Systemic inflammatory response conditions are associated with capillary leak and haemodynamic compromise. Fluid resuscitation to reverse the ensuing hypovolaemia is, however, complicated by the decreased endothelium reflection coefficient to albumin and other colloids. We developed PEG–Alb (albumin covalently linked to polyethylene glycol) as a potential resuscitative agent. PEG was covalently linked to human albumin at multiple sites on the protein. The modified protein was heterogeneous when examined by SDS/PAGE, size-exclusion chromatography and SELDI–TOF MS (surface-enhanced laser-desorption ionization–time of flight MS). Based on size-exclusion chromatography and osmotic pressure data, the effective volume of PEG–Alb is increased 13- to 16-fold compared with unmodified albumin. In an LPS (lipopolysaccharide) model of shock, rats treated with PEG–Alb showed better blood pressure, lower Hct (haematocrit) consistent with haemodilution and less lung injury than rats treated with unmodified albumin or saline. In a CLP (caecal ligation and puncture) model of sepsis, PEG–Alb was more effective than albumin or saline in maintaining blood pressure and in decreasing Hct. When fluorescein-labelled PEG–Alb and Texas Red-labelled albumin were administered to rats with LPS- or CLP-induced shock, PEG–Alb was retained within blood vessels, whereas albumin extravasates into the interstitial space. Based on these data, PEG–Alb appears to be retained within blood vessels in models of capillary leak. PEG–Alb may ultimately be effective in the clinical treatment of shock associated with capillary leak.

INTRODUCTION

Hypovolaemic states often lead to hypotension and hypoperfusion of vital organs, causing multi-organ dysfunction and ultimately resulting in morbidity and death [1]. Hypovolaemia can occur rapidly, as with haemorrhagic shock, or progressively due to capillary leak resulting from SIRS (systemic inflammatory response syndrome). In sepsis and other SIRS conditions, decreased myocardial contractility and NO (nitric oxide)-mediated dilation of arteriolar resistance vessels are additional important factors of the associated hypotension.

Key words: arterial pressure, capillary leak, colloid osmotic pressure, hypotension, hypovolaemia, systemic inflammatory response syndrome.

Abbreviations: COP, colloid osmotic pressure; CLP, caecal ligation and puncture; DTT, dithiothreitol; Hct, haematocrit; HR, heart rate; i.p., intraperitoneal; LPS, lipopolysaccharide; MAP, mean arterial pressure; Mn, number-average molecular mass; NO, nitric oxide; PEG, polyethylene glycol; PEG–Alb, albumin covalently linked to PEG; PEG–Hb, haemoglobin covalently linked to PEG; Rg, radius of gyration; SELDI–TOF MS, surface-enhanced laser-desorption ionization–time of flight MS; SIRS, systemic inflammatory response syndrome; $V_e$, elution volume; $V_v$, void volume.

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[2]. Resuscitation with plasma volume expanders remains a mainstay in treating hypovolaemia, but with mixed results. The efficacy and safety of volume expanders, including both colloids (e.g. albumin and starches) and crystalloids, continue to be topics of intense research and controversy [3,4]. The occasional ineffectiveness of albumin as a plasma expander may be linked to the severity of the underlying endothelial cell injury. If the endothelial integrity is compromised such that albumin can readily extravasate, the leaking albumin may exacerbate the oncotic gradient favouring capillary leak [5].

We hypothesized that modifying albumin by covalently attaching multiple PEG (polyethylene glycol) groups at lysine residues (PEG–Alb) would increase its effective radius and hydration, decreasing its extravasation during conditions associated with capillary leak. To test this hypothesis, we have compared the effectiveness of albumin, saline and PEG–Alb as resuscitative agents in two animal models of septic shock.

