INFECTION AND IMMUNITY, Jan. 1983, p. 7-14
0019-9567/83/010007-08$02.00/0
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Vol. 39, No. 1

Viral Hemorrhagic Septicemia of Rainbow Trout: Relation Between the G Polypeptide and Antibody Production in Protection of the Fish After Infection with the F25 Attenuated Variant

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Received 13 April 1982/Accepted 15 September 1982

A nonpathogenic variant of viral hemorrhagic septicemia virus has been selected which immunizes fish against a subsequent challenge with the wild-type virus strain. In this paper, we demonstrate that both the variant and the wild-type virus strains multiplied in spleen and kidney of infected fish, but the virus yield was lower for the variant and soon dropped below the sensitivity of our titration technique, which indicates that an early mechanism prevents the establishment of septicemia. This early mechanism could also be responsible for early protection since fish were already immunized 48 h postinfection with the variant. In a second step antibodies relayed that first defence mechanism to ensure long-lasting immunity. Antisera collected after immunization by the variant or the wild-type virus strain cross-reacted poorly in neutralization tests, which is in agreement with results obtained with plant lectins and proves that the variant is modified in its antigenic properties.

Viral hemorrhagic septicemia (VHS) virus type 1, which belongs to the Lyssa subgroup of rhabdoviruses (14), causes a systemic infection in rainbow trout. The wild-type strain replicates in cell cultures over the range 4 to 21°C, with an optimum at 14°C (6). A variant has been selected that replicates in cell cultures over the range 4 to 29°C, with an optimum at 14 to 25°C (3). It has lost most of its pathogenicity for trout but can be used as a vaccine (2).

In this paper, we demonstrate that the envelope of the variant virus grown at 25°C is deficient in glucose, mannose, fructose, and, to a lesser extent, glucosamine when compared with the wild-type virus strain. This result correlates with a difference in the serotype of the two virus strains. We also demonstrate that the sequence of events after infection of the host fish with the F25 variant is twofold: first, the establishment of a fatal condition is prevented, and second, neutralizing antibodies are synthesized and protect the fish against a subsequent challenge by the wild-type virus strain.

MATERIALS AND METHODS

Viruses and cells. Production of large amounts of VHS virus in epithelioma papulosum cyprini (EPC) cells was described elsewhere (6). The 07.71 VHS virus strain (wild type, serotype 1, pathogenic) was isolated from an infected rainbow trout and then passed four to five times in cell cultures. The F25 passage 21 (attenuated, thermoresistant) variant (F25-21) was obtained through several successive subcultures in EPC cells at progressively increasing temperatures (3).

Immunization, challenge, and virological and serological examination of fish. Young virus-free rainbow trout weighing between 1 and 2.5 g were immunized by immersion in aqueous suspensions of virus titrating at between 5×10^4 and 5×10^5 PFU/ml (4). When indicated, fish were challenged 20 days after immunization by the same method (2). For virological examination, 10 live fish of 120 were sacrificed at various intervals after infection with either the 07.71 or the F25-21 virus strain. The spleen and kidney of each fish were pooled and ground with a mortar and pestle. The suspensions were serially diluted and assayed on EPC cells seeded in Titertek (Flow Laboratories) wells (3×10^5 cells per well) (2). The plates were then maintained at 14°C, and the cytopathic effect was monitored for 7 days.

Seroneutralization tests were performed by using a 50% PFU number reduction technique after a 1-h contact of diluted sera and virus suspensions titrating at 200 PFU/ml in the presence of virus-free trout serum as a source of complement (8).

Purification of viruses. The EPC cells were seeded in either Multitray (Nunc) or Corning flasks (150 cm^2) containing Stoker medium supplemented with 10% tryptose and 10% fetal calf serum and maintained overnight at 25°C (6). Then the medium was removed and the cells were infected at a low multiplicity of infection. After adsorption for 1 h at 14°C, Stoker

TABLE 1. Comparison of plating efficiency and stability[a]

Virus strain	PFU ratio			
	EPC/RTG[b]	Interferon/ untreated[c]	Heated/ untreated[d]	Normal/ untreated[e]
07.71	1.55	0.50	0.57	0.45
F25-22	1.58	0.35	0.86	0.52

[a] After 1 h of adsorption, the cells from the four experiments were maintained at 14°C for 72 h and then stained, and the number of plaques was counted. Three dishes were infected with each inoculum so that the number of plaques counted for each untreated control was about 300.

