The Journal of Immunology

The $\alpha_4\beta_1$ (Very Late Antigen (VLA)-4, CD49d/CD29) and $\alpha_5\beta_1$ (VLA-5, CD49e/CD29) Integrins Mediate β_2 (CD11/CD18) Integrin-Independent Neutrophil Recruitment to Endotoxin-Induced Lung Inflammation

J. Adam Burns, Thomas B. Issekutz, Hideo Yagita and Andrew C. Issekutz

J Immunol 2001; 166:4644-4649; ;
doi: 10.4049/jimmunol.166.7.4644
http://www.jimmunol.org/content/166/7/4644

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

The $\alpha_4\beta_1$ (Very Late Antigen (VLA)-4, CD49d/CD29) and $\alpha_5\beta_1$ (VLA-5, CD49e/CD29) Integrins Mediate β_2 (CD11/CD18) Integrin-Independent Neutrophil Recruitment to Endotoxin-Induced Lung Inflammation[1]

J. Adam Burns,* Thomas B. Issekutz,* Hideo Yagita,† and Andrew C. Issekutz[2]*

The β_2 integrin cell adhesion molecules (CAM) mediate polymorphonuclear leukocyte (PMNL) emigration in most inflamed tissues, but, in the lung, other yet to be identified CAMs appear to be involved. In Lewis rats, the intratracheal injection of *Escherichia coli*-LPS induced acute (6-h) PMNL accumulation in the lung parenchyma (280×10^6 by myeloperoxidase assay; PBS control = 35×10^6) and bronchoalveolar lavage fluid (BALF = 27×10^6; PBS = 0.1×10^6). Parenchymal accumulation was not inhibited by a blocking Ab to β_2 integrins and only minimally inhibited (20.5%; $p < 0.05$) in BALF. We examined the role of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins and of selectins in this PMNL recruitment. Treatment with mAbs to $\alpha_4\beta_1$ or $\alpha_5\beta_1$, even in combination, had no effect on PMNL accumulation induced by intratracheal LPS. However, anti-α_4 combined with anti-β_2 mAbs inhibited PMNL recruitment to the parenchyma by 56% ($p < 0.001$) and to BALF by 58% ($p < 0.01$). The addition of anti-α_5 mAb to β_2 plus α_4 blockade inhibited PMNL accumulation further (by 79%; $p < 0.05$). In contrast, blockade of L-, P-, and E-selectins in combination or together with β_2, α_4, and α_5 integrins had no effect. LPS-induced BALF protein accumulation was not inhibited by treatment with anti-β_2 plus α_4 mAbs, but was prevented when $\alpha_5\beta_1$ was also blocked. Thus, while selectins appear to play no role, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ function as major alternate CAMs to the β_2 integrins in mediating PMNL migration to lung and to pulmonary vascular and epithelial permeability. ***The Journal of Immunology,*** **2001, 166: 4644–4649.**

A key pathological event during acute inflammation is the recruitment of leukocytes such as polymorphonuclear leukocytes (PMNLs)[3] to sites of inflammation. The infiltration of these cells into areas of inflammation involves several families of cell adhesion molecules (CAMs) including selectins, integrins, and their ligands, the Ig superfamily adhesion molecules (1, 2). The selectins (L-, P-, and E-selectin) mediate the initial tethering of leukocytes to the vascular endothelium, which sequesters them from the blood flow and brings them into contact with the endothelium CAMs. This leads to a firm adhesion of the PMNL to the endothelial wall, mediated by interactions of integrins, especially of the β_2 (CD18) family (LFA-1, CD11a/CD18 and Mac-1, and CD11b/CD18) with the Ig superfamily (ICAM-1 and ICAM-2) present on the endothelium. This is followed by transendothelial migration of the PMNL out of the vessel and migration of the cell to the site of inflammation via additional integrin interactions with the extracellular matrix (1, 3, 4). This multistep paradigm is based primarily on studies conducted on peripheral tissues involved by inflammation.

Several studies have examined leukocyte recruitment mechanisms in acute lung inflammation. The adhesion molecules implicated in PMNL recruitment have been varied depending on the stimulus (LPS, C5a, bacteria, immune complexes, etc.) used. For example, pulmonary IgG immune complex injury induces PMNL infiltration that is in part β_2 integrin dependent (5), whereas the response to intrapulmonary C5a deposition is largely β_2 integrin independent (6). In vitro studies of PMNL migration across pulmonary endothelium have also indicated that the adhesion molecule mechanisms are in part chemotactic factor dependent, IL-8 in particular inducing β_2 integrin-independent PMNL transmigration (7, 8). It has also been shown that the mechanisms involved vary with the chronicity of reactions. For example, PMNL recruitment to lung inflammation induced by a single infection with *Pseudomonas aeruginosa* is primarily β_2 integrin dependent but becomes β_2 integrin independent with repeated infection (9). Furthermore, PMNL infiltration induced by *Streptococcus pneumoniae*, *Staphylococcus aureus*, or HCl aspiration was shown to be β_2 integrin independent even on initial exposure (10–12). These findings are in marked contrast to PMNL infiltration in other tissues, which is highly β_2 integrin-dependent (12–14). Despite the accumulated evidence that PMNL recruitment to lung inflammation involves β_2 integrin-independent mechanisms, the alternate mechanisms remain unidentified.

Integrins are a large family of heterodimeric, transmembrane glycoproteins. Members of this family of CAMs have been found in all tissues and on all leukocytes. There are two types of integrin subunits (α and β), which associate to form over 20 different $\alpha\beta$ heterodimers (15). PMNLs are known to express several integrins in addition to the β_2 integrins (CD11/CD18). For example,

*Departments of Pediatrics, Microbiology/Immunology, and Pathology, Dalhousie University, Halifax, Nova Scotia, Canada; and †Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan

Received for publication September 22, 2000. Accepted for publication January 17, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants MT-7684 and GR-13298 from the Medical Research Council of Canada. J.A.B. is the recipient of studentships from Dalhousie University Faculty of Graduate Studies and the Dalhousie Inflammation Group.

