Soluble Complex of Complement Increases Hydraulic Conductivity in Single Microvessels of Rat Lung

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Abstract

We determined the effect of sera enriched with the soluble complex of complement (SC5b-9), on hydraulic conductivity (Lp) of single pulmonary venules (diameter 20–30 μm). Sera free of anticoagulants and blood cells were prepared from rat and human blood. Lp were determined by our split drop technique in isolated, blood-perfused lungs prepared from anesthetized rats (2% halothane; Sprague Dawley, 500 g; $n = 73$). Zymosan-activated (ZAS) and control sera were used for Lp determinations. In ZAS prepared from human serum, SC5b-9 concentration was > 300 μg/ml (control: < 1 μg/ml) as determined by ELISA. At baseline, Lp averaged $3.4\pm.4\times10^{-7}$ ml/($cm^2 \cdot s \cdot cm\ H_2O$), but it increased by 217±32% with undiluted ZAS ($P < 0.05$). The Lp increase correlated significantly with different ZAS dilutions for rat serum and with SC5b-9 concentration for human serum. Lp did not increase significantly with ZAS prepared from heat-treated sera, C6- and C8-deficient sera; or with ZAS in which SC5b-9 had been depleted by immunoprecipitation. The ZAS-induced increase of Lp was blocked completely by venular preinfusion with the arginine-glycine-aspartic acid (RGD) tripeptide (1 mg/ml, 10 min). We report for the first time that: (*a*) SC5b-9 increases lung endothelial Lp; and (*b*) the increase of Lp is attributable to an integrin-dependent mechanism. (*J. Clin. Invest.* 1993. 91:103–109.) Key words: endothelium • permeability • immunology • vitronectin • integrin

Introduction

The terminal complement pathway synthesizes two end products: the soluble complex (SC5b-9) and the membrane attack complex (MAC).[1] The soluble complex results from the binding of plasma vitronectin (VN) to complement proteins C5b through C7 to form SC5b-7, which then incorporates C8 and C9 to form SC5b-9 (1). MAC is synthesized when these complement protein complexes assemble on cell membrane instead of VN (1). Although the formation of these end products

is expected after complement activation, their effects on endothelial permeability have received little attention (2). With regard to lung, previous studies of microvascular flux following complement activation have been dominated by the hemodynamic and neutrophil mediated effects of the anaphylatoxins—C3a, C5a, and C5a-des-Arg (3). Although more recent work indicates that terminal pathway products may cause pulmonary edema (4, 5), the extent to which the end products affect endothelial barrier properties remains poorly understood.

Here we have considered the microvascular effects of SC5b-9 which is plasma soluble and is likely to achieve high plasma levels following complement activation (6). The resultant exposure of microvascular beds in lung and other organs to SC5b-9 may cause significant permeability effects, hence it is important to understand whether SC5b-9 affects the endothelial barrier. There are at least three reasons for expecting such a permeability effect: (*a*) SC5b-9 localizes at sites of endothelial injury (7–9); (*b*) an increase of plasma soluble complex levels predisposes to pulmonary edema (6); (*c*) SC5b-9 binds to endothelium through a VN–integrin interaction (10–12), and may thereby induce receptor-mediated permeability effects. SC5b-9's barrier effect may be quantified in terms of the endothelial hydraulic conductivity (Lp) which we recently determined in single pulmonary microvessels, by means of our split drop procedure (13, 14). Using these methods we report for the first time, that serum enriched with SC5b-9 increased Lp in venules of rat lung.

Methods

Chemicals used

SOD (Sigma Chemical Co., St. Louis, MO); the arginine-glycine-aspartic acid (RGD) tripeptide (Sigma Chemical Co.); purified human C6 (Quidel, San Diego, CA).

Experimental sera

Sera were prepared from rat and human blood. Heparin, which inhibits the terminal complement pathway (15), was avoided. Anticoagulant-free blood was collected by cardiac puncture in rats, and from the antecubital vein from healthy volunteers, then allowed to stand at 4°C for 1 h. The layer of separated serum above the buffy layer was removed, centrifuged (5,000 $g \times$ 10 min, RC2-B; Sorvall Instruments Div. [part of DuPont Co.] Newtown, CT) then injected through a 0.8-μm filter (Millipore Co., Bedford, MA) to prepare cell-free serum. The absence of cells was confirmed by microscopic examination of the sera in a Neubauer chamber, as also in serum smears prepared on glass slides and stained with Wright's stain (magnification, 1,000). All sera were stored as separate aliquots in plastic containers at −70°C and used within 4 wk of preparation. For experiments, an aliquot was slowly warmed to 37°C by immersion in a constant temperature water bath (Lauda RC20; Fisher Scientific Co., Pittsburgh, PA). Each aliquot was thawed only once to avoid complement activation due to repeated thawing (9).

