Induced Bactericidal Response in the California Spiny Lobster *Panulirus interruptus*[1] (34160)

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It has been suggested (1–3) that invertebrates do not exhibit adaptive or induced immune responses as defined by Good and Papermaster (3). It is true, however, that invertebrates do possess a variety of defense mechanisms, some of them fulfilling, in part, criteria of induced immunity. Investigations of these systems have been reviewed elsewhere (1, 2, 4–17). Recently, it has been shown (8) that invertebrates (Annelida) can reject xenografts and a crustacean, *Panulirus argus* has been shown to synthesize an inducible bactericidin (12). Because of the importance of such induced responses to an understanding of the evolution of immunoglobulins and cell-mediated reactions, it is imperative that investigations be initiated with key representatives of the major invertebrate phyla.

The present report describes primary and secondary induced bactericidal responses of the California spiny lobster, *Panulirus interruptus* together with preliminary observations on specificity of this potentially important system.

Materials and Methods. Adult specimens of *P. interruptus* were maintained in running sea water at the Marine Laboratory, Department of Biological Sciences, University of California at Santa Barbara. Weights of the lobsters ranged from approximately 1 to 16 lb. Injections were made into the pericardial sinus, and hemolymph samples were withdrawn from the same region both before and at various times after immunization. To prevent clotting of the hemolymph, it was necessary to add 0.2 ml of saturated sodium citrate/ml of hemolymph. Clotting was also prevented by stirring with glass beads. Hemolymph samples of 1 ml were removed at each bleeding from the smaller animals; 2 ml were removed from larger specimens. Cells were removed by centrifuging. The inoculum used was a formalin-killed suspension of gram-negative rods, strain EMB-1. This culture was isolated from the gut of a healthy spiny lobster (12).

Following a primary injection of 10^9 cells of EMB-1 in 0.5 ml of 0.9% sodium chloride solution containing 0.3% formalin (v/v), bleedings were made at 1, 2, 4, and 7 days. Two months after the primary injection, the animals were again bled ("60 days") and then given a secondary injection identical to the primary stimulus. They were bled at 12 hr, 1, 2, 3, 4, and 7 days. Similar experiments were conducted with other groups receiving (a) *Salmonella typhosa* H (STH) (10^9 cells); (b) bovine serum albumin (BSA) Armour, recrystallized, dissolved in 0.9% NaCl (10 mg/ml) and (c) 0.9% NaCl solution containing 0.3% (v/v) formalin. The bacterial inoculums were suspended in this latter diluent. All groups were injected with a volume of 0.5 ml. Sham controls received no material but were punctured with a hypodermic needle and bled along with other groups. The ability of serial twofold dilutions to kill the assay culture, EMB-1 was measured by the procedure of Schwab and Reeves (18). Although EMB-1 has not been completely identified, its biochemical properties have been investigated (19).

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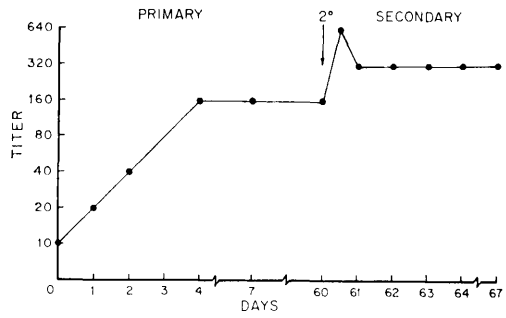

FIG. 1. Primary and secondary bactericidal responses after injection of EMB-1.

Results. The primary response of the *P. interruptus* in Fig. 1 was characterized by a gradual rise to a peak bactericidal titer of 160 at 4 days. The titer then remained at this level throughout the next 2 months. After secondary injection, there was a rapid rise of two $\log_2$ at 12 hr followed by a one $\log_2$ drop at 24 hr; the bactericidin remained on this plateau until termination of the experiment at 67 days.

The animal in Fig. 2 produced a somewhat more irregular primary response with a peak of 160 at 7 days. By day 60, this titer had declined two $\log_2$. After the secondary intracardial injection of EMB-1, there was a rather slow rise in bactericidal titer which did not reach a peak until day 63; the peak was one $\log_2$ above the primary maximum. One other animal (not shown) displayed a secondary rise of two $\log_2$ over the primary response. Two other lobsters failed to survive until the completion of the experiment.

The injection of STH also engendered a response that could be detected with the EMB-1 bactericidal assay system (Fig. 3).

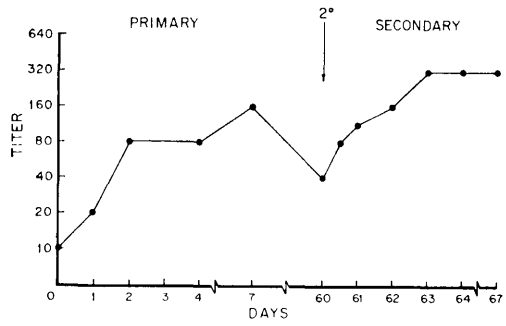

FIG. 2. Responses of another lobster to EMB-1.

