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THE EFFECT OF BOVINE AND HUMAN SERUM ALBUMINS ON THE MECHANICAL PROPERTIES OF HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY

Crystalline bovine serum albumin increased the mechanical resistance of fresh human erythrocytes to lysis by hydrodynamic shear forces. A saturation effect suggests that the bovine albumin molecules are adsorbed on to a finite number of "attachment sites" on the erythrocyte surface, possibly by displacing human proteins already occupying these sites. A heterogeneous fraction of human serum albumins does not exhibit the same marked protection effect, nor displace adsorbed bovine albumin molecules from the erythrocyte surface. The precise nature and extent of the interaction between any given concentration of either human or bovine serum albumin and the intact erythrocyte membrane depends upon the chronological age of the cell concerned.

INTRODUCTION

The normal human erythrocyte is able to withstand the large fluid shear forces it encounters during its passage through the circulation by virtue of its flexibility and the unique mechanical and physical properties of its cell membrane. By analogy with engineering studies, it has tacitly been assumed that the complex rheological behaviour exhibited by a given erythrocyte membrane is constant for given values of temperature and pH. However, recent studies[1] have indicated that the mechanical and rheological properties of normal erythrocyte membranes are altered reversibly by the presence or absence of some normal blood constituents (*e.g.* glucose and certain proteins). It is possible that *in vivo* administration of similar natural or synthetic materials, which can increase the mechanical strength of human erythrocytes *in vivo*, might provide at least a temporary remedy for certain haemolytic anaemias due to excessive red cell fragility[1].

The normal human erythrocyte membrane is viscoelastic[2–4]. High shear stresses are needed for lysis during short exposure times[5–7], while long exposures require lower shear stresses to rupture the cell membrane[3,4,8]. Unfortunately, the effect of the suspending medium on the mechanical properties of the erythrocyte membrane has largely been ignored, and has given rise to minor inconsistencies when comparing the results of different investigators: *e.g.* cells sheared in whole blood[3] give higher critical shear stresses than similar cells sheared for approximately the

same time in viscous saline solutions[8], after allowances have been made for changes in osmotic strength.

A clinical measurement of red cell fragility is obtained when blood is shaken with smooth glass or quartz beads for a given time[9]. Rous and Turner[10] and Heimpel *et al.*[11] showed that the presence of autologous plasma "protected" the cells from such mechanical trauma, although this did not occur when rough glass beads were used by Shen *et al.*[9] and Greenberg[12]. However, Indeglia *et al.*[13] demonstrated a pronounced "protective effect" of plasma on cells sheared in the Fleisch haemoresistometer[14]. Similarly, Katchalsky *et al.*[15] showed that low concentrations of albumin added to the hypotonic saline solutions used in rapid osmotic lysis exerted a clear-cut protective action on the cells either by preventing the entrance of saline at the beginning of haemolysis or by changing the mechanical properties of the membrane.

The above results indicate that some constituent of normal blood plasma exerts a measurable influence on the mechanical properties of the intact cell membrane. The present paper examines the effect of purified human and bovine serum albumins on the haemolysis of human erythrocytes in response to hydrodynamic shear stress.

MATERIALS AND METHODS

Four drops of fresh adult human blood were obtained by finger puncture and rapidly resuspended in 10 ml of hypotonic (185 mosmoles/l or the equivalent of 0.54% saline) unbuffered saline containing 15 mM glucose, 13% w/v Dextran 500 (Pharmacia) and 0 to 46 g/l of purified human (Koch–Light: Cohn fraction 5) or crystalline bovine (Sigma) serum albumin. Aliquots (0.6 ml) of this viscous cell suspension ($\eta = 28$ cP) were sheared for 5 min at the desired shear stress (0 to 7000 dynes$\cdot$cm^{-2}) at 25 °C by means of a Ferranti–Shirley cone and plate viscometer. The sheared cell suspension was removed with a syringe and 0.2 ml mixed with 2.5 ml of isotonic (0.9%) unbuffered saline and centrifuged at 3000 rev./min for 5 min to sediment the intact cells. The absorbance of the supernatant was measured at 540 nm in 1 cm glass cells using a Perkin–Elmer spectrophotometer model 124. Cell disruption (haemolysis)was expressed as the amount of haemoglobin released by the cells during shear as a percentage of the haemoglobin released by osmotic lysis of the same number of original cells. Haemoglobin release is negligible from intact partially swollen cells in the absence of membrane rupture.

