DE-ESTERIFICATION OF CHOLESTERYL ESTERS IN HUMAN PLASMA
α-LIPOPROTEIN (HDL) BY PREPARATIONS OF STAPHYLOCOCCAL ALPHA TOXIN

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SUMMARY

Chemical extraction and analysis of reaction mixtures of preparations of staphylococcal alpha toxin and human plasma α-lipoprotein (HDL) indicated that the cholesteryl esters which form an integral part of the lipoprotein molecules were de-esterified by the toxin preparations with no apparent destruction of the cholesterol moiety.

This report is the first of cholesterol esterase produced by staphylococcus. This activity is closely associated with alpha toxin and may be a manifestation of alpha toxin itself. In addition it is shown that soluble plasma lipoproteins are useful substrates for biological reactions involving lipids.

Certain bacteria produce extracellular toxic proteins which are cytolytic, causing damage to the structural and functional integrity of membrane systems of eucaryotic and in some cases, procaryotic cells (1). The alpha toxin of Staphylococcus aureus is one such agent. It is a heat labile protein whose monomeric form has a sedimentation rate of 3S, which aggregates easily to a soluble, less active 12S form and eventually to an insoluble, inactive form (2). Although the secondary effects resulting from the action of alpha toxin on a variety of natural and artificial membrane systems have been described (3), the primary site and mechanism of membrane damage has not been reported.

During investigations on the effect of membrane damaging agents on soluble lipoproteins, I have obtained data indicating that preparations of staphylococcal alpha toxin had a specific action of de-esterifying the cholesteryl esters which are an integral part of lipoprotein molecules (4). The cholesteryl moiety itself does not seem to be altered by the toxin.

Materials and Methods

Alpha toxin, produced by the Wood 46 strain of Staphylococcus aureus was
purified by ammonium sulfate precipitation and by successive column chromatography over G-50 fine and then G-100 super fine Sephadex gels (5,6). Analytical ultracentrifuge diagrams of all preparations showed either a single 3S peak characteristic of monomeric alpha toxin or the 3S peak accompanied by a 12S peak characteristic of aggregated alpha toxin. Preparations used in this study were free of proteolytic activity by the Azocol method (Calbiochem, La Jolla, California, Document No. 3505). Hemolytic activity was assayed using rabbit red blood cells (5).

Human plasma α-lipoprotein (HDL) was isolated and purified by differential flotation and equilibrium density gradient purification (7,8). It was quantitated using an ε_{1cm,280} of 5.5 based on dry weight measurements. Polyacrylamide gel electrophoresis of protein preparations was performed in 7-1/2% gels and at pH 8.9 (9) using a Buchler Polyanalyst apparatus. Lipids were extracted from lipoproteins by chloroform-methanol (10). Separation of extracted esterified and unesterified cholesterol was done by silicic acid chromatography (11) and the resultant fractions were analyzed spectrophotometrically (12).

**Experimental Procedure**

The experimental reaction mixtures contained a known amount of α-lipoprotein (HDL) (ca 10 mg/ml) and amounts of alpha toxin preparation containing from 3,000 to 15,000, 50% hemolytic units per mg. lipoprotein.

The mixtures were incubated at 37°C in phosphate buffer at pH 7.0 (5) for 2 hours. Controls contained the same concentration of α-lipoprotein (HDL) without added alpha toxin. At the end of the incubation period samples of the reaction mixture and of the control mixture were examined by electrophoresis in polyacrylamide gels, by analytical ultracentrifugation and by extraction and chemical analysis of lipids.

**Results**

The results of the chemical analyses showed that α-lipoprotein (HDL) treated with the alpha toxin preparations contained less esterified cholesterol and proportionately more unesterified cholesterol as shown in Table 1. The decrease in esterified cholesterol became more pronounced as the ratio of alpha toxin to α-
De-esterification of Cholesteryl Esters in Human Plasma

