Lymphatic Pumping in Response to Changes in Transmural Pressure Is Modulated by Erythrolysate/Hemoglobin

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Red blood cells and lysate products (erythrolysate) are observed consistently in lymph draining acute and chronic inflammatory reactions and from tissues subjected to trauma or surgical procedures. Using hemoglobin as a marker for erythrolysate, we have measured hemoglobin in lymph up to the $10^{-4}$ M range in a number of pathophysiological states. Data demonstrate that erythrolysate alters the pumping characteristics of lymphatic vessels. To test the effects of erythrolysate on lymphatic pumping, bovine lymphatics were suspended in an organ bath preparation with the vessels cannulated at both inflow and outflow ends. By raising the heights of the Krebs reservoir and the outflow catheters appropriately, a transmural pressure that stimulated pumping activity could be applied to the vessels. With a fixed transmural pressure of 6 cm H$_2$O applied to the ducts, sheep erythrolysate depressed pumping activity between 40% and 100%, with dilutions containing between $10^{-6}$ and $10^{-5}$ M hemoglobin. Although the active principle in the red blood cells has not been characterized, evidence from precipitation purification experiments suggests that hemoglobin is an important component. Once suppressed, pumping could be restored in many but not all vessels (often to control levels) by elevating the distending pressure above 6 cm H$_2$O. The relation between transmural pressure and fluid pumping is expressed as a bell-shaped curve, with pumping increasing up to a peak pressure (usually 8 cm H$_2$O) and declining at pressures above this level. By comparing pressure/flow curves, we were able to ascertain that hemoglobin shifted the lymphatic function curve to the right and, on average, reduced the maximum pumping capability of the vessels. We speculate that the presence of erythrolysate/hemoglobin in lymph may modulate the ability of lymphatic vessels to drain liquid and protein from the tissue spaces. (Circulation Research 1990;67:1097–1106)

Red blood cells (RBCs) are observed in lymph after vascular damage associated with inflammatory reactions, surgical procedures, or traumatic injury; however, this has not been considered to have any major significance in terms of lymphatic physiology. A number of recent observations have encouraged us to look into this issue in more detail. The ability of the lymphatics to contract and pump liquid and protein from the tissue spaces back to the bloodstream has been the major focus of our studies over the past few years. Many factors are capable of altering the contractile properties of these vessels, including neurohumoral and other agents.1,2 By isolating lymphatics in vivo from lymph input and applying transmural pressures to the ducts, several groups have been able to study lymphatic pumping without the complications of variable lymph inputs.3–6 These studies suggest that the host may have the ability to modulate pumping and, in this way, regulate the drainage of liquid and protein from the tissue spaces. Any factor that alters pumping could conceivably facilitate or interfere with this function. It is in this regard that we first became interested in erythrolysate/hemoglobin.

In previous studies, we observed that systemic endotoxin administration to sheep inhibited lymphatic pumping,3 and we obtained evidence that suggested a lymph-borne factor was at least partially responsible for this effect.6 The unknown factor or factors appeared to reduce the sensitivity of the

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vessels to changes in transmural pressure. Because RBCs and hemoglobin often were observed in lymph collected in these experiments, we began to investigate whether these elements could be contributing to this effect. There are numerous reports in the literature of the effects of erythrolysate on vascular smooth muscle7–11 and cardiac muscle,12,13 and in some cases, it would appear that hemoglobin is the active principle. Preliminary investigation revealed that erythrolysate prepared from sheep RBCs had a very similar effect to that of the endotoxin-induced unknown factor when tested on sheep vessels in vivo or on bovine vessels in an organ bath preparation.

The purpose of this study was to examine in more detail the effects of erythrolysate/hemoglobin on lymphatic pumping using the in vitro preparation. This included studying the dose–response effect of hemoglobin and determining its impact on the relation between transmural pressure and pumping activity. In addition, we have modified existing spectrophotometric techniques to permit assessment of hemoglobin concentrations in lymph.

