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IN VIVO RECRUITMENT OF NEUTROPHILS: Consistent Requirements for L-Arginine and Variable Requirements for Complement and Adhesion Molecules

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Abstract—The current studies examined the mechanisms of neutrophil recruitment into the rat peritoneal cavity following injection of glycogen and into rat lungs following alveolar deposition of IgA immune complexes or airway instillation of phorbol ester (PMA). Unexpectedly, in each model a requirement for L-arginine for neutrophil recruitment was demonstrated, since administration of the L-arginine analogue, N^G-monomethyl L-arginine acetate (L-NMA), greatly reduced neutrophil accumulation as assessed by quantitation of neutrophils in peritoneal exudates and bronchoalveolar lavage fluids, and by lung myeloperoxidase content. In the case of IgA immune complex deposition, lung recruitment of neutrophils was also suppressed by soluble recombinant human complement receptor-1 (sCR1) and antibody to CD18 but not by antibody to E-selectin. In contrast, neutrophil accumulation following airway instillation of PMA exhibited, surprisingly, no requirement for complement but requirements for both E-selectin and CD18. These data demonstrate variable requirements for complement, E-selectin and CD18 but a consistent requirement for L-arginine for neutrophil recruitment. These findings provide evidence suggesting that L-arginine or its derivatives regulate neutrophil recruitment.

INTRODUCTION

Recruitment of leukocytes into sites of inflammation has been extensively studied in vivo, including the use of antibodies that block leukocyte adhesion molecules. In general, blockade of β_2 integrins with antibody to CD18 is effective in inhibiting the recruitment of neutrophils into skin and lung following the local injection of proinflammatory mediators, living bacteria or bacterial lipopolysaccharide (1, 2). The requirement for CD18 is not invariable, since neutrophil accumulation in rabbit lungs in response to *Streptococcal pneumoniae* is apparently CD18-independent, in contrast to findings with *Escherichia coli*

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(2). The accepted process for neutrophil adhesion and transmigration includes the initial tethering of leukocytes by selectins and firm adhesion and transmigration mediated by interactions between leukocyte integrins and endothelial cell adhesion molecules of the immunoglobulin superfamily (3).

There is increasing evidence that L-arginine or its derivatives have important effects on leukocyte recruitment and on tissue injury. L-arginine can be metabolized by a variety of different cells to form nitric oxide (4). Nitric oxide (·NO) or its derivatives (peroxynitrite anion, $ONOO^-$, and the hydroxyl radical, HO·) appear to be highly toxic to cells and tissues, and the blocking of ·NO generation in vivo is protective against IgG immune complex-induced lung injury (5–7). Similar to prostacyclin, ·NO has been shown to inhibit the adhesion of leukocytes to endothelial cells (8, 9). However, superoxide anion (O_2^-), by interacting with ·NO to generate $ONOO^-$, can scavenge ·NO, thereby reversing the anti-adhesive effects of ·NO (9).

In the current studies we investigated determinants of neutrophil recruitment into the rat peritoneal cavity following instillation of glycogen and into the lung following deposition of IgA immune complexes or after airway instillation of phorbol ester. As assessed by the use of the L-arginine analog, N^G-monomethyl-L-arginine acetate (L-NMA), the data suggest a consistent requirement for L-arginine in the tissue accumulation of neutrophils, while requirements for complement, E-selectin and CD18 are variable among different inflammatory models.

MATERIALS AND METHODS

Materials. Oyster glycogen, phorbol 12-myristate 13-acetate (PMA) and L-arginine were obtained from Sigma Chemical Company (St. Louis, Missouri). L-NMA was obtained from Calbiochem (LaJolla, California). Soluble complement receptor-1 (sCR1) was produced by CHO cells transfected with the human cDNA for sCR1 and was kindly provided by Dr. Una Ryan (T Cell Sciences, Cambridge, Massachusetts). Murine monoclonal IgA antibody to dinitrophenol (DNP) as well as murine IgA that was non-reactive with DNP were obtained by implanting murine plasmacytomas, MOPC 460 and 315 (Litton Bionetics, Kingston, Maryland), respectively, into Balb/c mice (Jackson Laboratories, Bar Harbor, Maine), followed by collection of the ascites fluids. The IgA was then isolated and purified as previously described (10, 11). Conjugates of DNP to bovine serum albumin (BSA) were prepared such that the final preparation contained 50 DNP groups per molecule BSA.

