BLOOD GROUP REACTIVE SUBSTANCES IN SOME MARINE INVERTEBRATES [1]

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This paper reports the occurrence of substances in some marine invertebrates, the specific reactions of which can be closely identified with those of known vertebrate blood grouping systems. Such substances, while found in forms as diverse as vertebrates, spermatophytes and microbes (Boyd, 1962; Springer, Williamson and Readler, 1962), have only rarely been reported in marine invertebrates, even though it is known that erythrocyte agglutinins of broad specificity can be obtained from the body fluids of various invertebrate forms (Tyler and Metz, 1945; Tyler and Scheer, 1945; Tyler, 1946; and additional references in Cushing and Campbell, 1957). In fact, in addition to the reference to "mollusca" and the "lobster" in Boyd's (1956) tabulation of the occurrence of Forsmann antigens, only the "oyster" (Springer, Rose and György, 1954) and the lobster, *Homarus americanus* (Sindermann and Mairs, 1959), appear to have been investigated from this point of view.

Invertebrates used in this present study included the sipunculid, *Dendrostomum zostericolum* (Chamberlain), the inn-keeper worm, *Urechis caupo* (Fisher and McGinitie), the spiny lobster, *Panulirus interruptus* (Randall), and *Octopus bimaculatus* (Verrill) (or *O. bimaculoides* Pickford and McConnaughey, 1949). Additional animals are referred to in the text.

Materials and Methods

Invertebrates were collected at Santa Barbara, with the exception of the inn-keeper worm which came from Newport Beach, California. Whale erythrocytes came from individuals taken off San Francisco, basking shark serum from Santa Barbara, and sea lion serum from the Coronados Islands.

Sipunculid blood samples of 2 to 4 ml. were obtained by puncturing the posterior end of worms with a 25-gauge, $\frac{3}{4}$-inch needle. Hemerythrocytes in these samples were concentrated by centrifugation. Serum and sperm and egg layers were removed by aspiration. The few sex cells and those of other types (*cf.* Triplett, Cushing and Durall, 1958) that remained among the packed hemerythrocytes were observed microscopically not to be involved in the agglutinations to be reported. Hemerythrocytes were washed according to usual serological procedures in isotonic saline (3% sodium chloride). As relatively weak reactions were obtained if the cells used had been standing for several hours, all experiments were completed within two hours after bleeding.

Reactions between sipunculid cells and serums were determined by tube agglutinations. One drop of "2%" cell suspension was mixed with three drops of

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serum dilution, left for 10 minutes, shaken, centrifuged for one minute, and read macroscopically according to conventional methods. Symbols used to describe reactions are as follows: ++++ symbolizes complete agglutination, and +++, ++, +, and − symbolize progressively weaker through negative reactions. Identical symbols record degrees of hemolysis where this was studied. Saline controls showed that cells never autoagglutinated. Absorptions with sipunculid cells were made at room temperature, using two volumes of serum to one volume of packed cells. Such mixtures were kept agitated for 45 minutes, centrifuged, and reabsorbed with fresh cells for 30 minutes. Guinea pig complement was used in the hemolysis of sheep cells.

Octopuses were kept in the laboratory according to methods described by Schuyler (1961). Octopus serum was obtained by hypodermic puncture of one of the sinuses leading to the brachial hearts from animals anesthetized in MS 222. Serum samples could also be obtained from dead animals if fresh, one 60-lb. specimen being bled in this way. Cells and other suspended materials were removed by filtering or centrifuging. Agglutination tests were performed with a view to conserving serum by mixing capillary drops from tubes 1.8–1.2 × 100 mm. with a fine-point needle. Duplicate mixtures could be placed near others in a single depression on a glass plate and covered with glass sealed with Vaseline. These preparations were then put into a Petri dish with moist paper to prevent drying and shaken intermittently on a Yankee Rotator for twenty minutes. Readings were made at 10 × magnification and scored as noted above. Inhibitions were performed by mixing octopus serum and antiserum for 20 minutes, then adding cells and reading after an additional 20 minutes. Three per cent sodium chloride was used except for the washing and suspension of erythrocytes, where 1% sodium chloride was used. No effects of varying salt concentrations were observed between these two figures.

Whale erythrocytes were collected into equal volumes of glycerol-citrate during flensing and kept frozen at −20° C. until use, when they were recovered by dialysis (*cf.* Cushing, Fujino and Takahashi, 1959). Serum of the spiny lobster was obtained from the dorsal sinus, using an 18-gauge needle. Slide tests were used to observe agglutinations, which were recorded as noted above.

