The Murine Toxin of *Yersinia pestis*: Some Enzymatic Activities

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The phosphatase, phosphodiesterase, and phospholipase activities identified in this study in a highly purified preparation of the *Yersinia pestis* murine toxin should shed light on the mechanism by which this toxin acts on the target cells of plague-sensitive animals

Key Words: *Yersinia pestis; murine toxin; enzymes*

Immune or, more broadly, biological responses can be altered by certain compounds of bacterial origin, such as peptidoglycan, proteins A and B, aggregation factor, teichoic acids of streptococcal and staphylococcal cell walls, *Bordetella pertussis* adenylate toxin, endotoxins of gram-negative bacteria, and *Yersinia pestis* adenylate cyclase [1,13]. A special feature of these compounds is their heterogeneity, which has been demonstrated at several levels, including the structure of active molecules or their products, mode of action, and pharmacological and toxic properties [13]. The mechanisms of their action have not been fully elucidated, however. Although the structure of several compounds has been established, their cellular targets in the immune system are still uncertain. Some compounds may act in a more general way, intensifying or repressing all functions of specific and/or nonspecific cells.

The murine toxin (MT) of *Y. pestis* is an important determinant of the virulence exhibited by these bacteria and makes a sizable contribution to the development of infection in susceptible hosts.

The present study aimed to gain further insight into the biological properties of the *Y. pestis* MT, which became a subject of research in the early 1950s [7]. Presented below are the results of tests for possible enzymatic activities of this toxin, whose mechanism of pathogenic action has recently attracted attention [5].

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MATERIALS AND METHODS

The MT was derived from cells of the *Escherichia coli* strain DH5α carrying the recombinant plasmid pMB 188 with a cloned MT gene. The MT preparation had physicochemical and serological properties identical to those of the *E. coli* preparation. It was tested for homogeneity in a high-performance liquid chromatography (HPLC) system (Gilson) and found to have a single protein peak. Its analysis by polyacrylamide gel electrophoresis with sodium dodecyl sulfate revealed one protein zone with an electrophoretic mobility of 61 kD and a protein load of between 5 and 20 μg. The intraperitoneal LD_{50} for random-bred white mice was 0.4-1.2 μg in terms of protein.

Autophosphorylation of the toxin was recorded using γ-^{32}P-ATP. The amino acid attaching inorganic phosphate was identified on the radiogram obtained after subjecting the protein to hydrolysis with 6 N HCl (110°C, for 2 h) followed by separation of its components with thin-layer chromatography on a Silufol F 254 plate (Merck) in a water:isopropanol (3:7) system for 3 h. As markers, we used O-phospho-L-tyrosine, O-phospho-L-serine, and O-phospho-L-threonine synthesized in our laboratory by O. Yu. Ryabukhina.

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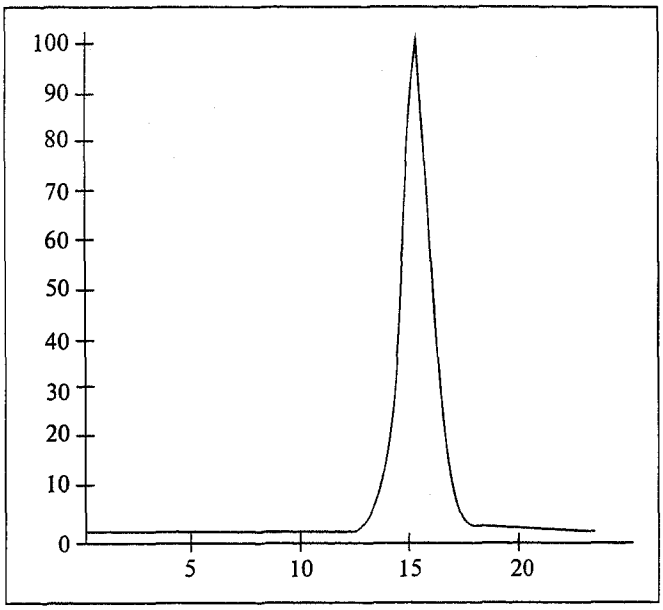

Fig. 1. Characteristics of the *Y. pestis* MT as revealed by HPLC (Bio–Gel TSK40, 0.5 ml/min; mobile phase: 10 mM Tris–HCl+20 mM NaCl, pH 7.2). Abscissa: time in min; ordinate: detector response.

