SHORT COMMUNICATIONS

Effect of Tryptophan Analogues on Synthesis of Staphylococcal β -Haemolysin

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INTRODUCTION

Amino acid analogues have been shown to exhibit selective action on the synthesis of proteins (Pardee & Prestidge, 1958; Mach, Reich & Tatum, 1963). Montie & Ajl (1964) reported the selective inhibition of murine toxin synthesis by tryptophan analogues in *Pasteurella pestis*. Leboeuf-Trudeau, De Repentigny, Frenette & Sonea (1969) studied the effect of tryptophan analogues on the tryptophan metabolism and α -toxin formation in *Staphylococcus aureus* Wood 46 strain and demonstrated a partial inhibition of growth and the loss of α -haemolysin activity.

In the present study, a number of tryptophan analogues were employed to see if a correlation between tryptophan metabolism and the synthesis of staphylococcal β -haemolysin also exists.

METHODS

Culture. Strain 681C of *Staphylococcus aureus* which produces β -haemolysin was used. It did not require tryptophan for growth or haemolysin formation.

Chemicals. 4-Methyltryptophan (4-MT), 5-methyltryptophan (5-MT), 6-methyltryptophan (6-MT), 5-fluorotryptophan (5-FT), 7-azatryptophan (7-AT), amino acids, anthranilic acid, shikimic acid, and indole were obtained from the Sigma Chemical Co., St Louis, Missouri, U.S.A. Vitamins were obtained from Calbiochem, Los Angeles, California, U.S.A.

Growth and β -haemolysin formation. Organisms were grown in the following chemically defined medium (g/l distilled water): L-alanine, 0.018; L-arginine, 0.105; L-asparagine 0.030; L-aspartic acid, 0.026; L-cystine, 0.024; L-glutamic acid, 0.0295; L-glutamine, 0.3; glycine, 0.015; L-histidine, 0.03; L-isoleucine, 0.052; L-leucine, 0.052; L-lysine, 0.058; L-methionine, 0.015; L-phenylalanine, 0.032; L-proline, 0.023; L-serine, 0.022; L-threonine, 0.048; L-tryosine, 0.036; L-valine, 0.046; choline Cl, 0.0015; folic acid, 0.0015; inositol, 0.003; nicotinamide, 0.0015; D-Ca pantothenate, 0.0015; pyridoxal HCl, 0.0015; riboflavin, 0.0015; thiamine, 0.0015; D-glucose, 5.0; sodium citrate, 0.420; Na₂HPO₄, 16.5; KH₂PO₄, 1.5; (NH₄)₂SO₄, 2.0; MgSO₄·7H₂O, 0.2; CaCl₂·2H₂O, 0.01; FeSO₄·7H₂O, 0.01. The complete medium was filter-sterilized (0.22 μ membrane filter, Millipore Corporation, Bedford, Massachusetts, U.S.A.) and dispensed in 300 ml Nephelo culture flasks (Bellco, Vineland, New Jersey, U.S.A.) with side arm. The medium was inoculated with 1.5 ml of the bacterial suspension having an extinction of 25 Klett Units (extinction was measured by Klett-Summerson Photoelectric Colorimeter with a no. 42 filter, Klett Manufacturing

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Fig. 1. Prevention of analogue inhibition of growth and toxin formation by L-tryptophan and its metabolic precursors. (A) Control culture; organisms grown in L-tryptophan-free medium. (B) As (A) but 5-methyltryptophan (5-MT, 100 $\mu\text{g/ml}$) was added after 6 h of growth. (C) As (A) but medium contained tryptophan (10 $\mu\text{g/ml}$) and 5-MT (100 $\mu\text{g/ml}$) was added after 6 h. (D) As (A) but tryptophan (10 $\mu\text{g/ml}$) and 5-MT (100 $\mu\text{g/ml}$) were added after 6 h. (E) As (A) but shikimic acid (20 $\mu\text{g/ml}$) and 5-MT (100 $\mu\text{g/ml}$) were added after 6 h. (F) As (A) but indole (20 $\mu\text{g/ml}$) and 5-MT (100 $\mu\text{g/ml}$) were added after 6 h. (G) As (A) but anthranilic acid (20 $\mu\text{g/ml}$) and 5-MT (100 $\mu\text{g/ml}$) were added after 6 h. $\square$, Growth; $\blacksquare$, toxin.

Co., New York, U.S.A.) prepared from overnight culture in the same medium. The flasks were incubated at 37 °C in air. After 6 h, when the organisms were in the logarithmic phase of growth, various analogues of tryptophan were added to separate flasks. At every 60- or 100-min intervals, the growth and the amount of β -haemolysin were measured.

The growth of the culture was followed by extinction measurements and organism-free filtrates obtained by centrifugation followed by membrane filtration.