Models of Inflammation. Specific pathogen-free male Long-Evans rats (Charles River Laboratories, Portage, Michigan) weighing 300–350 grams were used in all experiments. Anesthesia was induced with intraperitoneal ketamine (100 mg/kg) prior to all experimental manipulations. Glycogen-induced peritonitis was produced by intraperitoneal injection of 25 ml of 0.1% (wt/vol) oyster glycogen. Negative control rats received an equal volume of saline. Four h after injection, peritoneal exudates were collected and the cellular content analyzed by conventional microcytometry. Peritoneal exudates contained >95% neutrophils. IgA immune complex-induced alveolitis was

produced by intratracheal instillation of 1.2 mg anti-DNP IgA (in a total volume of 300 μl PBS) followed immediately by intravenous injection of 3.3 mg (in 0.5 ml PBS) DNP-BSA. Intravenous injection of DNP-BSA was omitted in negative control rats. Four h later, rats were sacrificed by exsanguination and lung tissues or bronchoalveolar lavage (BAL) fluids harvested for analysis. PMA-induced lung alveolitis was produced by intratracheal administration of 15 μg PMA in 300 μl PBS as previously described (12). Negative control rats received 300 μl PBS intratracheally. Four h later, rats were sacrificed by exsanguination and lung tissues or BAL fluids harvested for analysis.

Interventions. L-NMA was administered intraperitoneally with glycogen, or intratracheally with IgA antibodies or PMA, at a final concentration of 5 mM in the volumes indicated above. This dose of L-NMA has been shown to significantly reduce the level of NO_2^- and NO_3^- in BAL fluids during immune complex-induced lung inflammation (7). Hemolysis of red blood cells was blocked by sCR1 in the presence of human or rat serum and sCR1 blocks rat complement with an IC_{50} of approximately 150 pM (13). We administered sCR1 intravenously (15 mg/kg) 2, 2.5 and 3 h after induction of the inflammatory reactions. This dose of sCR1 is known to reduce IgG immune complex-induced lung neutrophil accumulation (14). Anti-rat E-selectin (clone CL-3) was generated as described previously (15). $F(ab')_2$ preparations were made using Immunopure $F(ab')_2$ (Pierce Co., Rockford, Illinois) and 45 μg were administered intravenously 2.5, 3 and 3.5 h after induction of injury. This dose of anti-E-selectin substantially reduced IgG immune complex-induced lung neutrophil accumulation (15). Anti-rat CD18 (clone CL-26) was generated as previously described (16). $F(ab')_2$ preparations were made and 33 mg were administered intravenously 2.5, 3 and 3.5 h after induction of injury. This dose of anti-CD18 greatly reduces neutrophil buildup in the model of IgG immune complex-induced lung injury (16). $F(ab')_2$ preparations of a mouse myeloma irrelevant IgG_1 (MOPC-21) served as the control for anti-CD18 and anti-E-selectin preparations.

Lung MPO Content. Whole lung MPO activity was quantitated as described previously (17). Briefly, whole lungs homogenates were diluted in 50 mM potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH, 6.0). After sonication and twice freeze-thawed, samples were centrifuged at 4000 × g for 30 min. The supernatants were reacted with H_2O_2 (0.3 mM) in the presence of *O*-dianisidine. MPO activity was assessed by measuring the rate of change in absorbance at 460 nm.

BAL Neutrophil Counts. BAL fluids were collected by instilling and withdrawing 5 ml of sterile PBS five times from the lungs via an intratracheal cannula. Neutrophils were identified and quantitated by microcytometry.

Statistical Analyses. All values are expressed as mean ± *SEM*. Data were analyzed with a one-way analysis of variance and individual group means were then compared with a Tukey's test. Differences were considered significant when $P < 0.05$. For calculations of percent change, negative control values were subtracted from positive control and treatment group values.