[2] Address correspondence and reprint requests to Dr. Andrew C. Issekutz, Department of Pediatrics, Division of Immunology, Rheumatology, and Infectious Diseases, IWK Grace Health Center, 5850 University Avenue, Halifax, Nova Scotia, B3J 3G9, Canada. E-mail address: aissekutz@iwkgrace.ns.ca

[3] Abbreviations used in this paper: PMNL, polymorphonuclear leukocyte; CAM, cell adhesion molecule; BALF, bronchoalveolar lavage fluid; VLA, very late Ag; i.t., intratracheal; CTAC, cetyltrimethylammonium chloride; MPO, myeloperoxidase.

0022-1767/01/$02.00

PMNLs also express several β_1 integrins, including $\alpha_2\beta_1$ (very late Ag (VLA)-2), $\alpha_4\beta_1$ (VLA-4), $\alpha_5\beta_1$ (VLA-5), $\alpha_6\beta_1$ (VLA-6), and $\alpha_9\beta_1$ (16–23). Although expressed at low levels on PMNLs, $\alpha_4\beta_1$ and $\alpha_9\beta_1$ have been shown to mediate human PMNL rolling or transendothelial migration in vitro, and $\alpha_4\beta_1$ can contribute to PMNL migration in rat models of inflammation (19, 22, 24). The $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_9\beta_1$ integrins may also mediate extravascular migration of PMNL (17, 20, 22).

In the analysis of the mechanisms of PMNL migration to lung inflammation, studies to date have examined the effect of blocking specific CAMs with Ab or knocking out CAMs by gene deletion. This strategy has eliminated the function of one or, in a few cases, two or three adhesion molecules. However, studies have not examined the role of multiple β_1 integrins possibly functioning in concert with β_2 integrins and selectins. In this study, we examined the role of these families of CAMs in PMNL recruitment to the lung in response to intratracheal (i.t.) injection of bacterial LPS in the rat, which, in this species, induces marked β_2 integrin-independent PMNL infiltration into the parenchyma with alveoli. Our results demonstrate a major role for $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, in addition to β_2 integrins, in this process.

Materials and Methods

Animals

Male Lewis rats, weighing 250–350 g, were purchased from Charles River Canada (St.-Contant, Quebec, Canada) and used in all experiments. The experimental protocols were approved by the Dalhousie University committee of laboratory animal care.

Reagents

LPS (*Escherichia coli* 0111:B4) was obtained from List Biological Laboratories (Campbell, CA). Cetyltrimethylammonium chloride (CTAC) and 3,5,5-tetramethylbenzidine-dihydrochloride were obtained from Sigma (St. Louis, MO).

mAb treatments

The mouse IgG1 mAb WT.3 (a gift from M. Miyasaka, Osaka University, Osaka, Japan) recognizes and functionally blocks the rat β_2-chain of the leukocyte CD11/CD18 integrins (25). The mouse IgG1 mAb TA-2 was generated in our laboratory and has been shown to react with and functionally block the rat α_4-chain of VLA-4 (26). The hamster IgG mAb HMα_5 reacts with and blocks the rat α_5 of VLA-5 (27, 28) and HRL-3 (a gift from D. Anderson, Pharmacia Upjohn, Kalamazoo, MI, and M. Miyasaka, Osaka University) reacts with and blocks rat L-selectin (29). The mouse IgG2a mAb RMP-1 recognizes and blocks rat P-selectin, and the mouse IgG1 mAb RME-1 blocks rat E-selectin (30, 31). These were generated in our laboratory. All mAbs to α-subunits and to selectins were used as F(ab′)$_2$ generated by pepsin digestion, as previously described (32). The mouse mAb B9 (IgG1) anti-pertussis toxin was used as a control mAb, as previously described (14, 19). Rats received 1 mg of each mAb i.v. as indicated. None of the mAb treatments used, even in combination, caused a decrease in the blood PMNL count. Measurement by immunofluorescent titration and flow cytometry analysis of the concentration of Ab in the serum of recipient animals at sacrifice indicated that this dose is sufficient to maintain serum levels of the Abs at more than five times saturation throughout the 6-h experiment.

Lung inflammation, perfusion, and bronchoalveolar lavage

Anesthetized rats (by Ketamine and Innovar i.v.) were given an i.p injection of 0.2 mg/kg atropine sulfate to reduce reflex coughing following LPS instillation. Then, the trachea was isolated, and 50 μg of LPS in PBS (total volume, 0.2 ml) or PBS alone (control) was injected via a 27-gauge butterfly needle. Within 5 min of i.t. administration of the LPS, the indicated mAbs were injected i.v.

Before sacrifice at 6 h, rats were pretreated with 80 mg/kg pentoxifylline in 0.45% NaCl i.p. 15–20 min before vascular perfusion. The perfusion was performed as previously described (33) with minor modifications. Briefly, a 25-gauge butterfly needle was inserted into the inferior vena cava below the diaphragm of anesthetized animals (Ketamine, 50 mg/kg, and Xylazine, 10 mg/kg, i.p.). The abdominal aorta was transected to allow perfusate to drain, and 25 ml Tyrode's solution with Ca^{2+} and Mg^{2+} was infused. Then, the butterfly needle was inserted into the inferior vena cava above the diaphragm, and 25 ml additional Tyrode's solution with Ca^{2+} and Mg^{2+} was infused. The perfusion was completed with the infusion of 10 ml PBS-0.1% EDTA into the pulmonary artery. Bronchoalveolar lavage was then performed by inserting a polyethylene catheter into the trachea, and the lungs were lavaged four times with a total of 28 ml cold PBS-0.1% EDTA. In all animals, 20–25 ml of BALF was recovered, and the cell concentration was determined by hemocytometer counting with crystal violet staining. BALF protein concentration was determined by UV absorbance at 280 μm using albumin as standard. The lungs were removed and immediately frozen at −70°C until myeloperoxidase (MPO) extraction was performed.