α-Lipoprotein by Staphylococcal Alpha Toxin

<table>
<thead>
<tr>
<th>Units of alpha toxin</th>
<th>Cholesteryl Moiety</th>
<th>Molar Ratio</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>mg. lipoprotein</td>
<td>µg/mg lipoprotein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unesterified</td>
<td>Esterified</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (i)</td>
<td>32</td>
<td>88</td>
<td>120</td>
</tr>
<tr>
<td>0 (ii)</td>
<td>24</td>
<td>73</td>
<td>97</td>
</tr>
<tr>
<td>Test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 x 10^3 (i)</td>
<td>46</td>
<td>80</td>
<td>126</td>
</tr>
<tr>
<td>8 x 10^3 (ii)</td>
<td>67</td>
<td>34</td>
<td>101</td>
</tr>
<tr>
<td>15 x 10^3 (i)</td>
<td>89</td>
<td>7</td>
<td>96</td>
</tr>
</tbody>
</table>

a: Each experiment used a different preparation of alpha toxin. Two preparations of α-lipoprotein were used, designated (i) or (ii). Each experiment was incubated at 37°C for 2 hours, pH 7. To convert the esterified values in column 3 into weight of the ester, these figures should be multiplied by 1.72 (4).

b: Rabbit Red Blood Cell hemolytic units (5).

c: The cumulative reproducibility of methods of extraction, separation and chemical analysis of cholesterol is, in my laboratory better than 5% over an extended period of time.

lipoprotein (HDL) was increased. Alpha toxin preparations held at 60° for 2 minutes or at room temperature for 12 hours had lost both their lytic activity on rabbit red blood cells and their de-esterifying activity on HDL. The data show that in experiments where the ratio of alpha toxin to lipoprotein was lower, i.e., 3 x 10^3 and 8 x 10^3 H.U./mg, the weight recoveries of the cholesteryl moiety (esterified + unesterified) in the test as compared with the control mixtures were essentially the same. This observation is evidence that the decrease in the amount of esterified cholesterol in the α-lipoprotein (HDL) which had been treated with alpha toxin preparations was not the result of some toxin-induced loss of the assay-reactive portion of the cholesteryl moiety or of the loss of intact cholesteryl ester. The lower recovery (80%) in the experiment containing 15 x 10^3 H.U. alpha toxin/mg...
Polyacrylamide gel electrophoresis in 7.5% gels at pH 8.9. a. bovine serum albumin, included as a reference. b. α-lipoprotein (HDL) MW ca 300,000. The broad band in this gel is due to both the high molecular weight and to the broad density and size distribution of pure α-lipoprotein (HDL)(4). c. α-lipoprotein (HDL) - alpha toxin mixture. The α-lipoprotein was incubated for 2 hours at 37°, with 8000 H.U. alpha toxin per mg. lipoprotein. d. alpha toxin, same concentration as in c. Note: The black spots at the bottom of gel c are artifacts.

lipoprotein may have been due to sampling difficulties due to denaturation and fragmentation of the lipoprotein as seen visually and in the analytical ultracentrifuge. Additional data indicate that as the reaction between α-lipoprotein (HDL) and alpha toxin preparations proceed the lipoprotein molecule begins to lose its integrity, releasing some smaller fragments and leaving a residual molecule(s) of lower sedimentation rate. The electrophoretic mobility of these fragments after polyacrylamide gel electrophoresis is increased compared to that of the unreacted lipoprotein control (Figure 1).
Discussion

The findings presented in this report indicate that our staphylococcal alpha toxin preparations de-esterify cholesteryl esters present as a part of plasma \( \alpha \)-lipoprotein (HDL). A result of this reaction is a gradual fragmentation of the lipoprotein molecule. The fact that the esterase activity has not been described previously may be ascribed to the emphasis placed on studying the various secondary effects of alpha toxin because these effects may be important in naturally affected membrane systems. Furthermore, studies using cholesteryl esters alone are difficult due to the insolubility of these molecules when present in aqueous systems.

Observations relevant to the role of cholesteryl esters in the stability of cell membranes have indicated that the red blood cell membranes of individuals suffering from lecithin-cholesterol-acyl-transferase (LCAT) deficiency have a lowered cholesteryl ester content. When serum containing normal LCAT activity is added to these abnormal cells the proportion of cholesteryl ester is increased to normal. The red cells of LCAT deficient blood are also abnormal in shape and have an increased fragility (13). Other studies have shown that red cells become more fragile as the content of cholesteryl ester decreases (14).

The task of proving that a single molecular species carries more than one biological activity is arduous. Detailed experiments are in progress in my laboratory designed either to prove or to disprove the identity of staphylococcal alpha toxin and the cholesteryl esterase described here. In either case, it is evident that \textit{Staphylococcus aureus} does produce a cholesterol esterase and that soluble lipoproteins provide a useful model system for the study of biological reactions involving lipids.

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References