ABNORMAL PHENYLALANYL-tRNA FOUND IN SERUM INHIBITED *ESCHERICHIA COLI*, STRAIN 0111

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1. Introduction

There is now a good deal of circumstantial evidence to suggest that iron-binding proteins can be of crucial importance in resistance to infection [1–5]. It has been proposed that specific antibody, acting in concert with transferrin, the iron-binding protein in serum, interferes directly with normal bacterial iron metabolism making iron unavailable to the cell and leading to an inhibition of bacterial growth [6]. Saturating the iron-binding capacity of transferrin overcomes this inhibition. Thus, the bactericidal and bacteriostatic effects of serum against *Escherichia coli* can be abolished by the addition of sufficient iron to saturate the transferrin [1].

The precise mechanism whereby antibody and transferrin interfere with bacterial multiplication, however, is unknown. Indeed, it has not yet been demonstrated that the inhibited bacteria are iron deficient. Recent work [7, 8] has shown that the inhibition of *Pasteurella septica* by specific antibody, complement and transferrin operates by affecting the biochemistry of the bacterial cell leading first to an inhibition of RNA accumulation and then to a cessation of multiplication. Although this process is reversed by iron compounds, it is not known how a lack of iron could specifically affect the accumulation of RNA. Gross interference with the energy supply of the cell is unlikely since the inhibition of net protein, RNA and DNA synthesis occurred at different times. Amongst other possible defects leading to a disruption of cellular physiology is an interference with tRNA production or function. Transfer RNA specifies a class of macromolecules that are of key importance to cellular processes. In addition, there are reports in the literature of iron dependent modifications of several tRNA species [9–11]. Thus, the chromatographic profiles of several tRNAs from *E. coli* grown in iron deficient media differ markedly from those of the iron replete cells. In view of these observations, it was pertinent to ask whether antibody and transferrin induced specific alterations in the tRNAs of bacterial cells. Such changes might be expected if bacteriostasis was in fact due to a derangement of the bacteria's iron metabolism.

The present communication describes an initial examination of the aminoacyl-tRNA in cells inhibited by serum. Since all previous reports mention iron dependent changes in phenylalanine tRNA, it was decided to start by looking at this particular macromolecule. In this work, the nature of phenylalanyl-tRNA in cells of the pathogenic bacterium *E. coli* strain 0111 growing under normal conditions and when inhibited by serum has been investigated. Results show that phenylalanyl-tRNA is indeed altered in the inhibited cells. Addition of iron to serum prevented the appearance of the abnormal phenylalanyl-tRNA and allowed the cells to grow. Similarly, adding haematin to the cells once inhibition had occurred resulted in the replacement of abnormal phenylalanyl-tRNA by the normal species and in the restoration of bacterial multiplication.