METHODS

Preparation of PEG–Alb

Methoxypolyethylene glycol cyanuric chloride (average $M_r$ 5000) was added with gentle stirring to human albumin (type V; Sigma Chemical Co., St. Louis, MO) dissolved in 50 mM potassium phosphate buffer (pH 7.5) at 50–60 mg/ml; four additions (0.2 mg/mg of albumin) were made at 10 min intervals at 22 °C. The reaction was stirred 40 min after the last addition of reagent. Modification was rapid, being complete in less than 15 min at room temperature with the extent of modification depending primarily on the amount of reagent added. PEG–Alb was applied on to Sephacryl S300 column (2.5 cm x 220 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4)/150 mM NaCl. The column was eluted at 40 ml/h and material eluting at a position with an apparent $M_r$ in excess of 200 000 was pooled and concentrated by ultrafiltration using a PM10 membrane (Millipore), followed by dialysis against several changes of 0.9 % saline. Analysis of the fluorescein- and Texas Red-labelled albumins by gel electrophoresis revealed fluorescence was associated with the protein; no fluorescence was detected at the positions of free dye. Steady-state fluorescence measurements were made on a QM4SE fluorometer (Photon Technology International, Monmouth Junction, NJ, U.S.A.).

In vitro characterization of PEG–Alb

SDS/PAGE

Samples of unmodified albumin and PEG–Alb were prepared for electrophoresis by adding 1 % (w/v) SDS and 5 % (v/v) 2-mercaptoethanol and heating in a boiling water bath for 1 min. Samples were subjected to electrophoresis on 7.5 % or 10 % (w/v) polyacrylamide gels [6].

Size-exclusion chromatography

Albumin and PEG–Alb were analysed by size-exclusion chromatography on a 24 ml bed volume Superose 6 column (Amersham Biosciences, Piscataway, NJ, U.S.A.). Samples or a mixture of standards (0.5 ml) were applied on to the column and eluted with 10 mM potassium phosphate buffer (pH 7.5)/150 mM NaCl at 0.5 ml/min. Absorbance at 280 nm was monitored continuously [7].

SELDI–TOF MS (surface-enhanced laser-desorption ionization–time of flight MS) protein analysis

SELDI–TOF MS was used to characterize the PEG–Alb and albumin resuscitation fluids. Samples (1 μl at 1–5 mg/ml) were air dried directly on a 2-mm² area of an aliphatically coated aluminium Protein Chip array (H4 Protein Chip; Ciphergen Biosystems, Palo Alto, CA, U.S.A.). Energy-absorbing matrix (EAM; 3,5-dimethoxy-4-hydroxyphenylacetic acid) was applied to the sample and allowed to dry. The Protein Chip array was transferred to a Protein Chip reader and a laser (N2 320 nm-UV) was focused on the sample. After two warming laser shots, proteins were ionized and desorbed from the array surface. Ionized proteins were detected and $M_r$ was determined using TOF analysis. TOF mass spectra were collected in the positive-ion mode with Protein Chip System (PBSII series; Ciphergen) using Ciphergen Peaks (version 2.1b) software. Real-time
signals of 65 laser shots were averaged to generate each spectrum [8].

COP (colloid osmotic pressure)
Albumin (50 mg/ml) was dissolved in 10 mM potassium phosphate buffer (pH 7.5)/150 mM NaCl and treated with 0.5 mM DTT for 1 h at 30 °C, and then incubated with 5 mM iodoacetamide for 1 h at 30 °C. The acetamidated albumin (5 ml at 50 mg/ml) was subjected to chromatography on a Sephacryl S300 column (2.8 cm × 40 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.5)/150 mM NaCl to remove albumin dimer and other low- and high-Mr contaminants that interfere with determination of osmotic pressure. Albumin and PEG–Alb were dialysed against several changes of 0.9 % saline. COP measurements were performed using the Model 4420 colloid osmometer (Wescor Inc., Logan, UT, U.S.A.). The instrument was zeroed with 0.9 % saline and calibrated with a 20.2 mosm albumin standard solution. The concentration of unmodified albumin was determined from absorbance at 280 nm (ε = 8.73 × 10³ M⁻¹ cm⁻¹) [9].