Reagents for these studies included anti-human blood typing lectins and serums (Hyland Laboratories, Los Angeles), the normal serums of various animals, and hetero-immune serums prepared by the injection of rabbits.

Observations were generally made at room temperatures of approximately 20° C. and the tests to be reported were repeated at least three and often several times.

Observations

Hemerythrocyte antigens of a sipunculid

A few invertebrates have blood cells comparable to the erythrocytes of vertebrates in that they are specialized for the carrying of respiratory pigments. The sipunculid, *Dendrostomum zostericolum,* is one of these, its cells containing hemerythrin (Prosser, 1952). Information concerning the classification, morphology and ecology of this species is given in Fisher (1952), Hyman (1959), and Peebles and Fox (1933).

TABLE I

Comparison of sipunculid and human cell reactions

Serum	Reciprocal of serum dilutions	Sipunculid	Human cells of types			
			A	B	AB	O
Rabbit, non-immune #4	2	−	+	−	+	−
	4	−	+	−	+	−
	8	−	+	−	−	−
Rabbit, anti-sipunculid #4	2	++++	++++	+	+++	−
	4	+++	+++	+	++	−
	8	+	++	−	+	−
	16	−	+	−	+	−
	32	−	+	−	+	−
Basking shark #60	0	++++	++	−	+++	−
	2	++++	++++	−	++++	−
	4	+++	++++	−	++++	−
	8	++	++++	−	++++	−
	16	−	++++	−	++++	−
	32	−	++++	−	+++	−
Sea lion #3	0	++++	++++	++	+++	++
	2	++++	++++	++	++++	+
	4	++	++++	++	++++	+
	8	+	++++	+	++++	+
	16	−	+++	−	++++	−
	32	−	++	−	+++	−
Anti-A	0	++++	++++	−	++++	−
	2	+++	++++	−	++++	−
	4	++	++++	−	++++	−
	8	+	++++	−	++++	−
	16	−	++++	−	+++	−
	32	−	++++	−	+++	−
Anti-B	0	+++	−	++++	++++	−
	2	++	−	++++	++++	−
	4	+	−	++++	++++	−
	8	+	−	++++	++++	−
	16	−	−	++++	++++	−
	32	−	−	++++	++++	−
Anti-white croaker #1	0	+++	−	−	−	−
	2	+++	−	−	−	−
	4	++	−	−	−	−
	8	+		−	−	−

Reactions of sipunculid hemerythrocytes, and human erythrocytes of various types with different kinds of unabsorbed serums. Explanation of method of testing and scoring will be found in the text.

Preliminary observations were made on the reactions of several serums with washed sipunculid cells. Of these, sheep, cow, and horse serums gave positive reactions, while lobster serum gave negative ones. Rabbit normal serums were very weakly positive, or negative, as was human Rh typing serum (anti-Rh'_0 (CD)), and rabbit anti-human M and N. The reactions of additional serums are given in Table I. These show that rabbits can produce antibodies reactive with sipunculid cells, and that these serums also react with human cells that carry the A specificity. Reactions with normal basking shark (*Celorhinus maximus* Gunner) and California sea lion serums (*Zalophus californianus* Dall) point to a similar correlation, as do those with human anti-A. The reactions of anti-B typing serums suggest that additional antigens occur, and rabbit anti-white croaker (*Genyonemus lineatus* Ayres) serum shows that sipunculid cells have at least one unique specificity within the framework of our observations.

Table II gives examples of the results of absorptions of basking shark serum with various cells which confirm a specific relation between the affinities of sipunculid and human type A cells for a fraction of the shark antibodies. Additional data showed that the antibodies involved did not distinguish between human cells phenotyped as A_1 or A_2.

Table III gives examples of the results of absorbing human typing serums. Absorptions of anti-A showed that human cells carrying A, B and O antigens removed almost all antibodies reacting with sipunculid cells (even when A absorptions were incomplete) while sipunculid cells did not remove any antibodies reacting with A cells.

