Evans blue dye in the assessment of permeability-surface area product in perfused rat lungs

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Dallal, Mohammed M., and Shih-Wen Chang. Evans blue dye in the assessment of permeability-surface area product in perfused rat lungs. J. Appl. Physiol. 77(2): 1030 1035, 1994.—Evans blue dye (EBD) has been used extensively as a marker of extravascular protein leakage. We assessed the utility of EBD as an albumin marker in the measurement of permeability-surface area product (PS) in perfused rat lungs and compared the results with PS values obtained using 125I-labeled albumin. In isolated perfused rat lungs, PS was measured by exposure to a perfusate containing EBD (600 μg/ml) and 125I-labeled albumin (1 μCi) for exactly 3 min, followed by washout of the intravascular space with fresh perfusate for 6 min. In lungs from normal rats, we found that PS obtained by EBD (PS-EBD) was fivefold higher than PS obtained by 125I-albumin (PS-125I) [1.92 ± 0.32 (SE) vs. 0.42 ± 0.03 × 10−3 cm²·min⁻¹·g⁻¹, P < 0.05]. Similarly, in rats pretreated with Salmonella enteritidis lipopolysaccharide (2 mg/kg iv), PS-EBD was much higher than PS-125I (2.01 ± 0.30 vs. 0.59 ± 0.08 × 10⁻³ cm²·min⁻¹·g⁻¹, P < 0.01). This discrepancy between PS-EBD and PS-125I was not explained by differences in the amount of free marker in perfusate, because the albumin-binding rate for both markers was very high. In addition, prolonged perfusion (40 min) with EBD did not significantly affect pulmonary vasoreactivity or water content in rat lungs. A detailed comparison of the kinetics of lung tissue uptake of the two markers showed an initial phase of rapid lung uptake of EBD, followed by parallel uptake of both markers up to 60 min of perfusion. We conclude that although EBD does not cause obvious lung injury, it is a nonreliable marker for measurement of vascular permeability in perfused rat lungs. This is most likely due to rapid binding of EBD to lung tissue proteins.

Radionuclide albumin is a standard marker for measurement of lung PS (6, 14). Recently, Evans blue dye (EBD), an inexpensive diazo dye that binds tightly to albumin (15, 19), has been proposed as a useful nonradioactive marker for albumin transfer in cultured endothelial monolayer as well as in isolated perfused lung (17), although the validity of these conclusions has been questioned (8). EBD, formerly known as T-1824, has been used extensively in the assessment of plasma protein leakage in intact animals (20, 22). Tissue uptake of EBD was found to correlate well with 125I-labeled albumin content in inflammatory skin lesions in the rabbit and guinea pig in response to a variety of injurious agents (22). Moreover, excellent correlation with 125I-albumin was also found in the airways of anesthetized guinea pigs treated with platelet-activating factor (20). However, the presence of a significant correlation does not imply the numerical equivalence of the data, and, to our knowledge, has used EBD in the formal assessment of pulmonary vascular permeability. Because of the many potential advantages in replacing 125I with an inexpensive nonradioactive dye as the albumin marker, we assessed in this study the utility of using EBD for PS measurement in isolated perfused rat lungs.

In lungs isolated from control and endotoxin-treated rats and perfused with a blood-free physiological salt solution (PSS) containing albumin, using the single-sample technique originally described by Kern et al. (13), we compared lung PS values measured with 125I-albumin (PS-125I) with PS measured with EBD (PS-EBD) as the albumin marker. Factors that are known to influence lung PS measurement, such as body size, lung blood flow, pulmonary microvascular pressure (Pmv), and the amount of free marker molecules in perfusate, were controlled for or measured. We also perfused normal rat lungs with EBD-containing perfusate for longer periods of time to assess any potentially deleterious effects of EBD on pulmonary blood flow or vascular reactivity. Finally, we compared the kinetics of EBD and 125I-albumin uptake by rat lung tissue.

