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Biological Properties and Identification of the Agent Causing Swim Bladder Inflammation in Carp[1]

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With 1 Figure

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Summary

The 10/3 isolate responsible for infectious swim bladder inflammation in carp was biologically characterized and compared with *Rhabdovirus carpio*. 10/3 virus replicated in a variety of cell cultures derived from fish, chicken and mammalian origin at 20° C. Its replication cycle in FHM cells lasted only 8—10 hours, and the virus was released slowly from the cells into the culture medium.

Plaque morphology of 10/3 virus and *Rhabdovirus carpio* was similar, and cross-neutralization tests showed a close relationship between the two virus strains.

10/3 virus produced a disease in carp (*Cyprinus carpio* L.) and Guppies (*Lebistes reticulatus* P.) similar to swim bladder inflammation after experimental infection. The clinical and pathological changes partly resembled the changes reported in carp after experimental infection with *Rhabdovirus carpio*.

The importance of the common etiology of spring viremia caused by *Rhabdovirus carpio* and swim bladder inflammation is discussed, since up to now the disease complexes and their etiology were considered to be separate entities.

1. Introduction

Economically the most important diseases in carp pisci culture caused by viruses are the acute form of infectious dropsy (IDC) or spring viremia (SVC) and the infectious swim bladder inflammation (aerocystitis). Whereas the agent of infectious dropsy has been isolated and identified by Fijan *et al.* (7) as *Rhabdovirus carpio*, the etiology of swim bladder inflammation remained subject of debate. Until recently, bacteria have been supposed as causative agents (10, 14, 23). In transmission experiments, however, a viral etiology was postulated, since the disease was successfully reproduced with ultrafiltrates from brain and swim bladder organ suspensions prepared from carp with symptoms of aerocystitis (2, 15, 16, 19). Inoculations of infectious organ material into cell cultures of carp origin were not successful at that time (22).

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Recently we isolated a rhabdovirus associated with aerocystitis in carp from outbreaks during summer 1971 and 1972 (4). This communication describes some biological properties of the isolate and its relationship to *Rhabdovirus carpio*.

2. Materials and Methods

2.1. Virus

Isolate 10/3 as described elsewhere (4) and *Rhabdovirus carpio* (RVC)[1] were used throughout the experiments as passage numbers 10 to 14 in the FHM cell line. Infectious hematopoietic necrosis virus (IHN)[2] and Egtved virus[3] were used for serologic comparison. IHN virus was grown in FHM cells at 15° C, Egtved virus in RTG-2 cells.

2.2. Cell Cultures

10/3 and RVC viruses were routinely grown in FHM cells [ATCC, CCL 42; (8)] at 20—22° C. In addition the 10/3 virus was cultivated in primary cultures of gonad tissue from carp (*Cyprinus carpio* L.) and rainbow trout (*Salmo gairdneri* Rich.) and in the RTG-2 cell line [ATCC, CCL 55; (25)].

Cultivation was also tried in primary chick embryo fibroblast cultures and in the following mammalian cell cultures: primary fetal calf kidney; BHK-21/13 cell line (hamster origin, ATCC, CCL 10); HEp-2 cell line (human origin, ATCC, CCL 23); Vero cell line (African green monkey origin, ATCC, CCL 81); MDCK cell line (canine origin, ATCC, CCL 34), and SK cell line [porcine origin (11)]. The non-fish cell cultures were infected with the 12th passage of 10/3 virus (titer $10^{6.8}$TCID$_{50}$/0.1 ml). Three successive virus passages were made with each cell system. Subsequent titrations were performed in FHM cells.

The medium used consisted of minimal essential medium (MEM) with Earle's or Hanks' BSS adding 3—5 per cent fetal calf serum.

2.3. Virus Titrations

All virus titrations were carried out in FHM cells unless stated otherwise. Dilutions of $\log_{10}$ were inoculated in 0.1 ml amounts into three tube cultures or microplate wells each, and then incubated at 20—22° C. Endpoints were read by evaluation of cytopathic effects four days after inoculation and calculated according to the formula given by Kärber (9).

2.4. Growth Curve

A single step growth curve for 10/3 virus was obtained by washing FHM monolayer cultures grown on coverslips in Leighton tubes twice with PBS and adsorption of 1 ml virus material for 60 minutes at 4° C.

