Vol. 10: 23–29, 1991 | DISEASES OF AQUATIC ORGANISMS Dis. aquat. Org. | Published March 8

Spectrophotometric method for titration of trout interferon, and its application to rainbow trout fry experimentally infected with viral haemorrhagic septicaemia virus

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ABSTRACT: The activity of a reference preparation of rainbow trout circulating interferon (IFN), induced by experimental infection of fish with viral haemorrhagic septicaemia virus, was detected on the basis of its non-specific protective effect for RTG-2 cells, grown in microplates and challenged with infectious pancreatic necrosis virus. The IFN was titrated by spectrophotometric assessment of the absorbancy (wavelength 595 nm) of dried cell monolayers, stained with crystal violet after virus challenge. By definition, the reciprocal of the IFN sample dilution giving a cell layer with 50 % of the dye absorbancy of the uninfected control cell layer represented the IFN titre. This spectrophotometric method of determining IFN titre appeared as sensitive as the plaque assay normally used for this purpose, but was better suited to the processing of large numbers of IFN samples, required by investigations on the pathogenesis of fish viroses. The method proved effective at detecting IFN in homogenates of whole rainbow trout fry, thus allowing individual screening for IFN of small fish which are the usual targets of systemic viroses.

INTRODUCTION

Synthesis of circulating interferon (IFN) in rainbow trout *Oncorhynchus mykiss* has been demonstrated following experimental infection with several viruses: Viral Haemorrhagic Septicaemia Virus (VHSV) (de Kinkelin & Dorson 1973), Infectious Haematopoietic Necrosis Virus (IHNV) (de Kinkelin & Le Berre 1974), and serotype Sp of Infectious Pancreatic Necrosis Virus (IPNV) (Dorson & Torchy unpubl.). Circulating IFN was also found during the natural course of VHS in farmed trout (de Kinkelin unpubl.)

Water temperature is critical in determining whether the above viruses will cause disease. Under natural conditions, they do not cause overt diseases above 14 °C even though the in vitro growth of the responsible viruses is optimal in the range 14 to 16 °C. This temperature dependence of the clinical infection suggests that natural immunity plays an important role in the pathogenesis of these viroses. The ability of the fish to produce IFN early in the course of infection may be involved in this immunity because it was demonstrated that young rainbow trout were protected against VHSV infection by injected IFN, the IFN being administered intraperitoneally at the time of viral challenge (de Kinkelin et al. 1982).

Investigating the role of IFN in the pathogenesis of fish viroses requires numerous titrations of individual samples of blood or organs. The plaque reduction assay previously used (de Kinkelin & Dorson 1973) is poorly suited to the processing of large numbers of samples. Therefore, we have developed an IFN microtitration technique using a cell culture system. The technique measures the degree to which IFN protects tissue culture cells against a viral challenge and is based on the differences in the absorbancies of infected cell monolayers treated with IFN and uninfected cell monolayers (controls), stained after virus challenge. Such a spectrophotometric technique has previously been used to assess the effect of cytotoxic factors (Flick & Gifford 1984).

Once the technique was perfected, it was applied to IFN detection in homogenates of whole rainbow trout fry during the course of experimental VHS. The

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homogenate approach is particularly useful because it can be applied to young fish that are too small to be individually blood-sampled and that are the main targets of septicaemic clinical viroses.

This paper describes the IFN microtitration technique and its use in IFN detection in young trout.

MATERIALS AND METHODS

The general principle of IFN titration lies in quantification of a nonspecific anti-viral protection of a virus-susceptible cell culture. Throughout our work, IFN synthesis was induced by VHSV, and cell protection was assayed using a rainbow cell culture challenged with IPNV.

Reference IFN. A pool of frozen (−60 °C) serum, prepared in 1975 from subadult rainbow trout injected intraperitoneally with 5×10^7 plaque forming units (pfu) of VHSV and dispensed in 0.2 ml aliquots, was used to develop the microtitration technique. This pool of serum, referred to as reference IFN, contained 1350 IFN units ml^{-1} (U ml^{-1}), as determined by the plaque reduction assay just prior to the start of the experiment.