Detection and quantitation of β -haemolysin. Formation of β -haemolysin was detected by its typical haemolytic action on sheep erythrocyte agar plates prepared by incorporation of washed sheep erythrocytes into Trypticase Soy agar. Wells were punched into agar plates and filled with culture filtrates. The plates were incubated at 37 °C for 1 h, refrigerated and then the typical haemolytic reaction of β -haemolysin was recorded. Quantitation of β -haemolysin was done by tube titration (Haque & Baldwin, 1964). The highest dilution showing 50 % lysis was recorded as the titre.

RESULTS

Effect of analogues on growth and β -haemolysin formation. 4-Methyl tryptophan, 5-MT, and 7-AT, when added to logarithmically growing culture were more inhibitory to growth and β -haemolysin formation than the 6-MT and 5-FT. In all cases, however, the formation of β -haemolysin was more sensitive to the inhibitory effect of these analogues than was bacterial growth. Formation of β -haemolysin was completely inhibited by 80 to 100 μg of 4-MT, 5-MT, or 7-AT/ml but most selectively inhibited by 5-FT (100 $\mu\text{g/ml}$) which gave 95 % inhibition of haemolytic activity with only 5 % growth inhibition. 5-Methyltryptophan and 7-AT, at the same concentrations, although inhibiting completely the formation of β -haemolysin, also resulted in 30 and 38 % inhibition of growth respectively. They thus provided an opportunity to study the reversal of both growth and haemolysin formation. 5-Methylprophan was selected for studies on the site of its action.

Reversal of inhibition of growth and β -haemolysin formation. Low levels of L-tryptophan (10 μ g/ml) when present in the medium allowed growth but did not prevent 5-MT inhibition of toxin formation (Fig. 1). L-Tryptophan, however, prevented inhibition of both growth and toxin formation when added simultaneously with the 5-MT to a logarithmically growing culture in the tryptophan-free medium (Fig. 1). Addition, at 20 μ g/ml, of either shikimic acid or indole, together with 5-MT, failed to prevent inhibition of growth or production of toxin (Fig. 1). In contrast, anthranilic acid prevented 5-MT inhibition of growth or toxin formation (Fig. 1).

DISCUSSION

The selective inhibitory action on β -haemolysin synthesis exhibited by 5-FT is similar to that reported toward murine toxin synthesis in *Pasteurella pestis* (Montie & Ajl, 1964). Together with the selective inhibition noted for 5-MT when L-tryptophan was present in the medium the findings indicate that formation of staphylococcal β -haemolysin, like other bacterial toxins so far studied (Montie & Ajl, 1964; Leboeuf-Trudeau *et al.* 1969), is influenced by the tryptophan metabolism of the cell.

Montie & Ajl (1964) proposed that methyl analogues of tryptophan exert their inhibitory effect on the synthesis of murine toxin in *Pasteurella pestis* by producing a tryptophan-deficient system. They were able to prevent the inhibitory action of these compounds by tryptophan, indole or shikimic acid but not by anthranilic acid, indicating that the methyl analogues probably act as false feedback inhibitors at sites prior to tryptophan synthetase. Moyed & Friedmann (1959) reported that 5-MT inhibited the conversion of shikimic acid-5-phosphate and glutamic acid to anthranilic acid but not to shikimic acid. This suggests that 5-MT inhibits the formation of β -haemolysin probably by blocking the enzymatic conversion of chorismic acid to anthranilic acid which results in the depletion of tryptophan. Leboeuf-Trudeau *et al.* (1969) have reported that, for the *Staphylococcus aureus* Wood 46 strain, tryptophan or anthranilic acid, but not shikimic acid, prevented inhibition by methyl analogues of tryptophan.

Selective inhibition of β -haemolysin synthesis can be explained on the basis that the synthesis of β -haemolysin requires more tryptophan than other bacterial proteins. This is supported by the finding that a low concentration of exogenous tryptophan (10 μ g/ml) resulted in complete restoration of growth but not of β -haemolysin synthesis. Obviously this exogenous supply met the requirements for the synthesis of proteins needed for growth but was not enough for the synthesis of β -haemolysin. The reports that murine toxin of *Pasteurella pestis* has higher tryptophan content than does the average bacterial protein (Montie & Ajl, 1964) and that α -amylase of *Bacillus stearothermophilus*, which contains no tryptophan, was preferentially synthesized in the presence of 5-MT (Welker & Campbell, 1964) lend further support to this view.

Of the analogues tested here 5-FT exerted the most selective inhibition of β -haemolysin formation. Sharon & Lipmann (1957) reported that 5-FT and 6-FT could be activated with purified tryptophan-activating enzyme. Thus, it is likely that the tryptophan-activating enzyme of *Staphylococcus aureus* recognizes 5-FT, activates it and probably incorporates it in place of tryptophan, and produces a defective toxin molecule.

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