# Latent Infection in Marine Fish Cell Tissue Cultures.* (30997)

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Approximately 6 years ago, a cell line, designated grunt fin (GF), was established in our laboratory from fin tissue of the blue striped grunt (*Haemulon sciurus*)(1). Two years after initiation of this line, at passage level 65, one bottle displayed a cytopathogenic activity which initially appeared as discrete foci and subsequently extended to destroy the entire cell population. From this culture was isolated an agent, designated GFA, which is capable of cytopathic replication in GF cells and which has many properties of a virus(2).

The present report describes the establishment in GF cells of a GFA-carrier status in which GFA replication occurs in the midst if unimpaired cell proliferation, a phenomenon which appears to be mediated by the production in the silently infected cultures of a substance akin to interferon.

*Materials and methods. Tissue cultures.* The GF cells have been maintained in Eagle's basal medium with modified Hanks' balanced salt solution (BSS) (sodium chloride concentration increased to 0.196 M) and with supplements of 10% human serum plus 10% calf serum. Rainbow trout gonad (RTG) cells, kindly provided by Dr. Kenneth Wolf, have been maintained in Eagle's basal medium prepared in Hanks' balanced salt solution (no additional NaCl), and supplemented with 15% fetal calf serum. Both cell lines were grown at 20°C as stationary cultures and subcultured at 7-10-day intervals by either trypsinization or mechanical cell dispersion.

*Viruses.* Stock suspensions of GFA were grown in GF cultures. Infectious pancreatic necrosis (IPN) virus(3), made available to us by Dr. Kenneth Wolf, was propagated in RTG cells. This virus replicates with concomitant cytopathic effects (CPE) with equal efficiency in GF cells. Stock preparations of both agents were stored at −65°C.

*Results.* Infection of GF cultures with GFA results in progressive CPE, but may culminate in the survival of a few cells which subsequently proliferate and give rise to cultures chronically infected with GFA. Similarly, carrier sublines can be established by challenge of GF cells with IPN. This phenomenon has been observed most frequently with large inputs of virus, as infections with dilute inocula of either GFA or IPN usually terminate in complete destruction of cultures.

One such GFA-carrier subline, designated G1A, has been cultivated serially for over 18 months with no evidence of CPE. This subline is indistinguishable from the parent uninfected GF cells as regards morphology, growth and karyologic characteristics. Repeated assays of cell-associated and free GFA were performed through the 42nd passage, employing cells and medium 7 days after seeding (with medium being replaced on day 3). These have consistently yielded infectivity levels of $10^{4.4}$ to $10^{4.7}$ $ID_{50}$/ml in both phases of the cultures. Cultures maintained for "insurance" purposes for 10 weeks without change of medium retain their viability, normal morphology and carrier state.

To test the status of susceptibility of the carrier cells to fish viruses, G1A tube cultures plus control GF cultures were challenged with varying dosages of GFA and IPN (the only two agents known to replicate in GF cells) and observed daily for CPE. The results shown in Fig. 1 clearly demonstrate resistance of the carrier cells to both agents.

The experiments with heterologous challenge were of especial interest in that serial subculture of IPN-challenged GFA-carrier cells resulted in a cell population doubly infected with GFA and IPN, as proved by host range and neutralization tests. Work now in