COP (π) as a function of concentration (c; in g/dl) was analysed using a non-linear least squares fit of eqn (1) to the COP data to extract $M_n$ (number-average $M_r$), the virial coefficient ($B$) and an exponent $\alpha$; the latter two parameters account for the non-ideal contributions of all virial coefficients [9].

$$\pi = RT(c/M_r) + Bc^\alpha$$  \hspace{1cm} (1)

This form of the equation, which is a slight modification of that traditionally employed [$\pi = RT(c/M_r) + Bc^2 + Cc^3 + \ldots$], avoids a priori assumptions of the number of virial coefficients; $R = 63.364$ mmHg/M, and $T$ is temperature (295K).

**In vivo animal studies**

**Animals**
Experimental protocols were approved by the Institutional Animal Care and Use Committee and the Academic Chemical Hazards Committee at the Medical College of Ohio. Animals were housed in an AAALACI (American Association for Accreditation of Laboratory Animal Care, International)-approved facility. Adult male Sprague–Dawley rats (Charles River Laboratories, Portage, MI, U.S.A.) weighing 400–480 g were used. They were provided standard rat chow and water ad libitum. Prior to experiments, animals were fasted overnight, but given water ad libitum.

**Physiological monitoring**
Rats were anaesthetized with i.p. (intraperitoneal) sodium pentobarbital (50 mg/kg of body weight), followed by intravenous sedation as a continuous drip (10 mg·h⁻¹·kg⁻¹ of body weight). A tracheotomy was performed using a tracheal cannula (2.2 mm inner diameter and 2.3 mm outer diameter; Harvard Apparatus, Holliston, MA, U.S.A.). An arterial catheter (Intramedic PE-50; Clay Adams, Palisades Park, NJ, U.S.A.) was inserted into the right carotid artery, connected to a pressure transducer, amplified and monitored continuously (sampling rate 100 Hz; MP 100; BioPac Systems Inc., Santa Barbara, CA, U.S.A.) and the data collected on a computer. An intravenous line (G24 Protective*Plus; Johnson and Johnson, Arlington, TX, U.S.A.) was inserted in the left jugular vein for infusion of fluids.

**LPS (lipopolysaccharide) model**
LPS-induced capillary leak was used to compare the resuscitative properties of PEG–Alb, albumin and saline; treatment was given before the endotoxin [albumin ($n = 9$), saline ($n = 6$) and PEG–Alb ($n = 12$)] [10,11]. Albumin and PEG–Alb were administered in normal saline at 40 mg/ml for a dose of 0.6 g/kg of body weight. A 1 ml blood sample was taken for baseline Hct (haematocrit) and replaced with the same volume of 0.9 % saline. Continuous MAP (mean arterial pressure) monitoring began prior to treatment with albumin, saline or PEG–Alb. After 30 min, 20 mg/kg of body weight of *E. coli* LPS (serotype 055: B45; Sigma Chemical Co.) dissolved in 1 ml of saline was administered, and the rats were monitored for 180 min thereafter. A blood sample was then taken for Hct, and rats were killed with 150 mg/kg of body weight of pentobarbital i.p. Once killed, the left kidney and the left lung were harvested and fixed in 10 % formalin for histological examination; the right lung was frozen fresh at −20 °C for immunofluorescence studies.

In some animals, 20 mg/kg of body weight of LPS was administered as a bolus prior to therapy with saline ($n = 6$), albumin ($n = 8$) or PEG–Alb ($n = 8$). The treatments, identical with that described above, were administered 2 h following LPS injection. Monitoring of MAP and Hct was performed from prior to LPS administration until 3 h after treatment.

**CLP (caecal ligation and puncture) model**
We also compared the resuscitative properties of PEG–Alb with those of albumin and saline using a CLP-induced sepsis rat model [12]. Rats were anaesthetized with 50 mg/kg of body weight of pentobarbital i.p. and a laparotomy was performed through a midline abdominal incision. The cecum was ligated just below the ileocecal valve with 3-0 silk ligatures such that intestinal continuity was maintained. The cecum was perforated with a 16-gauge needle in two locations and gently compressed until faeces were extruded. The bowel was returned to the abdomen, and the incision was closed with a
layer of proline sutures for the muscles and 3-0 silk for the skin. Sterile 0.9% saline (3 ml)/100 g of body weight was administered subcutaneously on the back for resuscitation. Sham rats underwent laparotomy, caecal exposure and manipulation, but no puncture. The rats were observed for 2 h. The rats were deprived of food, but had free access to water after surgery.