RESULTS

Requirement for L-Arginine in Glycogen-Induced Peritoneal Exudates. Rats received intraperitoneal glycogen in the presence or absence of 5 mM L-NMA with or without 20 mM L-arginine. Peritoneal exudates were harvested 4 h later

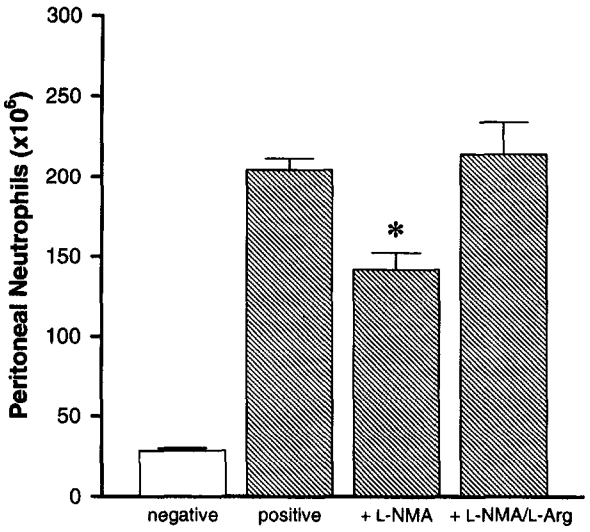

Fig. 1. Effects of L-NMA on peritoneal neutrophil accumulation during glycogen-induced peritonitis. Peritoneal exudates were harvested 4 h after intraperitoneal injection of PBS, glycogen, glycogen with 5 mM L-NMA, or glycogen with 5 mM L-NMA and 20 mM L-arginine. For each group, N = 6.

and the number of neutrophils in the exudates determined. In rats injected with PBS, few neutrophils were observed in peritoneal exudates, whereas glycogen injection resulted in significant neutrophil accumulation (2.0×10^8) (Figure 1). When glycogen was injected in the presence of 5 mM L-NMA, there was a 34% reduction ($P < 0.05$) in numbers of peritoneal neutrophils. However, when 20 mM L-arginine was co-administered with L-NMA, there was no reduction in the number of neutrophils in the exudates. These findings suggest that glycogen-induced peritoneal neutrophil accumulation is, at least in part, dependent on a requirement for L-arginine.

Requirements for L-Arginine for Neutrophil Recruitment during IgA Immune Complex-Induced Alveolitis. Effects of L-NMA on neutrophil accumulation induced by IgA immune complexes were assessed over a 4 h time course (Figure 2). IgA immune complex deposition resulted in progressive neutrophil accumulation in BAL fluids. Coadministration of L-NMA did not reduce neutrophil influx at the 1 h time point, but decreased neutrophil accumulation was found at 2, 3 and 4 h, with reductions of 38%, 50%, and 57%, respectively ($P < 0.01$). Lung MPO content was measured as another index of lung neutrophil accumulation. As shown in Figure 3, the presence of 5 mM L-NMA reduced lung MPO content at 4 h by 53% ($P < 0.05$), confirming the data in Figure 2. When

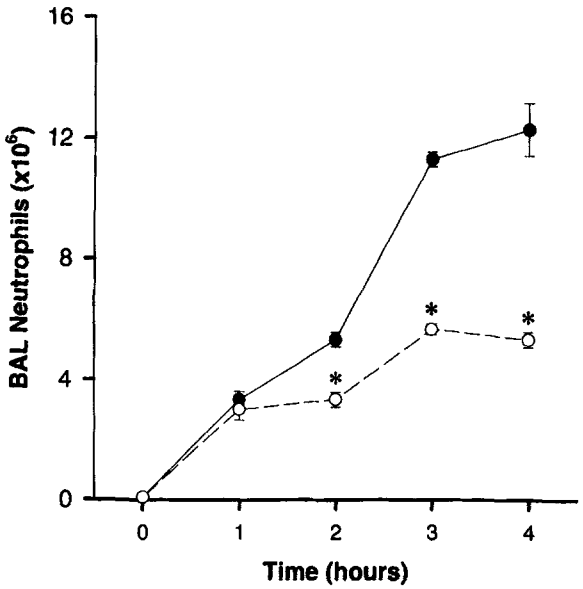

Fig. 2. Effects of L-NMA on BAL neutrophil counts after intrapulmonary deposition of IgA immune complexes. BAL neutrophils from rats developing deposits of IgA immune complexes (closed circles) or IgA immune complexes in the presence of 5 mM L-NMA (open circles) were quantitated at the times indicated. When L-NMA was used, it was given intratracheally together with the IgA anti-DNP-BSA. For each group, $N = 4$.

