structure ranging from a deletion of about 30 amino acids in proinsulin (*19*) to single amino acid changes in tyrosyl–transfer RNA synthetase (*20*) and dihydrofolate reductase (*21*). In each of these cases examination of x-ray crystal structure data has contributed to the design of the alteration. In modeling disulfide bonds, computer graphics analysis of crystal structure data may be especially important, since there appear to be important configurational as well as steric constraints on protein disulfide bonds (*1*).

Our results show that introduction of disulfides into proteins that have evolved without them is possible and that such derivatives can retain activity and also have enhanced stability. This has implications not only for studies of protein folding, structure, and function but also for commercial applications of engineered enzymes and other proteins.

While this work was in progress, Villafranca *et al.* (*21*) described a disulfide-bonded *E. coli* dihydrofolate reductase prepared by methods similar to those we have described. This derivative was fully active in the reduced state but had "significantly diminished" activity in the oxidized state. No data on the stability of this derivative was reported.

Note added in proof: T4 lysozyme ($Ile^3 \rightarrow Cys$) with a 3 to 97 disulfide link and a free thiol at Cys^{54} shows a thermal stability at 67°C, identical to the wild-type enzyme. The disulfide derivative with Cys^{54} blocked by reaction with iodoacetate has an activity half-life of about 6 hours under these conditions; in its reduced state, it loses stability in the same way as the material described in Fig. 3b. The two phases of activity decay for the mutant in Fig. 3a are thus due to two molecular forms, differing at Cys^{54} by the presence or absence of a blocking group (probably thiosulfate from the oxidant).

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Prophenoloxidase Activation: Nonself Recognition and Cell Cooperation in Insect Immunity

Abstract. *The mechanism of nonself recognition by the immune system of insects is unknown. In this report the activation of the prophenoloxidase system in the wax moth* Galleria mellonella *by a microbial product is shown to enhance the recognition of nonself material. These results explain previous observations of the interaction of two different blood cell populations in the cellular defense reactions of insects.*

An understanding of the interrelations between the insect host and parasite is important because numerous insects act as vectors of the most devastating diseases. Many of the disease organisms, such as those causing malaria and also possibly trypanosomiasis, have one or more stages in the hemocoel of their hosts, where they apparently develop and migrate unmolested (*1*). This is unexpected considering the speed and effectiveness of the insect cellular defense reactions in response to various parasites and foreign material (*2*).

Despite the efficacy of arthropod immune systems, insects and other invertebrates—in contrast to vertebrates—do not use immunoglobulins as recognition molecules and yet exhibit considerable selectivity and activity in their nonself reactions (*2, 3*). The body fluids of many noninsectan invertebrates, however, contain factors capable of acting as opsonins and stimulating the uptake of foreign particles by the blood cells (*3*). The most likely candidates for the invertebrate recognition molecules are the agglutinins, which are present in a wide range of species (*3–5*). However, in only two species, *Crassostrea gigas* and *Mytilus edulis*, have agglutinins been purified and shown unequivocally to have opsonic properties (*6, 7*). Insect hemolymph also contains agglutinins, but in the few species tested these did not enhance phagocytosis (*8*) either because the experimental systems used were suboptimal or because other factors may play a role in nonself recognition.

Melanin deposition around parasites is commonly associated with the cellular and humoral defenses of arthropods (*9–11*); in consequence, this substance and precursor enzymes used in its production, such as phenoloxidase, have been regarded as important components of these immune reactions. The prophenoloxidase-activating system has been reported to be triggered by microbial products (*10, 12, 13*), possibly through limited