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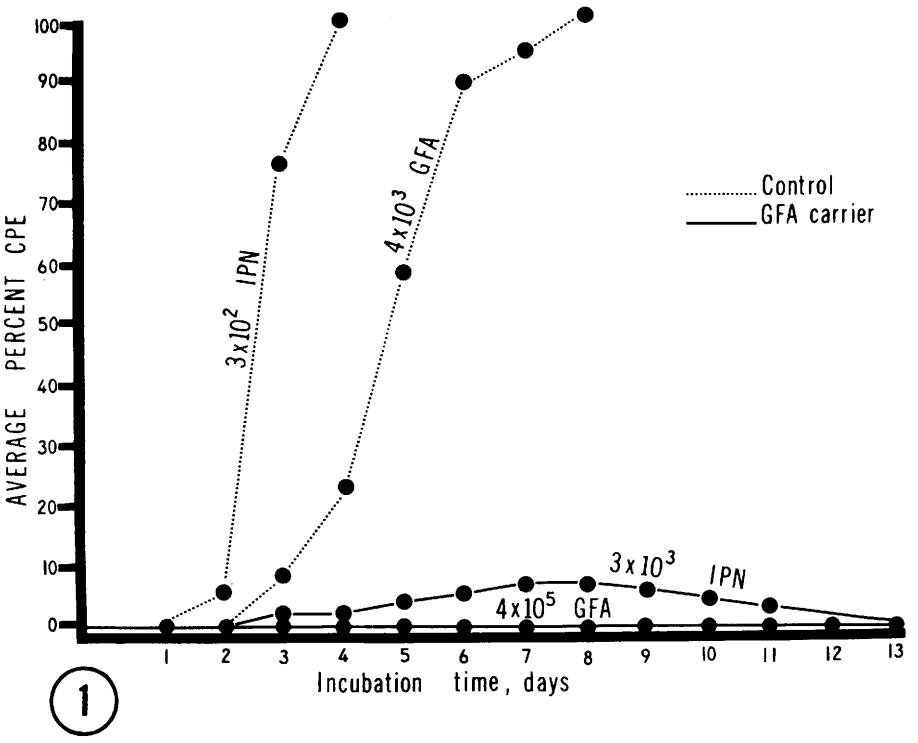


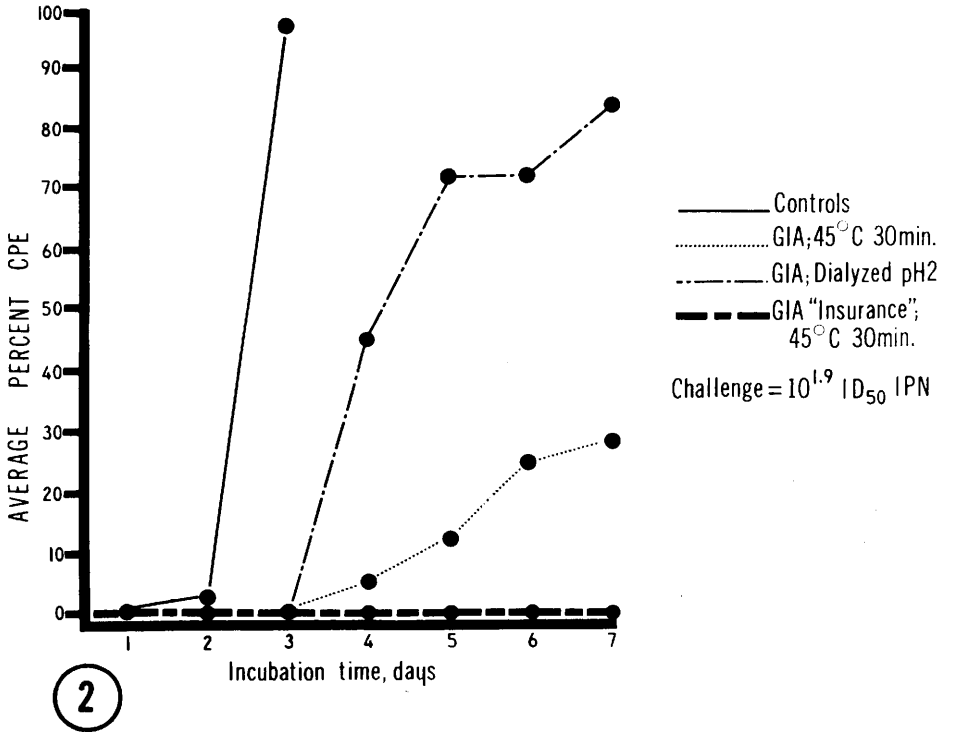


FIG. 1. Resistance of G1A cells to superinfection. FIG. 2. Protective effect of G1A supernatants.