RESULTS AND DISCUSSION

Human erythrocytes in artificial viscous media are deformed when subjected to hydrodynamic shear stress: the cells are extended to give prolate ellipsoids having their long axes aligned parallel to the direction of flow[16]. As the shear stress is increased, the deformation is increased until the surface area of the ellipsoid is only just enough to enclose the volume of haemoglobin present within the cell. Any further increase in shear stress or deformation must therefore develop tangential tensions within the cell membrane, since the haemoglobin solution is an incompressible fluid, and no water can leave the cell. If the shear stress is maintained, these tangential tensions lead to the eventual mechanical rupture of the cell membrane and release of haemoglobin.

Unfortunately, some haemoglobin is also released during microsphere formation: *i.e.* the spontaneous breakdown of a long thin stretched blood cell to give a large number of small haemoglobin-filled spheres of different sizes[8]. This behaviour is similar to the breakdown of an extended emulsion droplet to give a large number of small micro-droplets when the shear stress causing the extension is suddenly removed[17]. The microspheres formed by cells in isotonic media at high shear stresses are so small (less than 0.5 μm diameter) that they do not sediment during centrifugation and so scatter the incident light in the spectrophotometer, giving falsely high absorbence and haemolysis values. In the present experimental series, the number of microspheres produced during (or at the end of) shear has been greatly reduced by causing partial swelling of the cells by their immersion in hypotonic (185 mosmoles/l) saline/15 mM glucose: the microspheres which remain do not constitute a significant source of error below about 60% haemolysis.

Qualitative effect of bovine serum albumin

Fig. 1 shows two representative haemolysis profiles (*i.e.* cell disruption as a function of the applied hydrodynamic shear stress) after 5 min shear at 25 °C. The solid curve (A) was obtained with control cells (0 g/l albumin) and shows the typical pattern of an initial curved portion (up to 20% haemolysis) followed by a linear region which terminates in a plateau at about 110% haemolysis (Fig. 1A). This extra 10% is caused by the presence of non-sedimented microspheres and could be eliminated by further swelling the cells in a more hypotonic medium. However, this is undesirable since it makes the oldest cells spherical and distorts the shape of the curve. Curve B (interrupted line) in Fig. 1 shows erythrocytes sheared in the presence of 36 g/l of crystalline bovine serum albumin: all other experimental conditions were exactly the same as those of the control cells (Curve A). The experimental points obtained with all erythrocyte suspensions containing 12 g/l or more of bovine serum

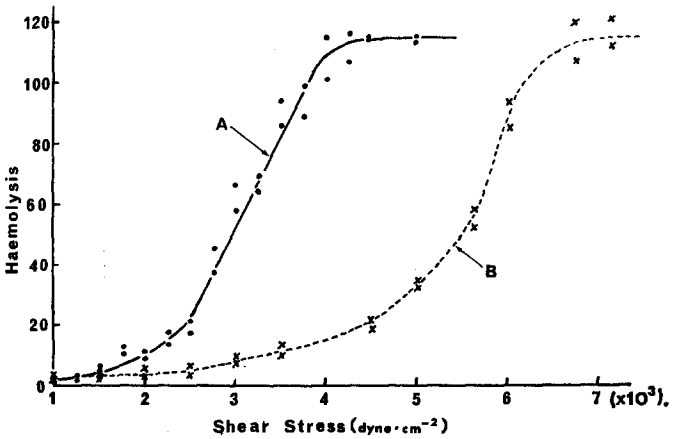

Fig. 1. Erythrocyte disruption in hypotonic medium (185 mosmoles/l) as a function of the applied hydrodynamic shear stress; showing the control curve (A) and similar cells in the presence of 36 g/l crystalline bovine serum albumin (Curve B).

albumin fit this curve (B), while suspensions containing less than 12 g/l gave curves having the same general shape as Curve B, but occupied a position on the shear stress axis between Curves B and A.

Fig. 1 clearly shows that bovine serum albumin increased the resistance of human erythrocytes to hydrodynamic disruption *in vitro*, *i.e.* it decreased the *in vitro* mechanical fragility of the cells. However, unlike glucose, which displaced the control curve to higher shear stresses without altering its shape[1], bovine serum albumin distorted the displaced curve (B in Fig. 1) so that the initial curved portion now extends beyond 20% haemolysis and has virtually eliminated the linear portion characteristic of the control curve (A).