Materials and Methods

Collection of Bovine Lymphatics

The lymphatic vessels were obtained from bovine mesentery 7–10 minutes after the cattle were slaughtered. The mesenteric lymph nodes were injected with Evans blue dye (0.1% in physiological phosphate buffered saline) to facilitate vessel identification. The larger vessels with diameters of 2 mm or more were carefully dissected and immersed in sterile saline solution (0.9% NaCl) at room temperature. After transport back to the laboratory, the vessels were cannulated at both ends using polyethylene tubing (Adams PE 160, i.d. 1.14 mm, o.d. 1.57 mm; Intermedic Clay Adams, Parsippany, N.J.). As a way of providing some standardization, lengths of vessels were cut that contained a minimum of four valves. After cannulation, the vessels were infusion tested for leaks with a 0.01% Evans blue solution, distending them with a transmural pressure of 12 cm H₂O. Vessels found to leak were discarded.

All cannulated vessels then were transferred to a custom-made water-jacketed organ bath14 and immersed in Krebs solution (millimoles per liter: NaCl 120.0, KCl 5.9, MgSO₄ 1.2, NaH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 15.0, glucose, 11.0). The Krebs was circulated continuously through the inner bath by a dual-channel peristaltic pump (MasterFlex Pump, Cole-Parmer Instrument Co., Chicago), maintaining a flow of 6.7 l/hr. The pH was maintained at 7.38±0.02 by continuous aeration with a mixture of 95% oxygen and 5% CO₂ (Accumet model 810, pH meter, Fisher Scientific Co., Pittsburgh). The solution temperature was kept constant at 38±0.2°C using a circulating water bath (12/TE-8D, Techne Inc., Princeton, N.J.). The inflow catheter to the lymphatic was connected to a valve that in turn was connected to a common fluid reservoir filled with Krebs solution (10 ml volume). A four-channel peristaltic pump (Buchler Instruments, Fort Lee, N.J.) was used to refresh the Krebs solution and to maintain a constant level of fluid in the supply reservoir. The flow rate was set at 10 ml/min.

The reservoir and the outflow catheters were mounted on a common adjustable stand. As soon as the vessels were immersed in the Krebs solution, a small hydrostatic gradient of 2–3 cm H₂O was applied for 15–20 minutes while the vessels equilibrated. After this period, the hydrostatic pressure gradient was removed. By raising the height of both the reservoir and the outflow catheter relative to the cannulated vessel, but keeping the heights of both equal to each other (i.e., no net driving pressure), a transmural pressure was applied to the ducts. The surface of the Krebs in the organ bath was made the zero reference level. All vessels were allowed to equilibrate for another 1-hour period with a transmural pressure of 6–8 cm H₂O applied to the vessels. In the majority of cases, this procedure elicited spontaneous pumping activity. Those vessels that did not demonstrate spontaneous contractile activity were discarded.

Collection of Sheep Blood and Preparation of Red Blood Cell and Platelet Concentrate

For the preparation of the RBC suspension, ovine blood was collected into acid citrate dextrose (trisodium citrate, 25 g; citric acid, 15 g; dextrose, 20 g, dissolved in 1 l distilled water; 6:1, vol/vol).15 The blood was centrifuged at 200g for 30 minutes, and the platelet-rich plasma and the Buffy coat were aspirated. The remaining erythrocyte concentrate was diluted with phosphate buffered saline and recentrifuged at 200g for another 30 minutes. Again, the platelet-rich plasma and Buffy coat were aspirated. The cells were diluted with phosphate buffered saline to 50 ml, spun a third time at 1,000g for 10 minutes, and the supernatant and Buffy coat were aspirated to remove most of the remaining leukocytes. One final phosphate buffered saline wash and spin was used to remove any plasma still remaining in the RBC concentrate. These procedures removed approximately 50% of the platelets. To remove the remaining platelets, the cell suspension was passed through a cotton wool column (Leukopak Filter, Fenwal Laboratories, Morton Grove, Ill.). Cotton wool (0.5 g) was compressed in the barrel of a 10 ml syringe. The filter column was rinsed with 100 ml of saline, and the erythrocyte concentrate was filtered through the column. After this filtration step, 95–99.5% of the platelets in the original collection had been removed.

