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Specific Hemagglutinin and a Modulator of Complement in Cockroach Hemolymph

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Natural hemagglutinin activity against vertebrate erythrocytes is present in the hemolymph of the cockroach *Blabarus craniifer*. The hemagglutinin titer against rabbit erythrocytes is high, whereas sheep and horse red cells agglutinate weakly. Hemagglutinin activity was depressed by the complement inhibitor, cobra venom factor. Cockroach hemagglutinin is heat-labile; all activity is destroyed by heating at 56 C for 1 hr. A humoral factor similar to the complement component 3 proactivator is also present in cockroach hemolymph. The formation of the cobra venom factor-hemolymph "complex" is dependent on the presence of divalent cations and will not proceed at 56 C. The hemolytic intermediate formed after treatment of cockroach hemolymph with cobra venom factor was active in the presence of serum treated with ethylenediaminetetraacetic acid to inactivate the early complement components.

In recent years, a renewed interest in invertebrate immunology and the immune response has augmented the data supporting the view that invertebrates possess the ability to differentiate between "self" and "nonself" and can respond to foreign substances with both humoral and cellular mechanisms. The importance of phagocytosis in the removal of foreign substances from the blood was first recognized by Metchnikoff at the turn of the 20th century (14). Cellular responses involving the encapsulation of large foreign bodies have been observed in several of the phyla of the protostomia (5, 17, 18). The blood of many invertebrates has been shown to contain various naturally occurring and inducible factors which, although mimicking the action of the vertebrate immunoglobulins, frequently lack specificity and fail to show memory (2, 4, 8, 11, 19). Hemagglutinins have been reported to be present in numerous invertebrates: Mollusca (3, 10, 15, 20), Sipunculida (19), and Arthropoda: Crustacea (15) and Insecta (1, 7).

The presence of the complement system or any of its components in the blood of invertebrates has not been as yet thoroughly investigated. It was generally held that no complement-fixing activity was present in invertebrate blood, including insect hemolymph (4). However, Day et al. (6) showed that, while lacking classical complement, the horseshoe crab and the sipunculid worm did possess components homologous to the complement components of mammals which were recognizable after activation with cobra venom factor (CVF).

The classical pathway of complement activation is initiated by an antigen-antibody complex, which is required for the sequential reaction of the complement components. Recent evidence indicates that complement may be activated by alternate pathways such as those initiated by the CVF (16; H. J. Müller-Eberhard, Fed. Proc. **26**:744, 1967; R. J. Pickering, M. R. Wolfson, R. A. Good, and H. Gerwurz, Fed. Proc. **28**:818, 1969) and endotoxin (9, 13). It was reported that a purified, nontoxic CVF eluted from a single band on polyacrylamide gel produced lysis of guinea pig erythrocytes in the presence of fresh serum (R. J. Pickering et al., Fed. Proc. **28**:818, 1969). It was demonstrated that CVF produces an inactivation of the complement system by acting through a serum intermediate on complement component 3 (H. J. Müller-Eberhard, Fed. Proc. **26**:744, 1967; R. J. Pickering et al., Fed. Proc. **28**:818, 1969). This serum factor was first referred to as a proinactivator (H. J. Müller-Eberhard, Fed. Proc. **26**:744, 1967) but has been renamed a proactivator (O. Götze and H. J. Müller-Eberhard, Abstr. 4th Complement Workshop, Johns Hopkins School of Medicine, Baltimore, Md., 1971). The present paper reports the presence of a factor similar to the proactivator in cockroach hemolymph. The term "cockroach proactivator" as used in this paper refers to this substance which is similar, but not necessarily

identical, to vertebrate proactivator in that it forms a hemolytic intermediate with CVF which functions via complement, is heat-labile, and requires divalent cations.

MATERIALS AND METHODS

Experimental animals. The West Indian Leaf Cockroach, *Blaberus craniifer* (Burmeister), was employed in these studies. Colonies were maintained in large cages at room temperature in the laboratory under an 8:16 light-dark photoperiod. Food consisted of ground dog meal supplemented with 1% corn oil and 5% dried yeast; chopped bananas were fed once a week. Water was constantly available.