For histological examination, bronchial lavage was omitted and, after vascular perfusion, 10 ml buffered formalin (3.7%) was infused via the pulmonary artery to fix the lung. Wedge segments from each lobe were paraffin embedded, and 5-μm sections were taken and stained with hematoxylin and eosin.

PMNL quantitation in lung parenchyma

Lungs were extracted as previously described (33, 34). Briefly, samples of lung tissue from each lobe were pooled (350–450 mg, ~10–15% of total lung weight) and freeze-dried. These samples were then homogenized in 50 mM HEPES (pH 8.0) at 0.5% dry w/v with a pestle homogenizer (Talboys Engineering, Emerson, NJ) in glass tubes at setting 30–40 until fully homogenized. Samples were then centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant was discarded. The pellet was then rehomogenized in the same volume of 50 mM HEPES (pH 8.0) and centrifuged again at 10,000 × *g* for 30 min. The resulting pellet was homogenized again in the same volume of 0.5% CTAC in distilled water and clarified by repeat centrifugation. The resultant clear supernatant was analyzed for MPO activity. The same protocol was used to extract MPO from BALF cell pellets.

The collected supernatant from the HEPES/CTAC extracts of lung tissue was diluted 10-fold with 10 mM citrate buffer (pH 5.0), and 75 μl of this solution was pipetted into 4 wells of a 96-well plate. As negative control, "stop solution" (4 N H_2SO_4) was added to two of the wells. Then, 75 μl of the substrate solution (3 mM 3,5,5-tetramethylbenzidine-dihydrochloride, 120 mM resorcinol, and 2.2 mM hydrogen peroxide in distilled water (H_2O_2)) was added to all the wells, and the reaction was stopped after 2 min at 22°C by adding stop solution to the test wells.

Determination of lung parenchyma PMNL content

A standard curve of MPO activity vs PMNL number was developed by homogenizing a known number of Percoll-purified blood PMNLs using the same technique used in MPO extraction from lung and also from BALF cells, as previously reported (34). The extract from these PMNLs was titrated, and a standard curve relating MPO activity to PMNL number was plotted. Aliquots of this standard extract were stored at −70°C, and a sample with known MPO content was tested in each assay as an internal standard and for calculation of PMNL concentration as previously described (34).

Statistical analysis

All data reported are arithmetic means. Error bars represent one SEM. Differences between means of indicated groups were analyzed by ANOVA with Bonferoni corrections for multiple comparisons.

Results

Effect of i.t. LPS on PMNL accumulation in the lung parenchyma and BALF

Rats received an i.t. injection of LPS or PBS as a control and were sacrificed 6 h later. In rats receiving i.t. LPS (control), the lung parenchyma PMNL content increased 9-fold over PBS control to $2.8 \pm 0.1 \times 10^8$ PMNL (Fig. 1). The PMNL content in BALF in PBS control animals was consistently $<1 \times 10^6$, but in rats receiving i.t. LPS, the PMNL content reached $2.7 \pm 0.1 \times 10^7$ (Fig. 2). The i.t. injection of LPS also led to a significant increase in BALF protein concentration compared with PBS control animals (Fig. 3). Protein concentration in the PBS control was 0.14 ± 0.03 mg/ml, and, in rats receiving i.t. LPS, the protein concentration increased to 0.30 ± 0.03 mg/ml ($p < 0.001$).

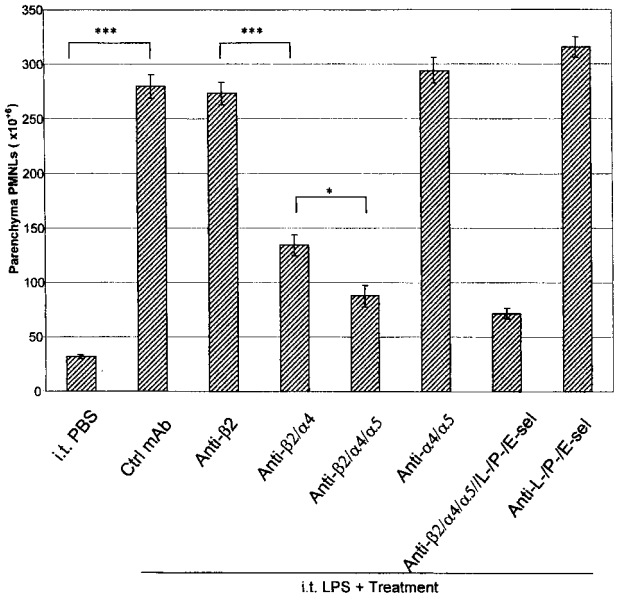

FIGURE 1. Effect of anti-integrin and anti-selectin treatment on either i.t. PBS (*left*), i.t. LPS plus control mAb (B9), or LPS-induced PMNL accumulation in the lung. Rats were given anti-β_2 mAb WT.3 or F(ab′)$_2$ mAbs to α_4 (TA-2), α_5 (HMa5), or L- (HRL-3), P-(RMP-1), and E-(RME-1) selectins. Total lung PMNL content was determined from MPO content of the parenchyma as described in *Materials and Methods*. Shown are means ± SEM of four to six animals per group.

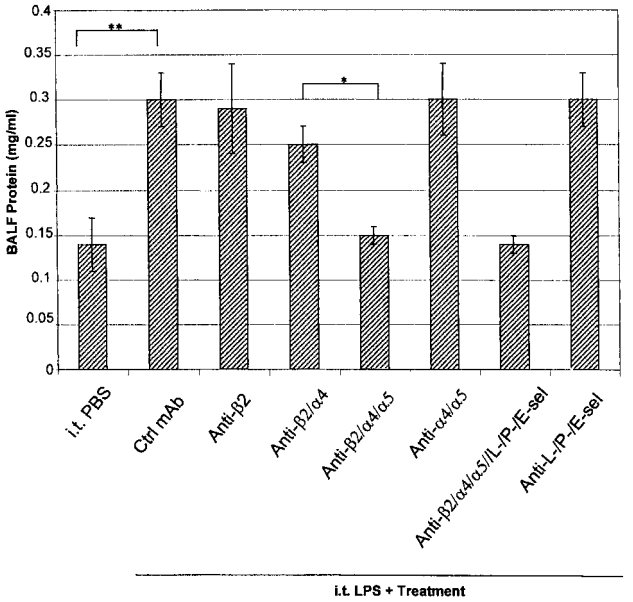

FIGURE 3. Effect of anti-integrin and anti-selectin treatment on protein concentration in the BALF following i.t. LPS. Rats were treated as in Fig. 1 by i.t. PBS (*left*) or LPS plus treatments in Fig. 1 as indicated. Protein concentration was determined in BALF supernatant. Shown are means ± SEM of four to six animals per group.