After adsorption, coverslips were again washed twice with PBS and changed to new sterile Leighton tubes containing 2 ml of virus medium. An input multiplicity of 5TCID$_{50}$/cell was chosen.

Samples from the whole culture (combined medium and cells), from medium (medium fraction) and from cells (cell fraction) were removed in two hour intervals up to 24 hours incubation at 20° C. The samples were frozen immediately at —70° C and titrated after thawing.

2.5. Plaque Production

FHM cell monolayers in 60 mm plastic Petri dishes (Fa. Greiner, Nürtingen, Germany) were washed twice with Earle's BSS and inoculated with 1 ml of different dilutions of the 10/3 isolate and RVC. After an adsorption time of 60 minutes at room temperature the cultures were washed twice again and overlayed with 5 ml of a 0.75 per cent methyl cellulose medium. The Petri dishes were incubated at 20—22° C for 72 hours, fixed and stained with a crystal violet-formol-alcohol mixture.

[1] Courtesy Dr. Fijan, Zagreb.

[2] Obtained from Dr. de Kinkelen, Thiverval-Grignon.

[3] Received from Dr. Vestergard-Jørgensen, Aarhus.

2.6. Immune Serum Production and Neutralization Tests

Supernatant from FHM cell cultures infected with 10/3 virus and RVC (infectivity titers between 10^6 and $10^7 TCID_{50}$/0.1 ml) was centrifuged without prior freezing and thawing at 2500*g* for 30 minutes. Again the supernate was saved and centrifuged in a Beckman Spinco L 65-2 at 40.000 r.p.m. for 180 minutes. The pellet was resuspended in PBS, mixed with complete Freund's adjuvant, and 0.25 ml of the suspension was given to rabbits intraplantar into each of the four extremities.

Four weeks after the first inoculation the rabbits were given 2 ml of the virus concentrate intraperitoneally, and bleeding was carried out 14 days later.

Neutralization tests were performed in FHM and RTG-2 cells by using serum dilutions and a constant $100 TCID_{50}$ of IHN, Egtved, RVC and 10/3 viruses. Virus and serum dilutions were mixed and allowed to bind at 4° C for 60 minutes. IHN and Egtved neutralization tests were incubated at 15° C, RVC and 10/3 N-tests at 20—22° C. Final reading was carried out 7 days post infection.

The immune sera had homologous neutralizing antibody titers for 10/3 virus of 1:8, and for RVC also of 1:8 after adsorption of the sera to noninfected FHM cells.

2.7. Animal Experiments

Carp (*Cyprinus carpio* L.) weighing about 200 g and Crucian carp (*Carassius auratus gibelio* Bl.) weighing ca. 150 g were kept in groups of five in different containers (50 to 75 l) at water temperatures between 14° and 17° C.

Guppies (*Lebistes reticulatus* P.) were held in small glass containers (500 ml) in groups of five at 20—22° C.

At the time of inoculation all animals were clinically healthy.

Infection of carp and Crucian carp was carried out by administering 2 ml of the 12th cell culture passage of the 10/3 virus intraperitoneally (infectivity titer $10^{6.5} TCID_{50}$/0.1 ml). Control animals received the same amount of non-infectious culture material.

Guppies were infected by adding 15 ml of the 10/3 virus to the water of the container. Controls were similarly kept in water with 15 ml non-infectious cell culture material.

Spleen, heart, kidney, brain, swim bladder, liver, intestine and muscle tissues were taken from carp and Crucian carp at different times after infection. The tissues were frozen for reisolation and titration of virus. Guppies were ground as a whole.

For reisolation 10 per cent organ suspensions were made with PBS, and after centrifugation at 2500*g* for 30 minutes, 0.1 ml of the supernatant was inoculated into each of three cell cultures.

3. Results

3.1. Growth of the 10/3 Isolate in Cell Cultures

The 10/3 virus replicated in a variety of different cell cultures with a cytopathic effect (CPE) provided that an incubation temperature between 20° and 22° C was observed. For infectivity titers obtained in different cell cultures see Table 1. The CPE which appeared at varying times post infection, depending upon the cell culture type used, consisted of granulation and rounding leading to a complete lysis of the cell sheet. After staining the infected cell cultures showed vacuolization of the cytoplasm, granulation and most of all margination of the nuclear chromatin.

