Biological Properties of a Plaque-Inducing Agent Obtained from *Acholeplasma oculi*

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A plaque-forming agent arose spontaneously during cloning of *Acholeplasma oculi* 19L. The agent produced plaques on *A. oculi* 19L and *A. oculi*-i, but not on *A. laidlawii*, *A. modicum*, or wild isolates of *A. oculi*. The agent required horse serum for plaque formation as well as for adsorption to the indicator lawn; however, it was extremely sensitive to an inhibitor in some horse sera. The agent retained infectivity after passage through a 50 nm filter and was heat-, Nonidet P-40-, and chloroform-labile, but relatively ether-stable. It was not determined whether the agent is a virus or a bacteriocin-like substance.

**INTRODUCTION**

Since the discovery of Acholeplasmavirus by Gourlay in 1970, mycoplasma viruses were isolated from *A. laidlawii*, *A. modicum*, and spiroplasma. These viruses are morphologically classified into three groups: rod-shaped, spherical, and polyhedral viruses from acholeplasma, and SVC1, SVC2, and SVC3 from *Spiroplasma* [1]. Recently, we noted the spontaneous appearance of plaques on the lawn of *A. oculi* which was cloned from *A. oculi* 19L. Plaques were more clearly visible after treatment with tetrazolium.

In this preliminary paper, factors necessary for plaque formation and some biological characteristics of the agent are described.

**MATERIALS AND METHODS**

The original strain of *A. oculi* 19L was received from Dr. J.J. Callis, Plum Island Animal Disease Center, Greenport, NY, through Dr. Ogata, Department of Microbiology, School of Veterinary Medicine, University of Tokyo, Japan.

*A. oculi* 19L was propagated and maintained for about two years in PPLO broth containing 20 percent horse serum. The serum originated in a butchery and was heated twice at 56°C for 30 minutes before storage. After repeated cloning of *A. oculi* 19L, a clone having the ability to produce plaques spontaneously was obtained and referred to as *A. oculi*-i. Lawns of *A. oculi* 19L and *A. oculi*-i were generally treated with tetrazolium chloride by the method of Fraser and Crum [2] in order to visualize plaques. The method of Smith [3] was used to measure colony-forming units (CFU) of *A. oculi*. Plaque titers were expressed as plaque-forming units (PFU)/ml.

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Biological characteristics of the plaque-forming agent were studied by the method of Gourlay and Wyld [5].

RESULTS

Spontaneous Production of the Plaque-Forming Agent in Broth

When *A. oculi*-i was cultivated at 37°C in PPLO broth containing horse serum, the plaque-forming agent was found in the supernatant fluid after 30–36 hours of cultivation. The relationship between the growth of cells of *A. oculi*-i and the production of plaque-forming agent was examined. Plaque-forming agent was separated and harvested from the acholeplasma cells by filtration at 220 nm.

Time of Infection of the Lawn of *A. oculi* 19L with the Plaque-Forming Agent

The age of the lawn of *A. oculi* 19L was critical for the appearance of plaques. When the agent was spread on the 24-hour-old lawn of *A. oculi* 19L, no plaques were produced. The highest yields of plaque were obtained when a two-hour-old lawn of *A. oculi* 19L was employed.

Host Range of *A. oculi*-i Plaque-Forming Agent

Host range of *A. oculi*-i plaque-forming agent was evaluated, together with the host ranges of other agents previously reported by others. This *A. oculi*-i plaque-forming agent produced plaques on *A. oculi* 19L and *A. oculi*-i but not on wild isolates of *A. oculi*. The results suggest that the host range of this agent is quite different from those of *A. laidlawii* viruses, but there are some similarities to that of *A. modicum* virus [4]; the agent does not make plaques on the lawn of *A. laidlawii* BN1 strain or on one strain of *A. modicum*, the PG 49 strain.

Effect of Horse Serum on Plaque Formation

Some horse sera have an inhibitor of plaque formation. Some sera partially or completely inhibited plaque formation but did not inhibit the growth of cells of *A. oculi* 19L. Therefore, it is recommended, for studies on this agent, that horse sera be tested first for inhibitors, and that a suitable horse serum be used at a dilution of 10 percent, which may eliminate minor amounts of inhibitor contained in the serum. Inhibitor-free horse serum should also be used in the agar base for making an indicator lawn.

Infection of *A. oculi* 19L

In order to characterize the agent spontaneously liberated from the *A. oculi*-i clone, a trial of infection of *A. oculi* 19L was carried out. Twenty-five hundredths ml of the agent containing 10⁶ PFU were added to *A. oculi* 19L cells 10⁸ CFU. After one hour, the mixture was diluted 100 times; then CFU as well as PFU were measured periodically. Plaque formation increased with time more than 1,000-fold at 12 hours post-infection. Therefore, it was concluded that the infective agent multiplied in the cells of *A. oculi* 19L.

Biological Characteristics

Biological properties of the agent were preliminarily examined. The agent was filtrable through a 50 nm (Millipore) filter, completely labile at 60°C, five minutes; and slightly sensitive to ultraviolet irradiation. In addition, the agent lost the plaque-forming ability after treatment with Nonidet P-40, as well as with chloroform, but lost it incompletely after ether treatment.
Morphology of the Agent

The plaque-forming agent was collected by centrifugation of approximately 200 ml of *A. oculi*-i culture fluid, and concentrated in 2 ml. The concentrated sample, which contained $10^{7.8}$ PFU/ml, was purified by ultracentrifugation in cesium chloride. Two bands were obtained. A sample of each was examined by electron microscopy after staining with 1 percent potassium phosphotungstate. No characteristic particles or features were recognized in either fraction. Therefore, it was not determined whether the plaque-forming agent of *A. oculi*-i was a virus or a bacteriocin-like substance.

DISCUSSION

Evidence is presented that the plaque-forming agent was recovered from a clone of *A. oculi*-i, which was derived from *A. oculi* 19L. The agent was produced spontaneously from the culture of *A. oculi*-i but not recovered from the original parent strain of *A. oculi* 19L. It is speculated that the cloned cells acquired the ability to show plaque formation, or that the agent was introduced from outside in the course of cloning. The origin of the agent is an interesting problem. It was found (data not presented) that plaque formation was not stimulated by treatment of *A. oculi*-i or provoked by treatment of *A. oculi* 19L cells with mitomycin c or ultraviolet irradiation. On the other hand, it could be postulated that the agent might have originated in the horse serum; the multiplication of the agent is extremely sensitive to some kind of inhibitor present in horse serum.

The biological characteristics of the agent are quite distinct from those of *A. laidlawii* viruses, but somewhat similar to those of *A. modicum* virus.

By ultracentrifugation in cesium chloride, the agent sample was concentrated and fractionated into two bands, having densities of 1.2 and 1.4 and infectivity of $10^{7.6}$ and $10^{5.0}$ PFU/ml, respectively. Electron micrographs, however, revealed no recognizable particles. Therefore, it was not determined whether the agent was a virus or a bacteriocin-like substance.

In the future, questions raised in this preliminary research, such as the morphology of the agent, one-step growth curve, biochemical properties of inhibitors present in horse sera, and the origin of the agent should be studied.

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