Collection of hemolymph. The insects were chilled (4 C for 30 to 60 min) and were bled in a refrigerated room to inhibit clotting. Blood was collected from the legs and from the hemocoel and diluted in normal saline or in glucose-gelatin-Veronal buffer (12) containing Ca^{2+} and Mg^{2+} ($GGVB^{2+}$). The dilute hemolymph was centrifuged for 5 min in an Adams Sero-Fuge to separate the cells. The cell-free hemolymph was then concentrated by using an Amicon Diaflow ultrafiltration apparatus with a filter that retained material of molecular weight greater than 1,000. Hemagglutinin and complement proactivator activity was contained in this fraction. The ultrafiltration was carried out at 0 C under 50 to 60 psi nitrogen pressure. All volumes used in the dilution and concentration procedures were carefully controlled to determine accurately the final hemolymph concentration.

Hemagglutination. Cockroach hemolymph was serially diluted in $GGVB^{2+}$ at 0 C in the wells of plastic Microtiter plates. An equal volume of a solution of 10^8 test erythrocytes was added to the wells, and the plates were incubated for 1 hr at 30 C. The cells employed were sheep, horse, and rabbit erythrocytes. After evaluation of agglutination, the Microtiter plates were centrifuged for 10 min at 2,000 rev/min (in Microtiter adaptors designed by the Cooke Engineering Co., Alexandria, Va.) to attempt to demonstrate a natural hemolysin (the supernatant material is colored by hemoglobin released upon erythrocyte lysis). The heat lability of the hemagglutinin was assayed by heating the hemolymph at 56 C for 1 hr before allowing it to react with the indicator cells.

Hemolysis. The Microtiter technique described above was used to search for natural hemolysins. A more quantitative spectrophotometric technique was also employed. Cockroach hemolymph was serially diluted in 0.5 ml of $GGVB^{2+}$ at 0 C, and 0.5 ml of 10^8 test red cells was added. The lysis of sheep erythrocytes was compared to the lysis of antibody-sensitized sheep cells (EA); EA are required to demonstrate complement-dependent lysis. The tubes were incubated for 1 hr at 30 C. The volume of the tubes was made up to 7.5 ml with normal saline and the tubes were centrifuged for 10 min at 2,000 rev/min. The optical densities of the resulting solutions were read at 421 nm on a Beckman spectrophotometer. By plotting the optical densities of the various samples on semilogarithmic paper, one may obtain by interpolation a value for the hemolymph concentration that will lyse 50% of the red cells present (CH_{50}).

Passive lysis. CVF, when combined with serum and a complement source, may lyse unsensitized red cells (16). CVF was purified by diethylaminoethyl column chromatography from Egyptian cobra (*Naja haje*) venom obtained from Ross Allen's Reptile Institute, Inc., Silver Springs, Fla. Cockroach hemolymph was serially diluted in 0.5 ml of $GGVB^{2+}$ at 0 C, and 0.1 ml of CVF was added (0.1 ml of $GGVB^{2+}$ added to controls); 0.5 ml of 10^8 indicator cells also was added. The mixture was incubated and diluted, and CH_{50} was calculated as described above.

"Proactivator" determination. Cockroach hemolymph and CVF (5:1) were incubated for 2 hr at 30 C. Frog serum was diluted 1:20 in 0.06 M ethlyenediaminetetraacetic acid (EDTA) in gelatin-Veronal buffer (12) lacking Ca^{2+} and Mg^{2+} (GVB^{2-}) to inactivate the early complement components, which are metal-dependent. The frog complement EDTA was serially diluted in 0.5 ml of 0.06 M EDTA in GVB^{2-}, and 0.1 ml of the hemolymph-CVF "complex" and 0.5 ml of 10^8 indicator cells were then added. The mixture was incubated, and the CH_{50} was calculated as above. The metal dependency of the "complex" formation was assayed by incubating the hemolymph and CVF in various concentrations of EDTA. The heat lability of the complex was determined by incubating the hemolymph and CVF at 56 C for 1 hr.