[b] The original virus suspension contained 200 PFU/0.1 ml as determined by titration on EPC cells.

[c] Trout cells (RTG) in 35-mm petri dishes were treated overnight at 20°C with several dilutions of trout interferon and then infected with 100 PFU of infectious pancreatic necrosis virus.

[d] The virus suspensions titrating at 100 PFU/0.1 ml were maintained for 15 min at 33°C in medium supplemented with 2% fetal calf serum and then were plated on EPC cells.

[e] One volume of virus suspension was mixed with 9 volumes of undiluted noninfected trout serum and maintained for 1 h at 11°C; then the suspension was diluted and plated on EPC cells. The titer of the mock-treated inoculum was 100 PFU/0.1 ml.

medium plus 2% fetal calf serum was added, and the cells were maintained at either 14 or 25°C until extensive cytopathic effect was obtained (72 h at 14°C; 24 h at 25°C). After cell lysis, the culture fluids were harvested and clarified. The virus was concentrated with polyethylene glycol 6000 (1), then layered over a discontinuous two-layer (45 to 15%) sucrose gradient in TNE (0.15 M Tris-hydrochloride [pH 7.6], 0.15 M NaCl, 0.001 M EDTA), and centrifuged for 90 min at 35,000 rpm in an SW40 Beckman rotor. The virus that sedimented on the 45% layer of sucrose was collected and diluted, pelleted by centrifugation, resuspended in TNE, and frozen at −70°C until used.

Polyacrylamide gel electrophoresis. A 1-volume sample was mixed with 1 volume of 2× ESB (0.06 M Tris-hydrochloride [pH 6.7], 1% sodium lauryl sulfate, 10% glycerol, 2% 2-mercaptoethanol, 1 mg of bromophenol blue per ml), boiled for 1.5 min in a water bath, and layered in the wells of a discontinuous sodium dodecyl sulfate-polyacrylamide slab gel (3) prepared as described by Laemmli (13). The electrophoresis buffer was also that described by Laemmli (13).

Agglutination by plant lectins. Given amounts of purified virus were diluted in 0.5 ml of lectin buffer (50 mM Tris-hydrochloride [pH 7.6], 100 mM NaCl, 1 mM $CaCl_2$), and the optical density was measured at a 500-nm wavelength. Then 100 μg of the lectin was added and the variation in optical density was recorded for 10 min at 1-min intervals. Wheat germ agglutinin from *Triticum vulgaris*, pokeweed mitogen from *Phytolacca americana*, garden pea agglutinin type III from *Pisum sativum*, peanut agglutinin from *Arachis hypogea*, concanavalin A (ConA) from *Conavalia ensiformis*, and limulin from *Limulus polyphemus* were all purchased from Sigma Chemical Co.

RESULTS

Stability of the variant virus. As already published (3), the F25 virus strain multiplies in EPC cell cultures but is scarcely pathogenic for rainbow trout. The virus was passed 100 times in rainbow trout gonad (RTG) cells before we received it and then 80 times in EPC cells during selection for thermoresistance. Thus, we verified whether the virus had lost either its ability to grow in trout cells or its stability as a result of subcultures.

Serial multiplication in heterologous cells did not modify the ability to grow in trout cells compared with the pathogenic virus strain since the EPC/RTG PFU ratios were similar for both F25-21 and 07.71 viruses. In both cases, the plating efficiency was higher on EPC than on RTG cells (Table 1), despite the fact that 07.71 is a recent isolate.

The nonpathogenic thermoresistant variant was slightly more thermostable than the virulent thermosensitive virus strain but was not more sensitive to rainbow trout interferon (5, 7) or to a 1-h exposure to undiluted and unheated virus-free trout serum than was the wild-type strain (Table 1).