METHODS

**Isolated Perfused Lungs**

Male Sprague-Dawley rats (250–350 g body wt) were purchased from Harlan (Indianapolis, IN) and given free access to food and water. The isolated lung preparation was performed as previously described (16). Briefly, after pentobarbital so-
dium anesthesia (70 mg/kg ip), the trachea, pulmonary artery, and left ventricle were cannulated, and the heart and lungs were removed en bloc from the chest cavity and suspended in a humidified chamber at 38°C. The lungs were ventilated using a Harvard small-animal respirator (model 646) with a humid gas mixture consisting of 5% CO₂-21% O₂-74% N₂ at 55-60 breaths/min with a tidal volume of 2.5 ml and a positive end-expiratory pressure of 2 cmH₂O. The maximal time period between the interruption of pulmonary circulation and establishment of artificial perfusion was 5 min, during which time the lungs were continually ventilated. Lungs were perfused at constant flow (0.03 ml·min⁻¹·g body wt⁻¹) with a PSS containing bovine serum albumin (PSS-albumin) at 4 g/100 ml (Sigma Chemical, St. Louis, MO) using a Harvard peristaltic pump (model 1203). The venous outflow pressure was elevated to 5 cmH₂O in all experiments to achieve zone 3 condition. The PSS contained (in mM) NaCl, 4.7 KCl, 1.17 MgSO₄, 22.6 NaHCO₃, 1.18 KH₂PO₄, 3.2 CaCl₂, and 5.5 glucose. The initial 50 ml of perfusate effluent from left ventricle were discarded, leaving 50 ml of cell- and plasma-free recirculating perfusate. Lungs were perfused undisturbed for 30 min to allow equilibration of temperature and pressure before any experimental protocol was conducted. Mean pulmonary arterial inflow pressure (Ppa) was measured continuously with a model P23 AA Statham transducer and recorded on a Soltec chart recorder. Pmv was measured at selected time points with use of a double-occlusion technique previously described by Dawson et al. (11). The perfusate temperature was monitored by a temperature probe inserted into the outflow cannula and maintained at 36.5-38°C throughout the experiment.

**Preparation of Test Markers**

A stock solution of EBD [6,6’-[3,3’-dimethyl[l,l’-biphenyl]-4,4’-dil]ibis(azo)]ibis[4-amino-5-hydroxy-1,3-naphthalenedisulfonic acid], Sigma Chemical] was prepared in water at a concentration of 2 g/100 ml, filtered, and stored at 4°C. To determine the relationship of dye concentration to optical density, a sample of the prepared EBD stock solution was serially diluted in PSS-albumin. Aliquots (300 µl) of these dilutions were placed in 96-well plates, and their optical density was measured using a microplate reader (Molecular Devices, Menlo Park, CA) equipped with a 620-nm wavelength filter. Optical density correlated linearly with EBD concentrations ranging from 0 to 50 pg/ml (linear regression coefficient, r = 1). All though we did not perform any experiments on the day on which EBD stock solution was prepared, we found our EBD stock solution to be stable over time. The optical densities of serial dilutions made from the stock solution at the beginning of our study were comparable to those obtained a few weeks later toward the end of the experiment (the latter densities were only 7-19% lower) ¹²⁵I-labeled human serum albumin was purchased from ICN Radiochemicals (Irvine, CA). To ensure low free iodine content, we purified ¹²⁵I-albumin every 2 days using the minicolumn technique described by Tuszynski et al. (21).