Absorptions of anti-B serum with these same cells had little or no effect on the antibodies reactive with sipunculid cells, nor did sipunculid cells remove any anti-

TABLE II

Sipunculid and human A cell reactions with absorbed basking shark serum

Cells tested	Reciprocal of serum dilutions	Serum absorbed by cells of				
		None	Sipunculid	Human types		
				A	B	O
Sipunculid	0	++++	−	+++	++++	++++
	2	+++	−	++	+++	+++
	4	++	−	+	+++	+++
	8	++	−	−	++	++
	16	+	−	−	+	+
	32	−	−	−	−	−
Human type A	16	++++	++++	−	++++	++++
	32	+++	+++	−	++++	++++
	64	+++	+++	−	+++	++++
	128	++	+	−	+++	+++
	256	+	−	−	++	++
	512	+	−	−	+	+

The agglutination of various kinds of cells by unabsorbed and absorbed basking shark serum. Explanation of methods of testing and scoring will be found in the text.

TABLE III

Sipunculid and human cell reactions with absorbed anti-A and anti-B sera

Cells tested	Reciprocal of serum dilutions	Serum absorbed by cells of				
		None	Sipunculid	Human types		
				A	B	O
Anti-A						
Sipunculid	0	++++	−	+	+	+
	2	+++	−	−	−	+
	4	++	−	−	−	−
	8	+	−	−	−	−
	16	−	−	−	−	−
Type A	16	++++	++++	+	++++	++++
	33	++++	+++	++	+++	+++
	64	++++	+++	++	+++	+++
	128	+++	+++	−	+++	+++
	256	++	++	−	++	++
	512	+	+	−	+	+
	1024	+	+	−	−	−
Anti-B						
Sipunculid	0	++++	−	++++	++++	++++
	2	++++	−	+++	++++	+++
	4	+++	−	+	+++	+
	8	+	−	−	+	−
	16	−	−	−	−	−
Type B	2	++++	++++	++++	−	++++
	4	+++	++++	++++	−	++++
	8	+++	++++	+++	−	++++
	16	++	+++	++	−	+++
	32	++	+++	++	−	++
	64	+	++	+	−	+
	128	−	+	+	−	+

The agglutination of various kinds of cells by unabsorbed and absorbed human blood typing serums. Explanations of methods of testing and scoring will be found in the text.

bodies reacting with B cells. Two serums from type AB humans failed to agglutinate sipunculid cells while a third did so very weakly. No observations were made on serums from type O persons.

That human cells removed antibodies which agglutinated sipunculid cells without themselves being agglutinated by these antibodies is not unexpected in view of related kinds of experiences by other workers (*e.g.*, Stormont and Suzuki, 1960; Cushing, Fujino and Calaprice, 1963; Stone, 1962; Pirofsky, Cordova and Imel, 1962). Further analysis of the reactions just reported should of course include considerations of the effects of temperature, the specific relation between serum and cell sources, the genotypes of persons providing these materials, and the multitude

TABLE IV

Forssman reactions of sipunculid cells and anti-sipunculid serums

(a) Hemolysis of sheep erythrocytes by anti-sipunculid hemerythrocyte antiserum

Rabbit #5, non-immune control serum		Rabbit #5, anti-sip. antiserum	
Dil.	Hemolysis	Reciprocal of serum dilutions	Hemolysis
0	–	0	++++
2	–	2	++++
4	–	4	++++
8	–	8	++
16	–	16	+
32	–	32	–

(b) Hemolysis of sheep erythrocytes by anti-Forssman antiserum

Anti-Forssman antiserum #1 not absorbed		Anti-Forssman antiserum #1 absorbed with sip. cells	
Dil.	Hemolysis	Dil.	Hemolysis
16	++++	16	++++
32	++++	32	+++
64	++++	64	+++
128	++++	128	++
256	++	256	–
512	+	512	–

(c) Agglutination of sipunculid cells by anti-Forssman antiserum

Non-immune control serum #1		Anti-Forssman antiserum #1	
Dil.	Agglutination	Dil.	Agglutination
0	–	0	++++
2	–	2	++++
4	–	4	++++
8	–	8	+++
16	–	16	+
32	–	32	–

Various tests showing that sipunculid cells have Forssman antigen. Explanation of methods of testing and scoring will be found in the text.

of complexities that have been shown to exist among agglutinogens and the heterogeneous antibodies used to identify them (*cf.* Owen, 1954, for a discussion of this subject).

Table IV shows that sipunculid cells conform with the criteria accepted by Stormont and Suzuki (1958) for establishing the presence of Forssman antigen. Their criterion is that cells carrying this antigen should cross-react with and be able to absorb out some (not necessarily all) of the sheep cell hemolysins present

in an antiserum prepared in rabbits by the injection of guinea pig organs. Such cells should also cause the production of sheep cell hemolysins in rabbits.