Sixty CLP rats were given fluid resuscitation: 20 received albumin, 20 received PEG–Alb and 20 received saline. CLP was performed on 71; 11 died prematurely, a mortality rate consistent with that reported by others [12–15]. At 24 h after surgery, animals were anaesthetized and instrumented for physiological monitoring. Two 1 ml blood samples were drawn for Hct and COP, one at time 0 (replaced with 1 ml of normal saline) and a second at 6 h following treatment. MAP (mmHg) and HR (heart rate; beats/min) were derived from arterial blood pressure data.

### RESULTS

#### In vitro characterization of PEG–Alb

##### Molecular size

The results of SDS/PAGE of albumin and PEG–Alb are shown on the right-hand side of Figure 1. Albumin runs as a homogeneous protein at the expected $M_r$, whereas PEG–Alb is heterogeneous, migrating with apparent $M_r$ ranging from approx. 200 000 up to material that does not readily enter the gel. The electrophoretic mobility of PEG-modified proteins is primarily a reflection of their extended structure, rather than of their $M_r$. The heterogeneity of the modified protein reflects varying extents of PEG modification at multiple residues. PEG–Alb was also examined by gel filtration. Consistent with its behaviour on SDS/PAGE, the modified protein is heterogeneous, eluting over an apparent $M_r$ range from 500 000 to several million (Figure 1, left-hand panel). Its behaviour on a size-exclusion chromatography column is also a manifestation of the extended nature of attached PEG rather than $M_r$. Using the $V_e/V_o$ data (where $V_e$ and $V_o$ are elution and void volumes respectively) for albumin and PEG–Alb, the corresponding mean $V_e/V_o$ are 2.11 and 1.59 respectively. Based on comparison with well-characterized standard proteins (Figure 1, inset), the apparent average $M_r$ for albumin and PEG–Alb were 77 000 and 1 000 000 respectively, or a relative size ratio of approx. 13. To examine the extent of PEG modification, albumin and PEG–Alb were analysed by SELDI–TOF MS (Figure 2). The spectra of albumin and PEG–Alb samples showed multiple peaks, reflecting the presence of monomers, multimers, singly charged ($z = 1$) and multiply charged ($z \geq 2$) species. The dominant singly charged albumin monomer peak was centred at an $M_r$ of 66 880 ± 2800. The PEG–Alb peak was heterogeneous, exhibiting multiple species ranging in $M_r$ from 77 400 to an excess of 100 000. These components reflected the number of PEG groups attached to lysine residues, since the mass separation is consistent with the size of the PEG (5000 $M_r$ average). Studies on other PEG-modified proteins indicate that the efficiency of ionization decreases with increasing extent of pegylation, such that calculations of

##### Statistical analysis

Values are presented as means ± S.D. unless otherwise indicated. Within a treatment group, data analysed at repeated time points (MAP and HR) were evaluated by repeated measures ANOVA using a post-hoc paired Student $t$ test employing correction for multiple comparisons. Differences among the treatment groups at comparable time periods were evaluated with ANOVA, followed by a post-hoc unpaired Student $t$ test also employing correction for multiple comparison. Statistical significance is reported at the $P < 0.05$ and $P < 0.01$ levels.
Fluid resuscitation with modified albumin

$M_n$ (number-average molecular mass) would be underestimated [16–18].