Comparison of virus multiplication in fish. We also compared the multiplication of the two virus strains in pooled serum and kidney of individual fish infected by immersion in contaminated water. The experiment was repeated three times with similar results. For both strains the number of fish which were positive on virological examination progressively increased during the first week postinfection (Fig. 1), but the individual yields were lower for fish infected with the attentuated F25-21 variant. Whereas fish infected with 07.71 began to die, with signs of septicemia, the others survived, as previously described (2), and the number of infectious particles recoverable from spleen and kidney dropped below the sensitivity of our technique (Fig. 1).

In other experiments, fish which had been infected with the nonpathogenic variant were challenged either 24 h (Table 2) or 20 days (Fig. 2) later with the 07.71 virus strain. In both cases the fish were protected against that challenge; late protection was not just due to aging of the fish, since most controls from the same batch were still sensitive (Fig. 2).

Moreover, in another experiment, 12 similarly F25-21-immunized fish which remained unchallenged until day 60 postimmunization were serologically checked for neutralizing activity against the F25-21 virus strain; 4 of them exhibited an average neutralizing titer of 100.

Comparison of antisera. Fish (mean weight, 150 g) were injected intraperitoneally with 10^8 PFU of one of the two viruses; then the survi-

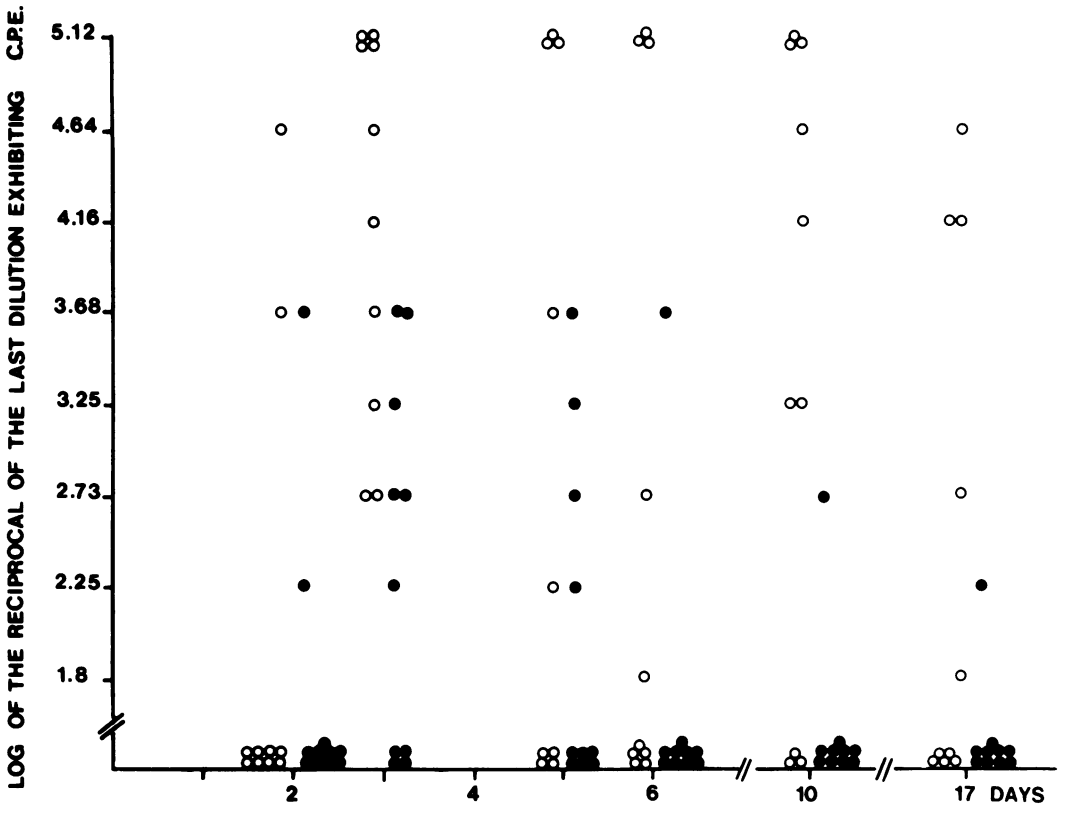

FIG. 1. Comparison of virus multiplication in fish. Young trout were infected with virus strain 07.71 (○) or F25-21 (●). At given intervals postinfection, 10 fish were sacrificed for virological examination. CPE, Cytopathic effect.