Platelets were removed from the pooled platelet-rich plasma obtained during the preparation of the RBCs. The pooled platelet-rich plasma was spun and washed three times at 200g for 30 minutes to remove RBCs and white blood cell contamination. The RBC- and white blood cell–free platelet-rich plasma then was spun for 20 minutes at 1,000g.
Preparation of Sheep Erythrolysate

RBCs were lysed by freezing in dry ice and thawing in a 37°C water bath. After centrifugation at 1,000g for 30 minutes, the supernatant was assessed for hemoglobin content using the cyanmethemoglobin assay (Sigma Chemical Co., St. Louis). In some experiments, the supernatant was dialyzed in a dialyzing membrane with a cutoff of 10,000 MW against a phosphate buffered solution (2.8 M PO₄, pH 6.8). When the volume of the dialysis bag was reduced to one third, the bag was cut and the contents were collected in a flask. The same phosphate buffered solution (2.8 M) was added slowly until precipitation occurred. The hemoglobin precipitate was spun twice at 10,000g for 30 minutes. The samples were assessed for hemoglobin content and an absorption spectrum obtained between 400 and 650 nm.

It was important to determine if any platelet products (from potential platelet contamination of the RBC preparation) could be contributing to the effects on lymphatics. To achieve this, we treated the platelets the same way as we treated the RBCs; that is, they were frozen and thawed, the lysate was centrifuged at 1,000g for 30 minutes, and the lysate was tested for effects in the pumping preparation.

Quantitation of Hemoglobin in Lymph Samples

The assay is based on spectrophotometric methods in current use for measuring hemoglobin concentrations in blood, plasma, urine, and cerebrospinal fluid (see References 17 and 18). The major problem in measuring hemoglobin in lymph is that it is essential to consider the other proteins (mainly albumin), as these absorb to some extent at the wavelength we use to measure hemoglobin. Also, in some lymph samples, for example intestinal lymph, a special problem occurs in that lipids cause dispersion of light. To reduce this latter effect, lymph was delipidated with the addition of butanol/disopropyl ether (40:60, vol/vol). Twenty milliliters of the solvent mixture was added to 10 ml of lymph. The mixture was shaken and centrifuged at 2,500g for 16 minutes to separate the aqueous and organic phases while leaving the lipids layered between the phases. The aqueous phase was removed and filtered through Whatman filter paper number 1 (Whatman Inc., Clifton, N.J.). Absorbance due to proteins was eliminated using a 4 g% albumin solution as a blank for the assay. This value represents the average protein concentration in sheep intestinal lymph. We used a wavelength of 410 nm, as this was the intersection of the absorbance of oxyhemoglobin and methemoglobin, both of which could be present in our lymph collections. Even after delipidation and deproteinization with 10% trichloroacetic acid, which also would remove any hemoglobin present, the sample still had a small absorbance (in the vicinity of 0.01). In all assays, this value was determined and subtracted from the overall absorbance. To prepare the standard curve, hemoglobin was isolated from sheep blood as described above, and its concentration was determined by the Drabkins method using the Sigma kit. The hemoglobin was added to saline containing 4 g% fat-free albumin, and the absorbance values were read at 410 nm against the 4% albumin blank. This process was repeated four times, and the average of all absorbances was used to prepare the standard curve. The lowest limit of detection was 1×10⁻⁷ M.

Collection of Lymph Samples for Hemoglobin Determinations

Female sheep weighing 25–35 kg were used. Plastic catheters were inserted into the efferent lymphatics 10–15 cm downstream from the terminal mesenteric lymph node as described previously. The catheter was externalized, and lymph was collected into sterile plastic bottles attached to the animal's flank. Lymph was collected continuously after surgery, and in some animals, endotoxin (33.0 μg/kg; O55:B5, List Biological Laboratories, Inc., Campbell, Calif.) was infused intravenously 4–5 days after catheter placement.