**Measurement of Lung PS**

We used a modification of the single-sample method of Kern et al. (13) to calculate lung PS. After the initial stabilization period, the lungs were perfused in a nonrecirculating fashion with 50 ml of PSS albumin containing ~1 μCi of ¹²⁵I-albumin and 600 µg/ml of EBD. Exactly 3 min later, the perfusate was switched to fresh PSS-albumin, and the lungs were perfused for an additional 6 min, without recirculation, to wash out the radioactivity and EBD from intravascular space. In our pilot experiments, 6 min of washout resulted in >99.5% reduction of the perfusate level of both markers. The lungs were then dissected free, lightly blotted, and weighed (wet lung weight). The radioactivity of lung and perfusate samples was determined in a gamma counter (Micromedic Plus, Irvine, CA). The optical density of perfusate samples was measured spectrophotometrically at 620-nm wavelength, and the concentration of EBD was calculated from a standard curve of EBD in PSS-albumin. To measure the amount of EBD in the lung, lungs were homogenized in 20 ml of formamide by use of a Tekmar Tissumizer at 50% output for 8 s and incubated at 50°C for 36 h. The homogenate was then centrifuged at 3,900 g for 10 min. The optical density of 1 ml of formamide extract was determined against formamide blank, and its EBD concentration was calculated by interpolation from a standard curve of EBD in formamide. Lung PS was then calculated as follows: PS = marker activity in lung tissue/marker activity in 1.0 g of perfusate × 3 min × wet lung wt).

In a separate experiment, we determined that our method of extracting EBD from lung tissue did not lead to a systematic error in the measurement of the amount present. We homogenized the lungs in formamide and centrifuged lung tissue as described above. A sample of our EBD stock solution was serially diluted in the lung homogenate supernatant. Over the concentration range we used in our experiments, we found that the optical densities of EBD diluted in the homogenate supernatant were <10% higher than those diluted in formamide. This could result in a <10% overestimation of lung EBD content.

**Specific Protocols**

**Lung PS in control and endotoxin-treated rats.** Sixteen rats were divided into two groups (n = 8 each) and given normal saline (control) or Salmonella enteritidis lipopolysaccharide (LPS or endotoxin; Difco, Detroit, MI; 2 mg/kg body wt iv) 2 h before lung isolation. On each experimental day, lungs from control and endotoxin-treated rats were studied using the same preparation of ¹²⁵I-albumin and EBD. PS, ¹²⁵I, and PS-EBD were compared in the same lung.

**Binding of ¹²⁵I and EBD to albumin.** ¹²⁵I binding to albumin was assessed daily. A small sample (1.5 ml) of the purified ¹²⁵I-albumin was placed on the proximal end of a 53 × 5-mm filter paper. The filter paper was allowed to dry before it was vertically suspended in a 20-ml vial, which contained 1.5 ml of 100% ethanol. The solvent was allowed to migrate for 5 min. The filter paper was then cut in half, and the radioactivity of the proximal and distal sections was measured separately. ¹²⁵I-albumin binding was calculated as follows: radioactivity of proximal half/radioactivity of proximal half + radioactivity of distal half). EBD binding to albumin was measured in four separate experiments by placing a length of dialysis tubing (Spectra/Per; 12,000-14,000 mol wt exclusion, Spectrum Medical, Los Angeles, CA) containing 4 ml of PSS-albumin in a bath of 50 ml of PSS-albumin containing EBD (600 µg/ml) for 24 h. The concentration of EBD in the dialysate was determined spectrophotometrically.

**Effects of EBD on pulmonary vascular reactivity.** To determine whether prolonged exposure to EBD has any adverse pulmonary vascular effects, isolated perfused lungs from eight normal rats were challenged with intra-arterial injection of 0.3 µg of angiotensin II (ANG II) followed, 5 min later, by ventilation with 0% O₂ 5% CO₂ 95% N₂ for 5 min (hypoxia). After a second set of ANG II and hypoxic challenges (each separated by 5 min), the perfusate was replaced with fresh PSS-albumin (control group, n = 4) or PSS-albumin containing EBD (600 µg/ml; EBD group, n = 4). Two additional sets (3rd and 4th) of ANG II and hypoxic challenges were then performed at 5-min intervals starting at 5 min after the change to a new perfusate. Mean Ppa responses to ANG II and hypoxia were measured. To account for the day-to-day and interanimal variability, the
TABLE 1. Comparison of PS values from perfused rat lung with 125I-albumin or EBD