2. Materials and methods

2.1. *Organisms and media*

E. coli strain 0111/B4/H2: details of the methods

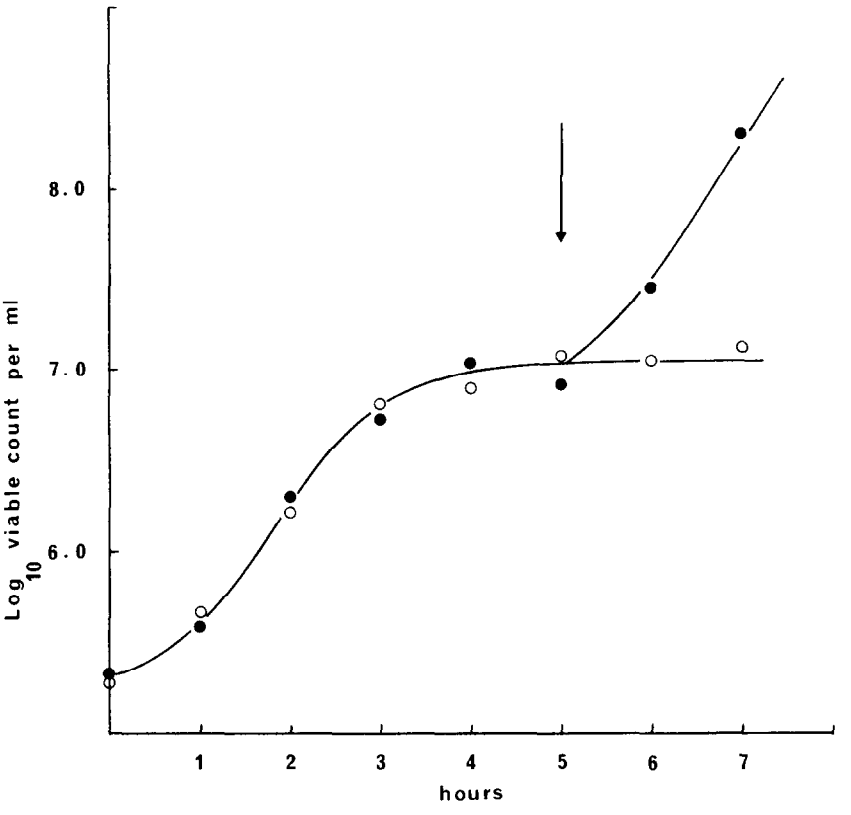

Fig. 1. Inhibition of *E. coli* 0111 by normal horse serum and the reversal of the inhibitory effect by haematin. (○—○—○) Normal horse serum; (●—●—●) normal horse serum with haematin hydrochloride (final concentration 10 mM, [8]) added at time 5 hr as indicated by the arrow.

used for passage and storage of organisms have been described before [12]. Bacteria for use as inocula were grown in papain digest broth for 3 hr at 37°. Methods for preparation of inocula and estimation of the viable counts are described by Griffiths [8]. To ensure that agglutination did not affect the measurement of the viable count, all samples were homogenized beforehand for 1–2 min [8].

'Carrier cells' were obtained by growing *E. coli* in papain digest broth at 37° for 4–5 hr; harvested bacteria were washed in 10% broth–saline [8] before use. The yield of cells was 1–2 g wet weight/l of culture.

For the aminoacylation of tRNA, *E. coli* was grown for 3 hr at 37° in papain digest broth. These 'broth grown cells' were harvested by centrifugation at room temp., washed in sterile 0.15 M NaCl, 0.01 M $NaHCO_3$ and 0.1% glucose (pH 7.6) and finally resuspended in the same medium.

2.2. *Growth of bacteria in serum under controlled conditions*

Growth experiments were carried out in 10 ml serum at 37° as described previously [8]. When larger quantities of serum grown bacteria were required for examination of the tRNA, the organisms were grown from an inoculum of about 10^6 cells/ml in 150–200 ml serum at 37° in a round bottomed flask fitted with a condenser and gas inlet. A sterile gas mixture [8] was passed over the surface of the stirred serum at 300 ml/min, maintaining the pH at 7.4–7.5. The bacteria were collected by centrifugation at room temp., washed in sterile 0.15 M NaCl, 0.01 M $NaHCO_3$, 0.1% glucose (pH 7.6) and finally resuspended in more of the same solution.