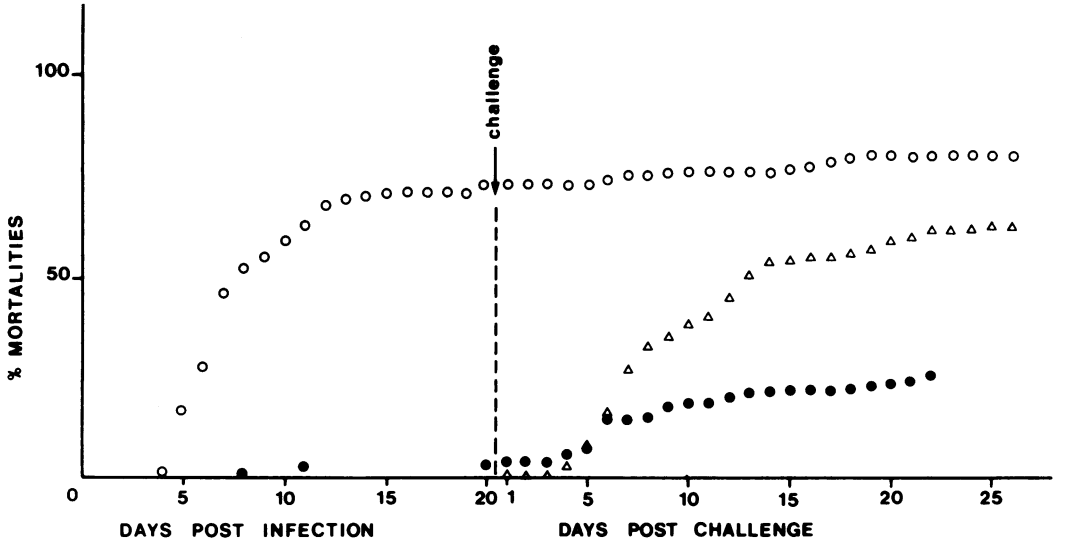

FIG. 2. Evolution of mortalities after infection and challenge. (Left) Young trout were infected with virus strains 07.71 (○) or F25-21 (●), and the cumulative mortalities were recorded daily. (Right) Twenty days postinfection, trout which had survived infection by F25-21 were challenged with virus strain 07.71 (●); aging controls from the same stock were also infected with the same virus suspension (△). Cumulative mortalities were again recorded daily.

TABLE 2. Early challenge on vaccinated fish

Infection with:	Challenge strain	No. surviving (n = 100)
F25-21	07.71	59
Control	07.71	17

vors were submitted to a second injection on day 150 and bled on day 180. With both virus strains neutralizing antibodies were present in detectable amounts in the serum of the survivors, but they cross-reacted poorly (Table 3), which means that at least some of the antigens were different. A parallel result was observed when fish were injected with 10 μl (1/30 of their weight) of either of the antisera and then immediately infected by the balneation route with the wild-type virus strain (Fig. 3) since we obtained 100% seroprotection with the anti-07.71 and 88% with the anti-F25-21 sera.

Agglutination by plant lectins. In previous experiments (3) we observed that no G polypeptide could be evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis when the F25 variant submitted to 22 passages at 25°C (F25-22) was labeled with [^{35}S]methionine and the Coomassie blue-stained gels were autoradiographed. However, such labeling and staining may not be sensitive enough in the case of the F25 variant since a band comigrating with the wild-type G polypeptide can be observed when the virus is labeled in vitro with [^{14}C]formaldehyde (not shown) or the gel is stained with $AgNO_3$ (Fig. 4). Since glycosylated polypeptides from viral envelopes have been shown to act as the antigen in neutralizing antibody synthesis (18) and the two virus strains cross-reacted poorly, it was necessary to verify whether a difference in glycosyl residues was correlated with a difference in serotype.

