

Characterization of rat lung ICAM-1

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Received 19 February 1998; returned for revision 25 March 1998; accepted by G. W. Carter 17 April 1998

Abstract. *Objective and Design:* We expressed soluble rat ICAM-1, generated a polyclonal anti-ICAM-1 antibody, and studied ICAM-1 upregulation in lung inflammatory conditions. Bacterial and baculovirus expression systems were employed.

Material: 250 g adult, male Long Evans rats were used. For in vitro studies, rat pulmonary artery endothelial cells (RPAEC), rat alveolar macrophages and aortic rings were stimulated (as described below) and evaluated for ICAM-1 expression.

Treatment: RPAEC and macrophages were stimulated with lipopolysaccharide (LPS) and recombinant murine tumour necrosis factor α (TNF α). In vivo immunoglobulin G (IgG) immune complex-induced lung injury was employed.

Methods: Enzyme-linked immunoassay (ELISA) Western and Northern blot analyses and immunohistochemical evaluations were performed. All experiments were done at least in duplicate. Data were analyzed by two-tailed Student's t-test.

Results: ICAM-1 expression of RPAEC was time- and dose-dependent, peaking at 6 h after LPS-stimulation. LPS and TNF α each enhanced ICAM-1 expression on alveolar macrophages (reaching a maximum at 2 h). In IgG immune complex-induced lung injury, ICAM-1 mRNA isolated from whole lung peaked at 4 h, while lung ICAM-1 protein peaked at 6 h.

Conclusions: Quantitation of ICAM-1 expression in vitro and in vivo suggests that ICAM-1 plays a central role in two lung inflammatory models. Furthermore, lung ICAM-1 upregulation involves at least two cell types: vascular endothelial cells and alveolar macrophages.

Key words: Rat ICAM-1 – Anti-ICAM-1 – Protein expression – Lung inflammation

Introduction

The adhesion molecule ICAM-1 (CD54), a member of the immunoglobulin superfamily, is constitutively expressed on endothelial and epithelial cells, and to a lesser extent on leukocytes. As shown in several lung models of pulmonary inflammation [1–3], ICAM-1 plays a major role in leukocyte recruitment to inflammatory sites [4]. In the animal models employed, one of the first steps in leukocyte migration into tissues is engagement of neutrophils with endothelial E-selectin, which facilitates leukocyte 'rolling' on the activated endothelium. This is followed by interaction of endothelial ICAM-1 with leukocyte LFA-1 (CD11a/CD18) (and perhaps Mac-1, (CD11b/CD18)), which induces a firm adhesion and blocks further rolling of neutrophils. Neutrophil transmigration through the endothelium is then aided by endothelial products such as platelet activating factor (PAF) and IL-8 or its homologues, which appear to stimulate adherent neutrophils. In addition, endothelial platelet endothelial cell adhesion molecule-1 (PECAM-1) facilitates leukocyte adhesion to basolateral junctions of endothelial cells [5]. Precisely what chemotactic factors facilitate neutrophil transmigration is still a matter under study. The extensive studies of Wawryk et al. [6] have helped to delineate the interactions between human ICAM-1 and leukocytes.

In order to precisely and thoroughly quantitate ICAM-1 message and protein in two lung inflammatory models, rat ICAM-1 was cloned. Cloned DNA (cDNA) for rat ICAM-1 was used to express ICAM-1 in two different cell expression systems and the purified product used to immunize rabbits to generate antibody that could be used in ELISA assays, in immunostaining methodologies, and in in vivo blocking experiments. Since rat cDNA and recombinant rat ICAM-1 are largely unavailable, we developed these reagents and applied them in vitro and in vivo in order to obtain detailed information about the amounts of rat-ICAM-1 message and protein expressed and the cell sources of rat ICAM-1. In all of these studies, precise, quantitative endpoints were used as indicated. The studies to be presented indicate that in the inflamed lung there are at least two different cell sources of

ICAM-1. Measurements of lung ICAM-1 message RNA (mRNA) and protein provide a detailed analysis of the response of the inflamed lung.

