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Identification of a Fish Serum Protein with Antibody Activity

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ABSTRACT

The golden shiner, *Notemigonus crysoleucas*, developed antibody titer of 1,280 in 59 days from multiple, intraperitoneal injections of a formalin-killed bacterin of *Aeromonas liquefaciens*. After four immunizing injections there was a significant increase in the time of survival of vaccinated fish over nonvaccinated fish challenged with viable *A. liquefaciens*. Analysis of golden shiner sera by paper electrophoresis produced a pattern of six protein components. Antibody activity was associated with a component which had a slow anodic electrophoretic mobility and solubility of gamma globulin. During the course of vaccination, increments in the serum protein component and in antibody titer coincided. Also, the amount of the protein component of vaccinated fish was decreased by specific adsorption of antibody by homologous antigen. Thus, a fish immunoglobulin was identified which had certain physical and chemical properties corresponding to classical definitions of gamma globulin.

INTRODUCTION

The ability of fish to form antibodies in response to a natural infection by a bacterium was reported by Babes and Riegler in 1903 (Nybelin, 1935). The nature of the immune response of fish has been studied (Duff, 1942; Krantz *et al.*, 1963; Nybelin, 1935), as well as the effects of temperature on formation of antibodies in fish (Cushing, 1942; Gee and Smith, 1941; Pliszka, 1939; Smith, 1940), with the conclusion that fish exhibit a typical immune response. Antibodies of humans and domestic animals are gamma globulins (Porter, 1960), however, the amount of protein with electrophoretic mobility of gamma globulin is reduced in certain freshwater (Deutsch and McShan, 1949) and marine osteichthyians (Engle *et al.*, 1958; Sindermann and Mairs, 1958; Woods and Engle, 1957). The variations in the electrophoretic patterns of fish serum proteins indicate that the function of the various proteins have not been described (Booke, 1964). Thus, because some fish lacked comparable quantities of a protein similar to human gamma globulin, Engle *et al.* (1958) assumed that fish antibodies did not migrate electrophoretically like gamma globulin. The present work reports the identification by physical and chemical methods of a serum protein in the golden shiner, *Notemigonus crysoleucas*, which has antibody activity against *Aeromonas liquefaciens*.

MATERIALS AND METHODS

The golden shiners were obtained from a fish farm in Jackson County, Illinois and were fed a "liver-cereal wet food" (Gordon, 1943) and a commercial pelleted food. Blood was withdrawn by direct puncture of the ventral aorta and heart with a glass capillary tube (100 mm long and 1.2 mm I. D.). The serum samples were subjected to electrophoresis within one week after collection. The optimum sample size of serum applied to the strips was 20 microliters.

The serum proteins were separated by use of the Beckman Model R, Series D, hanging-strip paper electrophoresis apparatus employing barbiturate buffer, pH 8.6, Schleicher and Schull 470 filter paper wicks and Whatman' 3 MM filter-paper strips. Use of a low current (2.5 or 15 milliamps), a dilute buffer (0.05 ionic strength) and refrigeration (6-15° C) controlled evaporation from the strips. The procedure for drying, staining (zinc sulfate-bromphenol blue) and rinsing the strips follows those outlined by Block *et al.* (1958). A Beckman Analytrol equipped with a B-3 cam was used to prepare a graph of the separated proteins. Following the procedure used by others studying fish serum proteins (Vanstone and Ho, 1961; Bouck and Ball, 1965) the protein components were designated with arabic numerals in order of decreasing electrophoretic mobility. Reconstituted human plasma (Versatol) provided a comparative standard in each run.

A bacterin was prepared by sterilizing a mass culture of *A. liquefaciens*, a natural pathogen of the golden shiner (Lewis and Bender, 1961). The culture was isolated locally from diseased fish and identified in our laboratory. The culture was formalin-fixed and a saline-thimersol (1:1000 thimersol) dilution was prepared containing 10^8 bacteria per ml. The immunization schedule consisted of weekly intraperitoneal injections of 0.1 ml quantities of the antigen containing 10^7 bacteria. Standard test-tube serial dilutions were used for determining antibody tier. Because of the small size of the fish, the first trial used pooled serum samples from five fish for antibody determination. In the second trial, individual response was studied by using only the larger females. Challenge tests were intraperitoneal injections of a viable subculture of *A. liquefaciens* used to prepare the vaccine; the number of bacteria was calculated to produce an LD_{100} in 10 days.

