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HUMORAL FACTORS IMPORTANT IN RESISTANCE OF SALMONID FISH TO BACTERIAL DISEASE. II. ANTI-*VIBRIO ANGUILLARUM* ACTIVITY IN MUCUS AND OBSERVATIONS ON COMPLEMENT

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ABSTRACT

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It has been demonstrated that heat-stable (presumably antibody) and heat-labile (presumably complement) components are necessary to prevent the growth of *Vibrio anguillarum* in in vitro experiments with trout immune serum and mucus. Anti-*V. anguillarum* agglutinins were found in the body mucus of intraperitoneally immunized rainbow trout (*Salmo gairdneri*) 3—6 weeks after serum agglutination titers of 131 072 or greater were attained. A component of rainbow trout body mucus was found to be indistinguishable from serum immunoglobulin by immunodiffusion and immunoelectrophoresis.

INTRODUCTION

There is still much that is not known about protective mechanisms against diseases in man and higher animals. Even less is known about the nature of such mechanisms in fish. Antimicrobial activity in body and intestinal mucus would be of apparent value to fish as a first line of defense, but little is presently known about it.

Fletcher and Grant (1969) were able to induce hemagglutinins in the surface mucus of plaice (*Pleuronectes platessa*) by parenteral immunization with human erythrocytes. They postulated that a specific secretory system was involved in the production of mucus antibody. Oral and parenteral routes were also used to induce antibody specific for *Vibrio anguillarum* in serum and in mucus secretions of the intestine and skin of *P. platessa* (Fletcher and White, 1973). Using immunoelectrophoresis, DiConza (1970) found that the slowest migrating component in immune catfish (*Tachysurus australis*) serum had specific antibody activity and that a substance antigenically related to this component also occurred in skin and intestinal mucus. Bradshaw et al. (1971) reported normal and induced hemagglutinins in the surface mucus of the gar

(*Lepisosteus platyrhinchus*).

In this report we describe an antimicrobial activity in serum and mucus of rainbow trout (*Salmo gairdneri*) that required the participation of a complement-like substance for its expression. The occurrence of complement, which acts in conjunction with antibody to exert its effects (e.g. lysis) on foreign cells, has been well established in lower animals (Day et al., 1970). Yet, much is still unknown about its structure and function. In the serum of salmonids, complement has been shown to participate in the destruction of foreign red blood cells (Chiller et al., 1969) and may also be involved in bacteriostatic or bactericidal activity (Harrell, 1973; Rohovec, 1974). Until now, however, the occurrence of a complement-like substance in salmonid mucus apparently has not been recognized.

MATERIALS AND METHODS

Experimental animals

Rainbow trout and rabbits were used as previously described (Harrell et al., 1975) to produce antisera; rainbow trout were used in the mucus studies.

Microorganisms

Strain 700-23 *V. anguillarum* (Harrell et al., 1975) was used in tests for mucus agglutinating antibody and for mucus and serum antibacterial activity.

Escherichia coli (Harrell et al., 1975) was used to determine the specificity of *V. anguillarum* agglutinins in immune serum and mucus.

Preparation of antisera

Anti-*V. anguillarum* sera were produced in large rainbow trout as previously described (Harrell et al., 1975).

To prepare an antiserum specific for rainbow trout immunoglobulin, rabbits were immunized with a mixture of complete Freund's adjuvant and immune precipitates composed of rainbow trout anti-dinitrophenyl (DNP) antibody and rabbit serum albumin conjugated with DNP. Preparation of DNP-conjugated rabbit serum albumin and preparation and purification of the trout antibody are described in detail elsewhere (Roubal et al., 1974). The rabbit anti-rainbow trout antibody serum was monospecific when reacted against normal rainbow trout serum in Ouchterlony and immunoelectrophoretic analyses.

Antiserum and mucus collections

Caudal blood samples collected from trout were allowed to clot at 7°C and were then centrifuged at 600 × *g*. Sera were either used immediately or frozen and stored at −20°C. Surface mucus was gently scraped from immune and

normal fish with a stainless steel spatula, diluted 1 : 4 with phosphate-buffered saline (0.15 *M* NaCl, 0.02 *M* phosphate, pH 7.5), and extracted by repeated passage through a 20-gauge hypodermic needle. After centrifugation at 90 × *g* for 5 min, the supernatant fluid (mucus extract) was removed and tested for agglutinating or antibacterial activity. In some instances mucus extracts were concentrated fivefold in Minicon-B Clinical Sample Concentrators (Amicon Corporation, Lexington, Mass.).*

Origin of mucus agglutinins

Four rainbow trout were used to determine if mucus antibody was derived from serum antibody. Each trout, weighing approximately 300 g, was given an intraperitoneal injection of 5 ml of trout anti-*V. anguillarum* serum, for which the agglutination titer was 16394. After injection, both serum and body mucus were tested for agglutinins at 2-day intervals over a period of 12 days.

Serological tests

Agglutination assays were performed as previously described (Harrell et al., 1975).

