal variation and geographical distribution, some clarification of the situation is required. Clearly, further studies are necessary on the comparative pathology of the two diseases and independent attempts at virus isolation from both diseases would be valuable in resolving the situation.

The serological identity of the SVC and SBI viruses suggests that there are only four distinct fish rhabdoviruses at the present time. The viruses can be classified into two groups on the basis of their polypeptide compositions, one group containing the VHS and IHN viruses and the other containing the SVC-SBI and PFD viruses. The same grouping is also found with regard to several biological properties. For example, the VHS and IHN viruses infect salmonids only, have a low optimal growth temperature (12 to 14 °C) in tissue culture and induce interferon. In contrast, SVC-SBI and PFD viruses infect non-salmonid fish but not salmonids, have a higher optimal growth temperature (20 to 22 °C) in tissue culture, and do not readily induce interferon (de Kinkelin & Scherrer, 1970; Wingfield, et al. 1969; de Kinkelin & Dorson, 1973; Fijan et al. 1971; de Kinkelin & le Berre, 1974a, b; de Kinkelin, et al. 1974). Further work will be necessary to ascertain whether this subgrouping applies to other properties of the fish rhabdoviruses.

REFERENCES


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