20 mM L-arginine was present with L-NMA, the inhibitory effects of L-NMA on neutrophil accumulation were lost (Figure 3). There was no effect of L-arginine alone on IgA immune complex-induced lung content of MPO. These data indicate that L-NMA suppresses neutrophil accumulation in lung during IgA immune complex-induced inflammation.

Effects of Complement and Adhesion Molecule Blockade on Neutrophil Recruitment During IgA Immune Complex-Induced Alveolitis. In order to determine whether complement and adhesion molecules contribute to neutrophil recruitment into lungs containing IgA immune complexes, rats were treated with sCR1, anti-E-selectin or anti-CD18 during the course of lung injury. As shown in Figure 4, treatment with sCR1 reduced IgA immune complex-induced increases in BAL neutrophil counts by 79% ($P < 0.01$). Treatment with anti-E-selectin had no effect on IgA immune complex-induced neutrophil recruitment, but treatment with anti-CD18 reduced neutrophil accumulation by 62% ($P < 0.05$) (Figure 4). Similar effects were observed when lung MPO content was measured (data not shown), indicating that in response to IgA immune complex-induced

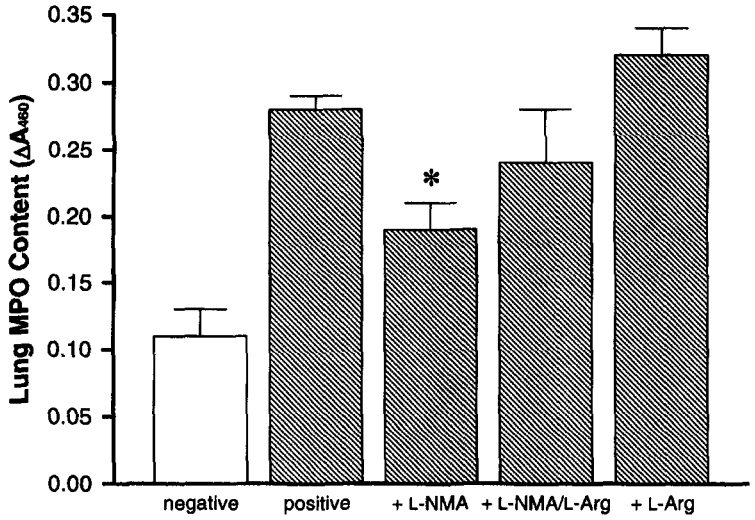

Fig. 3. Effects of L-NMA and/or L-arginine on lung MPO content after intrapulmonary deposition of IgA immune complexes. For each group, $N = 6$.

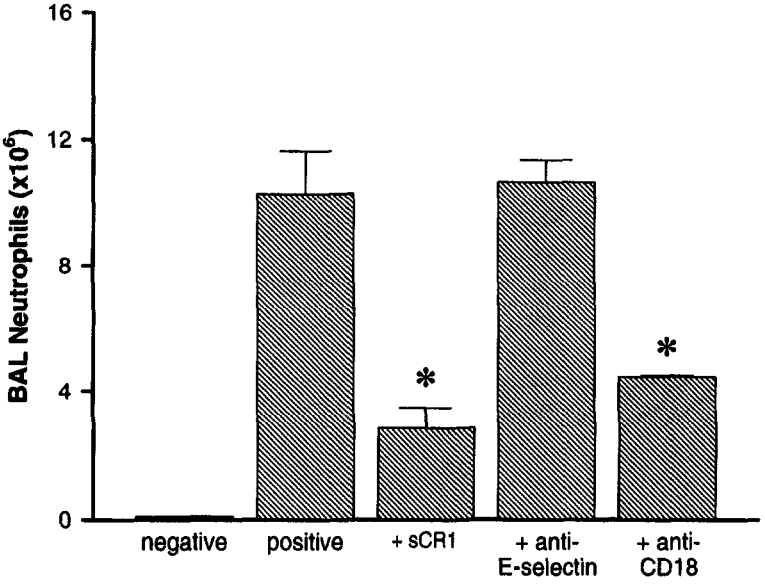

Fig. 4. Effects of sCR1, anti-E-selectin and anti-CD18 on BAL neutrophil counts after intrapulmonary deposition of IgA immune complexes. For each group, $N = 5$.