TABLE I. Infectivity of Intact and Lysed G1A Cells.

| Avg* No. of cells | CPE with intact cells | | $ID_{50}$ virus in lysate† | No. cells lysed per $ID_{50}$ |
|---|---|---|---|---|
| | Progressive[F] | Abortive[FF] | | |
| $9 \times 10^4$ | 0/13‡ | 13/13 | $10^{2.50}$ | 281 |
| $9 \times 10^3$ | 0/18 | 18/18 | $10^{1.54}$ | 257 |
| $9 \times 10^2$ | 4/18 | 14/18 | $10^{0.57}$ | 240 |
| $9 \times 10^1$ | 16/18 | 1/18 | $10^0$ | 89 |
| $9 \times 10^0$ | 6/18 | 0/18 | $10^{-0.46}$ | 26 |
| <1 | 0/14 | 0/14 | Noninfectious | |

* Average of 4 experiments with cell concentrations of 6.6, 9.6, 10 and 10 × indicated log.
† Lysates were assayed in 0.1 ml per culture tube; they were derived by disrupting (3 freeze-thaw cycles) cells in concentrations 10 times greater than shown in the first column.
‡ No. of culture tubes with CPE/No. inoculated.
F Progressive CPE = culminating in total destruction.
FF Abortive CPE = partial CPE, with subsequent recovery.

progress is directed toward determination of whether a single cell can simultaneously carry both viruses.

*Mechanisms of the carrier status.* Several experiments were performed to determine the proportion of cells in the general population which were infected with GFA at a given time. G1A culture monolayers were washed 3 times and the cells were dispersed by means of trypsin, centrifuged, washed once again and diluted to known concentrations, following which they were plated on GF monolayers. The development of CPE was followed over a period of 13 to 23 days. In addition, cells in different concentrations were lysed by 3 rapid freeze-thaw cycles, and their viral content was similarly assayed on GF cells. The cumulative results of 4 experiments are summarized in Table I. It is seen that infection was initiated with as few as 6 to 10 intact cells (average, 9 cells) in 6 to 18 instances. It is assumed that the infection was initiated by the cells acting as infectious centers rather than by free virus, even though immune serum was not present in the medium, because at dilutions corresponding to 6-10 cells no free virus could be demonstrated. In several experiments the level of free virus associated with $10^5$ washed cells/ml was on the order of $10^2$ $ID_{50}$; thus no detectable free virus would have been present when the cells were diluted by a factor of $10^4$. By extrapolation, it may be estimated that a minimum inoculum of 18 to 30 cells would have produced infection in all indicator cultures, implying that an average of one in 27 cells—or 4% of the population—was an active carrier. Assays of lysates indicated levels of infectious virus to be considerably below the infectivity registered by intact cells. One or more of several mechanisms may be responsible for the observed deficit. GFA is a highly labile agent and may have been partially inactivated in the process of lysis. Secondly, since infection in the G1A culture must be considered asynchronous, at a given time the infected cells contain GFA in various stages of maturation, *i.e.*, eclipsed or immature particles. Upon dispersal and plating of intact cells on indicator monolayers, the replication cycle would proceed in a normal fashion, whereas after lysis of the dispersed cells only complete mature forms of the agent could initiate infections. Further, it is to be noted that the deficit was greatest when the lysate was derived from high concentrations of cells, suggesting the release upon lysis of some interfering factor. We have previously demonstrated a similar phenomenon of diminished infectivity of lysates compared with intact cells, operative in a mammalian system of KB cells chronically infected with dengue virus (unpublished).

Table I also shows another interesting feature: the occurrence of abortive CPE following plating of high concentrations of carrier cells. Whereas small numbers caused clear, distinct and progressive cytopathic changes, high concentrations caused either no discernible effects or produced a transient aberrant CPE. In the latter case, the cells invariably underwent a spontaneous cure and at the end of the observation period looked normal for all intents and purposes. This

phenomenon has not been observed with free virus derived from spontaneous or artificial cell lysis. The abortive infections imply that some substance contained in the carrier cells was interfering with expression of the infectious process and suggest that at least some of the cells contained both virus and the protective substances. This speculation, which needs more decisive investigation, is strengthened by some observations on plaque formation by lysates and intact cells. Free GFA has produced distinct plaques whose number had a linear relationship to the concentration of agent, yet intact cells have thus far failed to give rise to such plaques in GF monolayer cultures.