In conformance with observations of Stormont and Suzuki, human A cells did not absorb antibodies from our Forssman antiserum. Sipunculid cells, however, did remove some of these antibodies, providing further evidence for the occurrence of Forssman specificity in this species. (Note that lysis of sipunculid cells did not occur with either Forssman or homologous antiserums.)

Hemocyte reactions in a second invertebrate: Urechis caupo

The inn-keeper worm, *Urechis caupo* (Fisher and McGinitie), is a burrowing worm of the phylum Echiuroidea. It is another of the few invertebrate animals whose blood contains pigmented blood cells. In this case, the respiratory pigment is a hemoglobin.

One specimen of this species was obtained through the courtesy of Dr. Albert Tyler, of the California Institute of Technology. Its cells were weakly agglutinated by normal serum from horse, pig, sheep, cow, and turkey. They were not agglutinated by normal serums from the sea lion, porpoise, basking shark, sipunculid, and rabbit. In addition, they did not react with anti-A and anti-B blood typing reagents or with rabbit anti-sipunculid hemerythrocyte antiserum. Therefore, it is apparent that *Urechis* cells differ markedly in their antigenic properties from those of *Dendrostomum.*

Agglutinins

The classical work on invertebrate agglutinins is that of Tyler and his associates in their studies on the serum of the spiny lobster (Tyler and Metz, 1945; Tyler and Scheer, 1945). Among their observations was the finding that several agglutinins were present in this serum, each capable of reaction with antigens on erythrocytes or sperms that were Class-specific among the wide range of organisms tested.

More recently, Sindermann and Mairs (1959) have shown that the unabsorbed serum of the eastern lobster (*Homarus americanus*) differentiates between the erythrocytes of individual Atlantic sea herring (*Clupea harengus harengus* Linnaeus), agglutinating the cells of some fish to titers of 1 in 128, and others only to 1 in 4. This serum has been utilized as a blood typing reagent in studies on herring.

We have found that serum from the spiny lobster is capable of differentiating individual whales within at least four different species. The blood group system involved appears to be the Ju system originally described by Fujino (1953) in finback whales and now known to be widely distributed through the Cetacea. This system consists of a major pair of antigens that determine the three phenotypes Ju1, Ju2 and Ju1·2. The two antigens are detected by properly absorbed rabbit heteroimmune serums. In addition, the Ju2 antigen reacts with "natural" agglutinins in the serums of whales and several species of domestic animals, exists as a series of subtypes, and, at least in the sperm whale, appears to have solubility properties not unlike those typified by the J substance of cattle. Recent papers introducing the literature on these antigens will be found in Fujino (1962), and Cushing, Fujino and Calaprice (1963).

TABLE V

Agglutination of whale erythrocytes by horse and spiny lobster serums

Species	Cells	Horse serum dilutions					Lobster serum dilutions				
		2	10	20	40	80	Und.	2	4	8	16
Sperm	25	++++	++++	++++	+++	++	+	+++	+++	++	−
	* 30	−	−	−	−	−	−	−	−	−	−
Humpback	65	++++	++++	++++	++++	+++	+++	++++		+++	+
	194	++++	++++	++++	++++	+++	++	++++		−	−
	36	++++	++++	++++	++++	+++	+	++		+	++
	153	++++	++++	+++	+	+	−	−		−	−
	132	+++	++	+	+	−	−	−		−	−
	*111	−	−	−	−	−	−	−		−	−
Sei	123	++++	+++	+++	++	+	+	++	++	+	−
	126	++++	++++	+++	++	+	−	+	++	−	−
	122	++++	++++	+++	++	+	−	−	−	−	−
	*136	−	−	−	−	−	−	−	−	−	−
Finback	198	++++					++++	++++	+++	+++	++
	26	++++					+	++	+++	++	+
	23	++++					+	+	+	+	+
	* 37	−					−	−	−	−	−
Blue	*	−					−	−	−	−	−

Examples showing the reactions of whale erythrocytes with horse and lobster serums. Cells reacting with horse serum have the Ju2 antigen, and reacted to different degrees with lobster serum. Cells that did not react with horse serum lack the Ju2 antigen and failed to react with lobster serum (cells marked with an asterisk are examples of all such negative cells). Total number of observations from which examples were taken in this series as follows: sperm whales, one Ju2 positive, nine negative. Humpback whales, five Ju2 positive, thirty-two negative. Sei whales, three Ju2 positive, five negative. Finback whales, eight Ju2 positive and twenty-three negative. Blue whales, one negative.