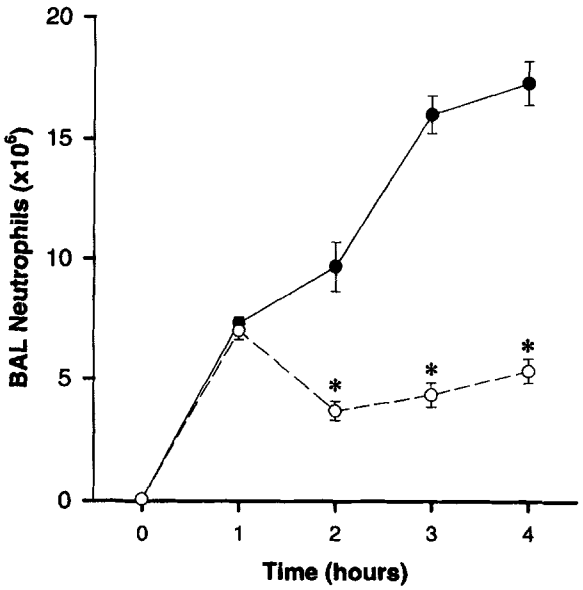

Fig. 5. Effects of L-NMA on BAL neutrophil counts after intratracheal instillation of PMA. BAL neutrophils from rats receiving PMA (closed circles) or PMA and 5 mM L-NMA (open circles) were quantitated at the times indicated. For each group, $N = 4$.

lung inflammation, complement and β_2 integrins, but not E-selectin are required for neutrophil recruitment.

Requirements for L-Arginine for Neutrophil Recruitment into PMA-Injured Lungs. Effects of L-NMA on neutrophil accumulation during PMA-induced lung injury was assessed over a 4 h time course (Figure 5). Lung instillation of PMA caused progressive increases in BAL neutrophil counts. Coadministration of L-NMA with PMA did not reduce the number of neutrophils recovered in BAL fluids 1 h after PMA instillation, but decreased neutrophil recruitment was found at 2, 3 and 4 h, with reductions of 63%, 73% and 70%, respectively ($P < 0.01$). Measurement of lung MPO content again confirmed BAL neutrophil counts as cotreatment with L-NMA reduced lung MPO values by 97% ($P < 0.01$) at 4h (Figure 6). The addition of 20 mM L-arginine with the L-NMA, reversed the inhibitory effect of L-NMA. L-arginine alone had no effect on PMA-induced increases in lung MPO content. Thus, it appears that L-arginine is required for the recruitment of neutrophils in PMA injured lung.

Effects of Complement and Adhesion Molecule Blockade on Neutrophil Recruitment into PMA-Injured Lung. In PMA-injured lung, blockade of com-

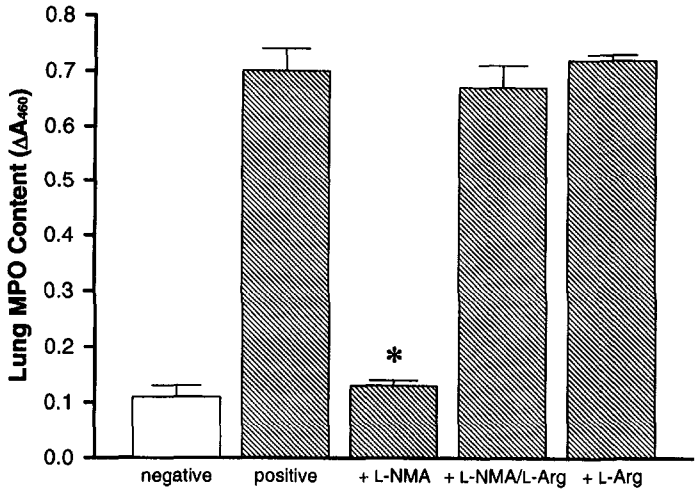

Fig. 6. Effects of L-NMA and/or L-arginine on lung MPO content after intratracheal instillation of PMA. For each group, $N = 6$.

plement using sCR1 had no effect on neutrophil influx (Figure 7), indicating that in this model of lung injury complement is not required for neutrophil recruitment. In contrast to IgA immune complex-induced lung injury, increases in BAL neutrophil counts induced by PMA were reduced by 42% ($P < 0.05$) by treatment with anti-E-selectin antibody and by 36% ($P < 0.05$) by treatment with anti-CD18 antibody (Figure 7). These data suggest that neutrophil recruitment to lung in response to PMA is dependent upon both E-selectin and β_2 integrins but not complement.