A variety of plant lectins, known to bind with glycosyl residues (9, 15), were used to study to what extent the variant was modified compared with the wild-type strain. Since absorption at a 500-nm wavelength is dependent upon light scat-

TABLE 3. Titers of anti-serum which neutralized 50% of PFU[a]

Serum	Titer with given virus strain	
	07.71	F25-21
Anti-07.71	6,400	800
Anti-F25-21	300	3,200

[a] Antiserum was serially diluted, mixed with one of the two virus strains, and then maintained for 1 h at 14°C. After treatment, the suspensions were diluted and plated on EPC cells, which were maintained at 14°C for 72 h. Numbers indicate the dilution of antiserum which inhibited formation of 50% of the plaques.

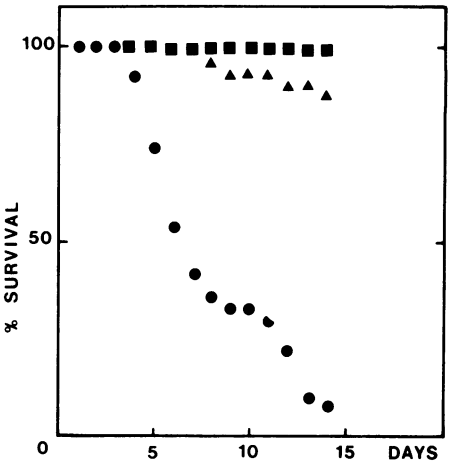

FIG. 3. Seroprotection. Young trout (mean weight, 0.3 g) were injected intraperitoneally with 10 μl of anti-07.71 (■) or anti-F25 (▲) sera and then immediately infected via the water route with the 07.71 virus. (●) Untreated infected controls.

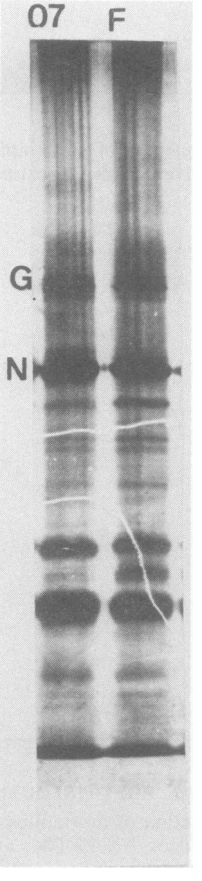

FIG. 4. Polyacrylamide gel stained with $AgNO_3$ (see text).