The rationale for inclusion of human serum in these studies was to enable use of commercially available human serum-specific products

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1. *Abbreviations used in this paper:* CH_{50}, quantity of serum required to lyse 50% of red blood cells in a standard hemolytic complement assay; Jv, endothelial flux; Lp, hydraulic conductivity; MAC, membrane attack complex; Pmv, microvascular pressure; VN, vitronectin; ZAS, zymosan-activated serum.

such as an ELISA kit for serum SC5b-9 quantitation and C6 and C8 deficient sera (see below). Rat serum was used in parallel, to control for heterologous effects.

The following special sera were prepared:

(a) Zymosan-activated serum (ZAS). To activate complement, serum was treated with zymosan (Sigma Chemical Co.) according to the method of Gawryl et al. (16). Zymosan was boiled (1 h), cooled to room temperature with saline washes, then dissolved in serum at 10 mg/ml. After incubation of the mixture (1 h at 37°C), the zymosan particles (diameter 3 μm, 17) were removed from the serum sample by centrifugation (20,000 $g \times$ 10 min) followed by injection through a 0.8 μm filter.

(b) Nonactivated serum. Control serum was prepared in parallel to the ZAS procedures, except zymosan was not added.

(c) Heat-inactivated sera. To inactivate complement, rat sera were incubated at 56°C for 30 min before the ZAS preparation procedures.

(d) Complement-deficient sera. Human sera immunochemically depleted of complement fragments C6 and C8 were obtained from Quidel Inc. (San Diego, CA).

(e) C6-repleted ZAS. In one batch, C6-depleted serum was replenished with purified human C6 (Quidel) at 80 μg/ml which is the normal C6 concentration for human serum (18). The repleted serum was then zymosan activated.

(f) SC5b-9–depleted sera. SC5b-9 was depleted in ZAS by the immunoprecipitation method of Chenais et al. (19), using protein A. An anti–human SC5b-9 monoclonal antibody (Quidel) was added to freshly prepared ZAS at molar ratio 1:1 and incubated first 22°C (2 h), then at 4°C (24 h) while the samples were continuously rotated. Protein A-Sepharose CL4B gel (Sigma Chemical Co.; 1 ml swollen gel binds 20 mg IgG) was added to the mixture and incubated for 3 h at 22°C with constant rotation of samples. Substrate-bound immunecomplexes were removed from serum by centrifugation (3,000 $g \times$ 10 min) and filtration (0.8 μm). Sham samples of serum were prepared by identical methods except antibody was not added. SC5b-9 was quantified by ELISA in the treated sera.

Serum analyses. In each serum sample we determined protein oncotic pressure by membrane osmometry (osmometer, model 4100; Wescor Inc., Logan, UT), total protein concentration by the biuret method (20) (spectrophotometer, model 6/20; Coleman, Maywood, IL) and osmolality by freezing point depression (The Advanced® Micro-Osmometer, model 3M0; Advanced Instruments, Inc., Needham Height, MA). In all samples, endotoxin levels were undetectable by limulus assay (limulus assay kit; Sigma Chemical Co.).

For human serum, in one batch each of control, zymosan-activated, SC5b-9–depleted, and C6-deficient sera, we determined SC5b-9 level by an ELISA kit (Quidel). In addition, we assayed functional hemolytic complement activity in these samples as the quantity or dilution of serum required to lyse 50% of the red blood cells in a standard hemolytic complement assay (CH_{50}) (21).

Experimental procedures

Lung preparation. The isolated, blood-perfused rat lung was set up according to our standard procedures which we have described in detail (14). Briefly, in each experiment lungs were removed from one anesthetized rat (500–600 g; halothane 2% inhalation followed by sodium pentobarbital 40 mg/kg, intraperitoneal; Harlan Sprague-Dawley, Inc., Indianapolis, IN). Then the lung was cannulated at the pulmonary artery, left atrium, and trachea, and immediately pump perfused in a perfusion circuit with blood drawn by cardiac puncture from a second rat. The perfusion blood was heparinized (500 U) and diluted with 4 g% albumin (rat albumin, Fraction V; Sigma Chemical Co.) in Ringer's lactate, to achieve hematocrit of 20%. The lungs were inflated with gas (30% O_2, 6% CO_2, 64% N_2) to maintain blood P_{O_2}, P_{CO_2}, and pH at, respectively, 140 Torr, 35 Torr, and 7.3 pH units; as determined every 30 min (178 pH/Blood Gas Analyzer; Corning Glass Inc., Corning, NY). A heat exchanger maintained perfusate temperature (44TD; Yellow Springs Instrument Co., Yellow Springs, OH) at 37°C.