COP

To evaluate the properties of PEG–Alb as an osmolyte compared with albumin, we examined the concentration-dependence of COP. Albumin and PEG–Alb showed non-linear dependence of COP with respect to solute concentration (Figure 3), reflecting colligative properties, the Donnan effect and effects arising from their molecular-excluded volumes. A fit of eqn (1) to the albumin data gave a value of 63 300 for $M_n$, 15.6 for the virial coefficient, and 2.04 for $\alpha$. Rg (radius of gyration) and excluded volume for albumin were 3.9 nm and 2070 nm$^3$ respectively, in agreement with published values [19,20]. PEG–Alb showed a larger non-ideal component compared with albumin with an $M_n$ of 129 000, virial coefficient of 62 and $\alpha$ of 2.40. Rg was 10.0 nm and excluded volume was 34 000 nm$^3$. The value of excluded volume for PEG–Alb is 16-fold larger than the value for albumin. The difference in the excluded volume and Rg between albumin and PEG–Alb inferred from the osmotic pressure data are comparable with the 13-fold difference inferred from size-exclusion chromatography.

In vivo studies

LPS model

Following LPS infusion, all groups developed a 40% decrease in MAP within 10–15 min (saline, from 135 ± 11 to 81 ± 30 mmHg; albumin, from 134 ± 14 to 85 ± 20 mmHg; PEG–Alb, from 125 ± 12 to 79 ± 19 mmHg). The time and magnitude of the acute fall in blood pressure following LPS treatment was the same in all groups. The recovery in MAP that followed was significantly better in PEG–Alb-treated rats (MAP 3 h after LPS = 120 ± 10 mmHg; $P < 0.01$) compared with both saline (99 ± 29 mmHg) and albumin (108 ± 14 mmHg) treatments. MAP recovery in albumin- and saline-treated rats was similar (Figure 4). Baseline Hct was similar in all groups [43 ± 3% (saline), 45 ± 2% (albumin) and 45 ± 3% (PEG–Alb)]. At 3 h after LPS treatment, Hct was elevated relative to baseline for albumin- and saline-treated rats (Hct at 3 h/baseline = 1.09 ± 0.11 and 1.19 ± 0.09 respectively), indicating a relative decrease in intravascular fluid volume. PEG–Alb-treated rats exhibited haemodilution after LPS administration (final Hct 3 h/baseline = 0.93 ± 0.07; $P < 0.01$).

Inflammatory histopathological changes consistent with severe acute lung injury, including hyalinization and interstitial lymphocytic infiltrates, were evident in most rats pretreated with saline or albumin, whereas these changes were not detected in rats pretreated with PEG–Alb. Acute lung injury scores were significantly lower for PEG–Alb (0.76 ± 0.47; range, 0–1) compared with saline- (2.0 ± 1.0; range, 0–3) and albumin- (2.4 ± 0.9; range, 1–4) treated samples (both comparisons $P < 0.01$). No significant histopathological changes were detected in sections of kidney (results not shown).
Figure 2  SELDI–TOF MS analysis of albumin (A) and PEG–Alb (B)
Spectra were obtained from 15 pM albumin and PEG–Alb. Note the presence of multiple peaks, suggesting multiple species with varying $M_r$, reflects the presence of protein monomers as well as multimers and the presence of singly ($z = 1$) and multiply ($z > 1$) charged molecules.

In rats treated 2 h after administration of LPS with saline, albumin or PEG–Alb, each of these volume expansion modalities was associated with an increase in MAP (Table 1). The increase in MAP following treatment was greater in the PEG–Alb-treated group compared with the saline and albumin groups ($P < 0.05$). Notably, MAP did not return to baseline in any of the treatment groups. Regarding the Hct measurements, LPS administration in the absence of pretreatment resulted in definite and comparable haemoconcentration in the three groups. However, whereas saline administration returned Hct to pretreatment values, both albumin and PEG–Alb caused significant haemodilution. Furthermore, the haemodilution observed with PEG–Alb was greatest ($P < 0.05$ compared with albumin and saline). These data are summarized in Table 1.

CLP model
Rats that underwent CLP showed signs of illness, piloerection, lethargy and early death (11 out of 71; 15.5 %), all indicative of sepsis, whereas sham rats did not.