Lysis was complete in primary cell cultures of *carp origin* three days after inoculation. Virus infectivity titers of about $10^6 TCID_{50}$/0.1 ml were recorded. Replication also occurred in *rainbow trout* cell cultures. Lysis, however, was demonstrated only after serial passages in the cell systems. The third passages titered between $10^{3.2}$ and $10^{4.2} TCID_{50}$/0.1 ml in FHM cells. Similar observations were

made after infection of the RTG-2 cell line. Here, best results were obtained, when cells were infected at the time of seeding.

Different results were recorded after inoculation of *mammalian* and *chick embryo fibroblast* cell cultures. It could be shown that compared to cell cultures of fish origin cytopathic changes appeared delayed for about 3—4 days post infection. Cultures of chick embryo fibroblasts, HEp-2, Vero- und MDCK cell lines did not show CPE after virus infection in the first passages, however, virus replication did occur (Table 1). Highest infectivity titers were read in primary fetal calf kidney cell cultures ($10^{6.8}$TCID$_{50}$/0.1 ml) after three successive passages. It appears from passages in the other mammalian cell types that in some instances an adaptation period is necessary, since higher infectivity titers can be obtained after further passages (BACHMANN and AHNE, unpublished results, 1973).

Table 1. *Infectivity Titers of 10/3 Isolate after Growth in Different Cell Cultures of Fish, Chicken and Mammalian Origin*

Cell culture origin	Infectivity titers[a] Passage No. 1	2	3
Rainbow trout			
heart	n.d.[b]	n.d.	4.2
gonads	n.d.	n.d.	3.2
swim bladder	n.d.	n.d.	3.2
RTG-2 cell line	n.d.	n.d.	3.8
Carp			
heart	n.d.	n.d.	6.5
gonads	n.d.	n.d.	6.2
swim bladder	n.d.	n.d.	6.0
FHM cells	6.5	6.8	6.8
Chick embryo fibroblasts	5.8	4.1	5.8
Mammalian			
fetal calf kidney	5.5	6.8	6.8
BHK-21/13 (hamster)	6.5	5.8	5.5
Vero (monkey)	6.5	4.5	5.2
HEp-2 (human)	5.8	5.1	5.5
MDCK (canine)	4.1	5.0	5.2
SK (porcine)	4.8	5.2	4.1

[a] $\log_{10}$TCID$_{50}$/0.1 ml.
[b] Not done.

The optimal cell culture system for the replication of 10/3 virus were *FMH* cells. Lysis of the cell sheet was complete between 48 and 72 hours after infection, depending upon the multiplicity input. Reproducible infectivity titers of between $10^{6.5}$ and $10^{7.5}$TCID$_{50}$/0.1 ml were obtained in these cells.

A single step growth curve, carried out in FHM cells showed that the first progeny virus was synthesized 4—6 hours after infection. Both cell-associated and cell-free virus reached a peak titer of $10^{8.3}$TCID$_{50}$ though at different times, *i.e.* at 10 and 22 hours postinfection (Fig. 1).

3.2. Plaque Production

10/3 virus and RVC both formed plaques after incubation in FHM cells under 0.75 per cent methyl cellulose. First cytopathic changes were delayed for about 5—8 hours compared with cultures without overlay. This finding is in agreement with other reports, where methylcellulose is used as overlay (5).

Plaque size increased with time, and reached a diameter of 1 mm after 72 hours of incubation. The plaque numbers decreased exponentially with higher dilutions. Morphology of the plaques was irregular, and the rim was diffuse. There is no difference evident between plaques produced by 10/3 and RVC viruses in size and morphology.

3.3. Serological Comparison

In cross neutralization tests, 10/3 virus was not related to IHN and Egtved virus. There was, however, a close relationship, if not identity with the RVC isolated by FIJAN *et al.* (7) from carp with spring viremia. The results are shown in Table 2.