Experimental Protocols

Fixed transmural pressure experiments. After equilibration of the bovine lymphatics, transmural pressures were reduced to zero. The transmural pressure and fluid pumping relation was obtained by elevating the pressure in increments of 2 cm H₂O from 0 to 14 cm H₂O. The vessels were allowed to pump for 10 minutes at each pressure with an equilibration time of 2 minutes between each pressure level. Pumping activity was assessed by measuring the weight of the liquid collected at the end of each 10-minute interval. After the maximum distending value was reached, the transmural pressure was decreased slowly over 10–15 minutes to a distending pressure that induced 75% of maximum pumping activity. The average distending pressure was in the range of 6 cm H₂O. In these experiments, transmural pressures were fixed at 6 cm H₂O, and the vessels were allowed to pump for 1 hour. At this point, the input reservoir was switched to that containing erythrolysate or platelet lysate, and the pumping was monitored for another 2 hours. To study the dose–response effect of the erythrolysate (in terms of hemoglobin concentrations), the same protocol was used except that the input reservoir was switched to a higher concentration at 1-hour intervals. All flows were averaged over 10-minute intervals and were expressed as a percentage of the mean pumping activity generated in the control period. Mean±SEM of the flows was plotted against time.

Variable transmural pressure experiments. To determine the ability of the lymphatics to respond to transmural pressure changes in the presence of erythrolysate, two transmural pressure and fluid pumping curves were generated for each vessel, one with Krebs and one with Krebs plus erythrolysate/hemoglobin. In some experiments, two curves were generated with Krebs to be certain that the genera-
tion of a second transmural pressure/flow curve from a given vessel had no deleterious effect on the pumping mechanism. All flows were averaged over 10-minute intervals and were expressed as a percentage of the maximum pumping activity generated in the first control curve. Mean±SEM of the flows at each pressure was plotted against the distending pressure values.

Analysis of Data

The graphs illustrate results from a minimum of three experiments conducted on separate days with vessels obtained from six or more animals. Data were assessed using analysis of variance and the Duncan's multiple range test. The analysis of variance that we use is based on the Statistical Analysis System, General Linear Model procedure, in which effects of groups are tested against the variability of time or transmural pressure within the group. We interpreted p<0.05 as significant.

Results

Response of Lymphatic Vessels to Transmural Pressure

The relation between transmural pressure and pumping activity in this model system has been described in our earlier publications.14,21 Once a transmural pressure was applied to the vessels, the majority of ducts (approximately 80% in this study) pumped fluid spontaneously. The average maximum pumping activity occurred at a distending pressure of 8 cm H2O, and as pressures were increased beyond this point, pumping declined. Maximum flow rates ranged from 0.35 to 1.4 ml/min. To study the effects of erythrolysate on steady-state pumping, the transmural pressure was fixed at 6 cm H2O and kept at this level for the duration of the experiment. This pressure level represented approximately 75% of the pressure that induced maximum pumping.

Effects of Erythrolysate on Lymphatic Pumping

When erythrolysate was added to the reservoir, a rapid decline in pumping activity was observed. Figure 1 illustrates the effects of erythrolysate diluted to contain 1×10⁻⁵ M hemoglobin. All 10 vessels to which erythrolysate was added stopped pumping within 80 minutes. In three vessels, pumping stopped within 30 minutes. Because some platelet contamination was inevitable, we also investigated the effects of platelet lysate (Figure 1). We diluted the platelet lysate by the same factor as the erythrolysate (approximately 100-fold) and found that it had no effect on lymphatic pumping. Considering that a maximum of only 5% of the original number of platelets (i.e., 5% of what we tested for effects on lymphatics) could have been present in our erythrolysate preparations, we felt justified in concluding that platelet products did not contribute to the erythrolysate effect.

In another series of studies, hemoglobin was partially purified from the erythrolysate by precipitation techniques and investigated for effects on pumping. As can be seen in Figure 2, the partially purified hemoglobin was effective at inhibiting pumping but was not as potent as the crude hemolysate because it required an order of magnitude higher concentration to achieve the same level of suppression. This suggested that hemoglobin in concert with some other factor in the lysate may have been responsible for the activity, although it is also possible that the precipitation procedures had altered the hemoglobin molecule and reduced its biological potential.