*Assay of anti-*Vibrio *activity*

The assay method described by Holmgren et al. (1971) was utilized to determine whether mucus had antimicrobial activity and whether complement played any role in the vibriocidal or vibriostatic activity of specifically immune serum and mucus. This assay was modified by increasing the concentration of DEAE-dextran (Pharmacia, Uppsala, Sweden) to 1 mg/ml to minimize anti-complementary activity. In this method, the substance to be tested for bactericidal/bacteriostatic activity is applied in drops to the surface of agar pour plates containing bacteria; activity is indicated by the presence of a clear bacteria-free area (plaque) in the plate culture. The immune trout serum and mucus extracts employed had agglutination titers of 512 and 32, respectively. Samples of each were heat-treated at 45° C for 30 min to destroy hemolytic complement activity (Chiller et al., 1969). Finally, the samples were diluted with fresh normal rainbow trout serum, which served as a complement source.

*Trade names referred to in this publication do not imply endorsement of commercial products by the National Marine Fisheries Service.

RESULTS

Agglutinins in mucus

Anti-*V. anguillarum* agglutinins in body mucus could be detected 3—6 weeks after maximum serum titers were attained. Mucus agglutinins were detected only in rainbow trout that had been injected with an emulsion consisting of heat-killed bacterin and Freund's complete adjuvant (Harrell et al., 1975), and in which serum titers of 131 072 or greater had been attained. It is recognized that agglutinating and bacteriostatic activities may not be associated with the same serological components. Yet, agglutinating activity in the mucus extract as well as in immune serum appeared to be specific for *V. anguillarum* since heat-killed *E. coli* were not agglutinated. Using the highly specific rabbit anti-rainbow trout immunoglobulin serum, serologically identical components of normal serum and normal mucus were shown in Ouchterlony reactions (Fig. 1A). Immunoelectrophoretic analysis provided additional evidence for

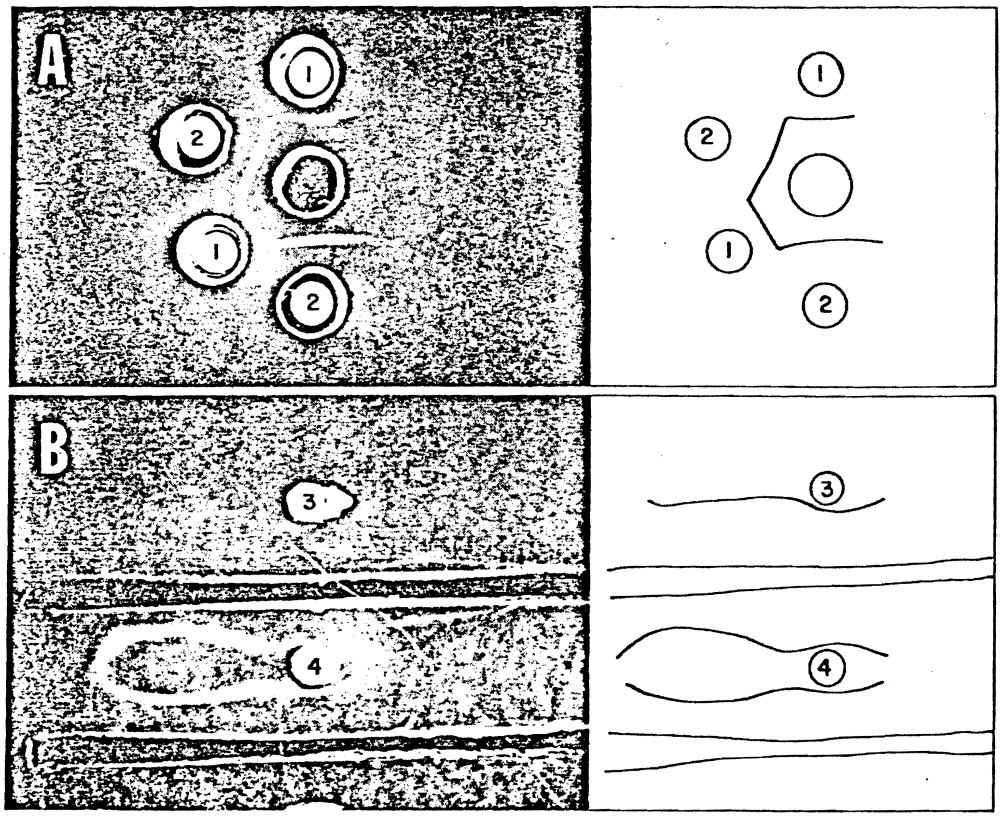

Fig. 1.

A. *Immunodiffusion.* Center well, rabbit anti-rainbow trout immunoglobulin serum; well 1, mucus extract; well 2, normal rainbow trout serum.

B. *Immunoelectrophoresis.* Troughs, rabbit anti-rainbow trout immunoglobulin serum; well 3, mucus extract; well 4, normal rainbow trout serum.

the presence of mucus immunoglobulin, since a mucus component exhibited mobility similar to that of serum immunoglobulin (Fig. 1B).