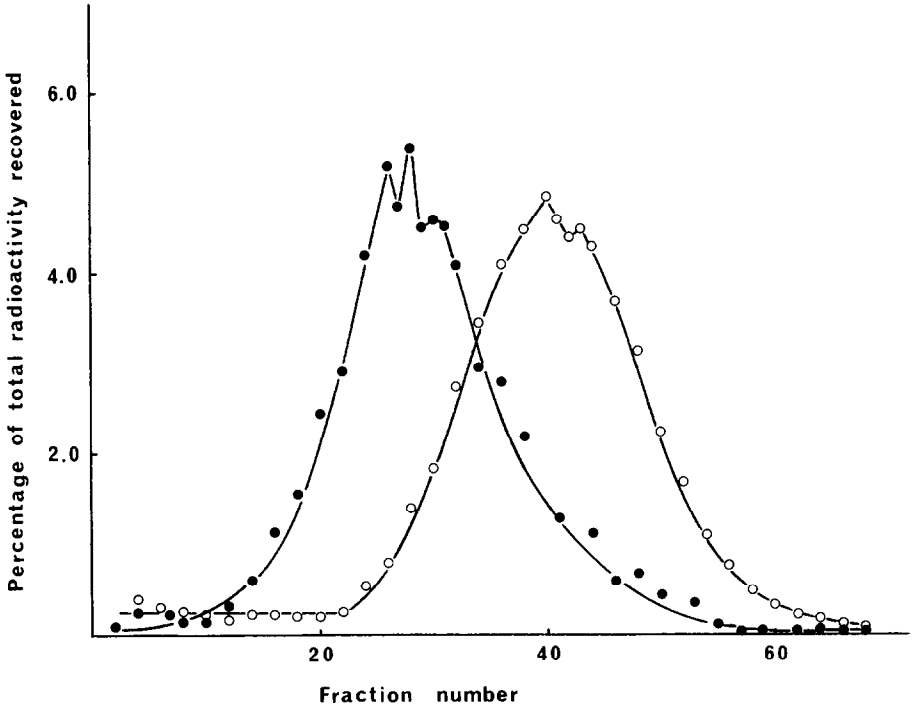

Fig. 2. Co-chromatography of phenylalanyl-tRNA from normal and inhibited bacteria on BD-cellulose. (●—●—●) [^{3}H] phenylalanyl-tRNA extracted from serum inhibited cells; (○—○—○) [^{14}C] phenylalanyl-tRNA extracted from broth grown cells.

2.3. *Aminoacylation and isolation of tRNA*

Transfer RNA was aminoacylated *in vivo* by a 3 min pulse with ^{14}C- or ^{3}H-labelled phenylalanine. The bacteria were suspended, at about 10^9 cells/ml, in 10 ml of 0.15 M NaCl, 0.01 M $NaHCO_3$, 0.1% glucose (pH 7.6) at 37° and [^{14}C] phenylalanine (10 μCi) or [^{3}H] phenylalanine (30–40 μCi) added. Three minutes later the mixture was cooled to 0° and the cells collected by centrifugation at 0–5°. The bacteria were washed once with ice cold 0.15 M NaCl, 0.01 M $NaHCO_3$, 0.1% glucose (pH 7.6) and finally resuspended in 2 ml of 0.05 M Na acetate, 0.01 M Mg acetate, 0.15 M NaCl (pH 5). For isolation of aminoacyl-tRNA, ^{14}C-labelled cells, ^{3}H-labelled cells and carrier cells (1–2 g), all suspended in 0.05 M Na acetate, 0.01 M Mg acetate, 0.15 M NaCl (pH 5) were mixed together; final volume 8–10 ml. The mixture was then extracted with an equal volume of phenol (80%) at 0–5° for 20 min and the aminoacyl-tRNA precipitated from the aqueous phase with ethanol in the usual way [13]. The precipitated material was re-precipitated from 0.05 M Na acetate, 0.01 M Mg acetate, 1.0 M NaCl (pH 5) as before and the final ethanol precipitate dissolved in 3 ml of 0.05 M Na acetate, 0.01 M Mg acetate, 0.15 M NaCl (pH 5).

In single label experiments, the two lots of labelled bacteria, mixed with carrier cells, were processed separately.

2.4. *Chromatography on BD-cellulose**

BD-cellulose (Schwartz Bioresearch, 20–50 mesh) columns were packed as described by Gillam et al. [14]. Aminoacyl-tRNA, dissolved in 3 ml of 0.05 M Na acetate, 0.01 M Mg acetate, 0.15 M NaCl (pH 5), was applied to a column (1.5 × 26 cm) of BD-cellulose previously equilibriated with 0.05 M Na acetate, 0.01 Mg acetate, 1.0 M NaCl (pH 5). The column was washed with 120 ml of 0.05 M Na acetate, 0.01 M

* Abbreviation: BD-cellulose, benzoylated diethylaminoethylcellulose.

