Characterization of a Defense Complex Consisting of Interleukin 1 and Phenol Oxidase from the Hemolymph of the Tobacco Hornworm, Manduca sexta*

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Hemolymph of fifth instar Manduca sexta larvae collected under non-sterile conditions exhibited the presence of a novel high molecular weight protein complex, which was absent from the hemolymph collected aseptically. The high molecular weight complex consisted of, at least, prophenol oxidase, phenol oxidase, and an interleukin 1-like molecule, thereby demonstrating the generation of this complex as a consequence of a host response, while the native phenol oxidase and the interleukin 1-like molecule possessed molecular weights of about 80,000 and 17,000, respectively, the complex had a molecular weight of about 400,000. Apart from prophenol oxidase, phenol oxidase, and interleukin 1, dopachrome isomerase and other, as of yet unidentifed, proteins may be part of the complex as judged by the presence of additional bands observed during SDS-polyacrylamide gel electrophoresis. The significance of the assembly of this defense complex for insect host defense strategies is discussed.

Invertebrate organisms have developed a plethora of defense reactions to fight invading parasites and pathogens. Insects and other arthropods in general do not possess immunoglobulins found in higher animals, although proteins containing immunoglobulin-like domains have been identified in insects (1). Synthesis and secretion of antibacterial and antifungal proteins, agglutination and nodule formation, encapsulation of foreign objects, and phagocytosis are a few of the defense mechanisms insects use to protect themselves against infectious agents (2–6). During defense reactions, invariably the foreign organisms are found to be encapsulated and melanized (5–12). Therefore, melanin and the enzyme responsible for its biosynthesis (i.e. phenol oxidase) are considered an integral part of insect host defense reactions. Phenol oxidase is present throughout the body of insects including the open circulatory system of hemolymph (5–8). Active phenol oxidase is deleterious as it can catalyze the oxidative polymerization of phenols and catechols, but in doing so, it can also polymerize proteins and other macromolecules, posing a potential threat to the host (7, 8). Hence phenol oxidase is preserved as an inactive proenzyme form (prophenol oxidase) and is specifically activated proteolytically when needed (5–8). We have been examining the control mechanisms associated with the prophenol oxidase cascade and discovered: (a) a protease inhibitor controlling the prophenol oxidase cascade (9), (b) quinone isomerase that inactivates deleterious 4-alkylquinones that are formed during phenol oxidase action (10), and (c) phospholipid-mediated activation of prophenol oxidase (11).

In insects and other arthropods, phenol oxidase also forms the terminal components of a cascade of reactions resembling the complement system of vertebrates (5–8, 11, 12). As a consequence, during invasion by a foreign organism, which is too large to be phagocytosed, inactive prophenol oxidase found in the hemolymph gets activated and participates in the encapsulation and melanization of the intruder. Thus damage that can be caused by the foreign object is limited by physical isolation. In addition, quinonoid compounds, being highly cytotoxic (13, 14), could participate in the killing process, but this hypothesis requires verification (7).

Numerous studies have been carried out on the activation of prophenol oxidase in insects (8). Some authors have suggested that it is part of a recognition system (6). This hypothesis needs to be proven although there is no doubt that the generation of phenol oxidase is a consequence of final reactions triggered by the host defense system.

Cytokines are polypeptide mediators released by a variety of activated immune and nonimmune cells and are major immunoregulatory proteins in mammals (15). Equally important are the effects of cytokines as mediators of nonspecific host defense mechanisms (15, 16). Biochemical characterization and studies of the molecular biology of cytokines from a number of vertebrate and invertebrate species have revealed basic similarities in the structure and biological properties of these important host defense molecules (15–18). Isolation of cytokine-like molecules from several invertebrate phyla attests to their importance to animal host defense systems (15, 18).

The cytokine interleukin (IL)1–1 was one of the first cytokines described (16, 17). It is an 18-kDa protein that has numerous host defense-related properties. The pivotal role of IL-1 in the immune response is demonstrated by its varied activities. Among its myriad activities, IL-1 elicits the release of neutrophils from the bone marrow, increases the expression of IL-2 receptors on lymphocytes, and stimulates T cell proliferation (16). In addition, IL-1 functions as a critical molecule in innate host defense responses. Thus, IL-1 stimulates acute phase protein release from the liver, causes the extravasation of neutrophils, increases vascular permeability, and regulates the production of fever (16, 17). We have characterized IL-1 and other cytokine-like molecules from invertebrates and studied the release of these critical mediators by invertebrate coelomocytes (15, 18).

In this communication we describe the assembly of a novel...
Defense Complex of M. sexta

EXPERIMENTAL PROCEDURES

Animals and Collection of Hemolymph—Larva of M. sexta were raised on a synthetic diet (19). Fifth instar larvae were used in all experiments. To collect hemolymph under aseptic conditions, the larvae were surface sterilized with 70% alcohol, and one of the posterior legs was cut with sterile scissors. The hemolymph was collected in a sterile tube and used immediately for biochemical analysis. To collect the hemolymph under non-sterile conditions, no such precautions were used. The hemolymph was collected without sterile technique in a non-sterile tube. Sterile hemolymph remained bluish green for more than 1 h, whereas the non-sterile hemolymph readily blackened, indicating activation of phenol oxidase in the sample and subsequent production of black melanin pigment (20).