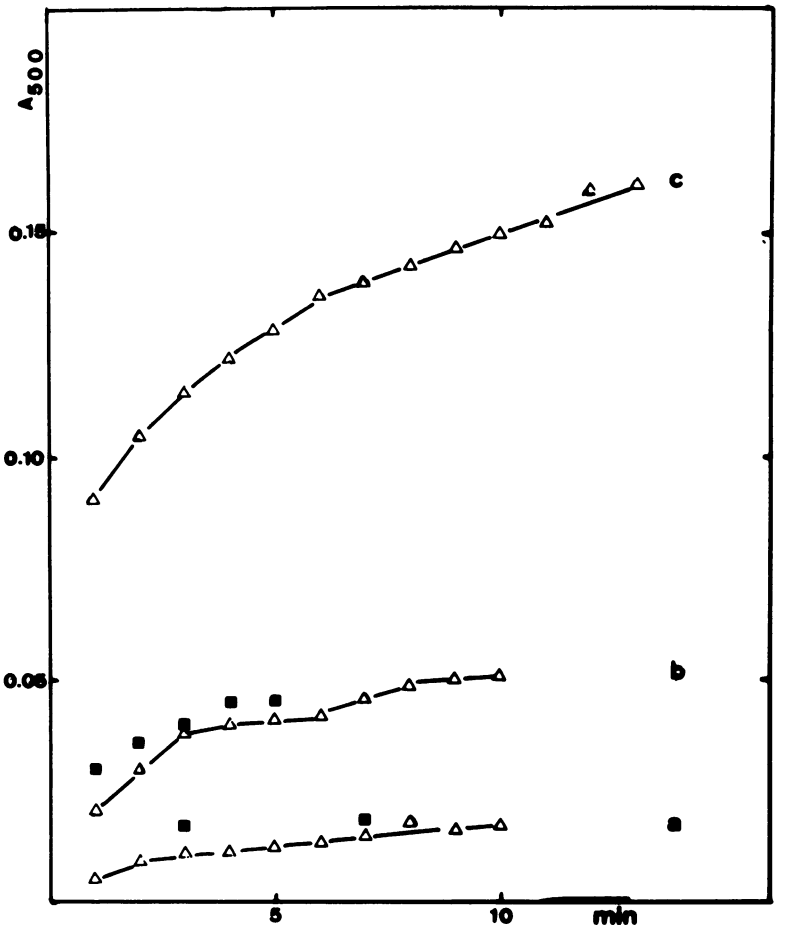

FIG. 5. Comparison of agglutination by ConA. A 100-μg portion of ConA was added to 0.5 ml of virus suspension, and the adsorption at 500 nm (A_{500}) was recorded at 1-min intervals. The value before addition of the lectin was subtracted. Strain 07.71 (△) was grown at 14°C and strain F25-21 (■) was grown at 25°C. (a) 48 μg of 07.71, 90 μg of F25-21; (b) 60 μg of 07.71, 150 μg of F25-21; (c) 120 μg of 07.71.

tering by the particles and thus upon their size, this method was chosen to measure aggregation of the virus.

Peanut agglutinin and limulin, which have binding sites for galactose and D-glucuronic acid, respectively (15), and pokeweed mitogen did not cause appreciable aggregation of either the 07.71 or the F25 virus (data not shown). ConA (fructose and mannose), wheat germ agglutinin (*N*-acetyl-glucosamine), and garden pea agglutinin (glucose and mannose) all agglutinated 07.71 with similar efficacy (Fig. 3 and 4). The size of the aggregates increased as a function of time until a plateau was reached, and by 10 min the turbidity was proportional to the virus protein content of the suspension (Fig. 5).

The F25-22 variant was only weakly agglutinated by ConA (Fig. 5) and garden pea agglutinin (Fig. 6) since the protein content had to be raised to 150 μg of variant virus to obtain the same result that we had observed with only 60 μg of the wild-type virus. Agglutination by wheat germ agglutinin was somewhat greater (Fig. 6). Such residual agglutination could be explained by cellular contaminants since unglycosylated rhabdoviruses are difficult to purify (10–12).

ConA agglutination of F25-22 was more easily observed when the protein content was raised to between 0.96 and 1.97 mg/ml (Fig. 7), which may indicate either the presence of very few glycosylated particles which could otherwise escape detection or that glycosylated material of cellular origin contaminated the virus as described for rabies virus (16). We did not observe any significant difference in ConA agglutination of F25 grown at 25°C or submitted to one passage at 4°C (Fig. 7).