Fish cells and virus strains. The rainbow trout gonad cell line (RTG-2) (Wolf & Quimby 1962) was grown at 20 °C in Stoker's medium (Stoker & MacPherson 1961), buffered with 0.16 M Tris HCl at pH 7.4, and supplemented with 10% tryptose phosphate, 10% fetal calf serum (FCS), and antibiotics (penicillin 100 international units (IU) ml^{-1} and dihydrostreptomycin 100 µg ml^{-1}). *Epithelioma papulosum cyprini* cell line (EPC) (Fijan et al. 1983) was grown in the same medium but at 30 °C. For virus production, the FCS content of the medium was reduced to 2% and the incubation temperature to 15 °C.

VHSV strain 07-71, belonging to serotype 1 (Vestergaard-Jørgensen 1972), an IHNV strain (courtesy of D. Amend), and a RTG-2 cell-adapted SVCV strain (courtesy of A-M. Hattenberger-Baudouy) were grown in EPC cells as previously described (Fijan et al. 1983). IPNV strain VR 299 (Wolf et al. 1960) was grown in RTG-2 cells.

The 3 virus strains were titrated by plaque assay and stored at −60 °C (VHSV and IHNV) or in 50% glycerol at −20 °C (IPNV).

IFN microtitration technique. ***Cell protection tests:*** Using a manual distributor (Titertek Multichannel), 2-fold dilutions of the reference IFN preparation were made in Stoker's medium containing 2% FCS (from 1:10 to 1:640), and placed in the wells of a 96-well microplate (Nunc) (0.1 ml $well^{-1}$, 4 wells $dilution^{-1}$). In addition, 4 wells representing the uninfected controls and 4 wells representing infected controls received 0.1 ml Stoker's medium per well but no IFN. For titrating IFN in fish homogenates, the first dilution was 1:8. Then, 95000 RTG-2 cells in 0.1 ml Stoker's medium with 2% FCS were seeded into each well and allowed to adhere to the plastic during their IFN treatment. The treatment and attachment phase was permitted to continue for 18 h at 20 °C in a moist chamber (plastic box containing damp paper napkins).

To challenge the attached cells, the medium was first removed from the cell culture wells by turning over the microplate and shaking it vigorously. The edges of the plate were then dried with a sterile paper napkin and IPNV was added to each of the wells (4000 pfu $well^{-1}$ in 0.2 ml Stoker's medium containing 2% FCS), except for the 4 wells corresponding to the 100% protection controls. Incubation was carried out at 20 °C until advanced CPE was evident in the infected, unprotected controls (usually within 2 d). The medium was then decanted into a vessel containing 0.1% chlorine solution, and the cell monolayers were fixed and stained for 10 min with a solution containing 1% crystal violet in 20% ethanol. Following this, the cell monolayers were rinsed with tap water and allowed to dry at room temperature.

Determination of the IFN concentration: The degree of cell protection was assessed by measuring the absorbancy of the stained cell monolayers at a wavelength of 595 nm. The measurements were made using the Titertek Multiskan spectrophotometer normally used for reading enzyme-linked immunosorbent assay (ELISA) tests (Flick & Gifford 1984). By definition, the IFN sample dilution resulting in a monolayer with an absorbancy equal to 50% of that of the uninfected cell monolayers provided a 50% protection of the cells and thus contained one IFN unit (U) in our system.

The optical density (OD) 50% was given by the formula:

$$\frac{\text{OD maximum} + \text{OD minimum}}{2}$$

where OD maximum = absorbancy of uninfected cell monolayers treated or untreated with IFN (protection 100%); and OD minimum = absorbancy of the infected non-protected cell monolayers (protection zero).