Effect of migration of PMNL into bronchoalveolar spaces on PMNL MPO content

To assess whether migration out of the vessel and through the lung parenchyma into the airspaces induced significant PMNL degranulation and release of cellular MPO, which may influence assessment of parenchymal PMNL content using the MPO assay, we assayed the MPO activity of PMNL in the BALF of rats 6 h following i.t. LPS and compared this to MPO in blood PMNL. As Fig. 4 shows, the BALF PMNL number predicted by MPO extraction, using blood PMNL MPO content as a standard, was not significantly different from the PMNL number in BALF determined by a visual hemocytometer quantitation of PMNL in BALF.

Effect of CAM blockade on PMNL accumulation in the lung parenchyma

The role of β_2, $\alpha_4\beta_1$ (VLA-4), and $\alpha_5\beta_1$ (VLA-5) integrins and of L-, P-, and E-selectins in PMNL migration into the lung during

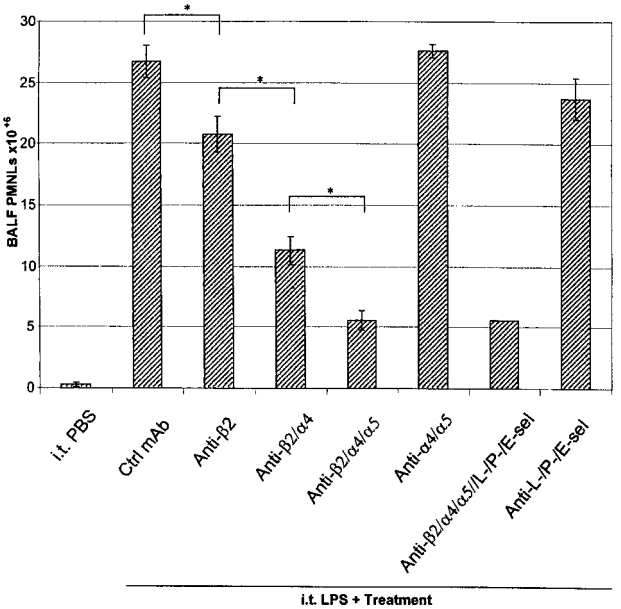

FIGURE 2. Effect of anti-integrin and anti-selectin treatment on LPS-induced PMNL accumulation in BALF. Rats were treated as in Fig. 1, and PMNL content of BALF was determined by hemocytometer counting. Shown are means ± SEM of four to six animals per group.

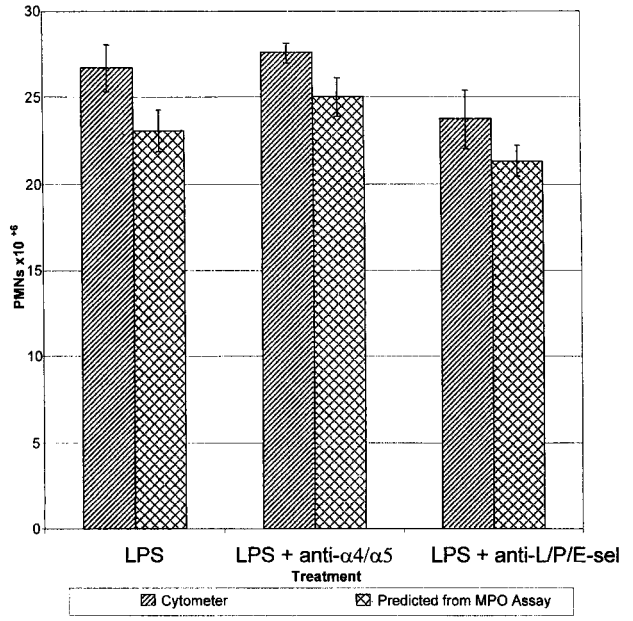

FIGURE 4. Comparison of PMNL content determined by hemocytometer counting with that predicted using MPO extraction. Rats were given F(ab′)$_2$ anti-α_4, anti-α_5, L-, P-, and E-selectin mAbs as indicated. Shown are means ± SEM of three experiments.

acute LPS-induced inflammation was studied using blocking mAbs. As Fig. 1 illustrates, mAbs to β_2 integrins, or α_4 plus α_5 integrins separately or to L-, P-, and E-selectins as a group, had no effect on PMNL accumulation in the parenchyma after i.t. LPS administration. In contrast, mAbs to β_2 in combination with $\alpha_4\beta_1$ integrin inhibited by >50% the PMNL accumulation in the parenchyma ($1.34 \pm 0.1 \times 10^8$ PMNLs; $p < 0.001$) as compared with LPS controls. Treatment of rats with mAbs to β_2 plus $\alpha_4\beta_1$ and also $\alpha_5\beta_1$ in combination inhibited PMNL accumulation significantly more ($8.8 \pm 1.0 \times 10^7$; $p < 0.05$) when compared with the β_2 plus $\alpha_4\beta_1$ mAb-treated group. To determine whether selectins may be involved in the remaining PMNL infiltration, rats were additionally treated with mAbs to L-, P-, and E-selectin in combination. However, no additional inhibition of PMNL accumulation was observed ($7.2 \pm 0.5 \times 10^7$) compared with rats with β_2, $\alpha_4\beta_1$, and $\alpha_5\beta_1$ blockade.