DISCUSSION

It has been shown during ischemia/reperfusion events in the mesentery, that infusion of L-NMA or L-NAME causes enhanced neutrophil adhesion to the postcapillary endothelium (18). In contrast, our studies examining neutrophil recruitment into an inflamed peritoneal cavity or lung have indicated that treatment with L-NMA causes a substantial reduction in the accumulation of neutrophils at these two sites. The apparent discrepancies may be explained by the different models for induction of inflammation, by the different endpoints employed (ex vivo microscopy versus direct cell counting of retrieved cells or MPO extraction), or by differences in the vascular beds under study. In the case of the lung, neutrophil extravasation occurs within the pulmonary capillaries, in contrast to

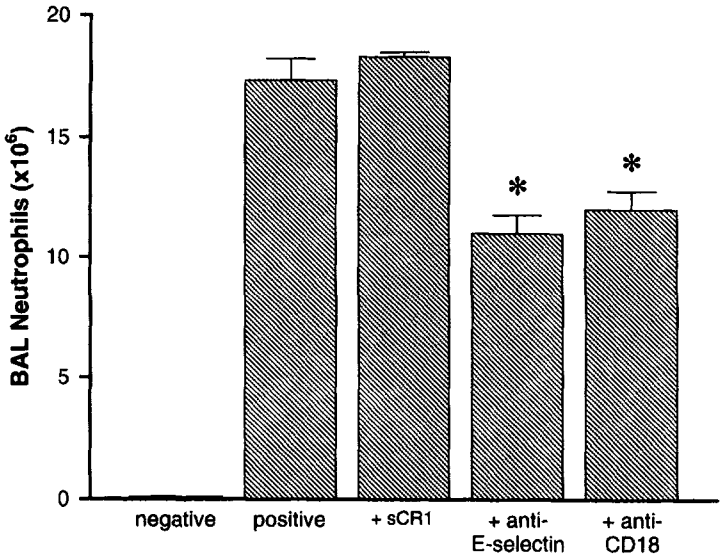

Fig. 7. Effects of sCR1, anti-E-selectin and anti-CD18 on BAL neutrophil counts after intratracheal instillation of PMA. For each group, $N = 5$.

venules in the mesenteric model of inflammation. Because the capillary diameter in lung is less than the diameter of the neutrophil, the rolling phenomenon well described in mesenteric venules cannot occur in pulmonary capillaries, especially if neutrophils are activated and relatively non-deformable (19). This may explain the differences noted in effects of L-NMA in mesenteric venules versus in the lung capillaries. In these various studies, there were also differences in the protocols for employment of the antagonist of L-arginine. In our studies L-NMA was injected locally (intraperitoneally or intratracheally), whereas in the studies involving ischemia-reperfusion the L-arginine analogues were infused directly into the vascular compartment. These technical differences might account for the divergent experimental results. It has been suggested that endothelial production of ·NO suppresses adhesion of neutrophils to activated endothelial cells and that there is a dynamic balance, such that the presence of O_2^- will "scavenge" ·NO, converting it to $ONOO^-$ and therefore removing the regulatory role of ·NO on neutrophil adhesion to endothelial cells (9). It is puzzling why in the current studies the presence of the L-arginine analog, L-NMA, resulted in reduced lung MPO content and BAL neutrophils. As pointed out above, it is important to note that in our experiments L-NMA was given intratracheally, which avoids the hypertension-inducing effects of intravenously or orally administered analogs of L-arginine. Alternatively, the different inflammatory conditions may trigger pathways that employ fundamentally different pathophysiological responses. Pul-

monary recruitment of neutrophils appears to be dependent upon the stimulus. As shown in the current studies, alveolar deposition of IgA immune complexes or airway instillation of PMA results in neutrophil accumulation that requires L-arginine. In contrast, in lung injury induced by IgG immune complexes, administration of L-NMA did not alter neutrophil accumulation in the BAL fluids or lung MPO content but was nevertheless highly protective (7). These protective effects of L-NMA in the IgG immune complex model were associated with a greatly reduced content of NO_2^- and NO_3^- in the BAL fluids, suggesting that injury in this model may be due directly to the generation from L-arginine of toxic products such as ·NO, $ONOO^-$ or HO·. In the current studies it is clear that L-arginine or its derivatives somehow positively affect tissue recruitment of neutrophils, even though in both IgA immune complex- and PMA-induced lung inflammation influx of neutrophils is unrelated to lung injury (the injury being linked to the generation of toxic products from lung macrophages) (10–12).