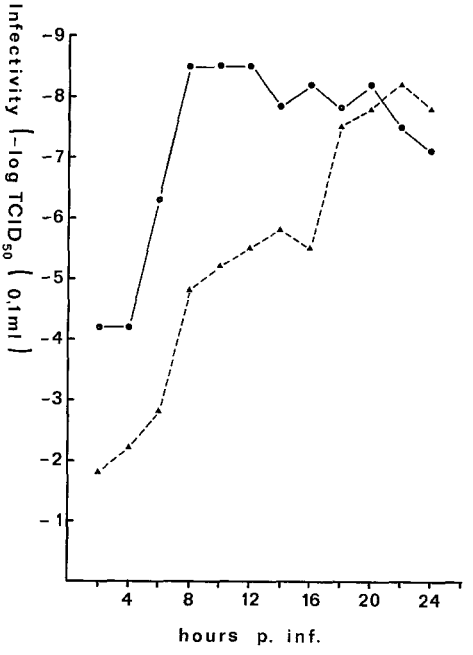

Fig. 1. Single step growth curve of 10/3 virus in FHM cells. A multiplicity input of 5 $TCID_{50}$ per cell was used. Incubation temperature was 20—22° C
●———● cell-bound virus; ▲ – – – ▲ medium phase virus

Table 2. *Cross Neutralization Tests between Salmonid and Carp Rhabdoviruses*

	Virus species			
Hyperimmune sera	10/3	RVC	IHN	Egtved
Anti-RVC	1:8	1:8	neg.	neg.
Anti-10/3	1:8	1:8	neg.	neg.

3.4. Animal Experiments

Carp that were infected with 10/3 virus showed first clinical signs three to four days after infection. They consisted of apathy, reduced reflexes, and in some cases loss of balance. In addition swelling of the anus and abdomen were observed. Some carp had petechiae on the skin and muscles. Animals with clinical signs rapidly died after onset of the disease, and first deaths occurred at the fourth day after infection. The last fishes died in most experiments at the 8th day p.i. None of the infected carp survived. Control animals remained normal during the observation time. 60 carp were used for these experiments.

Macroscopic post mortem changes of carp consisted of a catarrhal enteritis with formation of oedema and extensive petechiation in the swim bladder wall. Petechiae were also seen in the brain and pericard. Always present was ascitic fluid in the abdomen (1—3 ml per animal).

Virus could be reisolated regularly from all organs of infected carp, whereas control animals remained negative. Table 3 shows the virus titers found in various organs titrated in FHM cells.

Table 3. *Virus Titers in Different Organs of Carp after Experimental Infection with 10/3 Virus (organ pools of five carp)*

Organ	Virus titer[a]
Liver	5.8
Kidney	4.2
Spleen	4.5
Heart	4.2
Intestine	4.5
Swim bladder	4.8
Muscle	3.8
Brain	3.5

[a] $\log_{10}$ $TCID_{50}$/0.1 ml organ suspension.

Infected Crucian carp did not show clinical symptoms up to 21 days after infection. There were no gross changes at autopsy, and virus could not be recovered from organ pools of these fishes.

Fifteen guppies kept in virus-containing water died between 7 and 14 days after infection. Infected guppies developed similar clinical symptoms as carp after infection. Hemorrhage on gills and skin could be observed frequently. Virus could be reisolated from the whole infected fish, controls were negative. Virus titers in infected animals were on the average $10^{4.5}$ $TCID_{50}$/0.1 ml organ suspension.

4. Discussion

A rhabdovirus, isolated from carp with symptoms of swim bladder inflammation replicated not only in fish cell cultures but also in cells of mammalian and chicken origin. Highest infectivity titers were obtained with the FHM cell line, where titers of between $10^{6.5}$ and $10^{7.5}$ $TCID_{50}$/0.1 ml were recorded. With cell cultures

from carp gonads and carp swim bladders similar virus yields could be obtained, whereas the use of rainbow trout cell cultures (gonads, swim bladder, heart, RTG-2 cell line) resulted in much lower titers ($10^{3.2}$ to $10^{4.2}$ $TCID_{50}$/0.1 ml).

Surprising was the wide host cell spectrum of 10/3 virus. The agent multiplied with virus titers between $10^{4.0}$ and $10^{6.8}$ $TCID_{50}$/0.1 ml in chick embryo fibroblast cell cultures, in fetal calf kidney cells and in the cell lines BHK-21/13, Vero, HEp-2, MDCK, and SK. Infectivity titers in fetal calf kidney cells were in the same range as those obtained in FHM cells. This host cell spectrum compares well with the wide spectra of other rhabdoviruses (24).