Complement dependency of cockroach "proactivator." It was necessary to show that erythrocyte lysis obtained by using cockroach-CVF complex and EDTA-treated frog complement components was truly complement-dependent. The frog serum was either heated for 30 min at 56 C to destroy complement or treated with CVF (1:1) for 60 min at 37 C to inactivate complement. These complement-deficient sera were reacted with the cockroach hemolymph CVF complexes as described above, and the CH_{50} was calculated.

RESULTS

Naturally occurring hemagglutinin. Pooled hemolymph from both adult and larval (late instar) *Blabarus craniifer* was shown to contain hemagglutinin activity (Table 1). The hemagglutinin titer against rabbit erythrocytes was more than 10^3 times greater than the titer against sheep erythrocytes; horse erythrocyte hemagglutinin levels were intermediate. Preliminary evidence indicates that the agglutinin levels obtained with rabbit erythrocytes are higher in late instar nymphs than in adults. The hemagglutinin is not metal-dependent, since its activity is not greatly diminished by 0.04 M EDTA. The presence of CVF in the medium usually had an inhibitory effect upon hemagglutininination. Hemagglutinin titer decreased only slightly after storage of cockroach hemolymph at 0 C for 3 days; prolonged storage without loss of activity is possible

at −70 C. Cockroach anti-rabbit erythrocyte hemagglutinin is heat-labile and can be completely inactivated by heat (56 C for 1 hr).

TABLE 1. *Characteristics of Blabarus craniifer hemagglutinin*

Hemolymph source	Indicator cell[a]	Treatment[b]	Reciprocal of hemagglutinin titer
Adult	Rabbit E	None	5,120
	Horse E	None	320
	Sheep E	None	10
	Sheep EA	None	0
	Rabbit E	1 Day, 0 C.	1,280
	Rabbit E	1 Hr, 56 C.	10
	Rabbit E	0.04 M EDTA	2,560
	Rabbit E	CVF	1,280
	Rabbit E	1 Day, 0 C, CVF	640
Nymph (late instar)	Rabbit E	None	10,240
	Sheep E	None	80
	Sheep EA	None	10
	Rabbit E	CVF	10,240
	Sheep	CVF	20

[a] E, erythrocytes; EA, antibody-sensitized cells.
[b] In addition to incubation for 1 hr at 30 C. EDTA, ethylenediaminetetraacetic acid; CVF, cobra venom factor.

Naturally occurring hemolysins directed against rabbit, horse, or sheep erythrocytes were not present in *B. craniifer* adult or larval hemolymph. Enhanced lysis due to complement action was not observed when sheep EA were incubated with cockroach hemolymph.

Passive lysis using CVF. The addition of CVF to cockroach hemolymph did not initiate passive hemolysis of rabbit or sheep erythrocytes.

Complement component 3 proactivator. Both adult and larval *B. craniifer* hemolymph contained a factor which could complex with, or react with, CVF to form a complex which could lyse sheep erythrocytes via activation of the EDTA-treated frog complement (Table 2). Frog serum was previously shown to contain hemolytic complement which could be activated by hemolymph factors from various invertebrates (6). No lysis of sheep erythrocytes could be demonstrated when guinea pig complement terminal components were used. No hemolysis occurred in controls consisting of CVF and saline incubated at the same temperature and for the same time as the CVF-hemolymph mixtures. The factor could be demonstrated in hemolymph which had been stored at 0 C for 3

TABLE 2. *Frog complement component 3 proactivator in adult Blabarus craniifer hemolymph*[a]