When the value computed for OD 50% fell between 2 other absorbancy values, n and n + 1, as it generally did, the IFN titre was computed from the formula:

$$\text{IFN titre (U ml}^{-1}) = T_n + \left[(T_{n+1} - T_n) \times \frac{(OD_n - OD\,50\,\%)}{(OD_n - OD_{n+1})}\right]$$

where T_n = reciprocal of the IFN dilution corresponding to OD immediately higher than OD 50%; and T_{n+1} = reciprocal of the IFN dilution corresponding to OD immediately lower than the OD 50%. Because the titrations were made on volumes of 100 µl, the titre values given in the formula were multiplied by 10 so

that IFN concentration could be expressed in terms of IFN U ml^{-1}.

Rainbow trout fry infection schedule and fry sample processing. Young virus-free rainbow trout, 1800 degree-days old and weighing 2 to 3 g, were divided into 4 groups (96 fish per group) and held in 10 l flow-through aquaria. After 1 wk of acclimation at 6 ± 1 °C, 3 of the groups (1 to 3) were infected by immersion for 2 h in an aqueous suspension of VHSV, strain 07-71, containing 7×10^4 pfu ml^{-1}. Fish in Group 4 were mock-infected with the supernatant of a freeze-disrupted EPC cell culture.

Samples for IFN titration were collected from Groups 1 and 2 on Days 1 to 10 (5 fish d^{-1}), 12 and 14 (3 fish d^{-1}), and 21 and 33 (4 fish d^{-1}). Two groups were used for sampling because it was felt that mortalities due to VHS might decrease the number of fish available for sampling. Group 3 was used to determine the mortality rate resulting from the challenge and Group 4 to assess natural background mortality and to provide negative control samples on Days 10, 14, and 33.

The sampled fish were individually processed as follows: the head and caudal peduncle were removed and the trunk, diluted 1:4 in Stoker's medium containing 2 % FCS, was homogenised using an electric blender (Turrax) at maximum speed for 30 s. The resulting homogenate was centrifuged at 3500 × *g* for 15 min at 4 °C, and the supernatant was collected, heated at 45 °C for 30 min to inactivate the virus, and then stored at −20 °C until titrated for IFN. Prior to heating, aliquots from some samples were pooled and checked for the presence of virus by the plaque assay.

Characterisation of IFN activity in rainbow trout fry homogenates. Homogenates found to contain at least 50 IFN U ml^{-1} were pooled, sedimented at 100 000 × *g* for 4 h at 4 °C in an ultracentrifuge Beckman TL100, and processed as described previously (de Kinkelin & Dorson 1973).

Briefly, the range of antiviral protection conferred on the trout cells by IFN was established by challenging the cells after IFN treatment with the following viruses (4000 pfu $well^{-1}$): IPNV, SVCV, IHNV, and VHSV, the VHSV being the IFN inducer in the fish.

IFN activity in the pooled homogenate was also checked after treatment of the homogenate with RNAse, trypsin, or low pH. Enzymatic treatments of aliquots of the pooled homogenate were performed at 37 °C using either RNAse (Boehringer) (40 µg ml^{-1} for 30 min) or trypsin (Sigma) (0.4 mg ml^{-1} for 60 min). Trypsin digestion was stopped by addition of soybean trypsin inhibitor (0.4 mg ml^{-1}). Resistance of IFN activity to acidity was tested by adding 35 µl of 1 M HCl to 1.5 ml of sample to obtain a pH value of 2. Following incubation for 24 h at 20 °C, the acidity was neutralised with 1 M NaOH (35 µl ml^{-1}).

RESULTS

Determination of IFN concentration in reference preparation

Fig. 1 illustrates how IFN titres were determined using the spectrophotometric method. The stained cell monolayers (Fig. 1a) gave absorbancy values shown in Fig. 1b. Their arithmetic means were 1.613 and 0.235 for the the OD maximum and OD minimum, respectively. Thus OD 50 % was:

$$\frac{1.613 + 0.235}{2} = 0.924$$

This value fell between the mean OD values of the IFN dilutions used in the wells of columns 9 and 10 (OD values of 1.159 and 0.641, respectively).

The means of the absorbancy values of each series of 4 wells from 1 to 12 (1 and 2 not shown), when plotted against the logarithm of the reciprocal of the IFN dilutions (titres), gave an S-shaped curve with an intermediate linear portion. The positioning of OD 50 % on this linear portion of the curve indicated that the IFN concentration was ca 2200 to 2300 U ml^{-1}.