Phosphatase activity was assayed in a sample containing a 20 mM buffer solution of sodium acetate (pH 6.0) or Tris-HCl (pH 8.0) by previously described methods [12,14,16], while phosphodiesterase activity was assayed as detailed by Shapot [6]. The following commercial compounds were used: ATP, GTP, 3':5'-cAMP, 3':5'-cGMP, 2'(3')-GMP, phosphatidylcholine, p-nitrophenyl phosphate, bis(p-nitrophenyl) phosphate (sodium salt), dimethyl sulfoxide, alkaline phosphatase from calf intestine (all from Serva), 2':3'-cAMP (Boehringer Mannheim), glucose-6-phosphate (Calbiochem), and fructose-1,6-diphosphate (Reanal). Protein was determined by Lowry's method and by differential spectrophotometry [11].

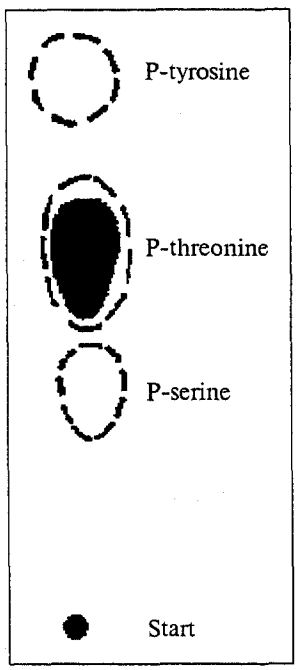

Fig. 2. Chromatographic identification of a phosphorylated amino acid in the *Y. pestis* MT molecule.

RESULTS

By now, a large number of proteins with two, three, or more enzymatic activities have been described. A feature of these proteins is the copresence in a single polypeptide chain of superdomains that can sometimes catalyze reactions proceeding in opposite directions, e.g., protein kinase and protein phosphatase reactions [2].

Enzymatic properties of the MT have been poorly characterized. For their thorough examination we had to have a preparation whose high degree of homogeneity would allow us to ascertain whether or not a particular activity were present. The results of HPLC and electrophoresis indicated that the MT preparation used met this requirement: possible impurities did not exceed 0.2% (Fig. 1).

The *Y. pestis* MT was found to possess autophosphorylating and dephosphorylating activities. The autophosphorylation phenomenon comprised phosphorylation of threonine residues by its own protein molecule (Fig. 2). The native MT appears to be in the phosphorylated state, given that its dephosphorylation by treatment with the alkaline phosphatase from calf intestine reduced the intraperitoneal LD_{50} of the MT preparation from 0.4 to 0.18 μg, probably as a result of conformational changes in the molecule itself.

The MT also exhibited tyrosine, serine, and threonine phosphatase activities (1500, 290, and 294 nmol/mg protein, respectively, pH 6.0). Its tyrosine phosphatase activity showed an unusual (inverse) dose dependence, peaking in the region of low (0.001 and 0.05 μg) doses. Such activity profiles are characteristic of bacterial products classed among immunomodulators and/or immunomodifiers [13].

In one series of tests, where a tyrosine phosphatase activity of 1500 nmol/mg protein corresponded to an LD_{50} of 0.4 μg for white mice, the toxin was no longer lethal for them when this activity declined to 700 nmol/mg and then to 375 nmol/mg during storage. The phosphatase inhibitor NaF suppressed the tyrosine phosphatase activity completely at 10 mM and by 53% at 1 mM.

To study total phosphatase activity of the MT (at 0.2 μg as protein) at pH 6.0 and 8.0, we used several substrates including a synthetic p-nitrophenyl phosphate, taking the MT activity toward the latter (750 nmol/mg protein) to be 100% (Fig. 3). All the substrates except cGMP proved sensitive to the MT predominantly at the acid pH value. With respect to glucose-6-phosphate and fructose-1,6-diphosphate, phosphatase activity of the MT was higher at pH 8.0