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<th>PS:125I</th>
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<tr>
<td>Control</td>
<td>8</td>
<td>0.42±0.03</td>
<td>1.92±0.32†</td>
</tr>
<tr>
<td>LPS</td>
<td>8</td>
<td>0.59±0.08*</td>
<td>2.01±0.30†</td>
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Values are means ± SE expressed X10⁻² cm⁻¹ min⁻¹ g⁻¹. Values were obtained after 3 min of exposure to 125I-albumin (1 μCi) or Evans blue dye (EBD; 600 μg/ml) with use of a modification of method described by Kern et al. (13). Rats were injected intravenously with normal saline (control) or S. enteritidis lipopolysaccharide (LPS; 2 mg/kg) 2 h before lung isolation. PS, permeability-surface area product. * P < 0.05 compared with control. † P < 0.005 compared with PS-125I.

pressor responses in each lung were expressed as a percentage of the second (baseline) ANG II or hypoxic response. At the end of each experiment, lungs were dissected free, blotted dry, and weighed (wet lung weight). The lungs were then placed in a 50°C oven until constant weight was achieved (dry weight). Wet-to-dry lung weight ratio was then calculated and compared between control and EBD groups.

Time courses of EBD and 125I uptake by the lung. Isolated lungs from 16 rats were divided into four groups (n = 4 each) and perfused with PSS-albumin containing EBD (600 μg/ml) and 125I-albumin (1 μCi) for 20, 40, 60, or 120 min. At the end of the perfusion period, the vascular space was washed free of markers with a fresh solution of PSS-albumin in a nonrecirculating manner for 6 min. Lungs were then dissected free, lightly blotted, and weighed. The radioactivity and EBD concentrations in samples of perfusate and lung tissue were determined as described above. Lung tissue extravascular marker uptake was expressed as follows: lung marker activity/marker activity in 1.0 g of perfusate X wet lung wt). From our earlier PS measurement study, we were able to calculate lung extravascular uptake of markers at 3 min (n = 8).

Statistical Analysis

Data are expressed as means ± SE. We used Student’s paired t test to compare the PS values obtained using 125I-albumin or EBD in the same lung and the PS values and other data from control and endotoxin-treated rats (paired for the day of experiment). Unpaired t test was used to compare lung vascular pressor responses in control and EBD-perfused lungs. Linear correlation and regression was performed using standard methods on an IBM personal computer. Differences are considered significant when P < 0.05.

RESULTS

PS:125I and PS-EBD in Control and LPS-Treated Rats

In control rats, PS-EBD was about fivefold higher than PS:125I from the same lungs (Table 1). Similarly, in LPS-treated rats, PS-EBD was significantly higher than PS:125I (3- to 4-fold). Moreover the variance was much higher in PS-EBD (17%) than in PS:125I measurement (7%). When the two measurements from each lung were plotted against each other (Fig. 1), no relationship could be discerned.

Table 1 also shows that PS:125I was more sensitive than PS-EBD in detecting LPS-induced lung injury. PS:125I was 40% higher in the LPS-injected group than in the control group, whereas PS-EBD was essentially unchanged between the two groups. The body weight, Ppa, and wet lung-to-body weight ratio were similar in control and LPS-treated rats (not shown). However, LPS did cause hemocencentration (hematocrit 51.3 ± 1.8 vs. 43.2 ± 0.7%, P < 0.01) and a small but statistically significant increase in baseline Pmv (4.3 ± 0.2 vs. 3.6 ± 0.1 mmHg, P < 0.05) in perfused lungs.

Binding of Markers to Albumin

The binding rate of 125I to albumin averaged 99.8 ± 0.1% (n = 8). This was not significantly different from the binding rate of EBD to albumin (99.96 ± 0.0%, n = 4).