Figure 3 illustrates the dose–response effect of hemoglobin. Levels down to 10⁻⁸ M (0.65 μg/ml) were capable of inhibiting pumping 40%, 10⁻⁷ M inhibited pumping 65%, and 10⁻⁶ M inhibited it more than 80%. Concentrations of 10⁻⁵ M (0.65 mg/ml) essentially reduced pumping to zero (data not shown). To determine if the effects of hemoglobin were reversible, the test solution in the reservoir was replaced with fresh Krebs and a hydrostatic pressure of 5 cm H2O was applied to the ducts for 10 minutes to wash the hemoglobin from the vessels. In most cases, pumping returned to control or near control levels over the next hour (data not shown). In those vessels in which high concentrations of hemoglobin had reduced the pumping to zero, flow could be
FIGURE 2. Effects of erythrolysate (hemoglobin content, 10^-6 M) (■, n=15) or hemoglobin precipitate 10^-7 M (□, n=9) on pumping activity of bovine lymphatics in vitro. Transmural pressure in all vessels was maintained at 6 cm H2O. After a 1-hour control period, erythrolysate or hemoglobin precipitate was added to the Krebs in the reservoir. For the control period, pumping was averaged over 10-minute intervals, with the mean of six measurements determined. Every 10-minute flow rate was expressed as a percent of the mean control value. Each point on the graph represents mean ± SEM. After the addition of the hemoglobin precipitate solution or the erythrolysate, pumping declined to less than 20% of control (≥80% inhibition) by approximately 140 minutes. Effects with erythrolysate or the precipitation purified hemoglobin were significant when compared with pumping in the absence of these agents. Ten times more of the partially purified hemoglobin was required to elicit the same level of depression caused by the crude erythrolysate.

FIGURE 3. Effects of hemoglobin (HB) at 10^-8 M, 10^-7 M, and 10^-6 M on lymphatic pumping activity in vitro. Transmural pressure was set at 6 cm H2O and maintained at this level. After a 1-hour control period, hemoglobin was added to the Krebs in the reservoir in increasing concentrations. During 60–120 minutes, the final hemoglobin concentration was 10^-8; between 120 and 180 minutes, 10^-7 M; and between 180 and 240 minutes, 10^-6 M. During the control period, pumping was averaged over 10-minute intervals, with the mean of six measurements determined. Every 10-minute flow rate (control and hemoglobin) was expressed as a percent of the mean control value. Each point on the graph represents mean ± SEM (data from 12 vessels). In each of the 12 vessels in which hemoglobin was added, pumping declined in direct relation to the hemoglobin concentration. Hemoglobin at 10^-8 M caused a 40% decrease in pumping activity; at 10^-7 M, a 65% decrease in pumping; and at 10^-6 M, greater than 80% inhibition. Analysis of variance revealed significant effects of hemoglobin on pumping activity. Analysis with the multiple range test indicated that pumping with each concentration of hemoglobin was significantly different from the control (pre-hemoglobin pumping level) and significantly different from the other doses of hemoglobin.

Effects of Hemoglobin on the Lymphatic Vessel Response to Transmural Pressure Changes

For each vessel, a transmural pressure and fluid pumping curve was obtained with Krebs in the reservoir and then repeated with the appropriate concentration of hemoglobin added to the Krebs. We previously determined (and it was confirmed in this study) that the generation of two transmural pressure and fluid pumping curves in the same vessel had no deleterious effect on the ducts. Figure 5 demonstrates three representative examples of the effects of hemoglobin. In all of these examples, 10^-5 M hemoglobin was used. In panels A and B, it is clear that the hemoglobin shifted the function curve to the right. In some cases, the maximum pumping activity was slightly more than that in the control curve (panel A), and in others, maximum pumping was somewhat less (panel B). Some vessels could not be induced to pump when pressures were elevated (panel C). Taking all the results together (Figure 6), it is clear that hemoglobin at 10^-5, 10^-6, and 10^-7 M shifted the function curves to the right and reduced the maximum pumping available to the vessels. With the two higher concentrations (10^-5 and 10^-6 M), the pumping tended to plateau beyond 10 cm H2O, with flows being maintained even with high levels of distending pressure.