Table 1. Effects of laminarin and endotoxin on phagocytosis of *Bacillus cereus* by *Galleria mellonella* blood cells. Monolayers of *G. mellonella* hemocytes were prepared for each of four experiments by bleeding seven final-instar larvae (0.28 to 0.30 g body weight) into 1 ml of Grace's insect medium (GIM, Gibco) on ice. Portions containing 5×10^4 cells were pipetted onto endotoxin-free cover slips, and the cells were left to attach in a moist chamber for 10 to 15 minutes. Unattached cells were washed away with fresh GIM, and monolayers were overlaid with 2.5×10^6 *B. cereus* (NCIB 9373) that had been killed by heat and suspended in GIM. For the experimental monolayers, the bacteria were mixed with laminarin (0.1 percent, P-L Biochemicals) or with *E. coli* 055:B5 endotoxin (100 μg/ml, Difco) in GIM. Control monolayers were overlaid with bacteria in GIM alone (control 1), with bacteria in GIM containing 0.01 m*M* inhibitor [*p*-nitrophenyl-p'-guanidobenzoate (*p*-NPGB)] (control 2), or with bacteria in GIM with laminarin (0.1 percent) or endotoxin (100 μg/ml) and 0.01 m*M* *p*-NPGB (control 3). All monolayers were placed on a rocking platform and incubated at 25°C for 60 minutes. Nonadherent bacteria were then washed off with GIM, and the monolayers were fixed in formaldehyde vapor for 30 minutes. Blind counts of at least 300 cells per monolayer were made, and the numbers of the different cell types enclosing one or more bacteria were scored. The percent phagocytosis in the experimental and control monolayers was compared by the Newman-Keuls test for multiple comparisons.

Item	Experimental		Control 1‡		Control 2‡		Control 3‡	
	With laminarin	With endotoxin	No laminarin	No endotoxin	No laminarin	No endotoxin	With laminarin	With endotoxin
All cells	7.69 ± 1.89*	3.93 ± 0.92†	1.20 ± 0.40	0.64 ± 0.32	1.37 ± 0.45	0.87 ± 0.52	1.41 ± 0.55	1.05 ± 0.47
Plasmatocytes	13.20 ± 3.78	7.95 ± 1.91	1.94 ± 0.73	1.15 ± 0.71	2.10 ± 1.29	1.64 ± 1.09	2.62 ± 1.43	2.02 ± 1.04
Granular cells	4.22 ± 1.41	1.49 ± 0.70	0.65 ± 0.49	0.34 ± 0.31	0.83 ± 0.45	0.33 ± 0.48	0.71 ± 0.41	0.42 ± 0.33

*Represents means of 20 monolayers from a total of 28 insects in four experiments ± standard deviation. †Represents means of ten monolayers from a total of 14 insects in two experiments ± standard deviation. ‡$P < 0.05$ compared to appropriate experimental.

proteolysis of the prophenoloxidase (*13–15*). However, not until activation of the prophenoloxidase system in the crustaceans *Astacus astacus* and *Carcinus maenas* was shown to result in elevated phagocytosis in vitro was the full significance of the activating cascade resulting in pigment deposition realized (*16*). The details of prophenoloxidase activation in the plasma of the lepidopteran *Bombyx mori* has been elucidated and its function considered, but no system for assaying its role in the immune system of the host was available (*13*).

We have now tested for the presence and role of the prophenoloxidase system in the recognition stage of phagocytosis by insect hemocytes using monolayer preparations of blood cells of the wax moth (*Galleria mellonella*) simultaneously overlaid with *Bacillus cereus* and either laminarin (a β-1,3-D-glucan extracted from fungal cell walls) or *Escherichia coli* 055:B5 endotoxin. Both laminarin and endotoxin have been reported to activate the prophenoloxidase system in arthropods (*11*). The monolayers were incubated with the test bacteria, and the numbers of particles ingested by the different hemocyte types were recorded. As controls, *B. cereus* were pipetted onto the monolayers either alone in Grace's insect medium (GIM) or simultaneously with dextran (a β-D-glucan present in certain bacterial capsules) or *p*-nitrophenyl-*p'*-guanidobenzoate (*p*-NPGB) [a potent inhibitor of serine proteases, which are activators of the prophenoloxidase system (*13*)], or with a mixture of laminarin and *p*-NPGB.