Baseline or pretreatment physiological measurements in sham and CLP rats are shown in Table 2. Baseline HR was similar for all CLP and sham subgroups irrespective of treatment. Compared with sham animals, CLP rats showed significantly lower MAP ($P < 0.01$) or hypotension, higher Hct ($P < 0.01$), indicative of haemoconcentration, and decreased COP ($P < 0.01$), indicative of net loss of protein.
Table 1 MAP and Hct responses to resuscitation fluids in animals pretreated with LPS
Data are means ± S.D. *P < 0.05 and **P < 0.01 compared with baseline, and †P < 0.05 and ††P < 0.01 compared with 2 h post-LPS. P values were determined using paired Student’s t tests.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>2 h post-LPS</th>
<th>3 h post-treatment</th>
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</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline treated</td>
<td>134 ± 14</td>
<td>72 ± 3</td>
<td>87 ± 3††</td>
</tr>
<tr>
<td>Albumin treated</td>
<td>139 ± 5</td>
<td>70 ± 4</td>
<td>86 ± 4††</td>
</tr>
<tr>
<td>PEG–Alb treated</td>
<td>128 ± 14</td>
<td>73 ± 4</td>
<td>92 ± 3††</td>
</tr>
<tr>
<td>Hct (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline treated</td>
<td>47.7 ± 2.6</td>
<td>51.7 ± 3.1†</td>
<td>47.5 ± 3.3†</td>
</tr>
<tr>
<td>Albumin treated</td>
<td>49.5 ± 2.3</td>
<td>51.5 ± 2.1†</td>
<td>43.5 ± 2.4††</td>
</tr>
<tr>
<td>PEG–Alb treated</td>
<td>48.8 ± 2.3</td>
<td>52.8 ± 3.2†</td>
<td>41.0 ± 3.7††</td>
</tr>
</tbody>
</table>

Fluid resuscitation effects
To evaluate the effects of saline, albumin and PEG–Alb fluid resuscitation, we examined HR, MAP, Hct and COP 6 h after initiation of fluid treatments relative to baseline values in sham and CLP rats (Table 2). Sham rats, 6 h after fluid resuscitation, exhibited similar increased HR and small, but significant, decreases in MAP irrespective of treatment. These changes were observed despite the different degrees of haemodilution, as indicated by the lower Hct (Table 2). Haemodilution was greatest in PEG–Alb-treated rats. COP decreased only in the saline-treated rats. Rats from all CLP groups had significantly lower HR at 6 h after beginning fluid resuscitation. Saline- and albumin-treated groups showed significant falls in MAP during the 6 h following initiation of fluid resuscitation, whereas MAP was only slightly decreased in the PEG–Alb-treated rats (Figure 5). All groups showed significant plasma expansion, as reflected in lower Hct (Table 2). The PEG–Alb-treated group showed greater haemodilution (final Hct = 34.0 ± 5.9 %) compared with the albumin-treated group (final Hct = 38.4 ± 4.2 %; P < 0.01). COP values in the serum of the saline- and albumin-treated groups was significantly lower than that seen in the PEG–Alb-treated group (Table 2).

As an additional control, six rats subjected to CLP were given a simultaneous infusion of albumin (1 g/kg of body weight) and PEG (0.5 g/kg of body weight). These doses were calculated to correspond with the total quantity of both albumin and PEG infused in the

Table 2 Haemodynamic, Hct and COP values in animals exposed to CLP
Data are expressed as means ± S.D. *P < 0.05 and **P < 0.01 compared with the corresponding baseline pretreatment value, as determined using paired Student’s t tests.