Origin of mucus agglutinins

Prior to passive immunization, no anti-*V. anguillarum* agglutinins were found in the serum or mucus of the four rainbow trout. However, following passive immunization, agglutinins were clearly evident in serum at the time of the first collection of mucus and remained so throughout the experiment. In contrast, agglutinins were never detected in the mucus extracts or in their fivefold concentrates.

*Anti-*Vibrio *activity*

Distinct plaques were evident using heat-treated anti-*V. anguillarum* serum diluted with normal trout serum after 30 h of incubation at 20°C (Table I). This antiserum, except when diluted 1 : 2, formed strong plaques indicating vibriocidal or vibriostatic activity to a dilution of 1 : 2048. The failure of 1 : 2 diluted antiserum to develop clear plaques may have represented a prozone effect.

TABLE I

Anti-*V. anguillarum* activity of trout serum

System	Vibriocidal/vibriostatic activity*
Normal trout serum**	–
Unheated anti-*V. anguillarum* serum	W+
Heated anti-*V. anguillarum* serum	–
Dilutions of heated anti-*V. anguillarum* serum in normal trout serum**	
1 : 2	W+
1 : 4 — 1 : 2048	+
> 1 : 2048	–

* – no growth inhibition; W+ weak growth inhibition; + strong growth inhibition.
** Complement source was fresh normal rainbow trout serum.

The immune mucus anti-*Vibrio* titer of 64 was essentially the same as the agglutinating titer (Table II). Normal mucus diluted in fresh normal serum was also capable of generating plaques, but its titer of 8 was lower than that of immune mucus. Neither heat-inactivated immune serum, heat-inactivated immune mucus nor fresh normal serum showed anti-*Vibrio* activity.

TABLE II

Anti-*V. anguillarum* activity of "normal" and "immune" trout mucus. Immune mucus was pooled from trout immunized with heat-killed *V. anguillarum*. The extract utilized in the agar plaque assay was concentrated fivefold and had an agglutination titer of 32. Normal mucus was pooled from non-immunized trout. The extract utilized in the agar plaque assay was concentrated fivefold and had an agglutination titer of zero

Agar plaques produced by immune mucus diluted in fresh normal trout serum (complement source)		Agar plaques produced by normal mucus (agglutination titer 0) diluted in fresh normal trout serum (complement source)	
System	Vibriocidal/vibriostatic activity*	System	Vibriocidal/vibriostatic activity*
Normal trout serum	–	Normal trout serum	–
Unheated immune mucus	+	Unheated normal mucus	+
Heated immune mucus	–	Heated normal mucus	–
Dilutions of heated immune mucus in normal trout serum		Dilutions of heated normal mucus in normal trout serum	
1 : 2 — 1 : 32	+	1 : 2 — 1 : 4	+
1 : 64	W+	1 : 8	W+
> 1 : 64	–	> 1 : 8	–

* – no growth inhibition; W+ weak growth inhibition; + strong growth inhibition.

DISCUSSION

In vitro experiments with immune trout serum demonstrated that heat-stable and heat-labile serum components were necessary to prevent growth of bacteria. It is reasonable to suggest, therefore, that an antibody-complement antibacterial system is important in this species in vivo.

The experiments also demonstrated the presence of heat-stable and heat-labile components in body mucus of trout (both immune and non-immune) which, together, were able to inhibit or kill *V. anguillarum* in vitro. Although no anti-*Vibrio* agglutinating activity was detected in mucus from non-immunized fish, it is possible that its heat-stable component was natural antibody capable of reacting with *V. anguillarum*. This postulation is supported by the finding that rabbit anti-trout immunoglobulin serum detected serologically and electrophoretically identical components in serum and mucus from non-immunized trout. The reason for our failure to detect agglutinating antibody in body mucus after intraperitoneal injection of anti-*V. anguillarum* serum is not clear. One possibility is that mucus antibody may not originate in serum. Another possibility, considering the high serum titers necessary to cause detectable levels of agglutinins in mucus of actively immunized trout, is that the passively transferred antisera may not have had the concentration of antibody necessary to result in detectable levels of agglutinins in mucus by the assay used.

Our suggestion that complement activity is present in trout mucus is reflected in the heat-lability of the anti-*Vibrio* activity observed in mucus, and in the ability of normal trout serum to restore this activity to heat-inactivated mucus. In addition, we have shown that extracts of normal trout body mucus can serve as a complement source in tube hemolysis tests with trout antibody. Thus, sheep red blood cells (SRBC) coated (sensitized) with specific SRBC antibody from trout were lysed after addition of mucus extract; non-sensitized sheep red blood cells were not lysed by the mucus extract.

This study clearly indicates that complement exists not only in serum but also in mucus of fish, and that it can have an antimicrobial function — at least in vitro. Our results do not allow us to conclude (1) whether agglutinating and sensitizing activities of serum and mucus are due to the same species of antibody molecule, and (2) whether mucus from non-immunized fish possesses an always-present, non-specific, low level antimicrobial system distinct from the readily inducible one in serum. These questions, and the significance of mucus antibody and complement in trout antibacterial defense, remain to be clarified.

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