Multiplication of Human Enteroviruses in Northern Quahogs[1] (35496)

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Shellfish have been incriminated as reservoirs of human enteric virus. Several outbreaks of infectious hepatitis were associated with ingestion of contaminated oysters and hard clams (1–7). Isolation of these viruses in the American oysters obtained from estuarine environments polluted by raw sewage has further supported this observation (8). It is assumed that shellfish are only passive carriers of virus, since viral replication in shellfish has never been demonstrated (8, 9). However, this question has not been completely resolved, because the techniques used previously could not detect the viral progeny in these animals if the replication was extremely slow and of low magnitude.

Hiatt and Moore (10) and Schaffer (11) showed that proflavine was bound into poliovirus particles when the dye was present in culture medium in which the virus was propagated. The poliovirus–proflavine complex was photosensitive. When such labeled virus was grown in tissue culture in absence of dye, its progeny contained no dye and were, therefore, insensitive to light. The objective of the present study was to use this technique in demonstrating the presence of viral replication, if any, in shellfish. This communication presents the findings when polio-, coxsackie-, and echoviruses and the Northern quahog, *Mercenaria mercenaria*, were used as a working model.

Materials and Methods. Virus. The LSc 2ab strain of type 1 poliovirus, the Farouk strains of type 1 echovirus, and the Nancy strain of type B-3 coxsackievirus, were used in these experiments. The virus seeds were obtained from the Reagent Reference Branch, NIH, Bethesda, Maryland. All virus stocks were propagated in primary African green monkey kidney cell (GMKC) cultures.

Tissue culture. In the initial experiments, primary GMKC was used for labeling virus and plaque assays of various samples. In some later experiments, virus assays were carried out in primary rhesus monkey kidney cell (RhMKC) cultures. The procedures used for preparation of monolayer tissue cultures in 3-oz prescription bottles were essentially those described by Hsiung and Henderson (12).

Virus assay. Plaque assay method was used. The procedures were those described by Hsiung and Melnick (13). Virus titers are expressed as total number of plaque-forming units (PFU) per milliliter of fluid or gram of tissue.

Quahogs. The Northern quahog was used throughout this study. The quahogs, each weighing 100 $\pm$ 15 g with shell, were stored in large aquariums supplied with flow-through sea water from Narragansett Bay, Rhode Island. It was critical in this study that the quahogs remained viable under the adverse conditions after inoculation until they were harvested for examination. In experiments presented, the majority of animals were kept alive throughout the observation periods and only living quahogs were used for viral determination.

Infection of clams with virus and preparation of extract. Previous experiments had shown that light waves in the 575A–600 Å range *i.e.*, yellow light, did not adversely affect the labeled virus. Therefore, all procedures in manipulating this virus were carried out under yellow light or in darkness.

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A hole was made at the ligament of the valves of a clam with a pointed object. A 0.5-ml inoculum containing 5×10^7 PFU of labeled poliovirus or 2×10^6 PFU of echovirus or coxsackievirus was injected through the hole into the mantle cavity of each quahog. Following injection, the quahogs were incubated in darkness at 37 or 25° in absence of sea water. At various times of incubation, the valves of clams were opened by severing the adductor muscles and the mantle cavity fluid was collected with a syringe. Each digestive diverticulum including stomach was dissected out and weighed. All organs were washed 3 times in phosphate buffered saline (PBS), ground with pestle and mortar, and sufficient Hanks' balanced salt solution (HBSS) was added to constitute a 10% (w/v) homogenate. Homogenates were clarified by centrifugation at 2000 rpm for 15 min.

Labeling virus with proflavine. A stock solution of proflavine containing 500 μg/ml was autoclaved at 15 lb for 15 min. One ml of the stock solution was added to 199 ml of Earle's maintenance medium (ELS 2%) containing 0.5% lactalbumin hydrolysate and 2% calf serum. Thus, the final concentration of proflavine was 2.5 μg/ml of the medium. Approximately 1×10^5 PFU virus in 0.1 ml were inoculated into a 3-oz bottle containing monolayer of GMKC. After 1 hr of adsorption, 10 ml of ELS 2% with proflavine were added to the bottle. The bottle was then wrapped in aluminum foil to exclude light and incubated at 37°. The infected cells and fluids were harvested after freezing and thawing for 3× *in situ* when advanced cytopathogenic effects (CPE) were observed. All harvests were clarified by centrifugation at 2000 rpm for 20 min. Seven serial passages of the virus were made in GMKC containing medium with proflavine.