Lung vascular pressures were regulated by adjusting the height of the venous outflow. Opening a shunt between the arterial and the venous cannulae, stopped lung blood flow during the split drop procedure. Pressure transducers (P23 ID; Gould Statham, Oxnard, CA) recorded large vessel pressures with respect to the micropuncture level. Airway pressure was held constant at 5 cm H_2O during micropuncture, but cyclically varied to induce ventilation during nonmicropuncture intervals lasting 10–15 min. Pressure recordings were displayed on a multichannel recorder (RS 3400; Gould Statham).

Preparative procedures for lung micropuncture

The micropuncture surface. The diaphragmatic or costal lung surface was positioned on a vibration free air table (Micro-G; Technical Manufacturing Corp., Woburn, MA) for stereomicroscopy (×150; Olympus model SZH), videoimaging (Panasonic WV-5410), and videorecording (Panasonic AG-6050) by means of a closed circuit TV system (Panasonic CCTV; camera WV-CD51). The micropuncture surface was layered with silicone oil (200; Dow Corning Corp., Midland, MI) at 37°C (Thermalert TH-5; Sensortek, Clifton, NJ). The lung elsewhere was covered by plastic wrap (Saran Wrap).

Micropipettes. Micropipettes (tip diameter $\sim$ 5 μm) were pulled (Sachs-Flaming Puller, model P-84; Sutter Instrument Co., Novato, CO) and bevelled (Sutter Instrument Co. beveller) from glass tubing (1 mm diameter; Glass Co. of America, Millville, NJ) which had been acid cleaned, sterilized, and siliconized. They were filled with either castor oil colored with carbon black B or the experimental serum ("split solution").

Split drop procedures

Venules (diameter 20–25 μm) were identified by noting the distribution pattern expected for venular blood flow (13, 14). The micropuncture steps of our split drop procedure are illustrated in Fig. 1. After stopping lung blood flow at vascular pressure of 5 cmH_2O, the experimental venule was micropunctured with the oil pipette to inject $\sim$ 100–200 μm of vessel length with oil. The serum pipette was then used to split the oil drop with an injection of the test serum (split solution) to separate the margins of the split oil drop by $\sim$ 80 μm. In the third step, more oil was injected through the oil pipette to displace the split drop $\sim$ 40 μm away from the micropuncture sites. This crucial step ensured placement of the experimental solution in a nonmicropunctured region of the vessel, hence leaks through micropuncture defects were prevented. Finally, both micropipettes were removed. We rejected split drop data if step 3 (Fig. 1) of the procedure was unsuccessful and if blood entered the split drop.

Every 10 s (videotimer: VTG-33; FOR-A Corp. of America, Newton, MA), microvessel diameter and minimum distance between the split drop margins (split drop length) were determined by microcaliper (Mitutoyo Corp., Tokyo, Japan), from a frame-by-frame replay of the videorecording (×400; resolution 1 μm). Split drop length decreased with liquid exit from the split drop (filtration), but increased with liquid entry (absorption). Microvessel diameter remained constant. We estimated liquid flux per unit surface area (Jv) at time zero according to our analysis described fully in previous reports (13, 14). Briefly, split drop length and diameter data were used in the cylinder formula to calculate split drop volume and surface area. Then Jv was calculated by differentiating at time zero, the slope of the exponential regression of split drop volume versus time in the first minute. The split solution maintained endothelial contact for < 2 min. The linear Jv-microvascular pressure (Pmv) relation was determined through Jv measurements at different Pmv. The line's slope gave Lp (13, 14).