Since it is well known that several types of mammalian carrier culture systems owe their status of latent infection to interferon, it was decided to determine whether the protection of the fish cells was mediated by an interferon-like substance. To test directly for the presence of such a substance several experiments were carried out in which GF cells were treated with supernates from G1A carrier cells, and subsequently challenged with GFA and IPN agents. For this purpose we have used the growth media of 7-day G1A cultures and pooled supernates from 8-10-week "insurance" cultures which had not been refed after the 3rd day following seeding. Portions of both preparations were heated at 45°C for 30 minutes to inactivate their infectious GFA. In addition, an aliquot of the 7-day growth medium was dialyzed overnight against 100 volumes of 0.05 M HCl-KCl buffer pH 2.0, followed by a 24-hour dialysis against modified Hanks' BSS. As control preparations, supernates from uninfected GF cultures of comparable vintage were similarly treated. Immediately prior to testing, all materials were supplemented with vitamins and amino acids at concentrations corresponding to those in the usual growth medium.

The various preparations were added to normal GF monolayer tube cultures in 0.9 ml volumes, totally replacing the initial culture medium. After 24 hours incubation, all cultures were challenged with GFA or IPN virus in an inoculum of 0.1 ml and observed daily for CPE. The supernates from the carrier cultures afforded protection against both agents. Fig. 2 illustrates results obtained with IPN, $10^{1.9}$ $ID_{50}$ per culture. The 7-day carrier-culture growth medium conferred on the GF cells a significant degree of protection, as exemplified by the relatively small extent of CPE (33%) after 7 days' incubation—in contrast to 100% destruction within 3 days in case of cultures treated with control preparations. The protective substance was resistant to treatment with a high hydrogen ion concentration as evidenced by complete protection on day 3 and 55% protection on day 4 by fluids which had been dialyzed 24 hours at pH 2. This Figure also shows that the amount of inhibitor increased with time: fluids collected 8 to 10 weeks after the last medium change exerted a greater protective effect than did the fluids collected 4 days after medium change. All subsequent experiments were therefore performed with supernatants from cultures maintained from 6 to 12 weeks without feeding.

In view of the fact that the growth media of the G1A cultures are known to contain substantial amounts of GFA, it was essential to rule out the possible direct interfering action of the agent. For this reason, large amounts of free GFA (inactivated by overnight incubation at room temperature followed by heating at 56°C for 10 minutes) were introduced into GF cultures which were subsequently challenged with IPN virus. In this experiment, the equivalent of $10^{6.5}$ $ID_{50}$ of GFA was added to one group of cultures while a control group received an identical volume of growth medium. Two and 24 hours later, the cultures were challenged with 100 $ID_{50}$ of IPN virus. At the latter challenge time, the growth media were harvested and replaced with fresh medium prior to administration of the challenge virus. As seen in Table II, free inactivated GFA exerted no interfering effect against IPN virus. The supernatants removed at 24 hours after addition of GFA were assayed in fresh GF cells for protective action against IPN. None was found.

*Properties of the protective factor.* The biological and some of the biophysical characteristics of the antiviral substance(s), uti-

TABLE II. Failure of GFA to Interfere with IPN Virus.

| Primary inoculum | Time of challenge | IPN CPE, day: 1 | 2 | 3 |
|---|---|---|---|---|
| Diluent | 2 hr | — | ± | 4+ |
| GFA | " " | — | ± | 4+ |
| *Idem* | 24 " | — | 1+ | 4+ |
| | " " | — | 1+ | 4+ |

lizing the more effective supernates from 6-11-week G1A cultures as test materials, were found to be as follows: (1) Inhibitory activity was retained following centrifugation at 105,000 × *g*. (2) The active factor was resistant to heating at 45°C for 30 minutes and was partially retained following exposure to 56°C for 30 minutes. (3) The substance had no neutralizing activity against either GFA or IPN virus. (4) The activity was undiminished by exposure to pH 2.0, as shown in Table III. (5) The protective activity was distinctly species specific, inasmuch as G1A supernatants were without effect in RTG cells. On the other hand the inhibiting action was nonspecific as regards the challenge virus: G1A supernatants were protective both against GFA and IPN. (6) The carrier supernates were capable of reducing plaque formation by GFA in GF cells, in addition to being able to prevent or reduce and delay gross GFA- or IPN-induced CPE. (7) The protective substance in the carrier cell medium inhibited virus replication as well as CPE subsequent to challenge. This is illustrated in an experiment (Table IV), wherein 2 groups of GF cultures were treated, respectively, with control and G1A supernates, challenged with IPN, and subsequently assayed after various time periods for infectious virus. After 4 days no virus was demonstrable in the inhibitor-treated cultures, while replication in the controls had reached $10^{6.47}$ $ID_{50}$ per culture. Even 6 days later, the amount of virus in the inhibitor-treated cultures was less than one percent of the maximum level in the controls.