Alternatively, the IFN concentration could be computed from the previously given formula in which the relationship between titre and OD was as follows:

$$\text{IFN (U ml}^{-1}) =$$

$$1600 + \left[(3200 - 1600) \times \frac{(1.159 - 0.924)}{(1.159 - 0.641)}\right] = 2326$$

Detection of IFN synthesis in rainbow trout fry infected with VHSV

IFN synthesis in rainbow trout fry undergoing experimental VHS at 6 °C is shown in Table 1. IFN activity was detected on Day 10 post-infection; it peaked between Days 14 and 21 and was still present on Day 33. IFN titres varied from fish to fish, as previously reported by de Kinkelin & Dorson (1973). The onset and course of mortality (and clinical signs of VHS) paralleled the appearance of IFN in the homogenates of the infected fry. By 8 wk post-infection, 72 % mortality had occurred in the infected control group (Fig. 2). Similarly, virus testing of the fish revealed that IFN synthesis coincided with virus multiplication. The mean VHSV titres (pfu ml^{-1}) in pooled homogenate supernatants from infected fish were 0, 8×10^1, 10^4, 10^6, and 5×10^5 at Days 1, 7, 14, 21, and 33 post-infection, respectively. No IFN was detected in the fry from the uninfected group at the lowest dilution (1:8) of homogenate tested.

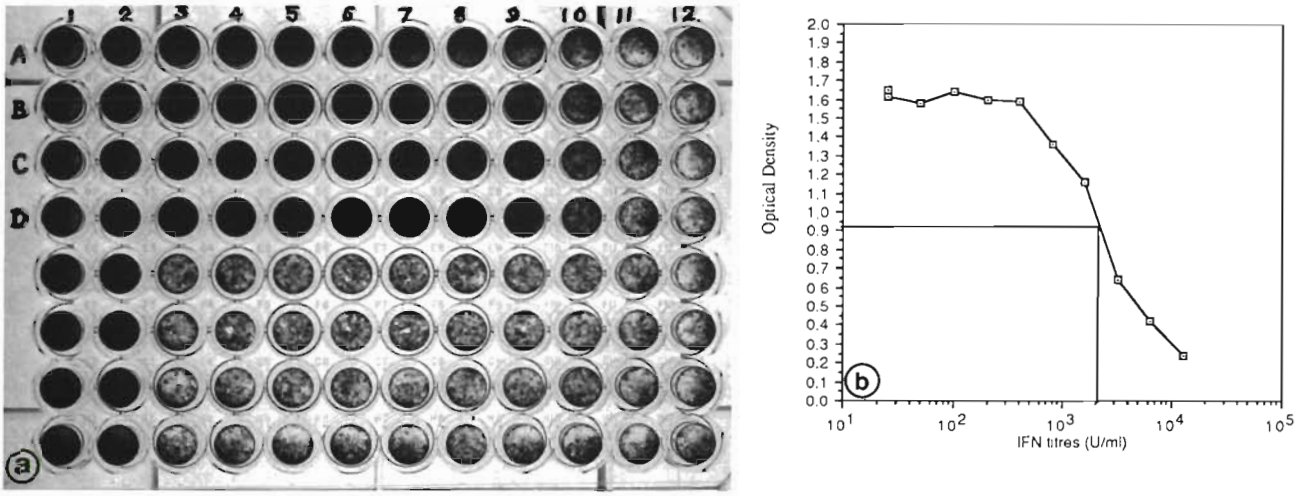

Fig. 1. Spectrophotometric titration of IFN. (a) The microtiterplate. After treatment for 18 h with different dilutions of IFN, the RTG-2 cells were challenged with IPNV strain VR 299 (4000 pfu well^{-1}), incubated at 20 °C, and stained 48 h later with a solution containing 1 % crystal violet in 20 % ethanol. The absorbancy of the stained cell monolayers was then read with an ELISA reader (Titertek Multiskan). Rows A to D only are considered here (rows E to H correspond to a serum sample lacking IFN activity and to its uninfected controls). Rows A to D: columns 1 and 2, uninfected cells; columns 3 and 4, uninfected cells treated by IFN dilutions 1:10 and 1:20, respectively (columns 1 to 4 represent protection 100 %). Columns 5 to 11 represent cells treated with serial 2-fold dilutions of the IFN samples, ranging from 1:10 to 1:640; column 12 represents untreated infected cells (protection 0 %). (b) Graphical determination of the IFN concentration. The spectrophotometric reading of absorbancy values of cells monolayers from rows A to D and the arithmetic means of these values were:

	1	2	3	4	5	6	7	8	9	10	11	12
	1.518	1.530	1.329	1.339	1.372	1.307	1.228	1.021	0.689	0.504	0.235	0.269
	1.408	1.571	1.699	1.629	1.673	1.570	1.693	1.426	1.364	0.677	0.320	0.244
	1.615	1.781	1.762	1.693	1.779	1.775	1.735	1.359	1.166	0.676	0.456	0.188
	1.716	1.727	1.679	1.656	1.749	1.730	1.700	1.621	1.420	0.710	0.401	0.240
OD	1.564	1.652	1.617	1.579	1.643	1.595	1.589	1.356	1.159	0.641	0.373	0.235

(Standard deviation values are not given because the extent of the variability is indicated.) The above absorbancy values (except 1 and 2 which were not needed for the figure) were plotted against the logarithms of the reciprocal (titres) of the IFN dilutions. Along the X axis, titres were multiplied by 10 so that IFN concentrations could be expressed in terms of IFN U ml^{-1}. OD 50 % and the corresponding IFN titre were obtained as described in the text

Characterisation tests on IFN in trout fry homogenates

The tests were run in parallel with the reference IFN preparation (serum IFN) that had been previously titrated for its IFN activity. Results are shown in Table 2. They indicate that the protective activity was non-sedimentable, independent of the challenge virus, trypsin-labile, RNAse-resistant, and stable at pH2. In addition, the protective activity was partially specific to the cell and it also resisted heating at 45 °C for 30 min (data not shown).

DISCUSSION

The anti-virus cell protection conferred by IFN on RTG-2 cells could be readily estimated from the degree of absorbancy of the stained monolayer of susceptible cells, the absorbancy being maximum for the fully protected virus-challenged cells or for uninfected cells and minimum for infected unprotected cells. The trout IFN was titrated by monitoring the OD (at wavelength

Table 1. *Oncorhynchus mykiss*. Kinetics of IFN production (U ml^{-1}) in rainbow trout fry following VHSV infection by the water route at 6 °C. (Assay performed on homogenized trout tissues)

	Sampling days[a] (post-infection)					
	0 to 9	10	12	14	21	33
IFN titres (U ml^{-1}) of sampled fry[b]	<8	22	56	1380	175	70
	<8	<8	56	260	260	60
	<8	<8	<8	<8	95	<8
	<8	<8			<8	<8
	<8	<8				

[a] Daily samplings were: from Days 0 to 10, 5 fish; on Days 12 and 14, 3 fish; and on Days 21 and 33, 4 fish
[b] IFN titres are in units per ml of fry homogenate

595 nm) given by crystal violet-stained infected monolayers, grown in microtiter plates. This method not only enables the detection of the IFN in salmonid blood but can also be used to measure IFN in homogenates of whole fry, which, because of their small size, are poor sources of blood.

A dye other than crystal violet, neutral red, was used in a spectrophotometric technique of fish cell IFN titration (de Sena & Rio 1975). Because it required additional handling to release the neutral red, we preferred to read the absorbancy of dry, stained cells.

Perfecting the technique

Our IFN titration technique utilized 95 000 RTG-2 cells well^{-1}, seeded as suspended cells into wells containing serial dilutions of IFN. They were allowed to react with IFN for 18 h at 20 °C and were then challenged with 4000 pfu of IPNV strain VR 299 for 48 h. Following this, they were fixed, stained with 1 % crystal violet and dried and read for absorbancy using an ELISA spectrophotometer.