We have established that in this preparation, Lp remains unchanged for up to 4 h (14). Therefore we completed all split drop determinations within this period. We have also established that large vessel pressures (e.g., pulmonary artery) are identical to split drop hydrostatic pressures (13), that neither our micropuncture procedures nor the endothelial contact with oil deteriorates the endothelial barrier, and that the Jv-Pmv line is highly correlated by linear regression (14). Based on our previous micropuncture measurements (14, 22), we assumed perivenular interstitial pressure to be 0 cm H_2O (13). After the experiment, the lung was homogenized for determination of extravas-
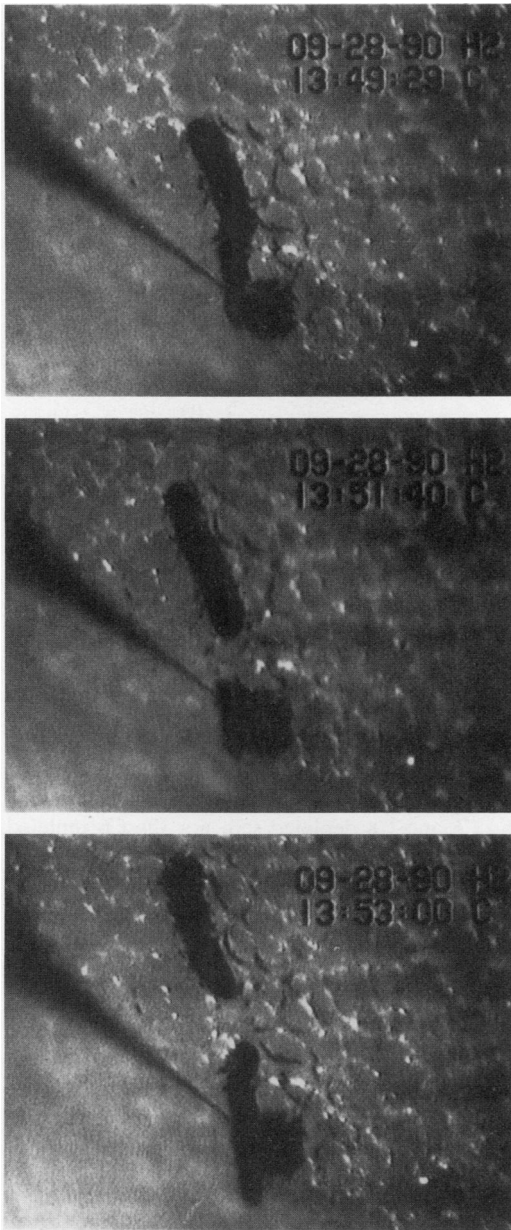
Figure 1. Sequential steps of split drop procedure. Frames show videorecordings of a pulmonary venule (diameter 30 μm). (*Upper panel*) The venule has been micropunctured and filled with castor oil stained with Sudan black. The oil pipette is visible on left. (*Middle panel*) The oil drop has been split by an injection of serum through a second pipette. The serum was colorless, hence the second pipette is not visible in the photograph. The micropuncture site of the second pipette is located at the bottom margin of the split segment. (*Lower panel*) By additional oil injections the segment containing serum has been displaced above both micropuncture sites. This step prevents leakage of experimental serum through micropuncture defects in the vessel wall. Before flux determination, both pipettes were withdrawn.

cular lung water content by our standard procedures (13, 22). Data were rejected if lung water exceeded 4 g/g dry.

Experimental Groups

L_p *responses to different dilutions of ZAS (rat sera).* ZAS was used either undiluted or in dilutions with nonactivated serum as 50, 20, and 10% solutions. In each experiment, paired, control measurements of

Lp were obtained using nonactivated serum. In one group, ZAS was prepared with heat-treated serum.

Complement-deficient experiments (human sera). In these experiments, we determined SC5b-9 concentrations by ELISA. In separate groups, Lp responses to ZAS were compared against responses for, respectively, ZAS prepared with C6 and C8 deficient sera, SC5b-9–depleted ZAS, and C6-repleted ZAS. In another group, we used a 10% vol/vol dilution of ZAS in nonactivated serum.

Preinfusion experiments (rat sera). We microinfused venules for 10 min with the arginine-glycine-aspartic acid (RGD) tripeptide dissolved in 4 g% albumin at a concentration of 1 mg/ml (23), then immediately followed with the split drop procedure using ZAS as split solution. Control Lp were determined in venules which were both non-preinfused, and in which preinfusions were carried out with only the vehicle, 4 g% albumin. The rationale for using 4 g% albumin was that a sufficiently high oncotic pressure was required to prevent rapid solution escape into the interstitium. To further prevent such escape during preinfusion, Pmv was held at 5 cm H_2O to ensure net absorptive conditions (13, 14).

Statistics. Data are presented as mean$\pm$SE. Paired differences were tested by the paired t test, and differences among more than 2 groups by the ANOVA-Neuman Keul's test. Significance was accepted at $P < 0.05$.

Results

Effect of ZAS. The plots shown in Fig. 2 for a single experiment, represent Jv-Pmv relationships that were obtained using ZAS and control serum as split solutions. The slope of the relationship gave Lp. In this experiment, Lp was 3.4×10^{-7} ml/(cm$^2 \cdot$ s $\cdot$ cm H_2O) for control serum, but it increased to 9.5 with ZAS.

ZAS prepared from fresh serum always increased Lp. These Lp responses to ZAS were assessed in different groups. In three groups, ZAS was diluted by addition of control serum as indicated (Table I). In a fourth group, undiluted ZAS was used. In each group, Lp responses were determined as paired comparisons of ZAS against control serum (Table I). The overall average for Lp from the 20 baseline determinations, was $3.4\pm.4 \times 10^{-7}$ ml/(cm$^2 \cdot$ s $\cdot$ cm H_2O). In each group, ZAS always increased Lp above baseline. This effect was most pronounced in the undiluted group, in which Lp increased on average by 217$\pm$32%. However as evident from the SE of the experimental groups in Table I, the Lp increases varied between experiments. As shown in Fig. 3, the means of the Lp
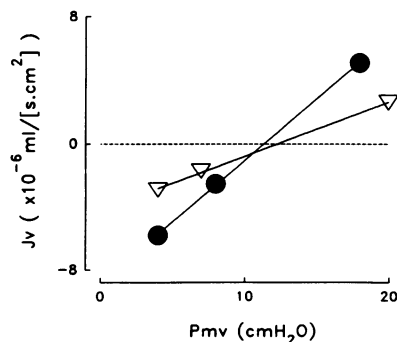