Exposure of virus to incandescent light. Tubes containing 1 ml of fluid with labeled virus were exposed to 100 W of incandescent light at a distance of 2.5 cm. Tubes and light bulk were immersed in ice water throughout exposure period.

Results. Multiplication of labeled poliovirus in GMKC. Before study of the shellfish, the validity of our approach was tested by using a known system, *i.e.*, primary AGMC.

TABLE I. Multiplication of Proflavine-Labeled Poliovirus in Primary GMKC Cultures at 37°.[a]

Time after inoculation (hr)	Virus yield (PFU/ml)	
	Dark	Light[b]
1	8.3×10^3	0
12	1.3×10^7	1.3×10^7
18	1.3×10^7	3.0×10^7
24	1.4×10^7	1.3×10^7
48	1.7×10^7	2.1×10^7

[a] GMKC cultures maintained in media containing no proflavine.

[b] Virus exposed to 100 W of incandescent light at a distance of 2.5 cm for 1 hr.

A number of 3-oz bottles with the GMKC were infected with labeled poliovirus and incubated at two temperatures. Two bottles were collected at each time and frozen and thawed 3× before the fluids were pooled. All pools including infected cells and medium were clarified by low speed centrifugation and stored at −20° before the assay for viral content. The results from the GMKC infected with labeled virus and incubated at 37° are summarized in Table I. As shown, the virus yield consisted only of light-sensitive virus at 1 hr following infection. The light-resistant virus yields, however, were detected from 12 to 48 hr. During this period, little difference in titers was observed between samples assayed in darkness and those exposed to light. Table II summarizes the results obtained from cell cultures infected with labeled virus and incubated at 25°. It is evident that light-resistant virus was not de-

TABLE II. Multiplication of Proflavine-Labeled Poliovirus in Primary GMKC Cultures at 25°.[a]

Time after inoculation (days)	Virus yield (PFU/ml)	
	Dark	Light[b]
1	9.0×10^3	0
3	1.0×10^4	5.0×10^2
4	1.0×10^4	8.0×10^3
6	9.0×10^3	7.0×10^3
8	1.8×10^4	9.6×10^3

[a] GMKC cultures maintained in media containing no proflavine.

[b] Virus exposed to 100 W of incandescent light at a distance of 2.5 cm for 1 hr.

TABLE III. Poliovirus Isolations from Northern Quahogs at 37°.[a]

After inoculation (days)	Clam no.	Virus yield: Dark, Mantle fluid (PFU/ml)	Dark, Dig. gld. (PFU/g)	Light[b], Mantle fluid (PFU/ml)	Light[b], Dig. gld. (PFU/g)
1	1	1.3×10^{4}	4.5×10^{2}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	2	5.0×10^{3}	9.0×10^{2}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	3	3.3×10^{4}	6.0×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	4	5.0×10^{4}	1.2×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
2	1	1.0×10^{2}	9.0×10^{2}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	2	5.5×10^{3}	1.0×10^{2}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	3	1.4×10^{4}	1.0×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	4	9.0×10^{3}	4.2×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	5	6.2×10^{2}	1.0×10^{2}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
3	1	3.8×10^{3}	2.0×10^{2}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	2	4.1×10^{3}	2.3×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	3	6.0×10^{3}	3.5×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$

[a] 5×10^{7} PFU of proflavine-labeled type 1 poliovirus were inoculated into each quahog.
[b] Virus preparation exposed to 100 W of incandescent light at a distance of 2.5 cm for 1 hr.

tectable until the third day of incubation. A gradual increase of light-resistant virus was seen from 3 to 8 days. The increase was minimal compared to the original inoculum, but real and clear-cut.