Table V gives examples of the reactions of whale erythrocytes with spiny lobster serum. These show that this serum has potential value as a sub-typing reagent for the Ju2 antigen, and give support to the prediction of Tyler (1961, p. 491) that lobster serum will continue to yield substances of immunological significance.

Inhibition of human anti-A serum by Octopus serum

Studies made in conjunction with transplantation experiments on *Octopus bimaculatus* (Trump and Cushing, reported by Cushing, 1963) showed that serum of this species did not agglutinate various human erythrocytes known to be carrying the A, B, O, M, and N antigens. Octopus serum was therefore tested for its ability to inhibit commercial typing serums (Hyland Laboratories) reactive with these antigens. No inhibition was observed with respect to anti-B, anti-M, anti-N, anti-H (against O and A_2 cells), or Anti-A_1 lectin (*Dolichos biflorus*). However, isoimmune anti-A was partially inhibited with respect to its reactions with type A human cells. Table VI shows examples of many protocols that consistently demonstrated this inhibition to involve both A_1 and A_2 cells. Relatively more inhibition

TABLE VI

Specific inhibition of human anti-A blood typing serum by octopus serum

	Reciprocal of serum dilutions						
	Not diluted	2	4	8	16	32	Saline control
Human type A_2 cells (individual one)							
Saline added	+++	++++	+++	++	+	+	–
Octopus serum added	+++	+++	+	–	–	–	–
Human type A_2 cells (individual two)							
Saline added	++++	++++	++++	++	+	+	–
Octopus serum added	+++	++	+	–	–	–	–
Human type A_1 cells (individual three)							
Saline added	+++	+++	++	++	++	++	–
Octopus serum added	+++	+++	++	++	+	–	–
Human type A_1 cells (individual four)							
Saline added	++++	++++	++++	+++	++	++	–
Octopus serum added	++++	++++	+++	+++	+	+	–

The above protocols are selected examples of many experiments that consistently showed that octopus serum partially inhibits the agglutination of human type A cells by human anti-A serum, and that relatively more inhibition occurred with A_2 cells than with A_1 cells.

for the agglutination of A_2 cells was consistently indicated, but quantitative confirmation of this point has yet to be made. The inhibitory power of octopus serum disappeared when the serum was diluted to 1 in 32, but was not destroyed when the serum was heated sufficiently to cause protein coagulation (70° C. for 12 minutes). Efforts to absorb and to conjugate the soluble substances onto guinea pig and type O human cells gave some indications of success, but were not carried to a conclusive point.

Other marine invertebrates have not been previously investigated for soluble inhibitors of blood typing reagents with the exception (references in the introduction) of an unidentified species of "oyster." Water extracts of this form contained a non-dialyzable substance capable of inhibiting isoantibodies for human A, and eel anti-H while not inhibiting anti-B. Some cursory examinations of invertebrate serums in our laboratory either yielded negative results or were complicated by the presence of agglutinins and could not be pursued within the time available.

Discussion

The observations reported above are significant not so much individually as collectively in that they support the concept of the ubiquitousness among organisms of substances with specificities and reactivities closely akin to those initially and classically described for the A, B, and O blood grouping antigens and isoantibodies. This concept continues to be involved with such intriguing questions as that of the physiological significance of these widely distributed substances, and that of the biochemical basis for the evolution of the antibody response. Additionally, a better understanding of these substances may lead to their use as genetic markers in invertebrate populations, such, for example, as among the plankton. Markers of this sort are already of established value in distinguishing genetically isolated populations within single species of marine animals, and have great potential value as aids in learning something of the ecological and evolutionary forces that influence these populations (*cf.* a "Symposium on immunogenetic concepts in marine population research" 1962, and Manwell, 1963).

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Summary

The occurrence of blood group reactive substances in some species of marine invertebrates is reported. These include:

1. Antigens on the hemerythrocytes of the sipunculid, *Dendrostomum zostericolum,* that react with various antiserums including human anti-A, human anti-B and anti-Forssman, and a comparison of these reactions with those of the hemocytes of the inn-keeper worm, *Urechis caupo.*

2. A substance in the serum of the spiny lobster, *Panulirus interruptus,* that agglutinates erythrocytes carrying the Ju2 antigen in whale species. These include the sperm, humpback, sei and finback whales.

3. A substance in *Octopus bimaculatus* serum that specifically inhibits the agglutination by anti-A serum of human cells carrying the A_1 and A_2 antigens.

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