Verotoxin and neuraminidase induced platelet aggregating activity in plasma: their possible role in the pathogenesis of the haemolytic uraemic syndrome

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SUMMARY Certain strains of Escherichia coli producing verotoxin have been isolated in the stools of patients with the haemolytic uraemic syndrome. A platelet aggregating activity has been found in normal plasma after incubation with verotoxin at 37°C for 24 h. This activity, unlike neuraminidase, has an effect that is independent of changing factor VIII related antigen, but requires the IIA and IIIB platelet surface glycoprotein (deficient in thrombasthenia) to mediate its effect. Prostacyclin totally inhibited this effect, but other antiplatelet drugs and heparin were without inhibitory effects.

The haemolytic uraemic syndrome is characterised by microangiopathic haemolytic anaemia, thrombocytopenia, and renal failure, usually preceded by a prodromal period of bloody diarrhoea. Although the aetiology of the disease is unknown, recent reports have implicated a cytotoxin, verotoxin, in the pathogenesis of this disorder. Verotoxin is present in culture filtrates of certain strains of Escherichia coli and can induce cytopathic changes in vero cells. These strains have been isolated from faeces, while several neuraminidase producing organisms have been isolated from blood of patients with the haemolytic uraemic syndrome. Explanations as to the pathogenesis of the platelet microthrombi and deposited fibrin in the haemolytic uraemic syndrome includes platelet endothelial cell interaction, with possible primary endothelial cell damage, or the presence of a platelet aggregating agent as reported in some cases of thrombotic thrombocytopenic purpura.

We report here the effect of culture filtrates from verotoxin producing E coli strains and Clostridium perfringens (a neuraminidase producing organism) on plasma and platelets.

Material and methods

Venous blood was collected from three normal adults, one patient with severe classical von Willebrand's disease, and three patients with inherited platelet defects (one with Bernard-Soulier syndrome and two with thrombasthenia). Nine parts of blood was added to one part of 0·11 M trisodium citrate. Platelet rich plasma (PRP) was prepared by centrifugation at 200 g for 8 min at room temperature. Platelet poor plasma (PPP) was prepared by centrifugation at 1500 g for 15 min. For platelet aggregation studies, the PRP was diluted with the subject's own PPP to give a final platelet concentration of 300 × 10⁶/l. Fresh washed platelets were prepared using the albumin layering technique described by Walsh. Platelet aggregometry was done using a Bio Data Corporation 4 channel aggregometer with stirring at 37°C.

Preparation of cultured bacterial filtrates.

Verotoxin producing E coli 026:K60 and 30979 were provided by Dr D Candy (Institute of Child Health, Birmingham Children's Hospital) and Dr B Rowe (Central Public Health Laboratory, Colindale) respectively. The haemolytic strain of E coli and Clostridium perfringens type A were isolated from clinical material at the Birmingham Children's Hospital. The E coli strains were incubated aerobically for 36 h in iso-sensitest broth (Oxoid Ltd) at 37°C and Clostridium perfringens was incubated anaerobically in the same broth using a GasPak system (Beckton Dickinson Limited). Cell free filtrates of the culture were obtained by centrifugation and filtration through a 0·2 µm millipore filter. The spun deposit of each culture was recultured to exclude bacterial contami-
nation, and the filtrate was subcultured on to blood agar and incubated aerobically and anaerobically to confirm sterility.

**VEROTOXIN ASSAY**
Two hundred microlitres of vero cell suspension in minimal essential medium (Gibco Ltd), with added fetal calf serum to a final volume of 10%, was added to each well of a microtitre tissue culture and examined daily until vero cell monolayers had been established. Fifty microlitres of the tissue culture medium was removed and replaced with an equal volume of heat or serially diluted filtrate (1/2 to 1/4096) in phosphate buffered saline, pH 7-4. The plates were resealed and incubated at 36°C, and the lowest titre at which vero cell cytotoxicity occurred was determined. Verotoxin was present to a titre of 1/80 with verotoxin producing E coli 026:K60 and 1/160 with verotoxin producing E coli 30979. No verotoxin was found with the control haemolytic E coli strain.

**STUDY OF THE EFFECT OF TOXINS**
Sterile filtrates prepared by verotoxin producing E coli strains, haemolytic E coli and Cl perfringens were tested as follows:

**On platelets**
1 Forty microlitres of filtrate was added to 200 μl of PRP from three normal controls, two patients with thrombasthenia, and one patient with Bernard-Soulier syndrome.
2 Two hundred microlitres of freshly washed platelets was incubated for 1 h with 40 μl of filtrate before adding 100 μl of fresh normal PPP. The effect on the platelets in each experiment was observed in the aggregometer.