Multiplication of proflavine-labeled enteroviruses in quahogs. Results of quahogs inoculated with labeled poliovirus, followed by incubation at 37° for 3 days are shown in Table III. Light-resistant virus was not detected for the entire period. Light-sensitive virus remained at 10^{3}/ml or g even at the end of incubation. Table IV summarizes the results obtained from the quahogs incubated

TABLE IV. Poliovirus Isolations from Northern Quahogs at 25°.[a]

After inoculation (days)	Clam no.	Virus yield: Dark, Mantle fluid (PFU/ml)	Dark, Dig. gld. (PFU/g)	Light[b], Mantle fluid (PFU/ml)	Light[b], Dig. gld. (PFU/g)
30 min	1	4.3×10^{4}	4.0×10^{2}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	2	2.5×10^{4}	2.0×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
3	1	6.0×10^{4}	3.6×10^{5}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	2	9.3×10^{4}	2.4×10^{4}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
4	1	2.6×10^{4}	1.4×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	2	9.6×10^{4}	4.2×10^{4}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	3	1.3×10^{4}	6.1×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
6	1	7.1×10^{4}	3.4×10^{4}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	2	1.6×10^{3}	3.3×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
8	1	4.0×10^{3}	4.5×10^{4}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	2	4.7×10^{4}	8.5×10^{3}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$
	3	4.0×10^{4}	7.0×10^{4}	$<1.0 \times 10^{1}$	$<1.0 \times 10^{1}$

[a] 5×10^{7} PFU of proflavine-labeled type 1 poliovirus were inoculated into each quahog.
[b] Virus preparation exposed to 100 W of incandescent light at a distance of 2.5 cm for 1 hr.

at 25°. No light-resistant poliovirus was detected up to 8 days following inoculation.

The actual data on shellfish inoculated with labeled echo- and coxsackievirus are not presented. Groups of quahogs were inoculated with labeled echovirus at 2–3 $\times$ 10^6 PFU/quahog and incubated at 37 and 25° for 3 and 7 days, respectively. No light-resistant virus was detected throughout the entire period. Also, virus titers remained unchanged until the end of observation periods. When labeled coxsackievirus was inoculated into quahogs at 2 $\times$ 10^6 PFU/quahog and the animals incubated at 37 and 25°, similar results were obtained. At no time was light-resistant coxsackievirus isolated. Also the virus titers had little reduction during entire observation periods.

Discussion. Results obtained in the study using proflavine-labeled virus in primary GMKC showed that the poliovirus replicated at the temperature investigated (Tables I and II). However, the rate of replication was significantly affected by temperature. At 37° the virus produced maximum progeny within 24 hr; whereas, at 25° progeny virus was low even after 3 days of incubation. The light-sensitivity technique, however, was capable of clearly distinguishing the new progeny from the input virus at both temperatures. At the latter temperature, virus was light sensitive from 1 to 3 days postinfection, representing input virus, and light resistant thereafter, indicating emergence of a new generation that had not incorporated the dye.

In the present study, digestive diverticulum and mantle cavity fluid were chosen for virus isolation. This choice was made on the basis of: (a) Liu *et al.* (9) and Metcalf and Stiles (8) had shown that, among all organs examined, the diverticulum was found to harbor great majority of virus. (b) Liu *et al.* (9) showed that the mantle fluid also retained significant amounts of virus when shellfish were polluted and maintained in stationary water aquaria.

Liu *et al.* (9) reported that in Northern quahogs poliovirus was not found intracellularly and also the virus was probably not chemically bound to any type of cells. These investigators based their findings on the fact that trypsinization of minced quahog tissues released 80% of the total input virus and only 20% of virus remained with the cells. Hedstrom and Lycke (14) and Metcalf and Stiles (8) also considered oysters as passive carriers of virus. These authors showed a gradual decline in virus titer in oyster organs under varying conditions of maintenance for a period of 6 to 38 days. In the present study, a different approach was taken. By inoculating proflavine-labeled and light-sensitive virus into quahogs and examining for light-resistant viral progeny, no progeny was found. Thus, one may conclude that at least a type 1 poliovirus a type 1 echovirus, and type B-3 coxsackievirus did not replicate in quahogs under these experimental conditions. The same procedure was used by Miller and Horstman (15) to study pathogenesis of poliomyelitis in monkeys. This method successfully circumvented the difficulties encountered by previous investigators in attempts to determine whether virus in shellfish tissue represented persisting input virus or progeny formed *de novo*.

It is interesting to note that the 3 enteroviruses survived for a long time in quahogs. At present, no explanation can be given to this observation. It is clear, however, that long storage of shellfish in dry state before consumption will not reduce the risk of transmitting these viruses to man.

Summary. Three proflavine-labeled human enteroviruses were used to determine whether or not viral multiplication occurred in Northern quahogs. The results indicate that no multiplication occurred in these animals under the conditions tested. The virus isolated from quahogs at varying time intervals after inoculation represented persisting input virus.

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