Effect of CAM blockade on PMNL migration into the BALF

As shown in Fig. 2, under nearly all conditions of integrin and selectin blockade, PMNL accumulation in BALF mirrored the changes in PMNL accumulation in the parenchyma. However, unlike the parenchyma, the pretreatment of rats with mAb to β_2 integrins slightly but significantly inhibited the migration of PMNLs into the BALF of rats receiving i.t. LPS as compared with control treated rats ($21 \pm 1.5 \times 10^6$; $p < 0.05$). This inhibition was further enhanced by the addition of mAb to α_4 to the mAb treatment ($11 \pm 1.1 \times 10^6$ PMNL; $p < 0.05$, as compared with anti-β_2 treatment alone). When anti-α_5 mAb was added to the β_2 and α_4 mAbs, inhibition was further and significantly increased ($5.6 \pm 0.8 \times 10^6$; $p < 0.05$, as compared with anti-β_2 and anti-α_4 treatment). As with PMNL accumulation in the parenchyma, no additional inhibition of PMNL accumulation was observed when L-,P-, and E-selectins were blocked in concert with β_2, α_4, and α_5 integrins ($5.6 \pm 0.01 \times 10^6$).

Effect of CAM blockade on protein concentration in the BALF

As an indicator of the increase in lung vascular and epithelial permeability resulting from inflammatory injury, BALF protein concentration was quantified. As illustrated in Fig. 3, protein concentration in the BALF of LPS-injected control mAb-treated rats was twice that of rats receiving i.t. PBS (LPS group = 0.3 ± 0.03 mg/ml; PBS = 0.14 ± 0.03 mg/ml; $p < 0.001$). Protein concentration in BALF from rats treated with anti-β_2 mAbs alone or in combination with anti-α_4 mAb was not significantly different from LPS control (0.27 ± 0.01 mg/ml). Inhibition of protein leak into the BALF was only observed when animals received mAb to β_2, α_4, and also α_5 integrin, irrespective of selectin blockade (0.15 ± 0.01 mg/ml; $p < 0.001$).

Effect of CAM blockade on lung histology

Examination of lung sections from animals receiving either i.t. PBS or LPS indicated that LPS induced a marked increase in leukocyte infiltration into the lung interstitium (Fig. 5). The predominant cells in the infiltrate at 6 h were PMNLs. In the lungs of animals treated with mAbs to β_2 and α_4 plus α_5 integrins, a marked decrease of PMNL infiltration was observed (Fig. 5*c*).

Discussion

The major objective of this study was to define the mechanisms of PMNL emigration in acute lung inflammation, because most studies have concluded that there are yet unidentified β_2 integrin-independent mechanisms involved in this process. Our data are consistent with these previous reports. We found that PMNL recruitment into the parenchyma during LPS-induced acute lung

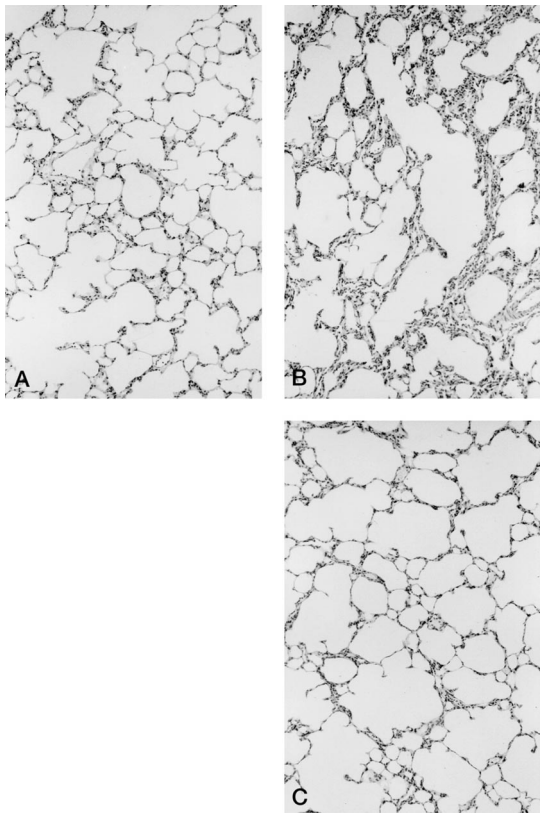

FIGURE 5. Histology of lung 6 h after i.t. LPS injection. Partially inflated, perfusion-fixed left lung lobes were paraffin-embedded, and sections were stained with hematoxylin and eosin. *A*, From an animal which received i.t. PBS; *B*, received i.t. LPS; and *C*, received i.t. LPS and anti-β_2 plus α_4 plus α_5 mAb treatment. Magnification, ×125.

inflammation in the rat was not inhibited by function blocking mAb to the β_2 integrins, and PMNL accumulation in the BALF was only slightly, albeit significantly, inhibited (by 20%). This is despite the fact that the anti-β_2 mAb used (WT.3) has been shown by us to inhibit PMNL recruitment to inflammation in skin by >80% and arthritic joints by >60% (19).

Here we show that multiple integrins participate in PMNL migration into lung parenchyma and into the bronchoalveolar space in the rat during LPS-induced lung inflammation. The $\alpha_4\beta_1$ integrin has recently been recognized on activated PMNL and to contribute to PMNL capture, rolling, and migration (19, 24, 35). Our findings demonstrate that it contributes to β_2-independent PMNL recruitment, because α_4 blockade in conjunction with β_2 blockade significantly inhibited PMNL accumulation in both the parenchyma and BALF (Figs. 1 and 2). Furthermore, the $\alpha_5\beta_1$ integrin, which is also expressed on PMNL at relatively low levels (19, 24, 36), also contributes to PMNL recruitment to the lung when β_2 and α_4 integrins are nonfunctional, because anti-α_5 mAb inhibited PMNL accumulation in the parenchyma and BALF significantly more than observed with β_2 plus α_4 blockade (Figs. 1 and 2). These mechanisms appear to be functionally redundant alternative pathways for PMNL recruitment to the lung, because neither β_2 blockade nor α_4 plus α_5 blockade reduced PMNL accumulation in the parenchyma and only minimally in the airspace (Figs. 1 and 2). The lack of effect of treatment with mAb to β_2 or to α_4 plus α_5 integrins or of a nonbinding control mAb (B9) on PMNL accumulation in parenchyma supports the conclusion that the inhibitory effects were not due to nonspecific effects.