The shape of the control curve is an index of the polydispersity of the initial blood sample, *i.e.* it is a measure of the distribution of membrane mechanical strengths among individual cells of different ages. Therefore a change in the shape of the displaced curve (B) means that some cells have become more resistant to mechanical rupture than others. This in turn implies that the same concentration of bovine albumin affects erythrocyte membranes of different ages to a different extent, or that membranes of different ages "interact" with different amounts of bovine albumin. This latter supposition is not unreasonable, since "old" red cells have a decreased electrophoretic mobility[18], and Danon *et al.*[19] have shown that they adsorb fewer charged colloidal iron particles. The same changes in the mucopolysaccharide surface coat which have decreased the number of sialic acid residues on the surface of the "old" erythrocyte could also have changed the number and possibly even the nature of the "attachment or interaction" sites of bovine serum albumin at the membrane surface.

Quantitative effect of bovine serum albumin

Fig. 2 shows the effect of increasing concentrations of bovine serum albumin on the hydrodynamic shear stress required to liberate 50% of the haemoglobin from the sheared cells (*i.e.* $\tau_{50\%}$). A curve of similar shape is obtained if any other degree of haemolysis is arbitrarily chosen. It can be seen that $\tau_{50\%}$ increased rapidly with increasing concentration of bovine albumin from 2950 dynes·cm^{-2} at 0 g/l and reached a maximum of 5500 dynes·cm^{-2} at about 10 g/l albumin (Fig. 2). A further

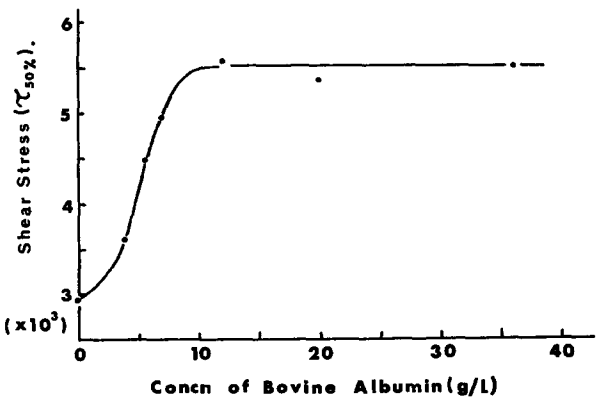

Fig. 2. This figure shows the shear stress needed to release 50% of the haemoglobin from cells sheared in the presence of various concentrations of bovine serum albumin.

increase in the concentration of bovine albumin (up to 36 g/l) caused no significant change in this maximum value of $\tau_{50\%}$ (Fig. 2).

Thus, Fig. 2 describes a saturation effect. This suggests that the bovine albumin is indeed being adsorbed by a finite number of "attachment" sites situated on the surface of the human erythrocyte membrane.

An order of magnitude estimate suggests that approx. 10^{-10} g or 10^9 molecules of bovine serum albumin are present for each red cell at the saturation concentration of 10 g/l (assuming an average molecular weight of 70000, and a final erythrocyte density of 10^8/ml). Each erythrocyte does not adsorb up to 10^9 molecules of bovine albumin, since cell suspensions containing 5 g/l bovine albumin still contain large quantities of protein (4.7 ± 0.5 g/l) after the erythrocytes have been removed by centrifugation. Thus, it seems likely that molecules of bovine serum albumin are competing for a finite number of attachment sites with other molecules, which are possibly protein in nature and are already adsorbed on to the erythrocyte membrane.

Qualitative effects of human serum albumin

The solid line in Fig. 3 (Curve A) shows the same control curve presented in Fig. 1 (Curve A) redrawn to a larger scale, and the experimental points have been omitted for the sake of clarity. The interrupted line in Fig. 3 (Curve B) shows the

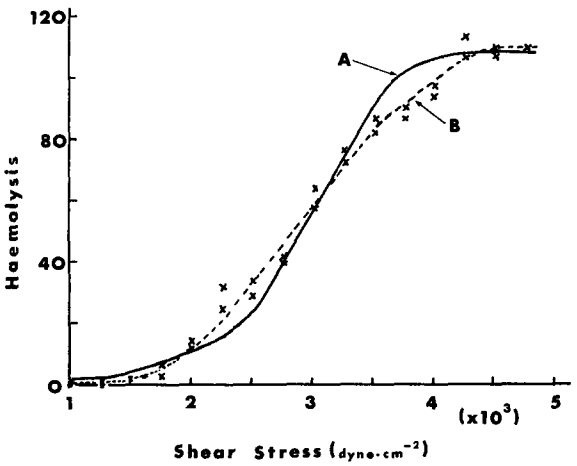

Fig. 3. Erythrocyte disruption as a function of the applied shear stress in the presence of 0 g/l (Curve A) and 11 g/l (Curve B) human serum albumins (Cohn fraction 5).

