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Failure to kill *Yersinia enterocolitica* by plasma diluted to the concentration found in red cell units

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Abstract

The possibility that the use of additive solutions for red cell storage might impair the ability of plasma to kill *Yersinia enterocolitica* was investigated by studying killing of *Y enterocolitica* by neat and diluted plasma. The ability of neat citrated plasma to kill complement sensitive organisms was lost at around 26%, the dilution typically found in red cell units. These results should be considered in the light of evidence that killing in plasma is important in the protection of donated blood against growth of *Y enterocolitica*, and the observation that the increase in frequency of transfusion reactions caused by *Y enterocolitica* coincided with the widespread introduction of additive solutions. Taken together, these points support the suggestion that the introduction of additive solutions may have precipitated the problem of growth of *Y enterocolitica* in stored blood.

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Keywords: *Yersinia enterocolitica*, blood transfusion, red cell storage, complement.

Sepsis and death resulting from transfusion of blood containing large numbers of *Yersinia enterocolitica* was first reported in 1975, but not reported again until 1982. Since then, the number of cases reported has increased with a total of 10 cases reported by 1988 and at least 33 by 1992.¹⁻³ It is thought that the *Y enterocolitica* found in donated blood originates as an asymptomatic bacteraemia in the donor. *Y enterocolitica* is able to multiply at 4°C, and can reach 10⁹ cfu/ml in blood at this temperature. Transfusion of blood containing large

numbers of *Y enterocolitica* has a high mortality (21/33; 64%), but is rare with an estimated one death per nine million transfusions since 1986 in the USA.

The increase in the frequency of *Y enterocolitica* induced sepsis following transfusion occurred at a time when the use of additive solutions for red cell storage was becoming more common. In the USA the use of additive solutions was approved by the Food and Drug Administration in 1983, and was adopted as standard in some states by 1985.⁴ This association may be significant as complement mediated killing in plasma is important in preventing the growth of *Y enterocolitica* in donated blood,² and as the use of additive solutions results in a reduced concentration of complement and other plasma components in the red cell storage medium. Details of red cell storage methods are not given in most case reports of *Y enterocolitica* induced sepsis following transfusion. Red cells in additive solution were reported to be involved in some of the early cases, and in both of the cases reported from Edinburgh.⁵ The use of additive solutions can also be inferred from reference to a 42 day expiration date in the largest reported series.³

We have proposed² that complement resistant phenotypes of *Y enterocolitica* in the blood of the donor become complement sensitive during the first few hours after storage, because of the known effect of low temperature on the expression of complement resistance in *Y enterocolitica*. In the presence of neat plasma the organisms are then killed, but in dilute plasma they could survive. Increased rate of cooling of blood, which may be a consequence of the use of red cell concentrates and additive solutions, also seems to contribute to survival

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Figure 1 Survival of complement sensitive *Y enterocolitica* strain 3175 after one hour at 22°C in citrated plasma from four donors. Blood was diluted in additive solution to the concentrations shown.

of *Y enterocolitica*, probably by reducing complement activity and favouring intracellular survival of the organisms.

When blood is stored in additive solution the concentration of remaining plasma is not accurately controlled. From the figures quoted in two studies,^{6,7} we calculated that the use of additive solution dilutes the citrated plasma to about 26% of its original concentration. Data from other studies⁸ suggest that the concentration of plasma may be as low as 9% of its original concentration. We therefore carried out experiments to determine whether dilution of plasma to these concentrations would affect killing of *Y enterocolitica*.

Methods

Blood (17.5 ml) from each of four donors was collected in 2.5 ml CP2D anticoagulant solution (citric acid, 16 mmol/l; sodium citrate, 89 mmol/l; sodium acid phosphate, 16 mmol/l; dextrose, 258 mmol/l; Tuta, Lane Cove, New South Wales, Australia). The blood was then centrifuged at 3000 rpm for five minutes and plasma separated and stored in aliquots at -70°C. For plasma killing experiments, freshly thawed citrated plasma was used neat or diluted in additive solution (citric acid, 2 mmol/l; sodium citrate, 20 mmol/l; sodium chloride, 123 mmol/l; sodium biphosphate, 20 mmol/l; adenine, 1.26 mmol/l; dextrose, 40 mmol/l; Tuta).

Eight strains of *Y enterocolitica* were used. Strains 3169 and 3172 were virulence plasmid positive, serotype O:9 strains from cases of transfusion associated *Y enterocolitica* sepsis.⁵ Strain 3202 was virulence plasmid positive, serotype O:3. Strain 3175 was a virulence plasmid negative derivative of 3169. Strains 3325-8 were plasmid negative strains of serotypes O:1, 2, 3; O:3; O5, 27, and O:20, respectively.³ *Escherichia coli* rough strain R2 was used as a complement sensitive control.