To our knowledge, this is the first report that PMNLs use $\alpha_4\beta_1$ for migration to lung inflammation. This leukocyte can be added to

the lymphocyte, monocyte, and eosinophil, which use α_4 in conjunction with β_2 integrins for migration to lung inflammation (33, 37). Further studies will be required to ascertain the major counterligands used by $\alpha_4\beta_1$ on PMNL. VCAM-1 is one candidate, because it is up-regulated in the pulmonary vasculature during inflammation (38, 39), although up-regulated expression on endothelium of ligands such as the connecting segment-1 (CS-1) isoform of fibronectin may also contribute (40). However, recent studies indicate that other counterligands yet to be defined may play a predominant role in vivo (41).

Our findings are the first evidence for a role of α_5 in PMNL recruitment in vivo. Previous studies have demonstrated the role of $\alpha_5\beta_1$ in PMNL adhesion to fibronectin and PMNL migration across fibroblast barriers (36, 42). The current in vivo results suggest that $\alpha_5\beta_1$ may also contribute to PMNL emigration from blood to some extent, because α_5 blockade inhibited PMNL accumulation in the parenchyma in the presence of β_2 and α_4 blockade. These ligands in vivo may involve cell-associated as well as extracellular matrix-bound fibronectin or possibly also the Ig superfamily member L1, which in the mouse is recognized by $\alpha_5\beta_1$ (43).

Another important finding is that these integrins may play a role in PMNL-mediated lung injury, as reflected in increased BALF protein accumulation. These integrins likely contribute to this process in concert, because blockade of β_2 alone or α_4 plus α_5 had no effect, but the protein leakage was prevented with simultaneous blockade of β_2, α_4, and α_5 integrins (Fig. 3). The $\alpha_5\beta_1$ integrin may be as important as β_2 or $\alpha_4\beta_1$ integrins in this response, because no attenuation of the protein exudation was observed unless α_5 was blocked.

The analysis of BALF protein accumulation revealed that this lung inflammatory injury may require a threshold of PMNL infiltration to develop or that it is a PMNL-independent response, because we observed either a maximum BALF protein concentration of 0.3 mg/ml or the normal PBS control level of 0.15 mg/ml, despite various degrees of inhibition of PMNL recruitment. For example, although β_2 plus α_4 blockade inhibited PMNL recruitment by 56–58%, this had no effect on BALF protein, whereas further inhibition of PMNL accumulation (up to 80%) with blockade of also $\alpha_5\beta_1$ eliminated the protein leakage (Figs. 1–3). Whether a critical threshold for PMNL transendothelial migration, activation, mediator release, and transepithelial migration are required to induce the vascular and epithelial permeability increase deserves further investigation because of the therapeutic implications.

Studies of the role of selectins in PMNL recruitment to lung inflammation have indicated either a partial or no role in PMNL recruitment to the lung, depending on the inflammatory stimulus (45–48). Because the mechanisms and mediators involved in different pulmonary reactions are likely to be varied, the results have not been directly comparable. Our finding that blockade of L-, P-, and E-selectin had no effect on PMNL accumulation or on BALF protein increase, even in rats treated with mAbs to β_2, α_4, and α_5 integrins, suggests that the selectins are not required for these responses to LPS, at least in the lungs of rats. This is likely because the predominant site of PMNL emigration in this model is at the alveolar level where close contact between PMNL and pulmonary endothelium occurs due to the small diameter of alveolar capillaries. This may minimize the requirement for selectin-mediated tethering and rolling (1, 49–51).

In summary, our studies indicate a major role for the β_2 family, $\alpha_4\beta_1$, and $\alpha_5\beta_1$ integrins, but no detectable role for selectins in PMNL migration into the lung during acute LPS-induced inflammation. Further studies examining the role of these integrins in other models of lung inflammation are required to define their contribution to PMNL trafficking to the lung, activation, and the associated endothelial and epithelial injury during pulmonary inflammation.

Acknowledgments

We gratefully acknowledge the gifts of Abs and compounds from colleagues listed and the excellent technical assistance of Carol Jordan and Derek Rowter. We are also grateful to the IWK Grace Health Center Department of Pathology, in particular to Marlene Henry and Connie Isenor for preparation of histology and photographs.