Figure 2. Plots of endothelial flux (Jv) versus microvascular pressure (Pmv) from one experiment. Jv was determined in single venules using serum as split solution. Slopes determined by linear regression ($P < 0.05$ for both) gave endothelial hydraulic conductivity (Lp). Lp for zymosan activated serum was 2.8 times higher than baseline Lp determined with nonactivated serum (3.4×10^{-7} ml/[cm$^2 \cdot$ s $\cdot$ cm H_2O]). Rat serum: $\triangledown$, baseline; $\bullet$, zymosan activated.

Table I. L_p Determined with Different Dilutions of ZAS

| | L_p ($\times 10^{-7}$ ml/(cm$^2 \cdot$ s $\cdot$ cm H$_2$O)) | |
ZAS dilutions	Control	Experimental
vol/vol %		
10	3.5±.8	6.4±.9*
20	3.8±1	7.2±1.4*
50	2.9±.4	7.7±1.1*
100	3.4±.7	10.5±2*

Mean±SE; n = 5 for each group; ZAS, zymosan-treated serum; L_p, hydraulic conductivity; vol/vol %, percent ZAS in nonactivated serum; * P < 0.05 compared with control.

Figure 4. The effects of complement depleted sera (mean±SE for human sera; *P < 0.05 by ANOVA-Neuman Keul's). Zymosan activation of control sera increased L_p above baseline. However, activation of C6 (C6(−)) and C8 (C8(−))-depleted sera did not affect L_p. C6 repletion followed by activation (C6RE) increased L_p. □, Baseline; ■, zymosan activated.

increases correlated linearly with log dilution of ZAS (P < 0.05 by linear regression).

In these 20 experiments, the extravascular water contents of experimental and control lungs were not significantly different (3.6±.2 versus 3.6±.1 g/g dry). Therefore our procedures did not cause pulmonary edema.

Heat treatment of serum completely abolished the L_p response to ZAS. In four experiments, L_p determined with ZAS prepared from heat-treated serum (3.5±.4×10^{-7} ml/[cm$^2 \cdot$ s $\cdot$ cm H$_2$O]) was not significantly different from baseline (3.2±.2 × 10^{-7} ml/[cm$^2 \cdot$ s $\cdot$ cm H$_2$O]). In contrast, in five concurrent experiments, ZAS prepared as usual significantly increased L_p above baseline (respective L_p for ZAS and baseline: 10.5±2 and 3.4±.7 × 10^{-7} ml/[cm$^2 \cdot$ s $\cdot$ cm H$_2$O]).

Addition of SOD did not affect L_p. In four experiments, we added 200 U/ml of SOD into both the perfusate and the aliquot of ZAS used as split solution. The mean L_p obtained with a split solution mixture of ZAS and SOD was 7.5 × 10^{-7} ml/(cm$^2 \cdot$ s $\cdot$ cm H$_2$O). This was similar to the paired value obtained with ZAS without SOD (8.1 × 10^{-7} ml/[cm$^2 \cdot$ s $\cdot$ cm H$_2$O]).

Complement-deficient experiments. We detected no differences in L_p determined with human and rat sera. Both the baseline L_p (3±.6 × 10^{-7} ml/[cm$^2 \cdot$ s $\cdot$ cm H$_2$O], n = 8) as also the L_p response to ZAS (Fig. 4, left extreme filled bar) did not significantly differ from the corresponding data for rat serum (NS by ANOVA-Neuman Keul's test).

Our ELISA determinations in six batches of human serum are summarized in Table II. In control serum, SC5b-9 was < 1 μg/ml, as consistent with previous reports (24). The control
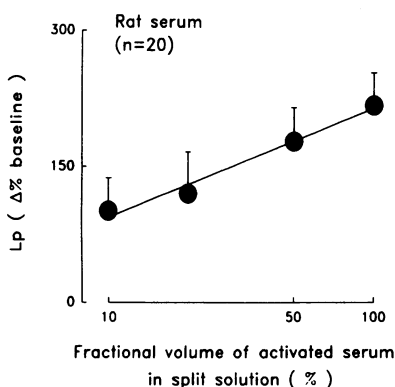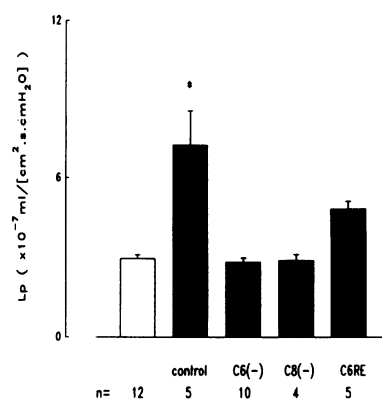
ZAS sample was markedly SC5b-9 enriched. However, after immunoprecipitation, ZAS was depleted of SC5b-9. As expected, SC5b-9 was undetectable in ZAS prepared with C6 and C8 deficient sera obtained commercially (data not shown). When we repleted C6 deficient serum with purified C6, zymosan activation increased serum SC5b-9 concentration. However this increase was considerably less than the increase of SC5b-9 concentration achieved in fresh serum. The discrepancy may be attributable to low levels of other complement factors in the commercially prepared C6 deficient serum.