Effects of EBD on Pulmonary Vascular Reactivity

Rats in control and EBD groups had similar body weights and baseline Ppa and Pmv. The baseline (2nd) pressor responses to ANG II were similar in the two groups (5.0 ± 0.2 vs. 5.3 ± 1.1 mmHg). Baseline hypoxic response was somewhat higher in the EBD group than in the control group (5.6 ± 0.4 vs. 10.3 ± 1.9 mmHg, P = 0.06). To account for this difference in baseline pressor response, subsequent pressor responses were expressed as a percentage of the baseline response. The addition of EBD to the perfusate had no effect on baseline Ppa or Pmv, and the subsequent pressor responses to ANG II and hypoxia were not altered (Fig. 2). At the end of the perfusion period, the lung wet-to-dry weight ratio was not different between control and EBD groups (6.3 ± 0.2 vs. 6.5 ± 0.2, respectively).

Time Courses of 125I and EBD Uptake by Lung Tissue

Figure 3 shows that the lung uptake of EBD was much more rapid than lung uptake of 125I during the initial 3 min of perfusion. After this initial period, EBD and 125I uptakes roughly paralleled each other for the next 60 min. Although both uptake curves appear to plateau after 1 h of perfusion, there was a small increase in lung EBD uptake between 60 and 120 min (not statistically significant). After 2 h of perfusion, the cumulative lung uptake...
uptake of EBD was 180% of $^{125}$I albumin uptake. When the data from the initial 3 min of perfusion were excluded, there was a significant correlation in the lung uptake of the two markers (Fig. 4).

**DISCUSSION**

In this study, we found that in isolated perfused rat lungs the use of EBD as a marker of albumin yielded lung PS values dramatically different from those obtained using the standard $^{125}$I-albumin marker. 1) PS-EBD was much higher than PS-$^{125}$I measured simultaneously in the same lung, and the measurements were not correlated. 2) PS-EBD, in contrast to PS-$^{125}$I, did not detect the increase in lung vascular permeability induced by treatment with LPS. 3) The variance in PS-EBD was much higher than the variance in PS-$^{125}$I. Because the PS-$^{125}$I values compared favorably with estimates of lung microvascular permeability with use of other independent techniques (14), we conclude that EBD is not a useful marker for PS measurement in perfused lungs.

We used the single-sample method described by Kern et al. (13, 14) with minor modifications by Czartolomna et al. (10) for measurement of PS in perfused rat lungs.

The control PS-$^{125}$I value obtained in this study was similar to that measured previously in our laboratory (10) and compares favorably with the result obtained by Kern and Malik (14) in rabbit lungs. The modest increase in lung PS-$^{125}$I after LPS treatment (~40%) was comparable in magnitude to the increase in lung “leak index” observed previously in rats given a similar dose of LPS (7) and to the increase in lung PS after administration of human recombinant tumor necrosis factor (10). That this increase in lung PS reflects changes in permeability and not vascular surface area is suggested by the following. 1) Rats in the two groups had comparable body weights and therefore are likely to have lungs of similar size. 2) All lungs were perfused at equivalent flow rates, and the pulmonary arterial perfusion pressures were comparable in the two groups. 3) The lungs were perfused under zone 3 condition, and therefore the small difference in Pmv could not have caused much additional vascular recruitment. Furthermore the presence of marked hemoconcentration in these LPS-treated rats and the previously documented elevation in lung eicosanoids and platelet-activating factor levels (2, 4, 7) suggest that this dose of LPS did cause acute lung vascular injury in the rat. Given this finding, the lack of difference...
leading to increased permeability. This also seems unlikely, because our \(\text{PS}^{125}\text{I}\), measured in the presence of \(\text{EBD}\), did not differ significantly from previous values obtained in the absence of \(\text{EBD}\). Moreover, prolonged exposure to \(\text{EBD}\) at the concentration used for \(\text{PS}\) measurement did not change \(Ppa, Pmv\), or lung wet-to-dry weight ratio and had no effect on pulmonary vascular reactivity to \(\text{ANG II}\) or hypoxia. Inasmuch as attenuation of hypoxic vasoconstriction is a sensitive indicator of pulmonary vascular injury, these results suggest that \(\text{EBD}\) did not cause acute microvascular injury.

