THE BACTERIAL FACTORS WHICH STIMULATE NEUTROPHILS MAY BE DERIVED FROM PROCARYOTE SIGNAL PEPTIDES

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1. Introduction

During the inflammatory response associated with most bacterial infections, neutrophils migrate into tissues containing viable bacteria and participate in their elimination from the tissue. The migration of neutrophils to the site of infection is probably due to the release by bacteria of factors which are chemotactic for neutrophils [1], as well as those arising from activation of the complement system. Bacterial chemotactic factors have been isolated from the filtrates of a variety of bacterial cultures and they appear to comprise small peptides which have a blocked N-terminal, are dialysable and heat-stable [2,3]. The origin of these chemotactic factors in bacterial metabolism has not been determined; they have been supposed to be a degradation product of denatured bacterial protein [4].

The naturally occurring bacterial chemotactic factors could not be purified to homogeneity [3], but a number of peptides have been synthesised which interact with specific receptors on the neutrophil cell surface [5–7] believed to be the receptor sites for the natural bacterial factors [5,7]. These peptides have the structure *N*-formyl-methionine followed by hydrophobic amino acids, the most active found being the tripeptide f-MetLeuPhe.

Here we consider the possibility that the bacterial chemotactic factors correspond to the signal peptides derived from bacterial membrane proteins and secretory proteins. In bacteria [8] as in other organisms [9,10], most such proteins are synthesised as precursors which contain an N-terminal extension of ~20 amino acids. This extension, known as a signal peptide, is believed to direct the transfer of the growing polypeptide chain across the appropriate membrane during its synthesis. The signal peptide is cleaved during transfer so that it is not found in the mature protein. In bacteria the synthesis of membrane and secretory proteins occurs at the cytoplasmic membrane so that the cleaved signal peptide could be released into the surrounding membrane.

2. Methods

Neutrophils were obtained from the rabbit peritoneal cavity 4–6 h after the infusion of 250 ml 0.1% glycogen in 0.15 M NaCl. They were suspended at 10^7 cells/ml in a buffered salts solution as in [11]. Reactions were initiated by adding 50 μl cells equilibrated at 37°C to 50 μl buffer containing the agent under test and $CaCl_2$ (final conc. 1.8 mM). All the experiments were carried out in the presence of cytochalasin B (final conc. 5 μg/ml) to enhance the extent

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of secretion. After 5 min the tubes were centrifuged and 50 μl supernatants was removed and assayed for β-glucuronidase.

β-Glucuronidase was determined using the chromogenic substrate *p*-nitrophenyl-β-D-glucuronide [12]. Secretion is expressed as the % of total cell β-glucuronidase (typically 0.3 $\mu mol \cdot min^{-1} \cdot 10^6 cells^{-1}$ at 37°C, measured after lysing the cells with 0.2% Triton X-100) found in the supernatant.

Proteins were synthesised in vitro using a cell-free coupled transcription–translation system derived from *Escherichia coli* and including ^{35}S-methionine [13]. DNA containing the penicillinase gene was isolated from a phage λ vehicle containing a DNA fragment from *Bacillus licheniformis* (λ_{pen}) as in [14]. DNA derived from bacteriophages T5 and T7 was purified as in [13].

Newly synthesised proteins were purified by SDS–polyacrylamide gel electrophoresis [15]. The gels were partially dried and autographed to identify the radioactive protein bands, as shown in fig.1. The areas of gel containing bands of interest were cut out, and the protein eluted from the gel by shaking for 16 h at 37°C in 1.0 ml water. The samples were then dialysed