FIJAN (6) reported also the replication of RVC in FHM and trout cell culture with similar results. RVC also seems to grow in a variety of mammalian cells at 20° C (CLARKE, F., personal communication, 1972).

Replication of the 10/3 virus is temperature-dependent (4). The growth cycle in FHM cells at the optimal temperature of 20—22° C lasts only 8—10 hours. After this time infectious virus is released slowly from the cells. RVC has a growth cycle very similar to that presented for 10/3 virus under comparable conditions (DE KINKELEN, P., personal communication, 1973). This optimal replication temperature of 10/3 and RVC viruses clearly differentiates these agents from the salmonid rhadoviruses, IHN and Egtved, and the agent of "red disease" in pike [*Esox lucius* L. 1766 (13)].

Virus 10/3 does not show any differences in plaque morphology when compared with RVC plaques. Plaques of both viruses in FHM cells were indistinguishable from each other in morphology, size and kinetics. They are, however, clearly different from plaques produced by the salmonid Egtved virus (12).

Finally, both viruses cross-neutralized completely. Possible minor antigenic differences could not be detected because of low antibody titers of the hyperimmune sera. 10/3 and RVC virus strains did not show any serological relationship to the salmonid rhabdoviruses, and RVC is antigenetically not related to the pike virus (13).

Swim bladder inflammation could be reproduced with 10/3 virus after inoculation of carp regularly. Infected carp showed typical symptoms when kept at water temperatures between 14° and 17° C. These symptoms partly resembled those described by FIJAN *et al.* (7) after experimental infection of carp with RVC. The only major clinical and pathological difference may be the strong involvement of the swim bladder wall after 10/3 virus infection.

Incubation time was dependent upon the virus dose administered. It is also related to the water temperature in case of RVC (7), similar observations may be expected also for 10/3 virus.

All carp infected with 10/3 virus died and virus could be recovered from the organs tested. Highest virus titers were found in the liver ($10^{5.8}$ $TCID_{50}$/0.1 ml) indicating that the infection results in a disease with systemic character. Crucian carp, a species closely related to carp, did not show any symptoms after infection with 10/3 virus. This finding is consistent with field observations, which claim that the Crucian carp is not susceptible to either swim bladder inflammation *and* spring viremia of carp (REICHENBACH-KLINKE, personal communication, 1973).

Guppies (*Lebistes reticulatus* P.) were highly susceptible to 10/3 virus and died with clinical symptoms and pathological lesions resembling those in carp. These

results suggest using guppies for animal experiments with this virus. They are easy to keep, and, in contrast to carp, available throughout the year. The high susceptibility of guppies further indicates that other species may carry the infection and play a role in the epizootiology of the disease.

Up to now, spring viremia or acute infectious dropsy of carp and the swim bladder inflammation have strictly been considered as two diseases caused by different etiological agents. This opinion has been supported by differences in seasonal incidence. Both disease complexes have saisonal character, spring viremia occurring mainly during early spring, and swim bladder inflammation being prevalent in early summer. During spring, swim bladder inflammation has been observed only in 0—5 per cent of carp with a rising incidence in June and July (17). These differences could, however, be due to different environmental conditions that may result in different pathogenetic pictures. This hypothesis is supported by findings of Avtalion *et al.* (3) that environmental temperatures influence the natural resistance and the immunologic response in infected fish, and complicate the course of a disease.

Our results show that the agents of spring viremia and swim bladder inflammation share many similar, if not identical properties. They strongly suggest a common etiology, and support observations of Lukowicz (18), who reported the simultaneous occurrence of both diseases, and Otte (21), who found similar histological lesions in SVC and aerocystitis.

Whether there are pathogenetic differences or different organ affinities of the two agents has to be shown in further experiments.

With 10/3 virus and RVC only the acute forms of SVC and aerocystitis can experimentally be reproduced. Whether the chronic forms, which are associated with the disease complexes, have a different etiology or are complications due to secondary infections remains to be open.

Note added in proof: A myxobacterium has been associated with chronic forms of the disease in a very recent paper: Bootsma, R.: An outbreak of carp *(Cyprinus carpio L)* erythrodermatitis caused by a myxobacterium. Aquaculture **2**, 317 (1973).

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