A fourth, and the most likely, explanation is that \(\text{EBD}\) or \(\text{EBD}\)-albumin complex binds to lung tissue proteins. Allen and Orahovats (1) demonstrated that \(\text{T-1824}\) could be extracted from albumin-containing solutions by cellulose. LeVeen and Fishman (15) studied the nature of \(\text{EBD}\) binding to albumin and concluded that \(\text{EBD}\) forms a dissociable compound with plasma albumin and that \(\text{EBD}\) could be fixed by tissue proteins. This process would continue until tissue proteins become saturated with the dye. Others (9) found that intravascularly injected \(\text{EBD}\) has an early phase of rapid disappearance from blood that could be explained by dissociation of \(\text{EBD}\)-albumin complex and fixation of dye to tissue proteins. Although the latter was an in vivo experiment, it is reasonable to expect such dissociation to occur ex vivo in the presence of tissue protein. Our time course study demonstrated an early phase of rapid lung uptake of \(\text{EBD}\) but not \(\text{125I}\)-albumin. This initial phase of rapid \(\text{EBD}\) uptake is not likely to reflect transvascular albumin leakage. More likely, it reflects fixation of the minute amount of free \(\text{EBD}\) in perfusate by lung tissue proteins with subsequent dissociation of the \(\text{EBD}\)-albumin complex and further binding of dissociated dye to lung tissue proteins. After this initial phase, and presumably with tissue proteins saturated with \(\text{EBD}\), the lung \(\text{EBD}\) uptake paralleled lung \(\text{125I}\) uptake with a kinetic pattern compatible with transvascular movement of albumin. As a result, there was a significant correlation between the lung extravascular contents of the two markers when the 3-min time point was excluded. Thus, even though \(\text{EBD}\) may not serve as a useful marker for transvascular albumin flux, the tissue content of \(\text{EBD}\) and \(\text{125I}\)-albumin may still be correlated, as reported in earlier in vivo studies (20, 22).

Recently, Patterson et al. (17) claimed that \(\text{EBD}\) is a "useful marker for quantitating albumin transfer... across the intact alveolar-capillary barrier in isolated, perfused rat lung." This conclusion was based on the finding of an excellent correlation between lavage \(\text{EBD}\) concentration measured spectrophotometrically and lavage protein concentration measured by Lowry assay in lungs perfused for 20 min with \(\text{EBD}\) under various conditions. Our interpretation of these data is that, in lavage fluid, \(\text{EBD}\) exists in a form that is tightly bound to albumin. However, there is no evidence that the \(\text{EBD}\)-albumin complex moves across the alveolar-capillary membrane as an intact molecule. On the basis of our finding in the time course experiment, it is likely that \(\text{EBD}\) rapidly saturates the lung tissue protein compartment, possibly in the interstitial space, which then gradually exchanges \(\text{EBD}\) across the alveolar epithelial membrane.
This could explain the time-dependent linear increase in lavage EBD concentration in lungs perfused under baseline conditions (17). With epithelial cell injury from oxidant stress, the “leakage” or exchange of EBD with the alveolar space is accelerated, reflecting increases in epithelial permeability. Thus, whereas lavage EBD concentration may still yield useful data, further studies are needed to characterize the kinetics of EBD exchange between the vascular-interstitial and the interstitial-alveolar compartments.

In summary, our study shows that, in isolated perfused rat lungs, EBD is not a valid marker of albumin for measurement of PS, because the early increase in tissue EBD concentration likely reflects fixation of dye by tissue proteins rather than diffusion of EBD-albumin complex.

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