The electrophoretic patterns of sera of nonvaccinated and vaccinated fish were compared before and after adsorption with homologous antigen. Sera with adsorbed antibody removed was obtained by using for electrophoresis the serum-saline fluid obtained from the first dilution of the antibody test. Bacteria and adsorbed antibody were removed by centrifugation at 15,000 rpm.

RESULTS

In trials 1 and 2, golden shiners developed antibody titers against *A. liquefaciens* as high as 640 in 42-45 days and 1,280 in 59 days (Table 1). Sera of nonvaccinated fish failed to react with the antigen at the lowest dilution. In trial 2 antibody titers varied between individual fish within treatment groups. All nonimmunized fish were killed when given a challenge dose of more than 1.25×10^7 viable bacteria. Discernible differences in mortality of challenged, vaccinated and nonvaccinated fish were noted after three weeks. However, after four or five immunizing injections there was an obvious increase in the survival of vaccinated fish compared with the nonvaccinated fish (Table 2).

Golden shiner sera was separated into six protein components by paper electrophoresis. Because of their solubility in ammonium sulfate and distilled water, it was determined that components 1 and 2 were albumins, components 3, 4, and 5 pseudoglobulins, i. e., water soluble globulins and component 6, a euglobulin or water insoluble globulin. The degree of sexual maturity of the fish influenced the

TABLE 1. *Antibody titers of golden shiners vaccinated with a killed suspension of* 10^7 *cells of* Aeromonas liquefaciens.

	Reciprocal of antibody titer on day shown[1]							
Trial 1								
Day	0	7	14	21	28	35	42	
Date	(5/3)	(5/10)	(5/17)	(5/23)	(5/31)	(6/7)	(6/13)	
Control (not vacc.)	0	0	0	0	0	0	0	
Vaccinated	0	0	20	40	160	320	640	
No. injections prior to titration	0	1	2	3	4	5	6	
Mean daily water temp. (°C.) during interval		19.8	20.4	20.4	20.4	23.9	24.4	
Trial 2								
Day	0		14	21		35	45	59
Date	(8/20)		(9/3)	(9/10)		(9/24)	(10/4)	(10/18)
Control (not vacc.)	0		0	0		0	0	0
Vaccinated	0		40	320		320	640	1280
No. injections prior to titration	0		1	2		3	4	5
Mean daily water temp. (°C.) during interval		25.6		24.6	24.5	21.3	21.3	

[1]The titers for trial 1 are for pooled serum from 5 fish; the titers for trial 2 represent the modal value of five individuals.

TABLE 2. *Mortality of vaccinated and nonvaccinated golden shiners challenged with intraperitoneal injections of viable* Aeromonas liquefaciens.

Number	No. immunizing doses prior to challenge	No. viable bacteria in 0.1 ml of challenge	Per cent mortality	
			vacc.	nonvacc.
0	0	2.57×10^8	100[1]	100[1]
		2.57×10^7	100	100
		2.57×10^6	100	100
1	1	1.26×10^8	100	100
		1.26×10^7	80	80
		1.26×10^6	20	40
2	2	1.25×10^8	100	100
		1.25×10^7	100	100
		1.25×10^6	80	80
3	3	1.95×10^8	100	100
		1.95×10^7	80	100
		1.95×10^6	20	60
4	4	1.56×10^8	80	100
		1.56×10^7	60	100
		1.56×10^6	20	60
5	5	1.65×10^8	80	100
		1.65×10^7	40	100
		1.65×10^6	0	80

[1]Ten fish were used here for challenge but the remaining data was calculated for mortality occuring from challenge of 5 vaccinated and 5 nonvaccinated fish.

appearance as well as the relative concentration of certain of the proteins. Component 4 from sexually mature fish was the most intensively stained band (Figures 1 A-F). A larger part of component 4 from mature females precipitated as a euglobulin rather than as a pseudoglobulin as in males and nonbreeding females. The decline in the breeding condition of females progressed during the course of study and this change was accompanied by a change in the appearance of the stained proteins. Figures 1 A through L represent the progressive decrease in the amount of component 4 and an improvement in the resolution of components 4 and 5 as the amount of component 4 decreased.

Vaccination resulted in changes in components 2 and 6. Component 6 corresponded in electrophoretic mobility to a gamma globulin, component 2 to an albumin. Before vaccination, component 6 appeared as a diffuse, heterogenous zone of endosmotic flow (Figures 1 A, B, D, F, H, J and K). In vaccinated fish, component 6 was darker, indicative of an increase in the amount of protein migrating to the anodic side of the point of origin, and the appearance of the component was more homogenous, forming a distinctly narrow band (Figures 1 C, E, F and L; to a lesser extent Figure 1 G).