<table>
<thead>
<tr>
<th></th>
<th>Sham rats receiving</th>
<th>CLP rats receiving</th>
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<tbody>
<tr>
<td></td>
<td>Saline (n = 10)</td>
<td>Albumin (n = 10)</td>
</tr>
<tr>
<td></td>
<td>Saline (n = 16)</td>
<td>Albumin (n = 19)</td>
</tr>
<tr>
<td>Pretreatment (t = 0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>397 ± 45</td>
<td>429 ± 37</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>123 ± 9</td>
<td>127 ± 6</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>49.5 ± 2.5</td>
<td>48.6 ± 2.1</td>
</tr>
<tr>
<td>COP (mmHg)</td>
<td>21.0 ± 1.2</td>
<td>19.2 ± 0.8</td>
</tr>
<tr>
<td>Post-treatment (t = 6 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>445 ± 21†</td>
<td>472 ± 47</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>106 ± 8**</td>
<td>109 ± 15**</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>42.0 ± 3.1†</td>
<td>39.4 ± 3.2**</td>
</tr>
<tr>
<td>COP (mmHg)</td>
<td>15.4 ± 0.9**</td>
<td>18.4 ± 1.7</td>
</tr>
</tbody>
</table>
PEG–Alb group. This PEG + Alb group had virtually identical blood pressure and Hct responses to the albumin alone group throughout the study. MAP reached a peak of 102 ± 5.6 % of pretreatment values at 20 min of fluid resuscitation whereas, by 6 h, MAP had fallen to only 80 ± 4.4 % (mean ± S.E.M.). In contrast, the PEG–Alb-infused rats reached a peak of approx. 125 % of the pre-infusion value by 60 min and values remained above the baseline value for the entire 6 h of observation (Figure 5).

Fluorescence studies
To demonstrate that PEG–Alb is retained within vessels while normal albumin leaks, we administered a mixture of fluorescein-labelled PEG–Alb and Texas Red-labelled albumin; co-localization of the dyes would indicate that both were retained, whereas the presence of one of the dyes in the interstitial space would indicate leak of the labelled species. Fluorescence microscopy of lung sections demonstrated co-localization of the Texas Red and fluorescein signals in rats subjected to sham surgery, whereas in the CLP rats, Texas Red fluorescence (albumin) was detected in the interstitial space and the fluorescein fluorescence (PEG–Alb) was detected only within vascular structures (Figure 6).

DISCUSSION
Significant resources have been expended to develop therapies for reversing hypovolaemia observed in sepsis or other manifestations of SIRS. In the United States, approx. 750 000 cases of sepsis are encountered annually, leading to 200 000 deaths [21]. The high mortality associated with SIRS is a result of multiorgan dysfunction. Capillary leak and subsequent organ oedema are a major component of the multiorgan dysfunction disease process. In addition to the appropriate antimicrobial and anti-inflammatory treatments, patients with significant capillary leak typically receive fluid resuscitation (crystalloids and/or colloids) and vasopressors to reverse the hypovolaemia and hypotension. The efficacy of crystalloid versus colloid fluid resuscitation in the presence of capillary leak remains a controversial issue [3,4]. Some groups advocate treatment with 5 % to 20 % albumin solutions to increase blood volume and to augment intravascular COP [22,23]. Albumin, which accounts for 80 % of the blood COP, normally extravasates at a low rate. During SIRS, the effective albumin extravasation rate increases substantially [4,5,24]. Clinical studies of the efficacy of albumin as a volume expander have been inconsistent [24–26], with some suggesting that albumin may be associated with increased mortality in SIRS [27]. In principle, such worsened outcomes with albumin may be expected if the decrease in the reflection coefficient of the capillary endothelium to albumin is large, i.e. increased albumin leak, as occurs with severe SIRS. This idea prompted our hypothesis that PEG modification of albumin to increase its effective molecular size would prevent extravasation and enhance its plasma-expanding properties.

Taylor and Granger [28] noted that large endothelial pores are less represented compared with medium pores in sepsis. Hubbard and Janssen [29] quantified the severity of capillary permeability in a canine endotoxic shock model from the concentration of large (apoferritin dimer, 900 000–1 000 000 Da; Rg = 12.1 nm) and small (albumin; Rg = 3.4 nm) proteins in the blood and lymph. An increase in leaked apoferritin dimer suggested increased endothelial injury. Based on osmotic pressure.

Figure 6 Representative fluorescence micrographs of pulmonary tissue in either normal rats (upper) or rats 20 h post-CLP (lower) treated with Texas Red-labelled albumin and PEG–Alb-labelled fluorescein
Note that in the normal rat lung tissue, both green and red fluorescence is limited to the pulmonary vascular compartment, resulting in predominantly a yellow colour. In contrast, the lung tissue from the rat subjected to CLP shows marked differences in the distribution of the Texas Red and fluorescein signals.

