USE OF PLANT HEMAGGLUTININS IN SEROLOGICAL STUDIES OF CLUPEOID FISHES

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ABSTRACT

As part of a general immunogenetic study of marine animals, the use of hemagglutinins in seed extracts has been investigated. Individual variations were found in reactions of alewife, blueback herring, and Atlantic herring erythrocytes with certain extracts, particularly those of lima beans. Reactions of Atlantic herring cells with one lima bean extract paralleled those found with certain rabbit antisera. Evidence was obtained for heterogeneity among four spawning populations of alewives with quantitative tests of erythrocyte antigens using an extract of one variety of lima bean.

Plant extracts, particularly those of legume seeds, have been useful in differentiating human erythrocyte antigens (Boyd and Reguera, 1949; Makela, 1957; Boyd, Everhart, and McMaster, 1958; Bird, 1959). This work has engendered interest in possible use of such extracts for blood-grouping studies of other animal species (Bird, 1953). The definitive separations of antigens possible with plant extracts suggest that plant hemagglutinins or lectins may prove useful in systematic and racial studies of fish, as an adjunct to current immunogenetic studies using sera and antisera of animal origin. Several laboratories are presently investigating the use of plant agglutinins in studies of teleosts, and Sprague (1961) has reported their use in studies of oceanic skipjack.

This paper describes the use of selected plant hemagglutinins to distinguish individual differences within species of clupeoid fishes and to characterize spawning populations of alewives.

METHODS

Blood samples were obtained from five species of clupeoid fishes (alewife, *Alosa pseudoharengus;* blueback herring, *Alosa aestivalis;* American shad, *Alosa sapidissima;* Atlantic herring, *Clupea harengus;* and Atlantic menhaden, *Brevoortia tyrannus*) taken in commercial trap nets at Belford, N.J., in April of 1960 and 1961. All fish were adults, although all species were not in the same condition of maturity in April. Blood samples from prespawning populations of alewives were obtained at Narragansett, R.I., in May, 1961; Bourne, Mass., in May, 1961; Damariscotta Mills, Maine, in May, 1961; and Tusket, Nova Scotia, in June, 1961. All samples were from upstream migrants, taken shortly after they had left the sea. Fish were bled by heart puncture with Pasteur pipettes. Whole-blood samples were refrigerated overnight, and serum decanted for other studies. Erythrocytes were washed from clots as needed, washed twice in 1.4 percent saline solution, and used in approximately 5 percent suspensions for testing.

Seeds from several species of Leguminosae were used: lima bean (*Phaseolus limensis*), lentil (*Lens culinaris*), and garbanzo bean (*Cicer arietinum*). Extracts were prepared by addition of 10 ml. of 1.4 percent saline to each gram of powdered seeds. After extraction for 3 hours at 37° C. and over-

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night at 4° C. the mixture was filtered and centrifuged. The supernatant fluid (the extract) was frozen in 3 ml. aliquots until use.

Tube agglutination tests used 0.2 ml. extract dilution and 0.05 ml. cell suspension. Presence and degree of reaction were observed macroscopically after 1 hour incubation at room temperature and 30 seconds centrifugation. Results were recorded conventionally as (++++) complete agglutination, (+++) strong agglutination with a few large clumps, (++) moderate agglutination with numerous smaller clumps, (+) weak agglutination with many very small clumps, and (−) no agglutination. Cells were tested within 72 hours from the time the sample was taken.

Absorptions of extracts in an attempt to obtain specific reagents were carried out with washed cells. One part extract was added to one part packed cells. The mixture was shaken briefly to suspend the cells and incubated at room temperature for varying periods up to 2 hours. Absorbing cells were then settled by centrifugation, and the supernatant absorbed extract tested with a previously removed aliquot of the cells used for absorbing.

RESULTS

Individual Variations in Erythrocyte Antigens

A battery of extracts, including several varieties of lima beans, was used to determine whether individual differences could be detected among fish of each of the species studied. Tests with cells of 10 individuals from each of the five clupeoid species produced results shown in table 1.

Most of the extracts agglutinated herring cells, some extracts agglutinated alewife and blueback cells, only large lentil extract agglutinated menhaden cells, and none of the extracts agglutinated shad cells.

Several extracts gave sufficient distinction among individuals to suggest further study. Individual differences were detected in alewives, blueback herring, and Atlantic herring with hemagglutinins from lima bean varieties 5, 21, 92, 106, and 121, as well as large lentils. Lima bean variety 21, provided in quantity by J. A. Harding, University of California, was selected to determine the extent of individual differences in the three species. Twenty-five individual blood samples from each species were tested, and examples of results obtained are presented in table 2.

Using three doubling dilutions of lima bean variety 21 extract, some individuals were positive at all dilutions, while others were negative at the same dilutions. Most definitive separation of individuals occurred in Atlantic herring. Possible subtypes or dosage effects may be indicated by differences in reactivity of individual fish.