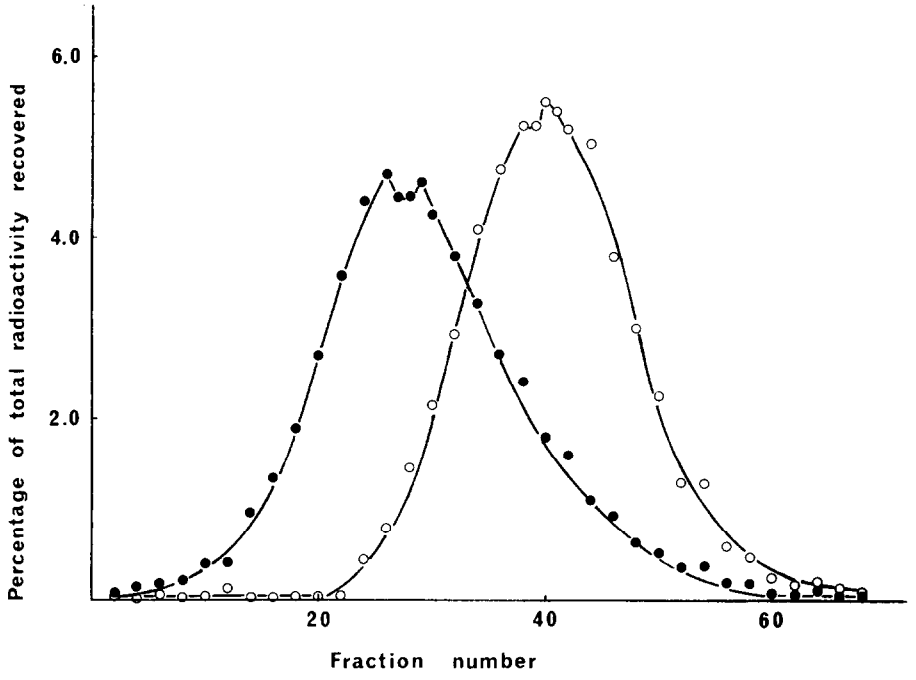

Fig. 3. Co-chromatography on BD-cellulose of phenylalanyl-tRNA from serum inhibited cells, (●—●—●): ^{14}C-labelled, and from cells grown in serum containing sufficient ferric ammonium citrate to saturate the transferrin [8], (○—○—○): ^{3}H-labelled.

Mg acetate, 1.0 M NaCl (pH 5) and the bound aminoacyl-tRNA eluted with a linear ethanol gradient (0–10% in 400 ml) in the same salt solution at 23°; 6 ml fractions were collected at a flow rate of 55 ml/hr. Radioactivity in the fractions was measured by precipitating the tRNA at 0° with trichloroacetic acid (final concentration 5% w/v) after adding 0.1 ml of a solution of serum albumin (5 mg/ml) to act as carrier. The precipitated material was collected on a Millipore filter (0.45 μm), washed with cold 5% trichloroacetic acid and dried. Filters were counted in a Beckman liquid scintillation counter using a scintillation solution containing 4 g 2,5-diphenyloxazone/l toluene.

2.5. *Materials*

Sterile normal horse serum was from the Wellcome Research Laboratories (Horse Serum No. 3) and kept at −20°. Ferric ammonium citrate (Laboratory reagent Fe 20–22% w/v) and haematin hydrochloride (Fe 8.7%) were from British Drug Houses (Poole, Dorset, UK). Uniformly labelled L-[^{14}C] phenylalanine (513 mCi/mmole) and L-[^{3}H] phenylalanine (1 Ci/mmole) were from the Radiochemical Centre (Amersham, Bucks., UK).

3. Results

3.1. *Bacteriostatic effect of horse serum*

Fig. 1 shows a typical growth curve for *E. coli* 0111 in normal horse serum. After a slight lag, the organisms grew rapidly before going into stasis between the 3rd and 4th hour. The addition of haematin hydrochloride to bacteria which were already in stasis restored their ability to divide and grow (fig. 1). As described previously [1], the addition of enough ferric ammonium citrate to saturate the total iron binding capacity of the serum transferrin abolished the ability of the serum to induce bacteriostasis and allowed the bacteria to grow normally.

3.2. *Comparison of the chromatographic profiles of phenylalanyl-tRNA from normal and serum inhibited cells*