Neither the C6 and C8 depleted sera nor the C6 repleted serum affected baseline L_p under nonactivated conditions (data not shown). Although ZAS prepared with fresh serum increased L_p as usual, ZAS prepared with C6-deficient or C8-deficient sera failed to increase L_p above baseline (Fig. 4). Using ZAS prepared with the C6-repleted serum, we recovered 49.7±5.7% of the control L_p response to ZAS. ZAS that had been sham immunoprecipitated as usual markedly increased L_p (6.5±.4 × 10^{-7} ml/[cm$^2 \cdot$ s $\cdot$ cm H$_2$O]) (P < 0.05). However, ZAS that had been SC5b-9 depleted, failed to increase L_p above baseline. Thus, with SC5b-9–depleted ZAS, L_p was 2.7±.3 × 10^{-7} ml/(cm$^2 \cdot$ s $\cdot$ cm H$_2$O), which was not significantly different from baseline (compared by ANOVA-Neuman Keul's against baseline L_p of other groups).

In Fig. 5 we have plotted the mean L_p determined for each serum type against the log of the corresponding serum SC5b-9

Figure 3. Effect of different dilutions of zymosan-activated serum (ZAS). L_p = hydraulic conductivity. ZAS was diluted in nonactivated serum then used as split solution. Each point represents mean (±SE) of five experiments. Mean L_p increased linearly with log of fractional ZAS content (P < 0.05).

Table II. Serum ELISA for SC5b-9

Serum	SC5b-9
	μg/ml
Control	<1
ZAS	333
ZAS-SC5b-9(−) (Sham)	525
ZAS-SC5b-9(−)	7
ZAS-C6(RE)	16
ZAS 10%	28

ELISA with monoclonal antibody to SC5b-9 (Quidel Inc.); ZAS, zymosan-treated serum; ZAS-SC5b-9(−), ZAS depleted of SC5b-9; ZAS-C6(RE), ZAS prepared with C6-repleted serum; ZAS 10%, 10% dilution of ZAS in control serum.
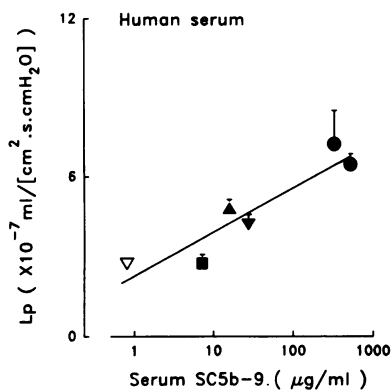
Figure 5. Effect of SC5b-9 concentration on L*p*. Mean (±SE) L*p* for different batches of serum have been plotted against corresponding SC5b-9 concentrations determined by ELISA. The sera batches are: nonactivated serum (*open inverse triangle, n = 8*), zymosan-activated serum immunodepleted of SC5b-9 (*filled square, n = 5*), C6-repleted serum (*filled regular triangle, n = 5*), 10% vol/vol dilution of zymosan-activated serum in control serum (*filled inverse triangle, n = 4*) and two batches of zymosan-activated serum (filled circles, *n = 5* each). L*p* correlated linearly with log SC5b-9 concentration (*P < 0.05 by linear regression*).

concentration from Table II. The plot correlated significantly by linear regression indicating an exponential relationship. An analysis of variance amongst the six serum groups indicated that L*p* was significantly higher than baseline for all groups, except for the immunoprecipitated group (*P < 0.05* by ANOVA-Neuman Keul's test).

In nonactivated human serum, we detected a CH_{50} score of 24 U/ml which is within normal limits (21). However, in ZAS, a CH_{50} score was undetectable (< 5 U/ml). This indicates that zymosan activation resulted in complete consumption of serum complement.

Fig. 6 summarizes results of the microinfusion experiments. As before, ZAS markedly increased L*p* above baseline by similar extents in both noninfused venules and venules infused with only vehicle (4 g% albumin) (*P < 0.05*). In contrast, in venules preinfused with the RGD tripeptide, ZAS failed to increase L*p* above baseline (NS). L*p* determined with control serum after microinfusions of RGD alone, did not differ from baseline (3.3±.1 × 10^{-7} ml/[$cm^2 \cdot s \cdot cm$ H_2O], *n = 5*).