Reactions of Atlantic herring cells with extract 21 were similar to those with certain dilutions of a rabbit antiherring serum (GBH5R), as illustrated in table 3. Since rabbit antisera such as GBH5R have been sources of reagents for detection of erythrocyte antigens of herring, particularly the the C antigen (Sindermann and Mairs, 1959; Sindermann, 1961), the similarity of reactions suggested that certain extracts might be useful as substitutes for antisera. A comparison of 70 individual herring blood samples whose C antigens had been previously determined disclosed that C-negative fish were negative at all dilutions of extract 21 and that C-positive fish were all positive with extract 21.

TABLE 1.—*Reactions of erythrocytes from individual fish of five clupeoid species with seed extracts*

Seed extract	Cells																											
	Alewife										Blueback herring										American shad									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Lima 121	+	+	++	+	+	+	++	+	++	−	−	−	++	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−
106	−	−	+	−	−	−	++	−	+	−	−	−	+	−	−	+	−	++	+	−	−	−	−	−	−	−	−	−	−	−
128	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
75	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	+++	−	++	++	−	+	++	+	++	−	−	−	++	−	−	+	−	++	++	−	−	−	−	−	−	−	−	−	−	−
21	+	−	++	++	−	+	++	++	++	−	−	−	++	−	−	++	−	+	+	−	−	−	−	−	−	−	−	−	−	−
92	+	−	+	+	+	+	++	+	++	−	−	−	++	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−
Garbanzo bean	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Large lentil	++	−	−	+	+	++	+	+	−	−	+	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Small lentil	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE 1.—*Reactions of erythrocytes from individual fish of five clupeoid species with seed extracts*—Continued

Seed extract	Cells																			
	Atlantic menhaden										Atlantic herring									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
Lima 121	−	−	−	−	−	−	−	−	−	−	+++	++++	++++	++++	+++	++	+++	++++	−	++++
106	−	−	−	−	−	−	−	−	−	−	++	++++	++	++	+++	+++	++++	++	−	+++
128	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
75	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−	−	++++	++++	++++	+++	++++	+++	++++	+++	−	+++
21	−	−	−	−	−	−	−	−	−	−	++++	+++	++++	+++	++++	+++	+++	++++	−	+++
92	−	−	−	−	−	−	−	−	−	−	+++	+++	++++	+++	+++	+++	++	++	−	+++
Garbanzo bean	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Large lentil	+	++	+	+++	+	+	++	+++	+	+	+++	++++	+++	+++	++++	++	+++	+++	−	+++
Small lentil	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE 2.—*Examples of reactions of alewife, blueback herring, and Atlantic herring erythrocytes with three dilutions of lima bean variety 21*

Lima bean extract	Alewife cells									
	11	12	13	14	15	16	17	18	19	20
21-undiluted	++	++	+	−	+++	++	−	++	++	−
1:1	+	+	−	−	++	++	−	++	+	−
1:2	+	−	−	−	++	−	−	++	+	−
	Blueback herring cells									
	11	12	13	14	15	16	17	18	19	20
21-undiluted	+	+	−	−	+++	+++	+	−	+	−
1:1	−	+	−	−	++	++	+	−	−	−
1:2	−	−	−	−	++	+	−	−	−	−
	Atlantic herring cells									
	11	12	13	14	15	16	17	18	19	20
21-undiluted	++++	++++	+++	++++	−	++++	++++	++	++++	++++
1:1	+++	++++	+++	+++	−	++++	+++	+	++++	++++
1:2	+++	+++	++	+++	−	++++	+++	+	+++	+++

TABLE 3.—*Examples of reactions of Atlantic herring erythrocytes with plant hemagglutinins (extract 21) and rabbit antisera (GBH5R)*

Reagent	Atlantic herring cells									
	11	12	13	14	15	16	17	18	19	20
Extract 21:										
1:1	+++	++++	+++	+++	−	++++	+++	+	++++	++++
1:2	+++	+++	++	+++	−	++++	+++	+	+++	+++
1:4	++	+++	++	++	−	+++	+	−	+++	++
Rabbit antiherring serum (GBH5R):										
1:128	++++	++++	+++	++++	−	+++	+++	++	++++	+++
1:256	+++	+++	++	+++	−	+++	++	+	+++	++
1:512	+++	++	+	++	−	+	+	−	++	+

Quantitative Studies of Agglutination Reactions in Alewife Spawning Populations

In view of the marked individual differences in reactions of alewife erythrocytes with seed extracts, particularly with lima bean variety 21, widely separated spawning populations were tested for reactions with three dilutions of this extract (1:1, 1:2, 1:4). Numbers of fish negative at all dilutions and positive at all dilutions are presented in table 4. A scoring system comparable to that used by Race, Sanger, and Lehane (1953) and Ridgway, Cushing, and Durall (1958), involving addition of reaction scores of individual fish, was also used. Average scores for each spawning population are presented in figure 1. A test for independence of scores (Snedecor, 1956), pooling

TABLE 4.—*Individual reactions of alewives from four spawning populations with lima bean variety 21 extract*

Location	Number sampled	Number positive at all dilutions	Number negative at all dilutions
Narragansett, R.I.	200	64 (32%)	66 (33%)
Bourne, Mass.	200	112 (56%)	28 (14%)
Damariscotta Mills, Maine	190	7 (4%)	112 (59%)
Tusket, Nova Scotia	73	12 (16%)	33 (45%)

frequencies of test scores of six and more (table 5) indicated that the distribution of scores was dependent on the population studied (chi square= 180.2, d.f.=18, $p<.001$). Only four out of many possible spawning populations were sampled, and a more definitive picture of the discreteness of spawning groups may emerge as more populations are studied.