The preceding conditions were the result of considerable preliminary work, the goals of which were to obtain, with in the shortest possible time and in a reproducible way, a large interval of OD values between the full protection point and that of zero protection, thus allowing a reasonably accurate determination of OD 50 %.

Cell density (95 000 cells well^{-1}) was found to provide suitable homogeneity of the monolayer. Higher cell densities led to cell clusters and patchy staining while lower numbers of cells resulted in unsatisfactorily low OD values (OD 0.9 to 1).

The state of the cells at the beginning of IFN treatment (cell suspension or preformed cell monolayer) did not significantly affect the titration results. Therefore, the cells were dispensed directly into IFN dilutions and treated for 16 to 18 h at 20 °C as per our plaque assay of IFN (de Kinkelin & Dorson 1973). This procedure allowed fair monolayer formation and shortened the time required for the titration.

The virus input inducing a consistent CPE within 48 h at 20 °C was 4000 pfu well^{-1} of the IPNV strain VR 299. This laboratory-adapted strain is stable at −20 °C in 50 % glycerol, and is thus very convenient for challenge. Incubation at 15 °C could also be used but the incubation time had then to be extended to 72 h.

OD monitoring of the dry, stained cell monolayers was chosen because monitoring performed on the eluted dye did not improve the sensitivity of the method. In fact, the dye dilution approach often hindered the OD determination because the OD values obtained with uninfected controls were frequently too high (> 2.0). Shorter staining times or higher dilutions of crystal violet could have overcome this difficulty but dye elution was not studied further as it involved an additional unnecessary step.

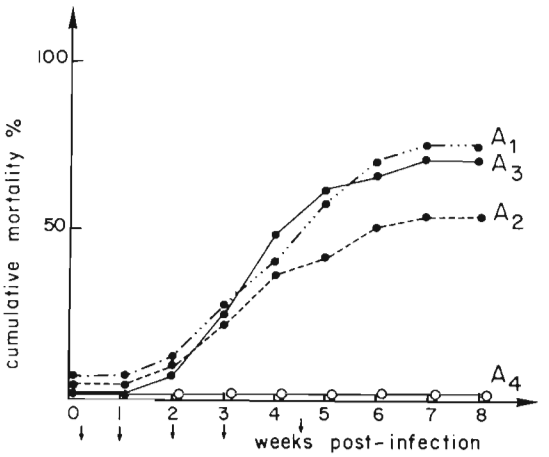

Fig. 2. *Oncorhynchus mykiss*. Experimental VHS in rainbow trout and resulting course of IFN synthesis. Three groups (A1 to A3; 96 fish group^{-1}) of rainbow trout (2 to 3 g, 1800 degree-days) were infected by immersion for 2 h in an aqueous suspension of VHSV containing 7×10^4 pfu ml^{-1} A fourth group (A4) was mock-infected with supernatant of freeze-disrupted EPC cells. Groups A1 and A2 were sampled for IFN as indicated in Table 1 and mortality in these groups was corrected for our IFN sampling. Group A3 shows the mortality rate undergone in the group of fish which was not sampled for IFN. Group A4 indicates the background (natural) mortality. Arrows indicate the days on which fish homogenates were checked for virus

Table 2. Results of IFN characterisation using the titration microtechnique

Characterisation test	IFN titre (U ml^{-1}) in: Reference serum sample	Trout fry homogenate
Sedimentation		
100 000 × *g*/4 h	4000	350
None	4000	370
Range of anti-virus protection[a]		
IPNV	4600	350
VHSV	3600	330
IHNV	4000	360
SVCV	3000	350
Enzymatic treatments		
Trypsin	300	6[b]
Untreated control	3000	110[b]
Ribonuclease A	3000	110[b]
Untreated control	3000	130[b]
Acidity		
pH 2 (24 h at 4 °C)	3100	360
Untreated control	3200	340