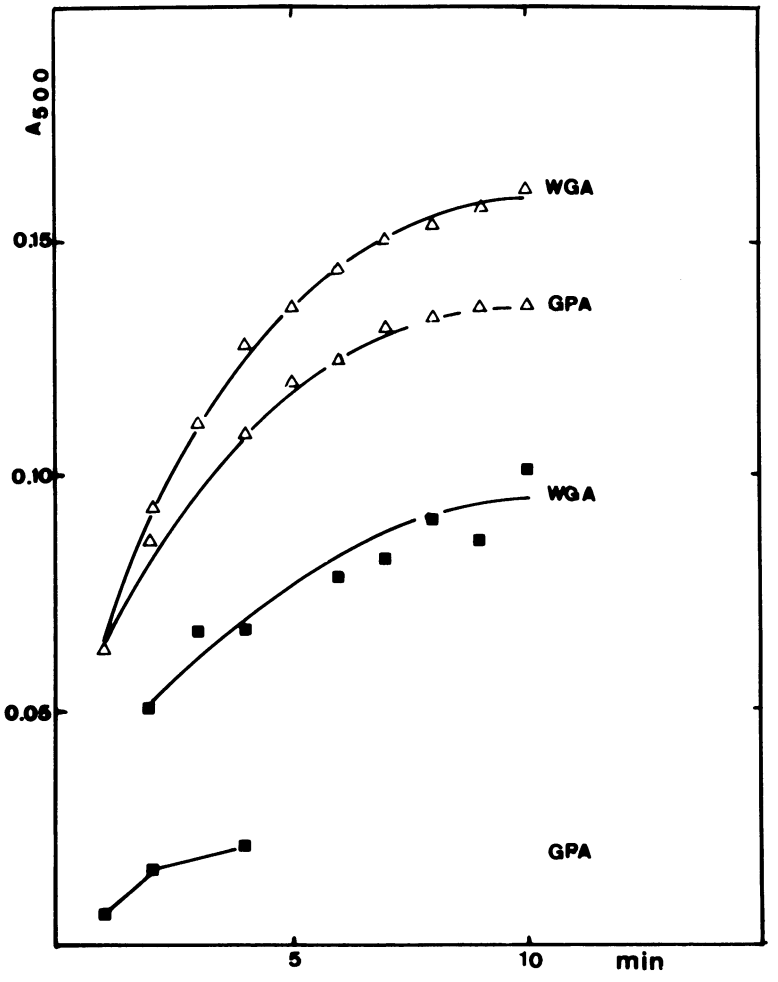

FIG. 6. Comparison of agglutination by wheat germ agglutinin (WGA) and garden pea agglutinin (GPA). A 100-μg portion of either lectin was added to 0.5 ml of virus suspension containing 120 μg of protein for 07.71 (△) or 150 μg of protein for F25-21 (■). Strain 07.71 was grown at 14°C and strain F25-21 was grown at 25°C. A_{500}, Absorbance at 500 nm.

DISCUSSION

A variant thermoresistant VHS virus has been selected (3) which can be used as a vaccine (2) since it is attenuated but still protects the host against a subsequent challenge by the wild-type virus strain (Fig. 2). Such a variant raises several questions about the mechanisms of attenuation of the virus and protection of the fish.

Variant F25 virus stocks grown at 25°C are deficient in glycosylated polypeptide (3; Fig. 5, 6, and 7). They are nevertheless efficient in primary infection since the virus can be recovered from spleen and kidney after submersion of the fish in contaminated water (Fig. 1) and can multiply in cell cultures (3). This observation raises a question about the role of glycosylated residues in infectivity. Glycosylation-deficient particles have been reported for other rhabdoviruses, and it seems that their infectivity depends upon the procedure by which they were obtained. Since noninfectious, spikeless particles were usually produced by enzymatic digestion with either proteases (12) or neuraminidase (17), it is not known whether constituents other than the glycosylated residues were modified. In contast, cells treated with tunicamycin, an antibiotic which prevents glycosylation of the nascent polypeptides, yield nonglycosylated vesicular stomatitis virus particles which are infectious (10). Since the F25 VHS variant grown at 25°C is also infectious, normal glycosylation may not be mandatory for infectivity.

In early days after primary infection of fish with either the F25 or 07.71 virus strain, the virus yield obtained from spleen and kidney was