References

1. Springer, T. A. 1995. Traffic signals on endothelium for lymphocyte recirculation and leukocyte emigration. *Annu. Rev. Physiol. 57:827.*
2. Gonzalez-Amaro, R., and F. Sanchez-Madrid. 1999. Cell adhesion molecules: selectins and integrins. *Crit. Rev. Immunol. 19:389.*
3. Roman, J. 1996. Extracellular matrix and lung inflammation. *Immunol. Res. 15: 163.*
4. Tsai, L. H. 1998. Stuck on the ECM. *Trends Cell Biol. 8:292.*
5. Mulligan, M. S., G. P. Wilson, R. F. Todd, C. W. Smith, D. C. Anderson, J. Varani, T. B. Issekutz, M. Miyasaka, T. Tamatani, M. Myasaka. 1993. Role of β_1, β_2 integrins and ICAM-1 in lung injury after deposition of IgG and IgA immune complexes. [Published erratum appears in *1993 J. Immunol. 150:5209.*] *J. Immunol. 150:2407.*
6. Hellewell, P. G., S. K. Young, P. M. Henson, and G. S. Worthen. 1994. Disparate role of the β_2-integrin CD18 in the local accumulation of neutrophils in pulmonary and cutaneous inflammation in the rabbit. *Am. J. Respir. Cell Mol. Biol. 10:391.*
7. Morland, C. M., B. J. Morland, P. J. Darbyshire, and R. A. Stockley. 2000. Migration of CD18-deficient neutrophils in vitro: evidence for a CD18-independent pathway induced by IL-8. *Biochim. Biophys. Acta 1500:70.*
8. Mackarel, A. J., K. J. Russell, C. S. Brady, M. X. FitzGerald, and C. M. O'Connor. 2000. Interleukin-8 and leukotriene-B_4, but not formylmethionyl leucylphenylalanine, stimulate CD18-independent migration of neutrophils across human pulmonary endothelial cells in vitro. *Am. J. Respir. Cell Mol. Biol. 23:154.*
9. Kumasaka, T., N. A. Doyle, W. M. Quinlan, L. Graham, and C. M. Doerschuk. 1996. Role of CD11/CD18 in neutrophil emigration during acute and recurrent *Pseudomonas aeruginosa*-induced pneumonia in rabbits. *Am. J. Pathol. 148:1297.*
10. Mizgerd, J. P., H. Kubo, G. J. Kutkoski, S. D. Bhagwan, K. Scharffetter-Kochanek, A. L. Beaudet, and C. M. Doerschuk. 1997. Neutrophil emigration in the skin, lungs, and peritoneum: different requirements for CD11/CD18 revealed by CD18-deficient mice. *J. Exp. Med. 186:1357.*
11. Motosugi, H., W. M. Quinlan, M. Bree, and C. M. Doerschuk. 1998. Role of CD11b in focal acid-induced pneumonia and contralateral lung injury in rats. *Am. J. Respir. Crit. Care Med. 157:192.*
12. Doerschuk, C. M., R. K. Winn, H. O. Coxson, and J. M. Harlan. 1990. CD18-dependent and -independent mechanisms of neutrophil emigration in the pulmonary and systemic microcirculation of rabbits. *J. Immunol. 144:2327.*
13. Arfors, K. E., C. Lundberg, L. Lindbom, K. Lundberg, P. G. Beatty, and J. M. Harlan. 1987. A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage in vivo. *Blood 69:338.*
14. Issekutz, A. C., and T. B. Issekutz. 1992. The contribution of LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) to the in vivo migration of polymorphonuclear leucocytes to inflammatory reactions in the rat. *Immunology 76:655.*
15. Aota, S., and K. M. Yamada. 1997. Integrin functions and signal transduction. *Adv. Exp. Med. Biol. 400B:669.*
16. Bohnsack, J. F. 1992. CD11/CD18-independent neutrophil adherence to laminin is mediated by the integrin VLA-6. *Blood 79:1545.*
17. Bohnsack, J. F., J. Chang, X. Zhou, and T. A. Yednock. 1995. Mechanisms of β_1 integrin-dependent adherence of granulocytic HL60 to fibronectin. *J. Leukocyte Biol. 57:592.*
18. Gao, J. X., and A. C. Issekutz. 1997. The β_1 integrin, very late activation antigen-4 on human neutrophils can contribute to neutrophil migration through connective tissue fibroblast barriers. *Immunology 90:448.*
19. Issekutz, T. B., M. Miyasaka, and A. C. Issekutz. 1996. Rat blood neutrophils express very late antigen 4 and it mediates migration to arthritic joint and dermal inflammation. *J. Exp. Med. 183:2175.*
20. Kubes, P., X. F. Niu, C. W. Smith, M. E. Kehrli, Jr., P. H. Reinhardt, and R. C. Woodman. 1995. A novel β_1-dependent adhesion pathway on neutrophils: a mechanism invoked by dihydrocytochalasin B or endothelial transmigration. *FASEB J. 9:1103.*
21. Werr, J., J. Johansson, E. E. Eriksson, P. Hedqvist, E. Ruoslahti, and L. Lindbom. 2000. Integrin $\alpha_2\beta_1$ (VLA-2) is a principal receptor used by neutrophils for locomotion in extravascular tissue. *Blood 95:1804.*
22. Taooka, Y., J. Chen, T. Yednock, and D. Sheppard. 1999. The integrin $\alpha_9\beta_1$ mediates adhesion to activated endothelial cells and transendothelial neutrophil migration through interaction with vascular cell adhesion molecule-1. *J. Cell Biol. 145:413.*
23. Shang, T., T. Yednock, and A. C. Issekutz. 1999. $\alpha_9\beta_1$ integrin is expressed on human neutrophils and contributes to neutrophil migration through human lung and synovial fibroblast barriers. *J. Leukocyte Biol. 66:809.*