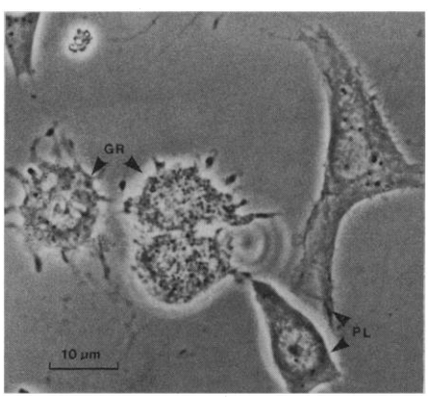

Fig. 1. Phase-contrast micrograph of *Galleria* monolayer showing typical plasmatocytes (PL) and granular cells (GR). Note the spikelike pseudopods and numerous granular inclusions of the granular cells and the flattened appearance of the plasmatocytes.

The two most common cell types in the *Galleria* monolayers are the granular cells and spreading cells termed plasmatocytes (Fig. 1). The granular cells form spikelike pseudopods and contain numerous granules that are rapidly discharged in vitro to induce hemolymph melanization and coagulation (*2*). The plasmatocytes are more stable cells in vitro that rapidly form lamellipodia and, in *Galleria*, are almost devoid of granular inclusions. In monolayer preparations, the plasmatocytes are the most actively phagocytic cell type in this species (Table 1).

The addition of laminarin or endotoxin to the monolayers significantly enhanced the uptake of *B. cereus* from 1.2 to 7.69 percent and from 0.64 to 3.93 percent, respectively, as compared to the GIM controls (Table 1). This represents a more than sixfold increase in the phagocytic rate as a result of addition of the two microbial cell wall extracts. No such stimulation was recorded with dextran (*17*), which does not activate the prophenoloxidase system (see below), or with the *p*-NPGB controls while *p*-NPGB added simultaneously with the laminarin or endotoxin clearly inhibited the stimulatory effect of these substances alone (Table 1).

In experiments to show that microbial products not only enhance phagocytosis but also specifically activate prophenoloxidase present in the *Galleria* blood cells, hemocyte pellets were homogenized and the supernatants were incubated with the appropriate substance (Table 2) before the addition of L-dopa substrate. No phenoloxidase activity was recorded with endotoxin or with the dextran and distilled water controls, whereas incubation with laminarin resulted in substantial activation of prophenoloxidase (Table 2).

Overall, these results show the en-

Table 2. Activation of prophenoloxidase in *Galleria* blood cells by various microbial extracts. Two batches of 15 insects were bled into EDTA-citrate buffer (*p*H 4.6) equiosmotic with *Galleria* hemolymph. The blood cells were washed with cacodylate buffer (*16*). The hemocytes were then homogenized on ice in cacodylate buffer and centrifuged, and the supernatants were subdivided and incubated with laminarin (0.1 percent), endotoxin (100 μg/ml), dextran (0.1 percent), or distilled water (control). L-Dopa was then added as a substrate, and the absorbance was read at 480 nm. Estimation of the protein content of the hemocyte lysate supernatants was by the standard Folin-Ciocalteau method.

Test system	Enzyme (units) (per milligram of protein)
Laminarin	7700
Endotoxin	0
Dextran	0
Control	0

hancement of the cellular defenses by certain microbial products. In some cases, as with laminarin, the activation of the prophenoloxidase system plays a vital role in the nonself recognition process; in other cases, as with endotoxin, although elevated phagocytosis was recorded no phenoloxidase was apparently generated. This may have resulted from the presence of a separate endotoxin-mediated prophenoloxidase activating system, as occurs in other arthropods (*18*). Unlike the laminarin-mediated complex, this system may be sensitive to the anticoagulant used in the enzyme assay or, alternatively, may be functional only in the presence of other factors such as agglutinins or specific ions that were not included in the assay. This point clearly merits further investigation, but even so the results represent the first step toward identifying recognition molecules in insects. These findings should provide a stimulus to research into vector species because it may now be possible to investigate ways by which parasites inhibit or manipulate the host defenses in order to survive in the hemocoel (*19*).