The relative concentration of protein in components 1, 3, 4 and 5 of vaccinated fish differed little from that in the nonvaccinated fish (Table 3) but the relative concentration of components 2 and 6 were conspicuously altered concurrent with the course of vaccination. With one exception (Table 3: period 9-3), the relative amount of component 2 in vaccinated fish was lower than the same component in the nonvaccinated fish. The relative amount of component 6 in vaccinated fish was always higher than the amount in the nonvaccinated experimental controls. No alteration resulting from vaccinations was noted in the electrophoretic mobility of the serum proteins.

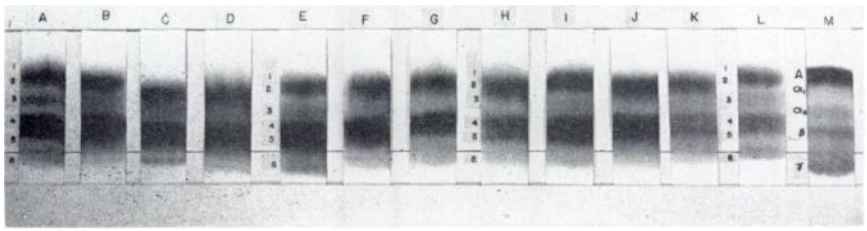

FIGURE 1. *Pattern of bromphenol blue-stained serum proteins of vaccinated and nonvaccinated female golden shiners;* A. *prior to vaccination* (8-10); B. *day of first vaccination* (8-20); C. *after a single vaccination* (9-3); D. *nonvaccinated control for C* (9-3); E. *after two vaccinations* (9-10); F. *nonvaccinated control for* F (9-3); G. *after three vaccinations* (9-24); H. *nonvaccinated control for* G (9-24); I. *after four vaccinations* (10-4); J. *nonvaccinated control for* I (10-4); K. *nonvaccinated control for* L (10-18); L. *after five vaccinations* (10-18); M. *human plasma control. See text for interpretation.*

TABLE 3. *Mean per cent composition of six serum protein fractions of vaccinated and nonvaccinated female golden shiners.*

Period	Number specimens	Number samples	Per cent composition of component number shown					
			1	2	3	4	5	6
			Before treatment controls (both groups)					
8-10	10	20	2.0	20.4	9.7	36.3	27.7	3.9
8-20	1	1	2.4	23.3	11.4	35.2	24.9	2.7
			Nonvaccinated Fish					
9- 3	5	5	2.1	27.2	12.1	35.6	18.4	4.6
9-10	3	6	2.9	25.0	11.4	32.6	23.9	4.2
9-24	5	7	2.9	30.7	10.7	28.7	22.0	5.0
10- 4	4	5	2.4	31.0	9.4	28.2	24.6	2.7
10-18	2	2	2.2	23.1	12.2	30.7	29.1	2.7
			Vaccinated Fish					
9- 3	9	11	3.3	29.2	11.9	28.9	20.0	6.6
9-10	4	5	1.6	18.1	10.4	33.4	28.4	8.4
9-24	6	6	2.1	20.3	10.1	32.0	27.5	7.9
10- 4	6	9	2.1	28.5	9.2	27.8	26.2	6.1
10-18	8	8	1.8	23.5	10.8	32.0	23.0	8.8

Increments in per cent composition of component 6 from 6.6 to 8.8 per cent (Table 3) corresponded to increments in antibody titer from 0 to 1,280 during the same period (Table 1). The largest concentration of component 6 and the highest antibody titers were obtained after five injections over a period of 59 days. Fluctuations in the relative concentrations of component 6 (Table 3) coincided with a drop in water temperature during the interval from 9-24 through 10-4 (Table 1).

The relative concentration of component 6 in sera of nonvaccinated fish after titration of antibody was always greater than the concentration of the same component in the sera of nonvaccinated fish before treatment (Table 4). The lower protein content in sera obtained from the saline dilution used in the antibody titration had the effect of reducing the relative amount of the faster moving proteins while increasing the amount of the slower moving proteins such as component 6. This occurred because of the greater adsorption of components 1 and 2 which have the longest migration path. However, the relative concen-

TABLE 4. *Per cent composition of serum protein component of vaccinated and nonvaccinated golden shiners before and after reaction with antigen.*

Period	Nonvaccinated Per cent composition			Vaccinated Per cent composition		
	Before	After	Diff.	Before	After	Diff.
9- 3	3.1	5.8	+2.7	5.1	6.4	+1.3
9-10	4.8	7.1	+2.3	6.0	3.0	-3.0
9-24	4.7	5.1	+1.4	7.9	2.8	-5.1
10- 4	4.0	6.5	+2.4	7.2	4.4	-3.8

tration of component 6 in the sera of vaccinated fish after adsorption of antibody was always less than in the nonvaccinated controls after similar treatment (Table 4). Removal of absorbed antibody was the apparent explanation for the reduction in the concentration of component 6 in the sera obtained from the agglutination reaction.

