An Attempt to Immunize the Blue Crab, *Callinectes sapidus*, with Vertebrate Red Blood Cells

The phylogeny of immune mechanisms has become an interesting area of research in recent years, but very few of the highly evolved invertebrates have been studied. More studies on the internal defense mechanisms of higher invertebrates could provide further insight into the evolution of vertebrate immune responses and a more comprehensive knowledge of these phenomena throughout the entire animal kingdom. Invertebrate animals, unlike vertebrates, do not produce specific antibodies of the gamma globulin type. However, invertebrates, particularly crustaceans, are capable of a variety of adaptive immune responses[1]. These include accelerated secondary antigen clearance[2,3], bactericidins[4–6], precipitins[7], opsonins[8], and passive transfer of xenograft recognition[9]. Additionally, crustaceans possess naturally occurring hemolysins[10] and agglutinins[11–13]. In general, these responses are not very specific, since cross-reaction with heterologous antigens is common[14]. Since many invertebrate animals, including crustaceans, possess natural agglutinins in their serum, a study of blue crab (*Callinectes sapidus*) hemolymph was undertaken to determine: 1. if natural agglutinins were present, 2. if titers could be increased by prior immunization, and 3. if there was cross-reaction with heterologous antigen.

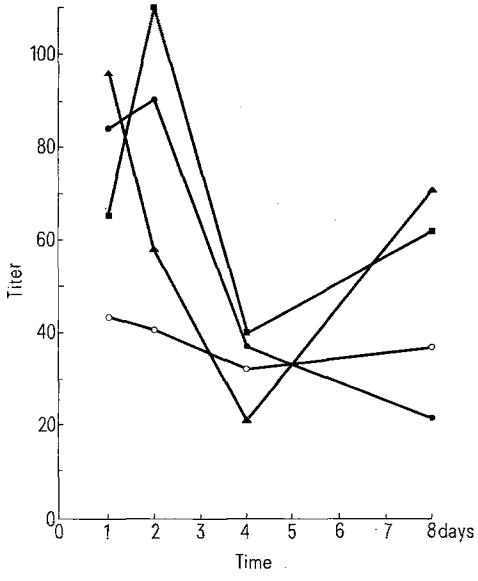
Materials and methods. After the shell was cleaned with 70% ethanol, a 10.0 ml syringe and a 20 gauge needle were used to remove whole hemolymph from the pericardial sinus of blue crabs. Hemolymph was placed in a test tube containing an applicator stick. After 2–4 h at room temperature (27 °C), a clot which formed around the applicator stick was discarded. The clot-free serum was filtered through a Swinnex-25 Millipore filter (0.45 μm pore size) to remove any remaining debris and bacteria. Serum from individual animals was kept separate and was not pooled.

Rabbit red blood cells (RBCs) were obtained by cardiac puncture and preserved in Alsevers solution. Chicken RBCs preserved in modified Alsevers solution were obtained from Flow Laboratories (Rockville, Maryland). Prior to agglutination assay or immunization tests, RBCs were washed 3 times in 0.15 *M* NaCl and centrifuged at 2500 rpm for 10 min at 4 °C before resuspending in 0.15 *M* saline.

For agglutination assay, 0.05 ml of a 2.0% (by volume) rabbit RBC suspension was added to Kahn tubes containing 1.0 ml of serial 2-fold dilutions of sterile crab serum in 0.15 *M* NaCl. Each agglutination series was run in duplicate and the degree of agglutination was determined after 24 h incubation at room temperature. Reactions were graded from very strong (++++) to very weak (+) with the aid of a dissection microscope (30 ×) and expressed as the reciprocal of the titer dilution. Control tubes contained 0.5 ml of 0.15 *M* saline inoculated with 0.05 ml of 2.0% rabbit RBCs.

Three experiments were performed attempting to immunize blue crabs. In each experiment, 0.5 ml of sterile saline containing 2% rabbit or chicken RBCs was injected into the crab's cheliped. Uninjected animals were utilized as one control group, while animals injected with 0.5 ml of sterile saline served as a second control group. Whole hemolymph from 5 different animals in each of the 4 groups was taken 1, 2, 4, and 8 days post injection and assayed for agglutination titer against rabbit RBCs.

Results and discussion. Naturally-occurring agglutinins for both chicken and rabbit RBC were found in the hemolymph of blue crabs. The normal titers ranged from 16–64. These titers appeared to be increased slightly by immunization (Figure). The modest increase was most noticeable in the first 2 days and subsequently declined. This immediate short-lived response is typical of invertebrates[15,16]. However, it should be noted that in at least one invertebrate, a sipunculid, an increased secondary immune response did not appear until 60 days after immunization[17]. Additional studies are needed to deter-



Blue crab hemolyph agglutinin titers following injection of vertebrate red blood cells. Each point represents the mean titer of 5 individual crabs, immunized with rabbit RBCs (■—■), chicken RBCs (▲—▲), and controls which are uninjected (○—○), or injected with sterile saline (●—●).