**On plasma**
Half a millilitre of filtrate was added to 1 ml of fresh normal PPP and also to 1 ml of PPP from a patient with severe von Willebrand's disease and incubated at 37°C for different times. Incubation for 24 h appeared to be the shortest incubation time to produce consistent results. Plasma obtained from two recent haemolytic uraemic syndrome patients at presentation was similarly treated with verotoxin filtrate.

Control samples of 1 ml of PPP with 0.5 ml of phosphate buffered saline and 1 ml of PPP with 0.5 ml of culture filtrate were similarly tested for platelet aggregation after 24 h incubation at 37°C.

**TESTS USING VEROTOXIN TREATED PPP**
Platelet aggregation induced by verotoxin treated PPP was quantitated by adding 20, 40, 60, and 75 μl of the treated PPP to 125 μl of fresh normal PRP. To investigate the nature of the platelet receptor implicated in this reaction, verotoxin treated PPP was added to PRP from two patients with thrombasthenia, one patient with Bernard-Soulier syndrome, and normal PRP treated with antiplatelet drugs. Normal PRP was incubated for 20 min at room temperature with aspirin (final concentration 60 μg/ml), sulphinpyrazone (final concentration 10 μg/ml), and dipyridamole (final concentration 80 μg/ml) before the addition of verotoxin treated plasma and subsequent platelet aggregation. Prostacyclin was added to PRP at a final concentration of 100 μmol/l before the addition of verotoxin treated normal PPP. The effect was monitored in the aggregometer. Normal PPP, after incubation with verotoxin, was incubated for a further 90 min at room temperature with sodium heparin (final concentration 170 units/ml) before the addition of normal PRP and testing for platelet aggregation. The same quantity of heparin added to 10 units/ml of thrombin appeared to neutralise all the thrombin, as after neutralisation no thrombin induced platelet aggregation was obtained.

**TESTS USING NEURAMINIDASE TREATED PPP**
As platelet aggregation was noted after the addition of Cl perfringens treated plasma, the effect of incubation of normal plasma with purified neuraminidase Sigma V (final concentration 1 mg/ml) was tested. After 24 h incubation at 37°C, the neuraminidase treated plasma samples were added to fresh PRP and platelet aggregation was observed.

**Results**
Direct addition of the filtrates of verotoxin producing E coli, a haemolytic E coli, and Cl perfringens strains to normal PRP failed to produce platelet aggregation. No aggregation was seen on the addition of 100 μl of PPP to washed platelets incubated for 1 h with verotoxin.

Fresh normal plasma incubated with the verotoxin producing E coli and Cl perfringens filtrates became capable of inducing platelet aggregation (Figure). The verotoxin producing E coli had a similar effect on the von Willebrand plasma (0% VIII:R:AG) whereas the Cl perfringens filtrate failed to change the von Willebrand plasma, suggesting a different mode of action. The action of the verotoxin producing E coli is independent of VIII:R:AG while that of Cl perfringens is dependent on the presence of VIII:R:AG. The same results as the Cl perfringens treated plasma samples could be obtained by incubating the normal and von Willebrand plasma with purified neuraminidase (Sigma V). The toxin
Table 1  Degree of platelet aggregation (recorded as % change in optical density) with different concentrations in verotoxin treated normal platelet poor plasma

<table>
<thead>
<tr>
<th>Volume of verotoxin treated plasma (µl)</th>
<th>Maximum change in optical density (%)</th>
<th>Lag phase (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>72</td>
<td>45</td>
</tr>
<tr>
<td>40</td>
<td>66</td>
<td>20</td>
</tr>
<tr>
<td>60</td>
<td>59</td>
<td>nil</td>
</tr>
<tr>
<td>75</td>
<td>49</td>
<td>nil</td>
</tr>
</tbody>
</table>

*In these experiments the volume of verotoxin treated plasma has been adjusted to a final volume of 75 µl with phosphate buffered saline (pH 7-4) and then added to 125 µl platelet rich plasma (final platelet concentration 300 × 10⁹/l).

Addition of 100 µl of verotoxin treated PPP to 125 µl of platelets resulted in maximum platelet aggregation of 48%. The ratio of platelets to verotoxin treated PPP appears to be critical, however, as greater changes in maximum aggregation were noted with lower volumes of verotoxin treated PPP (Table 1), but this increased aggregation was preceded by a slight lag phase. PRP from two patients with thrombasthenia (Table 2) failed to aggregate on the addition of verotoxin treated PPP, but PRP from patients with Bernard-Soulier syndrome did aggregate. Treatment of normal PRP with aspirin, dipyridamole, and sulphinpyrazole failed to alter the platelet aggregation caused by verotoxin treated plasma. Furthermore the platelet aggregating activity was not inhibited by heparin, thereby excluding a thrombin type platelet aggregating effect. Prostacyclin totally inhibited the effect of verotoxin treated plasma on normal PRP. Plasma from patients presenting with the haemolytic uraemic syndrome which had been stored at −20°C did not develop any platelet aggregating activity after treatment with verotoxin filtrate.