To produce the complement sensitive *Y enterocolitica*, bacteria were grown overnight at 22°C in brain heart infusion (BHI) broth. To produce the complement resistant phenotype, strain 3169 was grown overnight at 22°C, then 20 ml of this broth was added to 1 litre of tryptic soy broth (TSB), incubated for two

Figure 2 Survival of eight strains of *Y enterocolitica*, grown at 22°C to produce the complement sensitive phenotype, after one hour at 22°C in citrated plasma from a single donor. Blood was diluted in additive solution to the concentrations shown. p- indicates strain lacking the virulence plasmid. p+ indicates strains containing the virulence plasmid.

hours at 37°C, harvested by centrifugation, resuspended in 1 litre of TSB supplement with 20 mmol/l sodium oxalate and 20 mmol/l magnesium chloride and incubated for a further two hours at 37°C. *E coli* R2 was grown at 37°C overnight in BHI. The bacteria were washed twice by centrifugation in saline and the concentration was adjusted to approximately 5000 cfu/ml. For killing experiments 20 µl volumes of bacterial suspension were added to 1 ml volumes of plasma, plasma dilutions, and saline. After one hour at 22°C or 37°C, triplicate 20 µl samples were spread on blood agar plates and incubated overnight at 22°C. Colonies were counted and the count for each plasma dilution was expressed as percentage of the saline control count for each organism.

Results

Preliminary experiments showed that neat citrated plasma from one donor (APG) killed complement sensitive *Y enterocolitica* 3175 and *E coli* R2 in one hour at 37°C or 22°C. Heating the plasma at 56°C for one hour abolished its ability to kill these bacteria. Plasma diluted to 10% in additive solution did not kill either organism, but serum from the same donor diluted to 10% in complement fixation test buffer (CFT; Oxoid, Basingstoke, UK) did kill the organisms. Initial titration experiments at 37°C and 22°C showed that the ability to kill *Y enterocolitica* 3175 was lost at a plasma dilution between 40% and 20%.

Further experiments were carried out at 22°C with plasma from different donors (fig 1) and with additional strains of *Y enterocolitica* (fig 2). These confirmed that 20% plasma failed to kill the organisms, and that 26% plasma did not kill consistently. Strains of *Y enterocolitica* containing the virulence plasmid (p+ in fig 2) tended to survive at higher plasma concentrations than plasmid negative strains, des-

pite being grown at 22°C, conditions which do not favour plasmid expression.

Experiments with plasmid positive *Y enterocolitica* induced to express complement resistance confirmed earlier observations² that these organisms could be killed in neat plasma after four to six hours at 22°C. Prolonged incubation at this temperature allows the organisms to revert to the complement sensitive phenotype. Plasma at a concentration of 40% or more was able to kill the initially complement resistant organisms under these conditions, but failed to kill at 30% or less.

Discussion

We have shown that dilution of plasma in additive solution to 20% of its original concentration abolishes its ability to kill complement sensitive, virulence plasmid negative *Y enterocolitica*. This effect was fairly consistent between four different plasma donors. When strains containing the virulence plasmid were tested, 30% plasma did not kill the organisms consistently.

It is of note that 10% serum in CFT buffer in all cases was capable of killing the complement sensitive organisms, while 10% plasma in additive solution was not. We believe that this difference is related to the predominant activity of the alternative pathway in plasma, because of the low calcium concentration in the presence of citrate. Factor D, one of the key components of the alternative pathway, is present in much lower concentrations in blood than are the components of the classic pathway,⁹ and therefore alternative pathway activity may be more easily lost on dilution. We did find that the difference was not due to the presence of adenine in the additive solution (data not shown), but the effects of other components of the diluents were not directly compared because of the confounding effects of calcium and calcium chelators on clotting and complement activation.

Overall, these results support the hypothesis that dilution of plasma resulting from the use of additive solution may be partly responsible for the increased incidence of *Y enterocolitica* growth in donated blood. This does not exclude the possibility that more rapid cooling of blood may also contribute to the problem. An alternative explanation would be a greatly increased frequency of *Y enterocolitica* infection in the donor population, but there are no data to confirm this.

The experiments were not intended to reflect the exact timing of plasma dilution and cooling of plasma which would be encountered in practice. There is variation in the exact timing of these events in practice, so that attempting to model this accurately would not be practical. It was also thought not to be necessary, as plasma dilution might only have an effect on

survival of *Y enterocolitica* at one end of the range of conditions encountered in practice—that is, when blood is separated very soon after phlebotomy. This could still result in increased frequency of growth of *Y enterocolitica*.

Based on the present observations, it is reasonable to suggest that early dilution of plasma to low concentration would favour survival of *Y enterocolitica*. Delayed plasma dilution, probably at least six hours after collection (to allow time for *Y enterocolitica* to become complement sensitive at lower temperatures and then to be killed) would favour killing of *Y enterocolitica*. A higher residual concentration of plasma, probably at least 40%, would also favour killing of *Y enterocolitica*.

The mechanism involved in the increased incidence of *Y enterocolitica* growth in donated blood is unlikely to be established with certainty because it occurs rarely, and therefore practical steps to reduce this problem should be considered now. It is probably impractical to consider discontinuing the use of additive solution, or even to consider leaving 40% plasma in red cell preparations, because of the reduced yield of plasma for fractionation which would result. There is evidence to suggest that holding blood at 20°C overnight will favour killing of *Y enterocolitica*, but this gain may be offset by a rise in morbidity from more rapid growth of *Pseudomonas fluorescens*.¹⁰ Delay in dilution of the plasma for six to eight hours after collection could be introduced, and might significantly reduce the problem of growth of *Y enterocolitica*. A practical and reliable test for the detection of bacterial contamination in blood at the time it is issued from the blood bank would be extremely valuable.

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