*Discussion.* In the course of several years of invesitgation we have been impressed with the low frequency of virus diseases and malignant tumors in marine fishes. So far only two agents have been isolated from these animals: GFA, discussed here, and an agent recovered from lymphocystis tumors of snappers (to be published). The low incidence of viral diseases and tumors prompted us to undertake studies on the immune response and nonspecific resistance to infection in the lower vertebrates of the ocean. These investi-

TABLE III. Resistance of Inhibitor to High Hydrogen Ion Concentration.

| Exp No. | Challenge agent | Test preparation: TC source | Treatment | Avg % CPE, day: 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | GFA | Control | None | 0 | 0 | 1 | 4 | 37 | 97 | 100 |
| | | | pH 2.0 | 0 | 0 | 0 | 1 | 11 | 58 | 100 |
| | | G1A | None | 0 | 0 | 0 | <1 | <1 | 4 | 33 |
| | | | pH 2.0 | 0 | 0 | 0 | 0 | <1 | 4 | 32 |
| 2 | IPN | Control | None | 0 | 0 | 11 | 100 | | | |
| | | | pH 2.0 | 0 | 0 | 2 | 83 | 100 | | |
| | | G1A | None | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | pH 2.0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

TABLE IV. Effect of Inhibitor on IPN Virus Replication.

| Incubation time after IPN challenge | Parameter of interferon activity: Inhibition of IPN CPE, Avg % CPE: Control | G1A | Inhibition of IPN replication, $ID_{50}$ IPN/culture: Control | G1A | % inhibition |
|---|---|---|---|---|---|
| 2 hr | 0 | 0 | $<10^{0.67}$ | $<10^{0.67}$ | |
| 3 days | 69 | 0 | ND | ND | |
| 4 " | 100 | 0 | $10^{6.47}$ | $<10^{0.67}$ | >99.999 |
| 10 " | | 0 | | $10^{4.0}$ | 99.66 |

gations have already proved fruitful in the characterization of immunological phenomena, including the synthesis of immunoglobulins and the development of immunologic memory in relation to the phylogenetic status(4,5,6). The present study reveals that cells of fish are fully capable of withstanding the lethal action of an agent to which they are normally highly susceptible. Thus cells of *H. sciurus* can readily acquire the status of latent infection in which both cells and virus propagate with sufficient efficiency to permit undisturbed cell growth and high levels of virus production. Cultures of these cells have been shown to perpetuate two fish viruses, giving rise to a double virus carrier system. It is not known whether the two viruses are maintained in the same individual cells.

The factor associated with the establishment and maintenance of the carrier status has many properties of interferon. The role of interferon in determining recovery from virus diseases as well as in maintaining silent infections has been well documented for mammalian and avian cells(7,8,9,10). Recently Falcoff and Fauconnier(11) reported that an interferon-like inhibitor is also elaborated by tortoise cells. In describing the characteristics of a permanent cell line from the fathead minnow, Gravell and Malsberger(12) mention that these cells—which are susceptible to a large array of viruses, including some mammalian viruses—are capable of elaborating a protective factor possessing properties of interferon. It would therefore appear that the capacity of a cell to produce interferon had evolved early in phylogenetic development. The precise nature of the substance which possesses many properties of interferon remains to be determined as regards size and chemical composition.

*Summary.* GFA, an agent isolated from an established line of fish cells, was capable of causing in tissue culture cells a chronic infection which has persisted for a period of 18 months. These cells are resistant to superinfection by this and another fish virus, the latter derived from fresh water fish. The resistance and carrier status of the cells are attributed to the production of interferon.

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