24. Reinhardt, P. H., J. F. Elliott, and P. Kubes. 1997. Neutrophils can adhere via $\alpha_4\beta_1$-integrin under flow conditions. *Blood 89:3837.*
25. Tamatani, T., M. Kotani, and M. Miyasaka. 1991. Characterization of the rat leukocyte integrin, CD11/CD18, by the use of LFA-1 subunit-specific monoclonal antibodies. *Eur. J. Immunol. 21:627.*
26. Issekutz, T. B., and A. Wykretowicz. 1991. Effect of a new monoclonal antibody, TA-2, that inhibits lymphocyte adherence to cytokine stimulated endothelium in the rat. *J. Immunol. 147:109.*
27. Yasuda, M., Y. Hasunuma, H. Adachi, C. Sekine, T. Sakanishi, H. Hashimoto, C. Ra, H. Yagita, and K. Okumura. 1995. Expression and function of fibronectin binding integrins on rat mast cells. *Int. Immunol. 7:251.*
28. Noto, K., K. Kato, K. Okumura, and H. Yagita. 1995. Identification and functional characterization of mouse CD29 with a mAb. *Int. Immunol. 7:835.*
29. Tamatani, T., K. Kuida, T. Watanabe, S. Koike, and M. Miyasaka. 1993. Molecular mechanisms underlying lymphocyte recirculation. III. Characterization of the LECAM-1 (L-selectin)-dependent adhesion pathway in rats. *J. Immunol. 150: 1735.*
30. Walter, U. M., L. M. Ayer, B. A. Wolitzky, D. D. Wagner, R. O. Hynes, A. M. Manning, and A. C. Issekutz. 1997. Characterization of a novel adhesion function blocking monoclonal antibody to rat/mouse P-selectin generated in the P-selectin-deficient mouse. *Hybridoma 16:249.*
31. Walter, U. M., L. M. Ayer, A. M. Manning, P. S. Frenette, D. D. Wagner, R. O. Hynes, B. A. Wolitzky, and A. C. Issekutz. 1997. Generation and characterization of a novel adhesion function blocking monoclonal antibody recognizing both rat and mouse E-selectin. *Hybridoma 16:355.*
32. Parham, P., M. J. Androlewicz, F. M. Brodsky, N. J. Holmes, and J. P. Ways. 1982. Monoclonal antibodies: purification, fragmentation and application to structural and functional studies of class I MHC antigens. *J. Immunol. Methods 53:133.*
33. Schneider, T., T. B. Issekutz, and A. C. Issekutz. 1999. The role of α_4 (CD49d) and β_2 (CD18) integrins in eosinophil and neutrophil migration to allergic lung inflammation in the Brown Norway rat. *Am. J. Respir. Cell Mol. Biol. 20:448.*
34. Schneider, T., and A. C. Issekutz. 1996. Quantitation of eosinophil and neutrophil infiltration into rat lung by specific assays for eosinophil peroxidase and myeloperoxidase: application in a Brown Norway rat model of allergic pulmonary inflammation. *J. Immunol. Methods 198:1.*
35. Davenpeck, K. L., S. A. Sterbinsky, and B. S. Bochner. 1998. Rat neutrophils express α_4 and β_1 integrins and bind to vascular cell adhesion molecule-1 (VCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). *Blood 91:2341.*
36. Bohnsack, J. F., and X. N. Zhou. 1992. Divalent cation substitution reveals CD18- and very late antigen-dependent pathways that mediate human neutrophil adherence to fibronectin. *J. Immunol. 149:1340.*
37. Li, X. C., M. Miyasaka, and T. B. Issekutz. 1998. Blood monocyte migration to acute lung inflammation involves both CD11/CD18 and very late activation antigen-4-dependent and independent pathways. *J. Immunol. 161:6258.*
38. Manning, A. M., F. P. Bell, C. L. Rosenbloom, J. G. Chosay, C. A. Simmons, J. L. Northrup, R. J. Shebuski, C. J. Dunn, and D. C. Anderson. 1995. NF-κB is activated during acute inflammation in vivo in association with elevated endothelial cell adhesion molecule gene expression and leukocyte recruitment. *J. Inflamm. 45:283.*
39. Feng, C. G., W. J. Britton, U. Palendira, N. L. Groat, H. Briscoe, and A. G. Bean. 2000. Up-regulation of VCAM-1 and differential expansion of β integrin-expressing T lymphocytes are associated with immunity to pulmonary *Mycobacterium tuberculosis* infection. *J. Immunol. 164:4853.*
40. Munoz, M., J. Serrador, M. Nieto, A. Luque, F. Sanchez-Madrid, and J. Teixido. 1997. A novel region of the α_4 integrin subunit with a modulatory role in VLA-4-mediated cell adhesion to fibronectin. *Biochem. J. 327:727.*
41. Johnston, B., A. Chee, T. B. Issekutz, T. Ugarova, A. Fox-Robichaud, M.J. Hickey, and P. Kubes. 2000. α_4 integrin-dependent leukocyte recruitment does not require VCAM-1 in a chronic model of inflammation. *J. Immunol. 164: 3337.*
42. Gao, J. X., J. Wilkins, and A. C. Issekutz. 1995. Migration of human polymorphonuclear leukocytes through a synovial fibroblast barrier is mediated by both β_2 (CD11/CD18) integrins and the β_1 (CD29) integrins VLA-5 and VLA-6. *Cell. Immunol. 163:178.*
43. Ruppert, M., S. Aigner, M. Hubbe, H. Yagita, and P. Altevogt. 1995. The L1 adhesion molecule is a cellular ligand for VLA-5. *J. Cell Biol. 131:1881.*
44. Mulligan, M. S., S. R. Watson, C. Fennie, and P. A. Ward. 1993. Protective effects of selectin chimeras in neutrophil-mediated lung injury. *J. Immunol. 151: 6410.*
45. Mulligan, M. S., M. Miyasaka, and P. A. Ward. 1996. Protective effects of combined adhesion molecule blockade in models of acute lung injury. *Proc. Assoc. Am. Physicians 108:198.*
46. Doerschuk, C. M., W. M. Quinlan, N. A. Doyle, D. C. Bullard, D. Vestweber, M. L. Jones, F. Takei, P. A. Ward, and A. L. Beaudet. 1996. The role of P-selectin and ICAM-1 in acute lung injury as determined using blocking antibodies and mutant mice. *J. Immunol. 157:4609.*
47. Bullard, D. C., L. Qin, I. Lorenzo, W. M. Quinlin, N. A. Doyle, R. Bosse, D. Vestweber, C. M. Doerschuk, and A. L. Beaudet. 1995. P-selectin/ICAM-1 double mutant mice: acute emigration of neutrophils into the peritoneum is completely absent but is normal into pulmonary alveoli. *J. Clin. Invest. 95:1782.*
48. Mizgerd, J. P., B. B. Meek, G. J. Kutkoski, D. C. Bullard, A. L. Beaudet, and C. M. Doerschuk. 1996. Selectins and neutrophil traffic: margination and *Streptococcus pneumoniae*-induced emigration in murine lungs. *J. Exp. Med. 184:639.*
49. Drost, E. M., G. Kassabian, H. J. Meiselman, D. Gelmont, and T. C. Fisher. 1999. Increased rigidity and priming of polymorphonuclear leukocytes in sepsis. *Am. J. Respir. Crit. Care Med. 159:1696.*
50. Doerschuk, C. M., S. Tasaka, and Q. Wang. 2000. CD11/CD18-Dependent and -independent neutrophil emigration in the lungs: how do neutrophils know which route to take? *Am. J. Respir. Cell Mol. Biol. 23:133.*
51. Hogg, J. C., and C. M. Doerschuk. 1995. Leukocyte traffic in the lung. *Annu. Rev. Physiol. 57:97.*