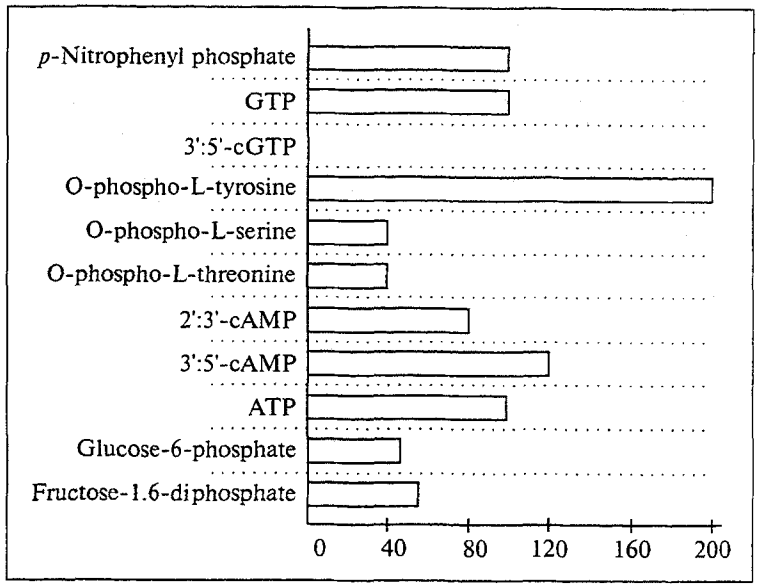

Fig. 3. Phosphatase activity of the *Y. pestis* MT with respect to various substrates (pH 6.0, 37°C, 30 min). Abscissa: phosphatase activity in %, the activity with respect to p-nitrophenyl phosphate being taken as 100%; ordinate: substrates.

(500 and 800 nmol/mg, respectively) than at pH 6.0 (350 and 400 nmol/mg).

Evaluation of the MT interaction with ATP and GTP was based on measurements, using malachite green or Fiske and Subarrow's method [12], of the inorganic phosphate produced by enzymatic hydrolysis. The MT displayed well-defined ATPase and GTPase activities, each of which, as in the case of tyrosine phosphatase activity, was considerably higher when the toxin dose was low (0.2 μg) than when it was much higher (20 μg), the activity values being 725 nmol/mg protein for ATPase and 750 nmol/mg protein for GTPase at 0.2 μg and 14 and 16 nmol/mg protein, respectively, at 20 μg.

The determination of inorganic phosphate using lecithin as the substrate suggested the presence of phospholipase activity in the MT. That this is so was confirmed by a qualitative reaction of the toxin with Florence's reagent that gave rise to crystals characteristic of choline periodide - a finding already reported by another author [4]. In this reaction, therefore, the MT most likely digested lecithin in a cascade manner to form phosphorylcholine (phosphorylated choline), from which inorganic phosphate then split off.

It is of interest that the MT manifested phosphatase activity with respect to the cyclic nucleotides 3':5'-cAMP and 2':3'-cAMP, but not toward 3':5'-cGMP. This suggested the presence of 3':5'-cAMP phosphodiesterase activity (cyclic AMP-5-nucleotide hydrolase, EC 3.1.4.17), which was confirmed by direct tests using sodium bis(p-nitrophenyl) phosphate with various MT doses (0.1-100 μg) in a pH range of 2.0-10.0. The highest phosphodiesterase activity was detected at pH 8.0 in the region of low doses (1400-210 μg p-nitrophenol/mg protein).

The detection of 3':5'-cAMP phosphodiesterase activity in the MT sheds some light on the possible mechanism of its action on eukaryotic cells. The hypothesis that the MT is a β-adrenergic receptor blocker [10] appears untenable, as this toxin can hydrolyze the cyclic nucleotide directly, a fact which may explain the large variations of cAMP levels in animals administered the MT [5].

Some of the activities we detected in the MT have been described as independent proteins in members of the genus *Yersinia*. Examples include phosphodiesterase, ATPase, tyrosine phosphatase, and reversibly phosphorylated proteins. The MT preparation used could be suspected of being contaminated with residues of other proteins or, on the contrary, of containing fragments imitating the actions of the aforementioned enzymes, especially since, as our analysis of its primary amino acid sequence revealed [3], the MT contains a number of PEST-like regions, i.e., regions rich in proline (P), glutamic acid (E), serine (S), and threonine (T) that are frequently delimited by clusters of several positively charged amino acids. These PEST regions are presumed to be involved in programming the half-lives of proteins *in vivo* [15]. However, the activities mentioned above were all found in the *Y. pestis* strain 556/106 devoid of the 65 MD plasmid coding for MT synthesis as well as of the Ca-dependence plasmid determining the synthesis of membrane Yop proteins, including YopH, which possesses a phagocytosis-blocking tyrosine phosphatase activity [8,9]. This bolsters the conclusion that the activities identified in the present study are related to the MT.

The *Y. pestis* toxin is thus a protein with several enzymatic activities which can evidently trigger a multiplicity of reactions that occur in a cascade fash-

ion to elicit different types of cell-mediated responses depending on the immunological status of the host.

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