TABLE 5.—*Frequencies of agglutination scores in four alewife spawning populations*

Scores	Numbers of fish				
	Tusket, Nova Scotia	Damariscotta Mills, Maine	Bourne, Mass.	Narragansett, R.I.	Total
0	33	112	28	66	239
1	15	41	17	27	100
2	11	22	31	30	94
3	3	6	15	22	46
4	1	7	20	14	42
5	6	1	44	15	66
6 or more	4	1	45	26	76
Total	73	190	200	200	663

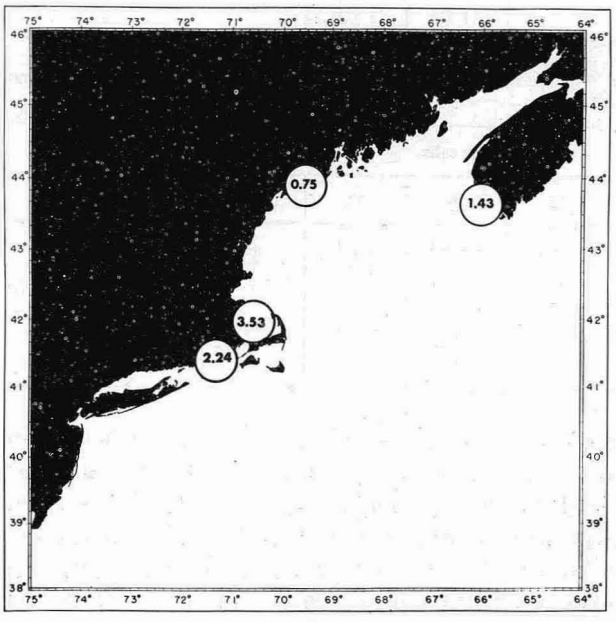

FIGURE 1.—Average reaction scores of four alewife spawning populations with lima bean variety 21 extract.

DISCUSSION

Immunogenetic studies of fishes have advanced rapidly during the past decade, so that quantitative information has already accumulated for subpopulations of several species (Ridgway, Cushing, and Durall, 1958; Sindermann and Mairs, 1959; Suzuki, Morio, and Mimoto, 1959). There must be, however, continuous exploration of new techniques, even as quantitive studies proceed, since adequate serological characterization of subpopulations depends on description and use of several different factors or blood-group antigens. An examination of the possible utility of plant hemagglutinins in serological research constitutes a phase of such exploratory work.

It is premature to attempt genetic explanations for the results of the present work, but the fact that reactions of clupeoid erythrocytes with certain extracts paralleled those obtained with specific rabbit antisera suggests that discrete antigenic factors are involved in the agglutinations. Also, the distributions of reaction scores for several alewife spawning populations lacked the continuous gradation characteristic of polygenic inheritance, but resembled distributions obtained with a series of alleles at a single locus. Individual differences in clupeoid species have been recognized, and this is significant, but information from fractionation of extracts, or from study of known crosses, should precede proposals of genetic systems that may control such differences.

There are advantages and difficulties in the use of plant extracts. Many varieties of many species of plants may be tested, relatively easily and inexpensively. Once a satisfactory variety has been found, any desired amount of extract may be prepared, whereas the amounts of specific antisera are dependent on the blood volume of the experimental animal used. Specific reagents for detection of individual antigenic variation are usually derived from absorptions of sera or antisera. Plant extracts have thus far proved resistant to normal methods of absorption with clupeoid fish cells. Use of unabsorbed extracts may provide valuable information, but further attempts to refine the extracts as reagents should be carried out. Also, the nature of the reaction between animal erythrocyte and extract that produces often highly specific agglutination is poorly

understood, and requires further study. Despite such limitations, plant hemagglutinins offer a promising approach to recognition and characterization of subpopulations of fishes. Plant extracts can form part of a wide spectrum of serological tools available to fishery research.

SUMMARY

Erythrocytes of the five dominant clupeoid species of the western North Atlantic: alewife (*Alosa pseudoharengus*), blueback herring (*Alosa aestivalis*), American shad (*Alosa sapidissima*), Atlantic menhaden (*Brevoortia tyrannus*), and Atlantic herring (*Clupea harengus*) were studied for reactivity with plant extracts. Individual variations were found in reactions of alewife, blueback herring, and Atlantic herring erythrocytes with hemagglutinins present in seed extracts. Several varieties of lima beans provided clear differentiation of individuals. Evidence for population heterogeneity was obtained when blood samples from four spawning populations of alewives were tested against an extract of one variety of lima bean.

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