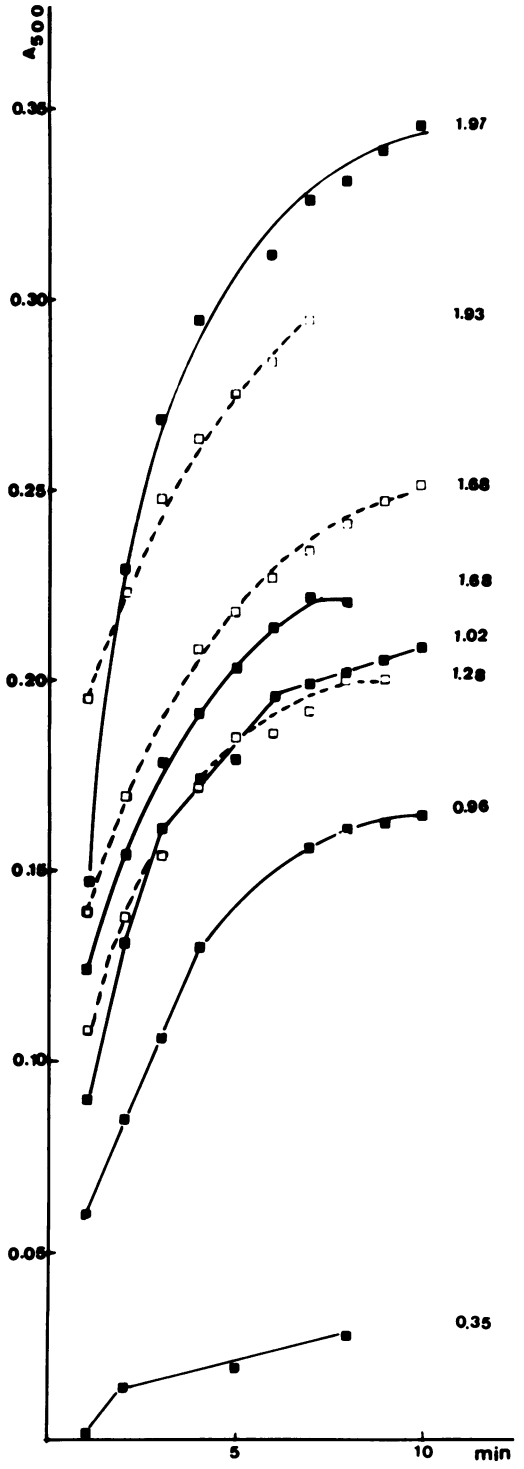

FIG. 7. Comparison of agglutination by ConA of F25-21 virus. A 100-μg portion of ConA was added to 0.5 ml of a suspension of virus grown either at 25°C (■) or for one passage at 14°C (□). The protein concentrations given at the right are in milligrams per milliliter. A_{500}, Absorbance at 500 nm.

always lower for the attenuated variant than for the wild-type virus strain (Fig. 1). On the other hand, the number of detectable F25 infectious particles dropped below the sensitivity of our diagnosis technique by 6 days postinfection (Fig. 1) and the fish did not die. Since stability of the variant toward external factors is not decreased when compared with that of the wild-type virus strain (Table 1), we have to imagine another limiting mechanism. If normal glycosylation is not necessary for infection of the primary target cells, it is very unlikely that its absence would prevent the establishment of septicemia. Thus, a second question is whether the lack of pathogenicity of the variant is due to an increased sensitivity toward a primary defense mechanism from the host fish or whether that variant is a better inducer of that primary defense mechanism than is the wild-type virus strain.

Fish vaccinated with the attenuated variant are protected against a subsequent challenge by the wild-type virus strain as early as 24 h after the primary infection (Table 2). The involvement of antibodies or of any specific immune mechanism is very unlikely after such a short period. Thus, two hypotheses can be considered: either secondary infection is prevented by interferon production, or the number of primary target cells is the limiting factor and, once they are infected, superinfection is impossible.

The question about late protection of the host fish is solved more easily since neutralizing antibodies were evident in the serum of fish after two injections of the F25 variant (Table 3) and seroprotection was efficient (Fig. 3). It is well known that in viral septicemia low titers of circulating antibodies are sufficient to protect the host against secondary infection, so that low levels of cross-neutralization are sufficient. Our antibody titration technique is limited by the toxicity of trout serum for cells in cultures, which explains why the fish had to be submitted to two injections in that experiment whereas one is sufficient for protection.

It should be noted that in seroneutralization experiments each virus cross-reacted poorly with the heterologous antiserum (Table 3). Such a result is in accordance with the difference we observed (Fig. 5 and 6) in glycosylation of the two virus strains.

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