Another finding that may be relevant to differences between pathophysiological events in lungs following deposition of IgG or IgA immune complexes is that, in the IgG immune complex model, TNFα is abundant in BAL fluids and blocking of TNFα with antibody sharply reduced neutrophil influx and the extent of injury (17). In contrast, in the IgA immune complex model of lung injury BAL fluids contain little if any measurable TNFα, and anti-TNFα is not protective against injury (20). Thus, neutrophil accumulation in lung following deposition of IgA immune complexes is independent of a requirement for TNFα, emphasizing the differences in the mediator pathways related to recruitment of neutrophils in these two models of lung injury. Similarly, we have been unable to demonstrate a role for TNFα in neutrophil recruitment induced by airway instillation of PMA. While mechanisms of lung neutrophil recruitment following deposition of IgA immune complexes or instillation of PMA share a requirement for L-arginine, divergent roles for complement, E-selectin and CD18 suggest complicated networking of pathways leading to intrapulmonary neutrophil recruitment.

The variation in requirements for E-selectin and CD18 for neutrophil recruitment into rat lung is not especially surprising, since it has been shown that neutrophil recruitment into lung can occur via CD18-dependent as well as independent pathways (2). At least in the case of the peritoneal cavity, optimal recruitment of neutrophils requires both P-selectin as well as E-selectin. In studies using P-selectin deficient mice, maximal blockade of neutrophil recruitment into the peritoneal cavity occurred only when antibody to E-selectin was also administered (21). Thus, there are nuances in factors required for neutrophil recruitment not only as a function of the organ under study but also as related to the circumstances of the inflammatory response.

The requirement for L-arginine for neutrophil recruitment may involve a product of L-arginine metabolism, such as HO·, which is ultimately derived from the interaction of ·NO and O_2^-. It has been shown that activation of the alternative

pathway of the complement system can occur following the generation of HO·, resulting in activation of C5 and formation of the membrane attack complex (22, 23). Although we observed that complement blockade by sCR1 did not interfere with neutrophil accumulation in PMA-induced lung injury, this does not eliminate the possible involvement of C5 products in the recruitment process, since there is evidence that HO· may directly interact with C5 to generate an activated form of C5 (22, 23). This reaction would not be blocked by sCR1 since sCR1 only blocks formation of the C3 and C5 convertases and does not cause consumptive depletion of C5 (13). The requirement for complement pathway activation in the model of IgA immune complex-induced alveolitis, as demonstrated by the effects of sCR1, may be explained by the fact that IgA immune complexes have limited complement activating properties (10) and that it is the combination of alternative pathway activation (caused by the IgA immune complexes) together with direct interaction of HO· with C5 to generate complement-dependent chemotactic products that are responsible for neutrophil recruitment.

Complement activation products and adhesion molecules are known to be necessary for neutrophil recruitment in a number of inflammatory systems (1, 2, 14, 15, 24). Other studies show that neutrophil accumulation during glycogen-induced peritonitis is dependent upon E-selectin and β_2 integrins (25). In contrast, neutrophil recruitment during IgA immune complex-induced alveolitis requires complement and β_2 integrins, but is independent of E-selectin. In PMA-induced lung injury, neutrophil accumulation appears to be independent of complement, but shows a requirement for both E-selectin and β_2 integrins. The requirement for CD18 is consistent with studies of PMA-induced pneumonitis in rabbits (2). The current report demonstrates the difficulty in predicting the determinants of neutrophil recruitment in vivo and suggest the existence of several different pathways that lead to tissue accumulation of neutrophils. The consistent ability of L-NMA to suppress neutrophil recruitment in the models studied suggests that L-arginine or product(s) of its metabolism may play an important role in the generation of a chemotactic factor or some other phlogistic product.

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