Hemoglobin Levels in Lymph

We have begun to estimate hemoglobin levels in lymph collections routinely. Figure 7 illustrates the hemoglobin levels in sheep lymph samples collected after the surgical placement of catheters in mesenteric lymphatics or after endotoxin administration. High hemoglobin levels are consistently present in lymph after surgery (into the 10^-6 M range). In the animals that received endotoxin (Figure 7, inset),...
there was a latent period between the administration of this agent and the presence of hemoglobin. Generally, we have found that hemoglobin becomes detectable with our assay 1 hour after endotoxin is injected, and levels climb to reach a maximum between 2 and 3 hours.

Discussion

Spectrophotometric analysis has demonstrated the presence of free hemoglobin in lymph in a variety of pathophysiological states. During the surgical placement of lymphatic catheters or the intravenous administration of endotoxin, hemoglobin concentrations up to the $10^{-6}$ M range can be achieved, and it seems likely that even higher concentrations may be found in lymph-draining areas of massive vascular damage. RBCs contain many elements that could affect lymphatics. However, data from the literature and from our own experiments suggest that hemoglobin is a major contributing factor. First, we have established that the effect is not due to contaminating platelets. Second, we know that the activity in the lysate must be greater than 10,000 MW because dialysis does not remove the effect on the lymphatics. In some experiments, hemoglobin was prepared from the erythrolysat by precipitation with 2.8 M phosphate buffered saline. This material was still active, although it was approximately 10-fold less potent than the crude hemolysate. Either some other factor in conjunction with hemoglobin is necessary for activity, or the hemoglobin molecule is altered in some way during the precipitation procedures. In any event, these results suggest that hemoglobin is an important component of the biological activity. Because freshly prepared hemoglobin is predominantly in the oxy-form (spectrophotometric analysis), we suspect that oxyhemoglobin is responsible for the effect on the lymphatic.

In addition, interest has been generated in the literature by the observation that RBC contents can constrict some vascular tissues, especially cerebral blood vessels, and it has been postulated that erythrolysat may contribute to the prolonged ischemia associated with cerebral hemorrhage.7-11 Analysis of

![Figure 4](http://circres.ahajournals.org/)

**Figure 4.** Example of the effects of hemoglobin on lymphatic pumping activity in an isolated bovine mesenteric lymphatic. Transmural pressure was set at 6 cm H2O and maintained at this level unless otherwise stated. After a 1-hour control period, hemoglobin was added to the Krebs in the reservoir to achieve a final concentration of $10^{-6}$ M. For the control period, flows were averaged over 10-minute intervals, with the mean of six measurements determined. After hemoglobin administration, pumping also was averaged over 10-minute periods and expressed as a percent of the prehemoglobin mean control value. At 1 hour posthemoglobin, distending pressure was increased above 6 cm H2O in 2 cm H2O increments. Vessels were allowed to pump for 20 minutes at each pressure level. This illustrates that hemoglobin-induced depression of pumping can be reversed by elevating transmural pressures.

![Figure 5](http://circres.ahajournals.org/)

**Figure 5.** Examples of the effects of hemoglobin on the response of the lymphatics to changes in transmural pressure. Bovine vessels were stimulated to contract in vitro with increasing transmural pressures. After a control distending pressure/pumping curve (□), hemoglobin was added to the Krebs solution supplying the same vessels to achieve a final concentration of $10^{-5}$ M (■). During the control period, pumping was averaged at each pressure point and expressed as a percentage of the maximum pumping activity. Values obtained posthemoglobin also were expressed as a percent of maximum control pumping. Panel A: Example of a vessel that responded to hemoglobin with a 2–4 cm H2O shift in the function curve to the right with a peak activity slightly above control peak pumping. Panel B: A different lymphatic vessel whose peak pumping activity was shifted right with a reduction in maximum pumping. Panel C: Pumping could not be reestablished by elevating transmural pressures.
dog erythrolysate by Sephacryl S-200 column chromatography and sodium dodecyl sulfate–urea polyacrylamide gel electrophoresis revealed that the active substance within the RBC had a molecular weight of approximately 60,000 Da and possessed a similar absorption spectrum to that of hemoglobin, although erythrolysate also may contain active substances of lower molecular weight.22,23 Studies on basilar artery preparations have demonstrated that oxyhemoglobin but not methemoglobin has vasoconstrictor properties.24,25