To make a detailed comparison possible nucleic

acid extracts from the normal and serum inhibited bacteria were co-chromatographed on a BD-cellulose column. Fig. 2 shows the elution profile of phenylalanyl-tRNA extracted from *E. coli* 0111 grown in broth and from the bacteria when inhibited by serum; inhibited cells were harvested after 4–5 hr in serum, just after they had entered stasis. It can be seen that the ^{3}H-labelled phenylalanyl-tRNA from the serum inhibited cells emerged ahead of the ^{14}C-labelled phenylalanyl-tRNA obtained from the broth grown cells. When the tRNA in both the normal and inhibited bacteria was charged with [^{14}C] phenylalanine and the extracted [^{14}C] phenylalanyl-tRNAs chromatographed separately on the same column, it was seen that phenylalanyl-tRNA from the inhibited cells again emerged before that of the normal cells. Similarly, reversal of the radioactive labels and co-chromatography produced an elution diagram similar to fig. 2. The difference in elution volume between the phenylalanyl-tRNA obtained from the two sources is, therefore, not an artefact produced by contaminants in the radioactive phenylalanine preparations [15]. It clearly shows the presence of abnormal species of phenylalanyl-tRNA in the serum inhibited cells. The behaviour of the phenylalanyl-tRNAs on BD-cellulose was highly reproducible, with the abnormal phenylalanyl-tRNA eluting with salt solution containing 3% ethanol and the normal phenylalanyl-tRNA with salt solution containing 5% ethanol. No attempt was made to separate individual isoaccepting species.

3.3. *Effect of iron compounds*

The co-chromatography on BD-cellulose of phenylalanyl-tRNA from serum inhibited cells and from cells grown for the same length of time in serum containing sufficient ferric iron to saturate the transferrin [8] is shown in fig. 3. As before, the serum inhibited cells contained the abnormal phenylalanyl-tRNA. Phenylalanyl-tRNA from bacteria grown in serum plus iron, however, behaved exactly like that obtained from broth grown cells and eluted from the column with salt solution containing 5% ethanol. The phenylalanyl-tRNA from *E. coli* 0111 first inhibited by serum and then exposed to haematin hydrochloride for 60 min before harvesting also behaved exactly like that from normal broth grown cells.

4. Discussion

E. coli grown in media containing less than about 10^{-7} M Fe^{3+} contain abnormal species of phenylalanyl-tRNA [9, 10]. The discovery of similar abnormal phenylalanyl-tRNA in *E. coli* 0111 when inhibited by serum therefore supports the hypothesis [6] that certain antibodies, acting in concert with transferrin, inhibit bacterial growth by directly interfering with an organism's iron metabolism. This view is strengthened by the observation that the appearance of abnormal phenylalanyl-tRNA could be prevented by saturating the transferrin with ferric iron and that the abnormal phenylalanyl-tRNA was replaced by the normal species when haematin was added to the inhibited cells. Both ferric iron and haematin abolished bacteriostasis.

The exact molecular basis for the alteration in phenylalanyl-tRNA in this instance is unknown. Although it is unlikely that incorrect esterification of another tRNA with phenylalanine has occurred *in vivo*, both this possibility and that of a real alteration in the structure of $tRNA^{Phe}$ itself will have to be investigated. Rosenberg and Gefter [10] have shown that iron is specifically involved in the synthesis of the 2-methylthio group on the isopentenyladenosine residue adjacent to the anticodon region in $tRNA^{Tyr}$ of *E. coli*. The N^6-(Δ^2-isopentenyl)-2-methylthio-adenosine residue is also present in $tRNA^{Phe}$ of *E. coli* [16–18] and it has been suggested [10] that all tRNAs containing this modification would be expected to show alterations during iron limitation. It is, therefore, tempting to speculate that the alteration in phenylalanyl-tRNA seen in serum inhibited *E. coli* 0111 is due to the absence of this particular nucleoside in $tRNA^{Phe}$.

The functional significance of the presence of abnormal phenylalanyl-tRNA and possibly other aminoacyl-tRNAs in bacteria inhibited by serum remains to be determined. It is known, however, that the presence of certain modified nucleosides is essential for the correct functioning of tRNA [19, 20]. Wettstein and Stent [9] found that when *E. coli* was grown in iron depleted media, both normal and abnormal phenylalanyl-tRNA occurred together in varying amounts. Sueoka and Kano-Sueoka [21] suggest that enough of the normal components are stil present to carry out protein synthesis at more or less

a normal rate under such conditions. Unlike serum, iron depleted media do not induce bacteriostasis [9, 22, 23]. In contrast, the present results show only abnormal phenylalanyl-tRNA in cells of *E. coli* 0111 when inhibited by serum. It may well be that bacteriostasis is induced not by the presence of abnormal phenylalanyl-tRNAs but by the absence of normal ones.

Acknowledgement

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