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mine positively whether the response observed in the blue crab is truly an adaptive immune phenomenon. Since both a heterologous antigen (chicken RBC) and a sterile saline evoked a slight increase in agglutinin titer, comparable to that of homologous antigen (rabbit RBC), this response may represent a simple variation of the normal titer range due to any stress. It is certainly not unusual to find an adaptive immune response completely lacking in invertebrates[18,19]. The current work indicates at best a very weak adaptive immune response and one without specificity, similar to that of other invertebrates. Studies of the blue crab agglutinin are now underway to ascertain its physicochemical properties and its function, since it has recently been shown that invertebrate agglutinins may play a significant role in the animals' defence by acting as opsonins[20].

Résumé. Nous avons essayé d'augmenter le titre de l'hémagglutinine naturelle qui se trouve dans le sérum du crabe bleu, *Callinectes sapidus*, par l'inoculation d'hématies de vertébrés. On a noté une augmentation légère de ce titre après 48 h, mais elle n'était apparemment pas spécifique, puisque on peut la provoquer avec l'antigène hétérologique et une solution saline ainsi que l'antigène homologique. Il appert qu'il s'agit là d'une réponse intérieure défensive et non-spécifique due à quelque force extérieure.

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Artero-Venous Differences in Blood Platelet Clumping by ADP

The contribution of the vessel wall (like that of different tissues) to the blood haemostatic and coagulative properties has as yet been scarcely studied[1–5]. The literature in this field is very conflicting; consequently we undertook experiments to verify whether there are some differences between arterial and venous blood in platelet aggregation induced, in vitro, by ADP.

Methods. Blood samples were collected from adult rats lightly anaesthetized with ether and then with urethan (Carlo Erba, Milano, 400 mg/kg body wt.) and Na nembutal (Abbot, Aprilia, 30 mg/kg body wt.) by a plastic syringe from different vessels: femoral vein, renal vein, abdominal aorta. Collection from the heart was performed from the right and left ventricle in openchest animals submitted or not to artificial ventilation. Blood was immediately added to the sodium citrate solution in a siliconized centrifuge tube (1 vol of 3.8% sodium citrate to 9 vol. of blood). The samples were centrifuged for 13 min at $100 \times g$. The supernatant platelet-rich plasma (PRP) was collected by siliconized Pasteur pipets and transferred into a plastic container; the platelet count was then estimated according to Rees and Ecker[6]. The remainder of the samples was centrifuged at $700 \times g$ for 15 min to obtain platelet-poor plasma (PPP).

PRP was diluted with PPP to a standard number of 700,000 platelets/μl. This PRP was divided into polystyrene tubes, stored at room temperature, and then ADP (Na_3ADP- C.F. Boehringer and Soehne H-Mannheim – final concentration $9.2 \times 10^{-6} M$) was added for the examination of the optical density (O.D.) by a 169 Platelet Aggregation Meter (Evans Electroseleniun Ltd), at a stirring rate of 2,800 rpm. The O.D. variations were recorded by Speedomax XL 690 Series Recorder (Lees and Northrup, North Wales and Philadelphia). PPP served as a blank to adjust the 100 point and PRP served to adjust the zero point on the optical density scale for each assay.

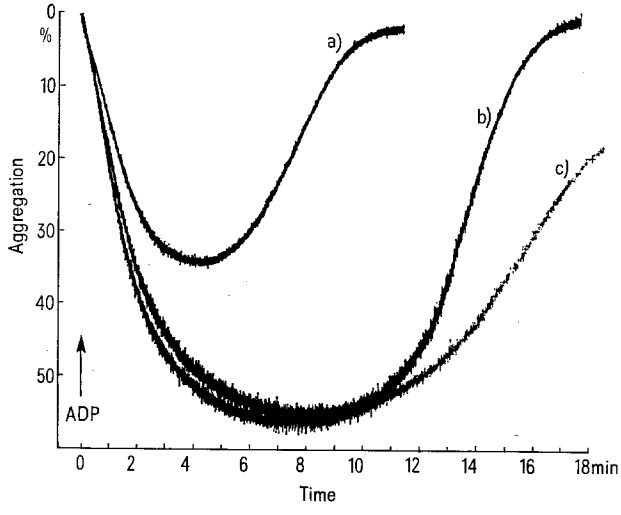

Fig. 1. Changes in O. D. induced by ADP (final concentration $9.2 \times 10^{-6} M$) in PRP (700,000 platelets/μl) obtained from aorta (a), femoral vein (b), renal vein (c).

Results. Figures 1 and 2 show the O.D. changes observed in PRP obtained from aorta (Figure 1a), femoral vein (Figure 1b) and renal vein (Figure 1c), from left ventricle of rats submitted to artificial ventilation (Figure 2a) or not (Figure 2b), and from right ventricle (Figure 2c).

The number of experiments was: right ventricle 18; left ventricle with ventilation 19; left ventricle without ventilation 18; aorta 15; femoral vein 9; renal vein 12.

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