The data further support our earlier hypothesis (*2*) that in the cellular defenses of *Galleria* two cell types interact in a biphasic process. Cytochemical and ultrastructural studies indicate that the granular cells, but not plasmatocytes, contain phenoloxidase (*20*) and that these cells react by violently discharging their contents over nonself material when in contact with foreign surfaces (*2*).

This release corresponds to the activation and appearance of the phenoloxidase observed in this study and constitutes the first or recognition phase in the cellular reactions. The second phase results from the identification of the invading parasite as foreign by the coat formed from factors derived from activation of the prophenoloxidase system. This phase is mediated mainly by the plasmatocytes, which contain little or no phenoloxidase (*20*) but respond to coated surfaces either by forming multicellular sheaths around large parasites or by ingesting smaller ones (*2*) (Table 1). Specific receptor interactions may be part of the mechanism by which plasmatocytes react toward coated material, but this phenomenon has yet to be explored.

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Isolation and Culture of a Tetraploid Subpopulation of Smooth Muscle Cells from the Normal Rat Aorta

Abstract. *Smooth muscle cells with 4C (double diploid) DNA content have been found in major arteries. The proportion of 4C cells increases with normal aging and with hypertension. These cells may represent a state of arrest at the G_2 phase of the cell cycle or may be examples of true tetraploidy. Flow cytometric cell sorting was used to isolate 4C smooth muscle cells from the rat aorta, and the cells were cultured. Flow cytometry, Feulgen microdensitometry, and karyotyping of the progeny of the 4C cells established the presence of true tetraploid cells. These findings demonstrate the presence of reproductively viable tetraploid cells in a normal mammalian tissue.*

Smooth muscle cell proliferation may be central to atherogenesis (*1*), and therefore characterization of smooth muscle cell growth is essential for an understanding of vascular disease. During the process of growth and aging, an increasing frequency of smooth muscle cells with 4C (double diploid) DNA content has been observed both in humans (*2*) and in experimental animals (*3, 4*). Hypertension has also been found to be associated with an increased frequency of 4C nuclei (up to 40 percent) in the aortas of rats (*4*). Over the past 30 years, cytophotometric techniques for quantitating DNA content in situ (*5*) have shown that nuclei with polyploid DNA content are present in various plant and animal cells. However, the isolation of a reproductively viable population of tetraploid cells from normal mammalian tissues has not been reported. We now report the isolation and culture of a tetraploid subpopulation of smooth muscle cells from the normal rat aorta. This suggests the coexistence of heterogeneous populations of viable smooth muscle cells with 42 and 84 chromosomes.

Smooth muscle cells were isolated from the thoracic aortas of 3- to 4-month-old normotensive male Sprague-Dawley rats (Charles River) as described earlier (*4*). A suspension of cells (pooled from ten animals) was stained with Hoechst dye 33342 (8 μM weight to volume) for 30 minutes at 37°C. Cells were sorted with a flow cytometer–sorter (Coulter Epics V) and were processed at approximately 1000 cells per second (*6*). Flow cytometric analysis revealed that 90 percent of the cells had the normal diploid (2C) DNA content and 10 percent had 4C DNA content. This distribution is consistent with other data for animals of this age (*3, 4*). The cells corresponding to the 2C and 4C peaks were separated, and the cells were collected in culture medium [minimal Eagle's medium supplemented with 20 percent fetal calf serum, D-glucose (0.9 mg/ml), sodium pyruvate (6.6 μg/ml), and antibiotics]. The cells were then washed several times by centrifugation and were placed in culture dishes. During the initial growth period of the primary culture, cells were shielded from light to the maximum extent possible. The primary cultures were allowed to grow to near confluency in 35-mm petri dishes for several weeks, and the culture medium was replenished every 3 to 5