**Discussion**

The above results show that treatment of normal plasma with verotoxin or neuraminidase will produce spontaneous platelet aggregating activity. Treatment of plasma from patients with von Willebrand’s disease with neuraminidase, unlike verotoxin, did not produce a platelet aggregating activity. This suggests that neuraminidase, unlike verotoxin, is dependent on changing VIIIIR:AG as the von Willebrand’s disease plasma lacks VIIIIR:AG. Purified factor VIIIIR:AG has been shown to be deasialated by neuraminidase to produce a platelet aggregating factor. In patients with the haemolytic uraemic syndrome, in whom neuraminidase producing organisms are thought to have an aetiological role, deasialated VIIIIR:AG may contribute to in vivo platelet aggregation and fibrin deposition.

The lack of response of verotoxin treated PPP with platelets from two patients with thrombasthenia suggests that the platelet membrane glycoproteins IIb and IIIa, which are deficient in such patients, are the site of action of the toxin induced

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**Table 2** Summary of effect of verotoxin treated plasma and platelet agonists (final concentrations shown) on normal and abnormal platelets

<table>
<thead>
<tr>
<th>Platelets tested</th>
<th>Platelet aggregation effect by</th>
<th>Verotoxin treated plasma</th>
<th>ADP (1 µmol/l)</th>
<th>Collagen (20 µg/ml)</th>
<th>Ristocetin (1-5 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal adults (×3)</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Thrombasthenia (1)</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Thrombasthenia (2)</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Bernard-Soulier</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Aspirin treated</td>
<td>N</td>
<td>(1)</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Dipyridamole treated</td>
<td>N</td>
<td>(1)</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Sulphinpyrazole treated</td>
<td>N</td>
<td>(1)</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

N = normal aggregation; O = no aggregation; (1) = 1st phase aggregation only; ↓ = reduced aggregation.
aggregating factor. Prostacyclin affects most aspects of platelet function preventing shape change and the expression of fibrinogen receptors on the platelet surfaces. Because fibrinogen receptors are critical for platelet aggregation, prostacyclin inhibits aggregation at an early stage. Prostacyclin may inhibit the verotoxin treated PPP by a direct effect on the platelet membrane—for example, preventing expression of fibrinogen receptors—but further studies are required to evaluate the exact way in which prostacyclin blocks the platelet aggregating effect of verotoxin treated PPP as prostacyclin inhibits the effect of all known platelet agonists. As aggregation occurred with platelets treated with antiplatelet drugs, the platelet aggregation effect of verotoxin treated plasma is probably independent of platelet release reactions. The verotoxin induced platelet aggregating factor is similar to the spontaneous aggregating activity reported in some cases of thrombotic thrombocytopenic purpura in that neither antiplatelet drugs or heparin inhibited the effect. The failure of verotoxin filtrate to make haemolytic uraemic syndrome plasma aggregate normal platelets may be due to denaturation of the plasma during storage. Alternatively, the plasma factor that may be altered by verotoxin filtrate has been totally destroyed or is deficient or an inhibitor may be present. Further study on fresh plasma from haemolytic uraemic syndrome patients at presentation is indicated. We have been unable to test the effect of verotoxin filtrate on thrombotic thrombocytopenic purpura plasma. The observed inhibitory effect of prostacyclin on verotoxin treated PPP platelet induced aggregation warrants further study to see if further information to substantiate or refute the aetiological importance of the lack of a stimulator for prostacyclin or the presence of an inhibitor to prostacyclin.

Recent reports suggest that verotoxin is a similar or even the same toxin as the previously reported Shiga toxin. The latter consists of an A chain with enzymatic activity and six or seven smaller molecular weight B chains, which may form the binding moiety of the toxin. Specific binding to a glycoprotein receptor on mammalian cells involving B1-4-linked N-acetyl-D-glycosamine oligomers has been reported. Interaction of the toxin and a plasma glycoprotein may be important in development of the platelet aggregating activity.

While accepting that the pathogenesis of the haemolytic uraemic syndrome is probably multifactorial, verotoxin induced plasma changes may result in platelet aggregation and contribute to the pathogenesis of the syndrome in some patients. Further study is required to establish the precise mechanism of toxin induced platelet aggregating effect. The failure of aspirin, dipyrindamole, and sulphipyrazone to inhibit this effect questions the therapeutic usefulness of antiplatelet drugs in the management of patients with the haemolytic uraemic syndrome.

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References

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