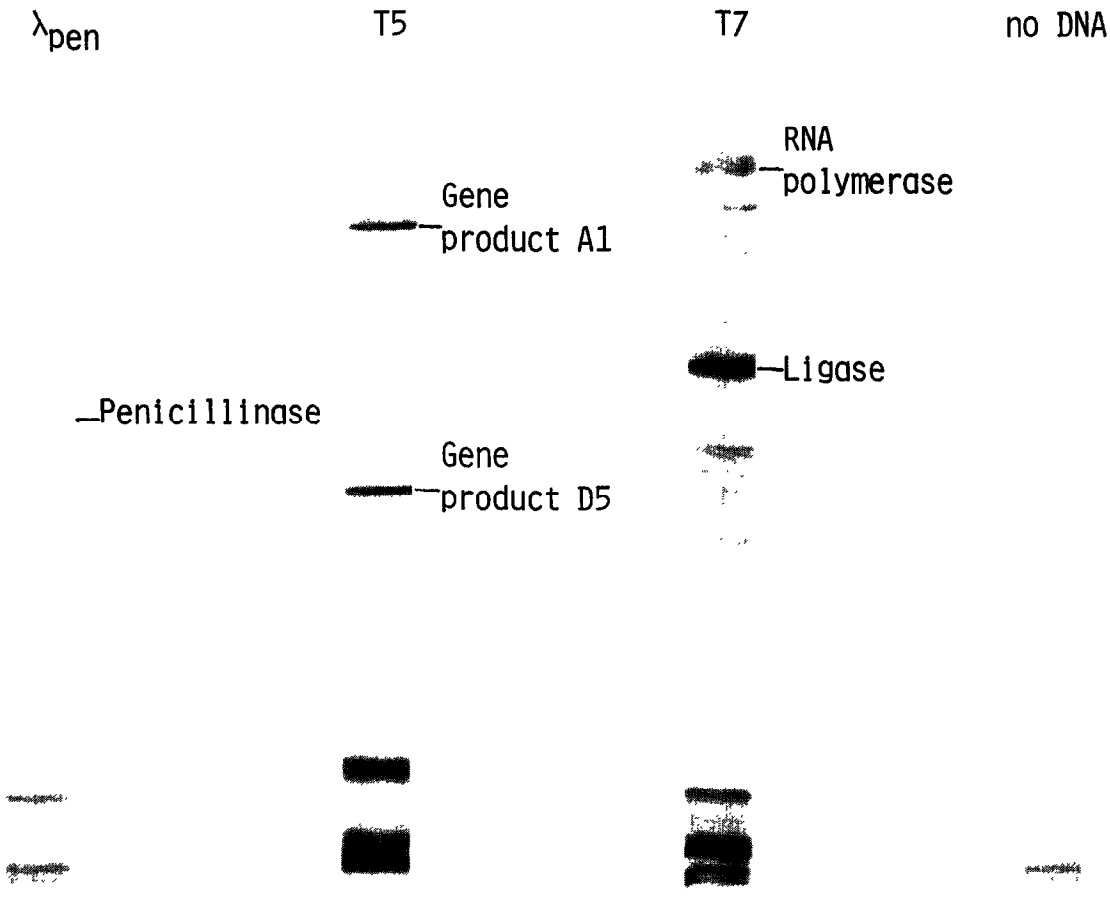

Fig.1. The products of in vitro protein synthesis using the cell-free coupled transcription–translation system containing DNA from λ_{pen}, T5 or T7. Control tubes contained no added DNA. The newly synthesised proteins (containing ^{35}S-methionine) were separated by SDS gel electrophoresis and autoradiographed. The position and identity of the proteins used for the experiment described in table 2 are indicated.

for 30 h against water (3 changes), freeze-dried and resuspended in a small volume of 0.15 M NaCl (buffered to pH 7.0 with 10 mM phosphate) for testing.

The membrane and secreted forms of mature penicillinase were purified as in [16]. They were denatured by adding SDS (final conc. 0.1%) and processed in the same way as the protein samples eluted from the gel. Enzymatic determination of penicillinase was as in [14].

3. Results and discussion

The penicillinase from *Bacillus licheniformis* is synthesised in vitro as a polypeptide of apparent relative molecular mass (app. M_r) 34 500 [14]. This precursor penicillinase contains an N-terminal peptide not present in the mature membrane form of the protein, which resembles by its location the signal peptide found in most membrane and secretory proteins [10]. This N-terminal extension is cleaved during cellular synthesis of the membrane form of the penicillinase. A second post-translational proteolytic cleavage removes the hydrophobic peptide anchoring the enzyme to the cytoplasmic membrane and converts membrane penicillinase (app. M_r 31 000) to the secreted form of the enzyme (designated 'ExoS', app. M_r 30 000, or 'ExoF', app. M_r 29 000) [16].

To examine whether a bacterial signal peptide may stimulate rabbit neutrophils, we compared the effect of the precursor form of penicillinase with that of the mature forms (table 1). Stimulation of neutrophils was measured in terms of secretion of the lysosomal enzyme β-glucuronidase in the presence of cytochalasin B. The secretion assay can be more accurately measured than chemotaxis (which is the physiologically relevant neutrophil response) using small quantities of material. The amounts of precursor protein which could be produced by cell-free coupled transcription–translation were only just sufficient to carry out these experiments. Secretion (in the presence of cytochalasin B) is a different expression of the cell receptor normally responsible for chemotaxis [6].