Discussion

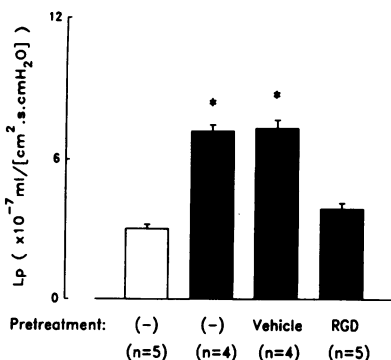We demonstrate for the first time that in single pulmonary microvessels complement-activated serum increased L*p* by a

Figure 6. The effect of the RGD (ARG-GLY-ASP) tripeptide on L*p* (means±SE; **P < 0.05* compared with baseline). Baseline determined with nonactivated serum in noninfused (−) venules. ZAS increased L*p* identically in noninfused and vehicle (4 g% albumin) preinfused venules. After RGD (1 mg/ml) preinfusion, ZAS failed to increase L*p* above baseline. Rat serum: □, baseline; ■, zymosan activated.

direct effect that was white blood cell independent and that occurred within 1 min. The response was attributable to a heat-labile complement product, because we inhibited the L*p* increase using sera which were heat treated or deficient in C6 or C8. This C8 dependence indicates that the product was formed in the post C8, terminal phase of complement activation. Post C8 products include the VN-incorporated soluble complexes (SC5b-8 and SC5b-9) and MAC. Although all of these products may form in the terminal phase, we have three principal reasons for believing that our results were not attributable to MAC. First, we ensured that all sera were cell free; hence, no membranes were available for MAC formation during zymosan activation. Second, our CH_{50} determinations indicate that complement was completely consumed in sera activated with zymosan. Therefore ZAS was free of MAC precursors and MAC could not have formed when we microinjected ZAS into the experimental venule. Third, we inhibited the L*p* response to ZAS by pretreating venules with the RGD tripeptide. This result rules out the involvement of MAC which acts directly on the membrane rather than through RGD recognition (25). Therefore our L*p* findings were attributable to a soluble product. Since complement was completely consumed in our samples of ZAS, the terminal complement pathway proceeded to completion. Hence we interpret that the soluble product was SC5b-9.

Our ability to inhibit the present L*p* increase by RGD pretreatment implicates an integrin-based mechanism in the response to ZAS. This possibility is supported by the presence of VN in SC5b-9. The RGD sequence of VN recognizes an endothelial integrin (10, 14, 26), and pretreatment of the integrin receptor with RGD inhibits the recognition (23, 27). An RGD based interaction between VN and a cell surface integrin has been implicated in the adherence of SC5b-9 to cultured myoblasts (28). Although the $\alpha_v\beta_3$ integrin is believed to be the endothelial receptor for VN (29), the involvement of this integrin in the present responses remains uncertain. Lampugnani et al. recently demonstrated that application of RGD to cultured endothelial monolayers induced the formation of large intercellular gaps (30). This effect was attributed to inhibition of the fibronectin-dependent intercellular adhesion which stabilizes the cultured monolayer. We were unable to confirm this finding because in our experiments with control serum RGD pretreatment did not detectably alter L*p* from untreated baseline values. This difference from the findings of Lampugnani et al. may be attributable to intrinsic differences of barrier properties between intact and cultured endothelium. We affirm that RGD pretreatment does not affect baseline barrier conductance; but the fact that it inhibits the L*p* response to ZAS indicates that endothelial integrins may subserve a barrier regulatory function.

In activated human serum, we quantified SC5b-9 by means of a recently described sandwich ELISA (9) for which a kit is commercially available. Although this ELISA is based on a monoclonal antibody that recognizes both SC5b-9 and MAC, in cell-free serum only SC5b-9 is assayed (9, 31). We confirm that serum SC5b-9 concentration is normally negligible (6), and that it increases to > 300 μg/ml after zymosan activation (16). Although immunodepletion of SC5b-9 abolished the L*p* response to ZAS, the log concentration of SC5b-9 correlated linearly with L*p* increases. Because of this exponential relationship, small increases of SC5b-9 concentration did not signifi-

cantly increase Lp. For instance, when we depleted SC5b-9 in ZAS by immunoprecipitation, the resulting SC5b-9 concentration was 7 μg/ml (Table II). Although this concentration was higher than control ($<$ 1 μg/ml), it did not detectably increase Lp. An ELISA kit is not available for rat serum in which we therefore did not assay SC5b-9. However, we point out that similar increases of Lp occurred with activated sera from both rat and human sources, and that different dilutions of activated rat sera also correlated nonlinearly with Lp. The similarity in the Lp responses to rat and human sera indicate that heterologous effects did not blunt the interaction of human SC5b-9 with rat endothelium. The concentration-dependent responses from rat and human sera are consistent with clinical reports that high (6) but not low (32) levels of serum-soluble complex predispose to barrier pathology. The abolition of the Lp response after immunodepletion of SC5b-9 and the concentration dependence of the Lp response (Fig. 5), also support our view that SC5b-9 was the critical factor in the present barrier response.