In these animals, there was a drop in titer by day 60, followed by a rapid rise within 1 day after the secondary injection.

The above experiment indicated that the response was not entirely specific. This was clarified by the data in Fig. 4 in which geometric mean titers are shown for groups of lobsters injected with four different substances. All of these primary responses were assayed with the EMB-1 system.

All animals had preexisting titers which suggested some previous contact and response to other bacteria, possibly gram-negative rods from gut flora. The 3 lobsters injected with EMB-1 reached the highest titer followed by the three receiving STH.

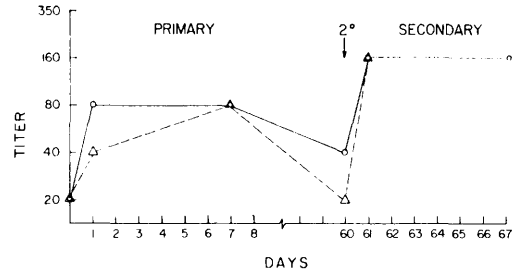

FIG. 3. Responses to injections of *S. typhosa* H (STH).

Responses of the four animals injected with BSA (5 mg) were not significantly different from controls receiving saline–formalin or the one sham control. Rises of one $\log_2$ or less were the rule in all three of these groups. This is generally conceded to be within the limits of experimental error for doubling dilution assays of this type.

Discussion. Previous failures to detect induced responses among the invertebrates may have been due in part to use of the wrong inoculum. In this case, a gram-negative bacillus isolated from the gut of a healthy lobster provided both the inoculum to induce a response and the assay system to detect it. It is true, of course, that somewhat diminished responses could also be produced by injections of STH. The rabbit produces antibodies which can distinguish sharply between EMB-1 and *S. typhosa*; hence, the specificity of the lobster material is less than mammal-

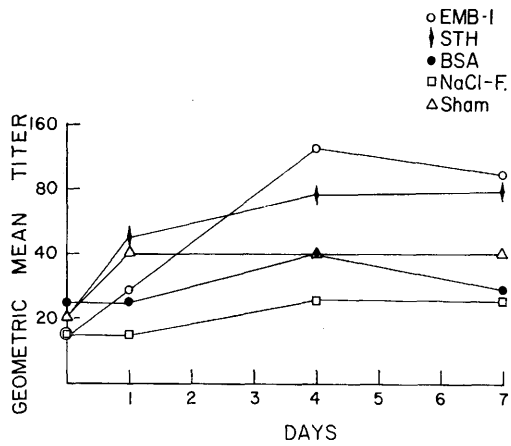

FIG. 4. Specificity of the response measured by the EMB-1 assay system. All titers are geometric means except the sham. The following numbers of animals were used in each group: three *S. typhosa* H (STH); four bovine albumin (BSA); four NaCl-formalin; one sham control.

ian divalent antibody. The lobster response has some degree of specificity, however. There were only insignificant bactericidal rises following the injections of BSA or NaCl–formalin.

Little is yet known about the structure or valency of the lobster material, although further experiments are in progress. It may be precipitated by ammonium sulfate without loss of activity and cannot be inactivated at 60° for 20 min; it coagulates at 65°. It does not agglutinate the bacteria used for induction. This may indicate that it is univalent.

The bactericidal responses of *P. interruptus* are sufficiently suggestive of the primary and secondary responses of mammalian antibody production with respect to rate, level and duration to warrant further investigation. It is probably significant that a single injection of inoculum will trigger a rise which reaches a plateau in some animals where it may remain for at least 60 days. The bactericidal responses of other invertebrates are transient (5–7, 12) and frequently remain elevated for only a few days. Currently, our attention is focused on structural aspects of this bactericidin to see if the analogy between it and mammalian antibody is evident at the molecular level.

Summary. When killed gram-negative bacilli (EMB-1) were injected into California spiny lobsters (*Panulirus interruptus*) an induced bactericidin appeared in the hemolymph. This reached a maximum level within 7 days after the primary injection and persisted without further stimulation for at least 60 days. Some elevation of bactericidal titers followed secondary injections of the same bacteria after 60 days. This rise was reminiscent of the anamestic response in antibody synthesis of higher vertebrates. Specificity of the response was not absolute, but the degree of induced reactivity decreased in the order: homologous inoculum (EMB-1) > *Salmonella typhosa* > bovine serum albumin. The response to bovine serum albumin was no greater than a sham control or those controls inoculated with sodium chloride–formalin solution.

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