The effects of hemoglobin on the lymphatic vessel should be considered in the context of the transmural pressure and pumping curve. With a fixed transmural pressure (chosen from the ascending portion of the function curve), the addition of hemoglobin depressed pumping activity in a dose-related manner between $10^{-8}$ and $10^{-5}$ M. However, even in the presence of hemoglobin, pumping could be restored in most ducts by elevating the transmural pressure above the level originally chosen for the experiment. This suggested that hemoglobin reduced the sensitivity of the lymphatic vessel to changes in distending pressure but still permitted pumping activity. The data demonstrated that hemoglobin (at least at higher concentrations) altered the range of transmural pressures over which the lymph pump was active, with pumping more effective in the higher pressure ranges compared with controls. This concept is supported by the data illustrated in Figures 5 and 6 in which the application of hemoglobin resulted in a shift of the transmural pressure and pumping curves to the right, although the pressures that induced peak pumping were significantly different only with $10^{-6}$ M hemoglobin. Not all vessels responded in this manner, however. For reasons not clear to us, the application of hemoglobin to some ducts (approximately 20%) resulted in an irreversible inhibition of pumping, and this effect did not appear to be related to the hemoglobin concentration. In addition, the lowest dose of hemoglobin used in the data illustrated in Figure 6 ($10^{-7}$ M) (panel A) consistently produced a different effect. It did not increase the range of transmural pressure over which the vessels were active, but rather, depressed pumping at each pressure tested.

If one accepts the contention that contractions of the lymphatic vessels play an important role in regulating the drainage of liquid and protein from tissue spaces,26 it follows that any factor that alters this function may have an effect on interstitial dynamics. In resting states, the regulation of lymphatic pumping appears to be myogenic, with increases in transmural

Figure 6. Effects of hemoglobin on the response of the lymphatics to changes in transmural pressure. Bovine vessels were stimulated to contract in vitro with increasing transmural pressures. After a control distending pressure/pumping curve (□), hemoglobin was added to the Krebs solution supplying the same vessels to achieve a final concentration of $10^{-7}$ M (panel A, n=7); $10^{-6}$ M (panel B, n=9), or $10^{-5}$ M (panel C, n=9) (■). During the control period, pumping was averaged at each pressure point and expressed as a percentage of the maximum pumping activity. Values obtained posthemoglobin also were expressed as a percent of maximum control pumping. Data represents mean±SEM. In the analysis of the data, we compared differences between the maximum pumping activity between prehemoglobin- and posthemoglobin-treated vessels and assessed transmural pressures at which peak flows were obtained. With each hemoglobin concentration, maximum pumping activity was reduced significantly compared with prehemoglobin levels, but the pressure at which maximum pumping was obtained was different only with the $10^{-6}$ M hemoglobin concentration (panel B).

Figure 7. Measurements of hemoglobin in lymph after surgical procedures (catheter placement) or after endotoxin administration (inset). Samples were collected and hemoglobin was quantitated as described in “Materials and Methods.” Data represent mean±SEM of three experiments (postsurgery) and seven experiments with endotoxin. [Hb], hemoglobin concentration.
pressure stimulating an appropriate response. If one argues that the transmural pressure stimulation (or equivalent forces) remain constant, one might expect that the presence of hemoglobin in the lymph would depress lymph flow rates. However, this seems unlikely in vivo. Many situations in which erythrolysat/ hemoglobin enters the lymph occur in conjunction with increases in filtration and lymph formation (for example, endotoxin-induced increases in vascular permeability or damage to the microcirculation). Therefore, variable increases in filtration in concert with a less sensitive lymph pump could produce increases, decreases, or no change in lymph flow rates, depending on the magnitude of the filtration changes and the concentration of hemoglobin in the lymph. In vivo, it is likely that alterations in pumping activity represent part of a continuum, as the lymphatic vessel responds to progressive changes in hemoglobin concentration and other factors and changes in tissue and transmural pressures. Therefore, the curve shift concept does not imply that lymph flow rates will decline. By altering the sensitivity of the lymph pump, lymph flow is still maintained, but an increase in flow rates after an elevation in filtration may not be as large as that occurring in the absence of erythrolysat. The most important consequence of this may be that tissue water may increase in such a circumstance, because the normal balance between filtration and lymphatic drainage may be subtly altered by the presence of hemoglobin.