effect of 11 g/l of human serum albumin on cells which were otherwise identical with the control cells. Fig. 3 shows that human serum albumin did not markedly increase the mechanical strengths of human erythrocyte membranes, as did the addition of similar concentrations of bovine albumin (Fig. 1). Instead, human albumin "smoothed out" the control curve, giving more haemolysis at low shear stresses and less haemolysis at high shear stresses (Fig. 3B). Thus, the slope of the linear portion has been decreased while the shear stress causing 50 to 60% haemolysis has remained virtually unchanged. This indicates that some of the various proteins comprising the human albumin fraction (Cohn fraction 5) interact with human erythrocytes in such a way

that old cells are made weaker and young cells are made more resistant to mechanical lysis (Fig. 3). This again suggests that a quantitative and/or qualitative change has occurred in the protein adsorbing capacity of the human erythrocyte membrane as the cell ages.

Quantitative effects of human serum albumin

The shear stress value obtained when the linear portion of each curve is extrapolated to zero haemolysis is called the critical shear stress (τ_{crit}), and is a measure of the stretching force required to rupture the weakest erythrocytes. Fig. 4 shows τ_{crit} as a function of the concentration of human serum albumin present in the suspending medium. These same experimental points gave a good straight line when τ_{crit} was plotted against the logarithm of the concentration of human serum albumin. Fig. 4 also shows that increasing concentrations of human serum albumin caused a progressive decrease in τ_{crit}, while $\tau_{50\%}$ remained approximately constant: *i.e.* the fall in τ_{crit} is a measure of the change in slope of the linear portion of the curve.

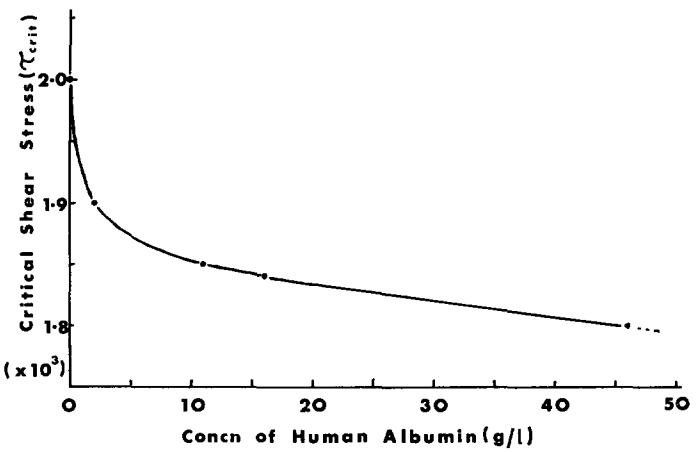

Fig. 4. This figure shows the critical shear stress (see text) as a function of the concentration of human serum albumin present during shear; *i.e.* it is a measure of the decrease in slope of the linear portion of the haemolysis curve.

In contrast to the effect of bovine serum albumin described above, the qualitative and quantitative changes observed with human serum albumin do not show a saturation effect. This suggests that the human proteins are not competing for a finite number of attachment sites.

Conclusions

This preliminary study has shown that crystalline bovine serum albumin increased the resistance of fresh human erythrocytes to lysis by hydrodynamic shear (Fig. 1). A saturation effect (Fig. 2) suggests that some of the bovine albumin molecules are adsorbed on to a finite number of attachment sites, possibly in competition with other molecules already adsorbed on to these sites. The adsorbed molecules presumaably interact with each other or with components of the erythrocyte membrane thus causing a structural re-organization and consequently altering its mechanical prop-

erties. Subsequent experiments (to be published) have shown that the bovine molecules which have been adsorbed on to the human cells are not easily removed by washing with saline, and are apparently not displaced by any of the proteins present in the heterogeneous (Cohn fraction 5) human albumin fraction.

None of the proteins present in this heterogeneous human albumin fraction cause a similar dramatic increase in the mechanical resistance of human erythrocytes (Fig. 3). Instead, the weakest and oldest cells are made more fragile, while the strongest and youngest cells are made slightly more resistant to mechanical rupture (Fig. 3). This effect does not exhibit saturation (Fig. 4) and is presumably due to a different type of interaction.

These results show that the mechanical properties of the intact human erythrocyte membrane can be altered *in vitro* by the addition of bovine serum albumin. Clearly, this is not of immediate clinical use, but it suggests that future research may lead to the discovery of non-antigenic protein fragments or synthetic molecules which could be tolerated *in vivo* and which could safely be used to increase the mechanical strength of the human erythrocyte membrane *in vivo*.

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