Column Chromatography—The hemolymph was chromatographed on a Sepharose 6B column (91.5 × 2.5 cm) in a volume of 5 ml and eluted using 50 mM sodium phosphate buffer, pH 6.0, at a flow rate of 1.2 ml/min at 4 °C. The effluent was monitored at 280 nm, and 5-ml fractions were collected. The standards used to calibrate the column were: thyroglobulin (669,000), apoferritin (443,000), β-amylase (200,000), alcohol dehydrogenase (150,000), and bovine serum albumin (66,000) (Sigma).

Assay of Phenol Oxidase and Prophenol Oxidase Activity—Phenol oxidase activity was assayed as described previously (21) using a reaction mixture (1 ml) containing enzyme protein, 2 mM dopamine, and 50 mM sodium phosphate buffer, pH 6.0. The mixture was incubated at room temperature, and the absorbance increase due to dopamine-oxidase isomerase was visualized on the gels by the staining procedure for phenol oxidase (25). In insects, this enzyme catalyzes the activation of phenol oxidase in the sample and subsequent production of black melanin pigment (20).

Native PAGE was performed as described previously (18). For Western analysis, gels were electroblotted in Towbin's transfer buffer and stained individually for both phenol oxidase and prophenol oxidase with dopamine and 3-methoxyindole and providing the indole for further oxidation by tyrosinase. Repeated attempts to detect this enzyme in the complex did not yield consistent results. In only 10% of our experiments could we observe the association of phenol oxidase in this peak. The native Mr, of prophenol oxidase is about 80,000 (21). Since the active enzyme is produced by the proteolytic severing of a peptide from the proenzyme (5, 21), one would naturally expect the active enzyme to possess a Mr, of less than 80,000. In contrast, the approximate Mr, of the phenol oxidase generated under non-sterile conditions was determined to be about 400,000 (Fig. 1a), indicating the self-polymerization of phenol oxidase and/or complex formation with other hemolymph proteins.

RESULTS AND DISCUSSION

Fig. 1 shows the elution profile of M. sexta larval hemolymph proteins collected under both non-sterile and sterile conditions. From the figure it is evident that a high molecular weight (Mr) complex is present in the hemolymph collected under non-sterile conditions (Fig. 1a, fractions 64–73), which is noticeably absent in the sterile hemolymph (Fig. 1b). Phenol oxidase is known to be activated during non-sterile conditions in M. sexta (20) as well as most other arthropods (8). Therefore, we reasoned that the new peak is due to active phenol oxidase. Accordingly, activity measurements confirmed the presence of phenol oxidase in this peak. The native Mr, of prophenol oxidase is about 80,000 (21). Since the active enzyme is produced by the proteolytic severing of a peptide from the proenzyme (5, 21), one would naturally expect the active enzyme to possess a Mr, of less than 80,000. In contrast, the approximate Mr, of the phenol oxidase generated under non-sterile conditions was determined to be about 400,000 (Fig. 1a), indicating the self-polymerization of phenol oxidase and/or complex formation with other hemolymph proteins.

Melanin production in animals is generally enhanced by the enzyme, dopachrome isomerase (25). In insects, this enzyme catalyzes the conversion of L-dopachrome to 5,6-dihydroxyindole and provides the indole for further oxidation by tyrosinase. Therefore, the presence of dopachrome isomerase usually enhances melanin production. Repeated attempts to detect this enzyme in the complex did not yield consistent results. In only 10% of our experiments could we observe the association of dopachrome isomerase with the large Mr, peak (data not shown). Therefore, the association of dopachrome isomerase with the complex is unclear at this time.

We then focused our attention on the characterization of an
IL-1-like molecule. During a comparative search for IL-1 molecules in different arthropods, we analyzed the hemolymph of *M. sexta* for IL-1-like activity. As shown in Fig. 3, the presence of an IL-1-like protein with a *M* r close to that reported in both vertebrates and invertebrates (~20 kDa) (15–18) could be readily demonstrated in the hemolymph of *M. sexta* (fractions 173–186). But what is more intriguing is the association of cytokines by the plasmaprotease inhibitor (26). It usually entails the binding of cytokines to the plasma protease inhibitor (26). It usually entails the binding of cytokines to the plasma protease inhibitor (26). It usually entails the binding of cytokines to the plasma protease inhibitor (26). It usually entails the binding of cytokines to the plasma protease inhibitor (26). It usually entails the binding of cytokines to the plasma protease inhibitor (26). It usually entails the binding of cytokines to the plasma protease inhibitor (26). It usually entails the binding of cytokines to the plasma protease inhibitor (26). It usually entails the binding of cytokines to the plasma protease inhibitor (26). It usually entails the binding of cytokines to the plasma protease inhibitor (26).