Although many previous reports have examined the effect of complement activation on lung liquid balance (recently reviewed in 2, 3, 33), ours is the first to quantitate the direct barrier effect of a terminal complement product. In previous studies, complement was activated either in the airway or in the circulation (2, 3, 33, 34). The resulting lung microvascular flux responses were attributed to the effects of neutrophil activation and pulmonary hemodynamic changes caused by the anaphylatoxins C3a, C5a, and C5a-des-Arg (3, 33). Seeger et al. attributed an increase of filtration coefficient in rabbit lungs given human serum, to a Forssman type reaction (26). Their response, which occurred with a $>$ 30 min delay, is clearly different from ours in which Lp increased within 1 min.

Our principal aim here was to quantify endothelial barrier properties in terms of Lp in single lung microvessels exposed to SC5b-9–enriched serum. We took advantage of the fact that in the absence of cell membranes and heparin, serum complement activation causes pure SC5b-9 formation (9). We chose the single microvessel approach which by our recently developed methods allows accurate determinations of Lp (13, 14). We recognize that single vessel data must be interpreted with caution with regard to the whole organ. However, our approach afforded several advantages. The Lp determination was precise because our approach uniquely makes possible in situ estimates of the critical surface area of filtration in the observed microvessel (1, 12, 13). We avoided interference from hemodynamic factors which have traditionally complicated permeability interpretations in lung.

In interpreting our data, we considered possible leukocyte effects. Although lung resident leukocytes have been shown to localize in alveolar septal capillaries which are two vascular generations upstream of our experimental venules (35), marginating leukocytes may have been resident in the split drop segment of the venule. Such leukocytes could have been activated by complement-activated sera and thereby could have induced independently oxidative damage to the venular endothelium (3, 33). However, we have three reasons for believing that leukocyte activation did not affect the present Lp responses: (a) leukocyte-generated oxidative effects occur independently of RGD recognition mechanisms. Therefore, our ability to inhibit completely the Lp response to ZAS with RGD pretreatment strongly argues against leukocyte activation as

being a mechanism for the increase of Lp. (b) Leukocyte activation is expected to induce SOD inhibitible oxidative damage. However, in four experiments, when we added SOD at a dose sufficient to inhibit a possible toxic radical effect attributable to leukocyte activation, the Lp response to ZAS was not affected. (c) Anaphylatoxins such as C5a are strong activators of leukocytes (3, 33). Although anaphylatoxins were probably formed in zymosan activated sera which were, respectively, C6, C8, and SC5b-9–deficient, none of these sera increased Lp significantly. Therefore, either the venules were leukocyte free or the leukocyte activation was not sufficient to evoke Lp changes in the 1-min experimental period. Moreover, because of its large size (kD $>$ 900; 1), it is unlikely that the SC5b-9 complex was transported across the endothelium within the response time of 1 min, to activate leukocytes lying in the perivenular interstitium. Taken together, these considerations rule out the importance of leukocyte activation as being a significant factor in our experiments.

Because our single microvessel technique is relatively new, some procedural considerations may be relevant. We determined Lp on the established principle that when Jv relates linearly to Pmv, the slope equals Lp (14, 36). Here and previously we demonstrated that the Jv-Pmv relation determined with three or more points gives a highly correlated linear relationship with positive slope (14). We have also established that the luminal hydrostatic pressure in the split drop equals large vessel pressures during stop flow conditions (13), that the oil-endothelial contact and the double micropuncture procedure do not detectably damage the endothelial barrier (14), and that oil viscosity is not a significant factor in the estimation of Jv (unpublished observations). Therefore we do not believe that our procedures affected the present data. As compared with the present baseline Lp for serum, previously we reported a higher baseline Lp for albumin solution (5.5 $\times$ 10^{-7} ml/[cm^2·s·cm H$_2$O]). This difference may be attributable to the known barrier-enhancing properties of serum (37).

In conclusion, our results indicate for the first time that SC5b-9–enriched sera rapidly increased Lp of single lung microvessels. This response demonstrates that SC5b-9 affects the endothelial barrier, hence it questions the prevalent notion that the sole biological function of SC5b-9 is to prevent the lytic effects of MAC (1, 25). Since the Lp increase was RGD inhibitible, we report the first evidence of an integrin-dependent regulation of the endothelial barrier. These new findings raise the significant possibility that SC5b-9 through a blood-borne effect, may diminish the lung endothelial barrier even when complement activation is extrapulmonary.

Acknowledgments

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