[a] 4000 pfu well^{-1}
[b] Diluted 1:3 before treatment

Cell absorbancy rating

Four absorbancy values were measured for each sample dilution (Fig. 1a). When the arithmetic means of the OD values were plotted against the corresponding log dilution values of IFN or their reciprocal, a sigmoid curve was produced which could be used to estimate the IFN titre graphically (Fig. 1b). As evident from Figs. 1a and b, however, low OD values, due to a defect of the monolayer homogeneity, were recorded for the contents of wells 3 to 9 in row A. However, it was observed throughout numerous titration assays that the aberrant OD values could be dismissed. In fact, it appeared from the example given in Fig. 1b that errors of this type in estimates of IFN titre (2326 computed with 4 values versus 2529 based on 3 values) could be overlooked as they were well within acceptable limits for interferon assays (Finter 1981) where differences of up to 2-fold are expected and accepted.

IFN in infected rainbow trout fry

The protective activity demonstrated in the trout fry homogenates was consistent with the conclusion that it was due to IFN. Further, its resistance to acid conditions characterized it as an α and/or β *Oncorhynchus* type I IFN (see nomenclature of Anonymous 1980). Specific abolition of the protective activity by antibodies to IFN has not yet been achieved because of a lack, thus far, of the reagents.

The IFN synthesis in VHSV-infected fry ran in parallel with the course of infection. This is consistent with the fact that the IFN synthesis was triggered by the infection. It is obvious that the virus counts made from the pools of homogenates from infected fry were not ideally suited to studying the possible correlation between virus growth and IFN synthesis and that virus titrations from individual fish would have been required. The aim of the present work was, however, primarily the designing of an IFN microtitration test for fry. We were therefore content to follow virus production using pooled fry homogenates.

Characteristics of the microtechnique

The sensitivity of the technique seems to be of the same level, if not better, than that of the plaque assay. Indeed, the titre of the reference IFN stock was found to range between 1000 and 1600 U ml^{-1} (average 1350) based on 15 plaque assays performed since 1976. By comparison, the reference IFN titres found by the microtechnique were mostly located between 2000 and 4000 U ml^{-1}. Our goals, however, were not to compare 2 titration techniques but to design a system enabling us to screen the hundreds of IFN samples required in fish virus pathogenesis investigations. Thus, no further comparative studies on the respective sensitivities of these techniques were conducted.

The reproducibility of the microtechnique is an extremely important characteristic to ensure. Reproducibility is largely a function of the cells' sensitivity to the challenge virus – a sensitivity that can vary from one assay to another. To check the reproducibility of the IFN titration procedure and allow comparison between results obtained between successive IFN titration tests, there are international reference preparations of IFN for mammalian and avian IFN (Finter 1981). For our fish system, there is no international IFN standard available and the reproducibility of our titration technique has to be ensured by the use of a laboratory reference IFN. The reference IFN permits corrections to be made for variations occurring between successive assays. The correction factor is determined as follows: all the IFN titration assays performed over a defined period must include a sample of a reference IFN stock. The arithmetic mean of the reference IFN titres is computed and the values obtained for the IFN titres of a specific set of titrations are multiplied by the ratio:

$$\frac{\text{Arithmetic mean of reference IFN values}}{\text{Value of the reference IFN in a particular assay}}$$

This procedure enables comparisons between successive titrations of different IFN samples and is currently used on a large scale in our laboratory.

The present microtechnique was set up with VHSV-induced IFN but it can be extended to other IFN induction systems. For example, preliminary results (not shown here) obtained with IPNV were satisfactory as long as the infectious viral particles present in the sample were eliminated by ultracentrifugation (Airfuge, Beckman). Inactivation of the virus by heating was not possible because it resists heating at 45 °C.

Our spectrophotometric technique for titrating IFN is a valuable investigative tool for studying the pathogenesis of fish viroses and it can also be used for titrating other cytotoxic factors.

Acknowledgements. We thank Dr M. Dorson and Dr B. J. Hill for reviewing the manuscript and D. Dumonteil for editorial assistance.

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Responsible Subject Editor: Dr T. Evelyn, Nanaimo, B.C., Canada

Manuscript first received: February 21, 1989
Revised version accepted: October 2, 1990