Assay	Complement source	Indicator cell	Complex formation	CH_{50}[b]
Demonstration of complement component 3 proactivator	Guinea pig	Sheep E	CVF[c]-hemolymph; 2 hr, 30 C	0
		Rabbit E		0
		Horse E		0
	Frog	Sheep E	CVF-hemolymph; 2 hr, 30 C	75
		Rabbit E		152
		Horse E		38
	Frog	Sheep E	CVF-saline; 2 hr, 30 C	10
		Rabbit E		23
		Horse E		12
Metal and temperature dependency of "complex" formation	Frog	Sheep E	CVF-hemolymph; 2 hr, 30 C	55
	Frog	Sheep E	CVF-saline; 2 hr, 30 C	20
	Frog	Sheep E	CVF-hemolymph; 0.05 M EDTA[d]; 2 hr, 30 C	46
	Frog	Sheep E	CVF-hemolymph; 0.1 M EDTA; 2 hr, 30 C	41
	Frog	Sheep E	CVF-hemolymph; 1 hr, 56 C	29
Complement dependency of complement component proactivator	Frog	Sheep E	CVF-saline; 2 hr, 30 C	35
	Frog	Sheep E	CVF-hemolymph; 2 hr, 30 C	63
	Frog (CVF, 37 C, 1 hr)	Sheep E	CVF-hemolymph; 2 hr, 30 C	21
	Frog (56 C, 1 hr)	Sheep E	CVF-hemolymph; 2 hr, 30 C	1

[a] Representative data from at least five separate experiments in each major grouping.
[b] Hemolymph concentration required for 50% complete hemolysis of erythrocytes (E) after 1 hr of incubation at 30 C.
[c] Cobra venom factor.
[d] Ethylenediaminetetraacetic acid.

days. The complex in concert with frog terminal component lysed rabbit, horse, and sheep red cells. The assay was run using sheep erythrocytes because the hemolymph contained an agglutinin against rabbit erythrocytes which could have interfered with the test; also, lysis of horse erythrocytes was less extensive than was lysis of sheep erythrocytes.

It was shown that the CVF-hemolymph reaction was dependent upon Ca^{2+} and Mg^{2+} in the medium during complex formation. By chelating the divalent cations with various concentrations of EDTA, it could be demonstrated that the CH_{50} varied directly with the concentration of metals present at the time of complex synthesis. If the CVF-hemolymph mixture was incubated at 56 C, no subsequent lysis attributable to complex formation could be detected.

The lysis of sheep erythrocytes by the complex and a suitable complement source is complement-dependent, and is not attributable to the generation of some nonspecific lytic agent during CVF-hemolymph incubation. If the complement in frog serum was inactivated by CVF or by heating, no lysis of indicator erythrocytes could be induced by cockroach proactivator.

DISCUSSION

A species-specific hemagglutinin has been demonstrated in cockroach hemolymph. The hemagglutinin is present in both late instar nymphs and adults; however, the transition from larva to adult is not abrupt in cockroaches as they undergo incomplete metamorphosis. *Blaberus craniifer* hemagglutinin shows more specificity, and retains its activity longer during storage, than that reported by Feir and Walz (7). It will be interesting to determine whether the hemagglutinin shows specificity towards human erythrocyte antigens, as has been reported for molluscs (10). In vitro phagocytosis of pathogenic and nonpathogenic bacteria by cockroach hemocytes is currently being carried out in this laboratory, and the possible role of cockroach hemagglutinin in opsonization is being investigated. Tripp (20) was the first to suggest such a relationship in invertebrate species.

Day et al. (6) have shown the existence of the complement component 3 proactivator in several protostomes including the primitive arthropod *Limulus*. This report provides evidence that a similar humoral factor is also present in a representative insect. At the present time, it is impossible to show directly the presence of a lytic complement system in the cockroach; however, more sensitive techniques may reveal its presence. It is possible that at least some of the terminal complement components (or a system homologous to these) may exist in insect hemolymph. Attempts to sensitize erythrocytes with antibody-like factors in cockroach hemolymph are presently being made in this laboratory.

Cockroach complement component 3 proactivator shares the characteristic of that described in other invertebrates by Day et al. (6), namely, metal dependency and heat lability during formation and complement dependency of its lytic properties. Subsequent studies are needed to define its molecular nature.

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