Table 1 shows that the precursor form of *B. licheniformis* penicillinase will stimulate secretion by neutrophils whereas the mature forms of the protein are completely inactive. Purification of penicillinase precursor from the in vitro transcription–translation system was necessary since the *Escherichia coli* cell-free system itself stimulated neutrophils; it was accomplished by elution of the appropriate band from a polyacrylamide gel (see section 2). Any components of the cell-free system which comigrate with penicillinase precursor by SDS gel electrophoresis did not stimulate neutrophils (table 1). One other protein coded for by λ_{pen} DNA, the O protein, will be a minor contaminant of penicillinase precursor since it migrates similarly on SDS gel electrophoresis [14]; we think that the secretion we observe is unlikely to be due to contaminating O protein since in the experiment shown in table 2 the ability to evoke secretion from neutrophils appears to belong to at least one other bacterial protein which is likely to contain a signal sequence.

Table 1

Sample	Release of β-glucuronidase (% total)
Buffer control	7
Penicillinase precursor	
(~0.4 nM)	60
(3 × dil.)	16
Gel control	8
Mature penicillinase	
Membrane form, 5 nM	7
ExoS form, 5 nM	6
f-MetLeuPhe	
1.0 nM	76
0.2 nM	24

Release of β-glucuronidase from rabbit neutrophils due to application of the precursor form of penicillinase. The concentration of penicillinase precursor was estimated from the radioactivity due to ^{35}S-methionine in the sample, assuming 5 methionine residues/polypeptide chain. The value agreed with an estimate of the amount of penicillinase synthesised in vitro calculated from the enzymatic activity. For the gel control, the area of gel in the same position as penicillinase precursor was taken after electrophoresis of the cell-free system with no added DNA and carried through the same procedure as for the penicillinase precursor (see section 2). The samples of mature penicillinase were denatured with SDS and carried through the same procedure. The data represent means of duplicate determinations from a single experiment; similar stimulation due to penicillinase precursor (compared with a gel control) was observed in 3 other experiments using different preparations

In this second experiment DNA from λ_{pen}, T5 and T7 was used to direct protein synthesis in the cell-free system. Five different proteins were purified by SDS gel electrophoresis (see fig.1) and tested for their ability to stimulate neutrophils. As indicated in table 2,

Table 2

Sample	Relative amount	β-glucuronidase release (%)	Cellular location of mature protein
Penicillinase precursor	1.0	28	Membrane/secreted
Phage T7, RNA polymerase	1.0	3	Cytoplasmic
Phage T7, ligase	13.6	0	Cytoplasmic
Phage T5, gene product A1	13.4	43	Membrane
Phage T5, gene product D5	17.2	0	Cytoplasmic

Release of β-glucuronidase from rabbit neutrophils due to the application of 5 procaryote proteins in the form synthesised in the cell-free system. In each case the value for the appropriate gel control (see table 1) has been subtracted. The relative amounts of the proteins refer to the radioactivity (as ^{35}S-methionine) in each sample. T7-RNA polymerase and T7-ligase are found in the soluble fraction after cell disruption [17,18]; T5-gene product D5 is similarly implicated in DNA replication and probably also has a cytoplasmic location [19]. T5-gene product A1 is the major bacteriophage membrane protein [20]

the proteins showing a clear ability to stimulate neutrophils were those for which there is evidence that they are membrane or secretory proteins. The proteins would presumably be synthesised as their precursor forms (i.e., including the signal peptide) in the cell-free system.

In these experiments the ability to cause secretion from neutrophils is associated with those proteins containing or likely to contain a signal sequence. It is not due solely to the presence of an N-terminal formyl-methionyl residue, since this will presumably also be present on cytoplasmic proteins synthesised in the cell-free system (table 2). We have considered the possibility that the apparent stimulatory activity could be attributable to detergent from the SDS gel electrophoresis purification remaining specifically associated to the relatively hydrophobic signal sequence. However the lowest concentration of SDS to cause release of β-glucuronidase from neutrophils is 10^{-5} M (J. P. B., unpublished): i.e., $>10^4$ molecules of SDS would have to be associated with the 20 or so amino acid residues of the N-terminal extension to cause the observed stimulation. This seems highly unlikely.

The signal peptides which are cleaved from newly synthesised membrane and secretory proteins after transfer across the bacterial membrane would be excellent candidates for the natural bacterial chemotactic factors. They will be specifically released by bacteria (eucaryote signal peptides do not contain f-Met), they are of low molecular weight so they are diffusible, and like the active synthetic peptides they contain predominantly hydrophobic amino acids with an N-terminal f-Met. There does not seem to be any close sequence homology for signal peptides [10] so that the released peptides (which may also be subject to further proteolysis) would form a heterogeneous population, as was found for the purified bacterial chemotactic factors [3].

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