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data, PEG–Alb has an average size of 10 nm, which then should be well retained in the vascular space in moderate-to-severe capillary leak.

Physical characterization of the PEG–Alb indicated that it is heterogeneous with a large average hydrodynamic radius. Based on the analysis of this material by gel-permeation chromatography and the concentration dependence of osmotic pressure, the effective molar volume is 13- to 16-fold larger than albumin with an $M_r$ of 129,000. We think the larger effective volume of PEG–Alb contributes to its retention within blood vessels under conditions of capillary leak. We characterized the biophysical properties of a novel colloid (PEG–Alb); PEG–Alb had increased osmotic activity and a hydrodynamic radius that was an order of magnitude larger than that of unmodified albumin. Our hypothesis is that this greater size would make it less likely to extravasate across the injured endothelium in conditions where systemic capillary leak occurred.

To test PEG–Alb as a plasma volume expander, we compared it with saline and albumin in two models of systemic capillary leak, LPS treatment and CLP. Rats resuscitated with PEG–Alb before LPS-induced endotoxic shock showed better recovery in blood pressure, haemodilution rather than haemoconcentration (a characteristic of capillary leak) and decreased lung injury compared with rats treated with albumin or saline. The superior effects of PEG–Alb compared with albumin or saline in the pretreatment model were only evident 2 h following LPS administration, after the initial non-capillary leak hypotension phase. In a separate study examining PEG–Alb following LPS administration, similar superior effects were noted; however, it should be stressed that each of the resuscitation strategies, including saline, had beneficial effects in this model. We feel that this indicates a relatively mild vascular injury in this model. Favourable physiological effects of PEG–Alb on blood pressure and Hct compared with saline and albumin were also seen in the rats subjected to the more severe CLP model. CLP rats resuscitated with saline, albumin or PEG–Alb differed significantly from one another following resuscitation. Hct values were decreased significantly in resuscitated groups indicative of volume expansion. However, the extent of haemodilution (percentage change in Hct) was lower in sham compared with CLP rats and was progressively greater for saline, albumin and PEG–Alb treatments. The beneficial effects of PEG–Alb could not be duplicated by simply infusing PEG along with albumin. Imaging studies with fluorescently labelled albumin supported the concept that, in the CLP rats, systemic capillary leak occurred which allowed albumin, but not PEG–Alb, to leak out of the vascular compartment.

Our approach to alter albumin has been used for other proteins [30–32]. Pegylated haemoglobin (PEG–Hb) has been evaluated as a blood substitute in hypovolaemic shock. PEG–Hb, constituting up to 80 % vascular volume, was effective in maintaining haemodynamics and oxygen delivery in pigs [33]. Although a detailed discussion is beyond the scope of this paper, we reasoned that PEG–Alb would be a more appropriate plasma volume expander than PEG–Hb in treating conditions such as sepsis, where there is no need for the additional oxygen carrying capacity provided by PEG–Hb. It would also be less likely to exert deleterious effects by binding of NO or exerting renal tubular toxicity as seen with extracellular haemoglobin [34]. However, we did not compare PEG–Alb with PEG–Hb in these studies. We should also point out that pegylation of proteins generally results in a prolongation of their clearance [30,35,36]; moreover toxicity from PEG is not generally observed, as it has been reported to be excreted unchanged in the urine [37–39]. Unfortunately, we have not yet studied either the pharmacokinetics or the toxicity of PEG–Alb, but we certainly feel that these studies are essential as we evaluate further the therapeutic potential of this agent.

In summary, we have developed a method of modifying albumin through pegylation. PEG–Alb demonstrated a considerably greater hydrodynamic radius as well as better retention within the vasculature in two models of systemic capillary leak. We propose that this PEG–Alb may ultimately be a useful therapeutic approach to volume expansion in patients with systemic capillary leak.

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REFERENCES


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