Of course, one might turn these arguments around and take the position that, as liquid accumulates in tissue spaces, transmural pressures may be applied to the ducts at levels beyond that required to induce peak pumping, that is, on the downside of the transmural pressure and pumping relation. A curve shift to the right permits the lymphatic to pump more effectively in the higher pressure ranges. If transmural pressure increases are not imparted to the initial vessels or collecting ducts, the lymphatics simply may become passive conduits, and the maintenance of lymph flow would have to be solely due to extrinsic forces or to pressure gradients from the interstitium to the initial lymphatics.

The mechanism of action of hemoglobin is unknown. There are a number of clues from the literature in terms of its effect on blood vessels, and some of these may be applicable to lymphatics. For example, hemoglobin is known to generate free radicals and induce lipid peroxidation,27–29 to affect prostaglandin synthesis,11,30 to inhibit endothelium-derived relaxing factor,31,32 and to suppress vasodilator nerve activity.10,33 We believe we can exclude the nerve effect for the following reasons. Some blood vessels have nonadrenergic, noncholinergic vasodilator innervation. Electrical field stimulation in vitro activates transmural nerves to produce dilation. RBC hemolysate products (most likely oxyhemoglobin) inhibit this response.10,33 Whereas lymphatics have noradrenergic innervation and contain α- and β-receptors, tetrodotoxin (a nerve blocker) has no effect on the pumping activity of isolated vessels,34,35 illustrating that nerves do not play a role in the regulation of spontaneous contractile activity. Therefore, it seems unlikely that hemoglobin acts on the lymphatic through an effect on nerve function.

Pharmacological responses to some agents are mediated by the production of factors from the endothelium. As one example, the dilator responses to acetylcholine are mediated by endothelium-derived relaxing factor.36 Hemoglobin has been demonstrated to inhibit dilation in endothelium-dependent reactions such as those induced with acetylcholine. Endothelium-derived relaxing factor binds to the heme group of guanylate cyclase to cause enzyme activation and stimulation of cyclic GMP.31 The endothelium-derived relaxing factor receptor may therefore contain a heme group, and it has been postulated that hemoglobin competes with the heme receptor. In our first attempts to study the effects of hemoglobin on lymphatic contractions, we used a ring preparation and observed that hemoglobin increased contractions in some preparations, depressed contractions in others, and in some cases, had no effect (unpublished observations, 1989). Part of the problem may have been that the endothelium was being damaged by the wires used to suspend the vessel segments. It is interesting to note that a similar variation was observed in studies designed to test the effects of acetylcholine on vascular preparations, and it turned out that the presence or absence of the endothelium was key to this effect.36

There is some experimental evidence that prostaglandin synthesis is involved in mediating the effects of hemoglobin on some vascular smooth muscle preparations.36,37 This perhaps is not surprising because heme is a cofactor in the synthesis of prostaglandins and is believed to be involved in the step in which molecular O2 is attached to unsaturated fatty acids.38 In support of this, cyclooxygenase inhibitors such as aspirin and indomethacin can markedly alter the response of basilar artery preparations to hemoglobin.30,37 Further confirmation of this relation comes from superfusion studies that demonstrated that hemoglobin was capable of causing the release of various prostaglandin types from vascular preparations.11 The possibility that arachidonic acid metabolism may be involved with the hemoglobin effect seems to warrant consideration in our model system, especially because we have obtained evidence in the past that the synthesis of cyclooxygenase and lipoxygenase products within the lymphatic vessel may be important in regulating the spontaneous contractions.39–42

It has been suggested that free radical reactions may be responsible in part for the effects of hemoglobin on cerebral vascular smooth muscle preparations and contribute to the vasospasm and ischemia after subarachnoid hemorrhage. Whether this effect is due to free radical production24 or whether it is secondary to the generation of various lipid peroxides by these reactions27,28 is unknown. In this regard, the oxidation of oxyhemoglobin to methemoglobin (if
this occurs in vivo or in the organ bath preparation) could result in the generation of free radical reactions, and these products conceivably could alter lymphatic pumping.

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