DISCUSSION

Intraperitoneal injections of killed suspensions of *A. liquefaciens* produced a linear increase in antibody titer for four weeks, on an arithmetic scale, following which, in spite of additional vaccinations, the titer increased at a decreasing rate. However, maximum antibody titers were equal to or higher than titers reported for other fishes in approximately the same time (Cushing, 1942; Duff, 1942; Krantz *et al.*, 1963).

The amount of serum protein in nonvaccinated golden shiners having an electrophoretic mobility of gamma globulin was small (2.7-5.0 per cent). However, the relative amount of this protein was equal to that previously reported for rainbow trout (*Salmo gairdneri*) by Meisner and Hickman (1962). Moreover, the amount of the component apparently may be considered independent of possible antibody activity, contrary to previous implications by others (Becker *et al.*, 1958; Engle *et al.*, 1958) because in the present work the amount was increased by vaccination; mean values of 6.6 to 8.8% in vaccinated fish compared to 2.7 to 5.0% in nonvaccinated fish. Also, although the amount of protein with an electrophoretic mobility like a gamma globulin may be reduced in some osteichthyians, as reviewed above, other investigators (Chandrasekhar, 1959; Flemming, 1958; Magnin, 1958; Meisner and Hickman, 1962) have reported a sizeable amount of a gamma globulin in other species of fishes.

The relative amount of a fast-moving albumin was always less in vaccinated fish than in nonvaccinated fish. This response is analogous to observed decreases in albumin associated with many infectious diseases (Dunn and Pearce, 1961; Wuhrmann and Wunderly, 1960). Flemming (1958) observed a similar decrease in the relative and absolute amount of an albumin in "acutely ill" carp.

As a conclusion from the foregoing data and discussion, antibody activity was associated with a water insoluble protein with anodic electrophoretic mobility. Flemming (1958) observed an increase in a slow moving electrophoretic protein in sera from carp with "stomach dropsy" caused by *Pseudomonas punctata*, although the changes were not demonstrated experimentally. Sorvachev *et al.* (1962) reported a larger amount of a gamma globulin component in nonimmunized fish. Therefore, the evidence for antibody activity of component 6 permits designation of an immunoglobulin (Nomenclature of Human Immunolglobulins, 1964) in fish although further classification can not be made without additional information on immunological crossreactivity, molecular weight, and carbohydrate content.

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New Wild Bird Hosts For Pox Viruses

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ABSTRACT

Pox lesions were described in three new species of wild birds: Swainson's thrush (*Hylocichla ustulata*), gray-cheeked thrush (*Hylocichla minima*) and brown creeper (*Certhia familiaris*). These birds were cellected during bird banding activities on Long Point, Ontario.

Individual cases of pox infection in wild birds have been observed occasionally during bird banding operations, and epizootics of pox have been described in chipping sparrows (Musselman, 1928) and mourning doves (Kossack and Hanson, 1954). Probably pox virus infection is more prevalent in wild birds than these accounts indicate. The present study was undertaken to determine if there were additional hosts for the virus.

During bird banding activities on Long Point, Lake Erie, Ontario, in the spring and fall of 1965, approximately 16,000 birds were captured and banded. A small number of birds with skin abnormalities were found. Three new avian hosts of pox viruses were captured at the same location, on a small ridge which crossed the peninsula approximately eight miles from its base.

On April 23, 1965 a brown creeper (*Certhia familiaris*) with a pea-sized dark brown nodular growth on the hind toe of the left foot was trapped in a mist net. The weather was cold and wet, and of the more than 700 birds trapped that day, about thirty died either in the nets or immediately after removal from the nets. The affected bird, which appeared otherwise healthy, was kept for further observation in a small holding box, but it was found dead several hours later. The carcass was kept five days at cool outdoor temperatures before it was brought to the laboratory.

Part of the lesion was fixed in 10% formalin. The rest was ground with sterile saline to which antibiotics (750 units penicillin, 750 mg. neomycin, and 750 mg. streptomycin per ml.) were added and used for inoculation of the cho-