Further confirmation for the presence of IL-1-like proteins in the complex comes from Western blot analysis of the hemolymph proteins probed with antibodies to human IL-1. As shown in Fig. 2, the high *M* r complex peak readily cross-reacted with the antibodies to human IL-1, confirming the presence of an IL-1-like protein in the complex.

To identify further the participation of an IL-1-like protein in the complex, the antibodies to human IL-1α and -β were used in the bioassay for IL-1. The high *M* r peak (Fig. 3, fractions 64–73) was incubated with antibodies for 30 min before the addition of the cells. As can be seen in Table I the antibodies to human IL-1, alone or in combination, inhibited the insect IL-1-like activity. As we have observed in previous studies using human antisera with invertebrate IL-1-like proteins, a maximum of ~60% inhibition in the A375 bioassay was observed (18). Negative controls (column buffer, fractions 4–14; and nonspecific proteins, fractions 130–145) incubated with the antibodies had no activity. In addition, a nonspecific control antiserum (rabbit antibody to human IgG) had no effect when added to the high *M* r peak (data not shown).

Large *M* r host defense-related complexes have been described in mammalian systems (26). It usually entails the binding of cytokines by the plasma protease inhibitor (26). It usually entails the binding of cytokines by the plasma protease inhibitor (26). It usually entails the binding of cytokines by the plasma protease inhibitor (26). It usually entails the binding of cytokines by the plasma protease inhibitor (26). It usually entails the binding of cytokines by the plasma protease inhibitor (26). It usually entails the binding of cytokines by the plasma protease inhibitor (26). It usually entails the binding of cytokines by the plasma protease inhibitor (26). It usually entails the binding of cytokines by the plasma protease inhibitor (26).

The function of this large *M* r complex in *Manduca* is only informative at this time. The assembly of this complex in the activated hemolymph attests to its host defense-related activity. When the prophenol oxidase cascade is activated, the phenol oxidase should be deposited on foreign (non-self) matter so that melanization can take place (30). The generation of toxic quinonoid products in the circulatory system would be deleterious to the host. Therefore, the open circulatory system of insects dictates localization of phenol oxidase action at the desired sites only. One way this could be achieved is by the generation of a complex consisting of, at least, phenol oxidase with subsequent deposition on the foreign matter. The recently identified thiol ester sequence of *Manduca* prophenol oxidase (21) may be involved in covalent binding to cell surfaces (i.e. the complex may possess characteristics to allow covalent binding to the foreign object). In addition, the complex may have effects on the distribution, availability, and clearance of the IL-1-like molecule. This would direct the cytokines to the site of infection rather than distributing them throughout the hemolymph. It may also be involved with inhibiting the degradation of the cytokine(s) as has been shown for cytokineα2-M complexes (27, 29).

In conclusion, these data indicate the formation of a high *M* r complex during an experimental insect host defense response.

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**Table I**

| Sample                   | Rabbit antiserum | Percent inhibition |
|--------------------------|------------------|--------------------|
| Buffer control (pooled fractions 4–14) | Anti-rhIL-1α   | 0%                 |
| Buffer control           | Anti-rhIL-1β     | 0%                 |
| Control (pooled fractions 130–145) | Anti-rhIL-1α and -β | 0%               |
| Control                  | Anti-rhIL-1α     | 0%                 |
| High M r, peak (pooled fractions 64–73) | Anti-rhIL-1α | 43.06 ± 5.69a,b,c |
| High M r, peak            | Anti-rhIL-1β     | 51.64 ± 5.58b     |
| High M r, peak            | Anti-rhIL-1α and -β | 61.33 ± 6.34a     |

* a Contains no IL-1-like activity.
* b Mean ± S.D.
* c p < 0.01.

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**Fig. 2.** Native and PAGE analysis of the *Manduca* high *M* r complex. Pooled fractions 64–73 from the Sepharose 6B column (see Fig. 1a) were concentrated by ultrafiltration and run on a 4–20% gradient native PAGE gel. The gel was stained individually for both phenol oxidase (lane 1) and prophenol oxidase (lane 2). A Western blot (lane 3) was performed using a mixture of rabbit antisera to human rhIL-1α and -β. Using SDS-PAGE, the peak contained only two bands when stained with Coomassie Blue.

**Fig. 3.** Presence of IL-1-like activity in the high *M* r complex. Concentrated hemolymph from the fifth instar larvae of *M. sexta* was chromatographed on a Sepharose 6B column (see Fig. 1a). Column fractions were tested in the A375 assay. The data presented are for the 1:64 dilution. ○, IL-1 activity; ●, A20. The positions of the *M* r markers are shown across the top of the figure.

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The complex is made up of at least an IL-1-like molecule, prophenol oxidase, and phenol oxidase. At this time, other components of this complex are being sought. Further experiments on the physiological role as well as the biochemical composition and metabolic fate of this novel complex are ongoing in our laboratories.

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