Materials and methods

Protein expression in the bacterial system

The cDNA from one of the selected full-length rat ICAM-1 cDNA libraries was sequenced and compared with already published data [7]. A partial length construct of 618 bp was used, containing the ligand binding sites for LFA-1 and Mac-1. DNA was amplified by polymerase chain reaction (PCR) adding the primers for the expression vector. Restriction enzyme sites, *Nde*I 5' and *Xho*I 3', were incorporated into the following sequences: 5'-CAT ATG *Nde* CCT CTG CCC GCC TGG-3' and 3'-TGG GAC TCG GTC GGC ACT *Xho* GAG CTC-5'. The DNA was ligated into the pET 14b vector (Novagen Inc., Madison, WI, USA) and closed into *E. coli* pLysS (Invitrogen, Inc., Carlsbad, CA, USA). For protein expression the cells were stimulated with 0.4 mM isopropylthiogalactoside (IPTG) (Gibco Co., Grand Island, NY, USA) at different bacterial densities (0.6–0.8) and for different periods (1 h–3 h) to achieve the optimal protein production. The soluble protein was purified over a nickel column (His bind resin, Novagen, Inc.) and eluted with several imidazole concentrations (100 mM, 200 mM, 300 mM). Dialysis in phosphate-buffered saline (PBS), pH 7.0 at 4 °C was performed and the product concentrated using Centricon Concentrators® (Amicon Co., Beverly, MA, USA).

Protein expression in the baculovirus

This procedure is similar to one described previously [8–10]. Briefly, the same template (as described above) was created for this vector but the restriction sites were changed to *Xma*I 5' and *Bam*HI 3' according to the vector pPBac (Stratagene Inc., LaJolla, CA, USA): 5'-CCC GGG *Xma* CCT CTG CCC GCC TGG-3' and 3'-TGG GAC TCG GTC GGC ACT *Bam*H CCT AGG-5' [11]. For transfection, the ICAM-1 bearing transfer vector was mixed with linear wild-type autographa californica nuclear polyhedrosis virus (AcMNPV) and SF-21 cells (Invitrogen Inc.) (2×10^6 cells in a 60 mm plate). Three plaque purification assays were performed. Amplification of the virus was achieved by infecting SF-9 cells. For optimal protein expression, SF-9 cells were infected and incubated for 4 days [9, 10]. The cells and supernatant fluids were harvested and centrifuged (1500 rpm, 10 min), washed in 50 mM Tris/Cl (pH = 7.0) containing 1 mM dithiothreitol and 250 mM sucrose, resuspended in 0.1 ml Tris/Cl (50 mM, pH 7.0), 1 mM dithiothreitol and 0.5 mM NaCl and lysed by freezing and thawing 5 times in liquid nitrogen. DNA shearing was performed by five passes through a 25-gauge needle. The supernatant fluids and cell lysates were analyzed by Western blot and run over an affinity column loaded with the polyclonal ICAM-1 antibody, followed by elution under acidic conditions.

Western blot analysis

Characterization of proteins was performed using sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) under both reducing and nonreducing conditions. 5 µl of Laemmli buffer were added to 20 µl of protein sample and loaded on gels (7.5 or 15%). After electrophoresis, the gels were either stained with Coomassie blue [12] or transferred to nitrocellulose membranes (Bio Rad Inc., Hercules, CA, USA). The membranes were blocked with 3% bovine serum albumin (BSA) in tris-buffered saline (TBS), incubated with the murine or rabbit ICAM-1 antibody at a dilution of 1:500 and then incubated with a 1:3000 dilution of goat anti-murine or goat anti-rabbit alkaline phosphatase-conjugated antibody (Bio Rad Co. Inc.), followed by color reagents A and B.

ELISA quantitation of ICAM-1 on RPAEC and alveolar macrophages

This assay was performed as described previously [13]. Briefly, 96 well microtiter plates were coated with 5×10^6 RPAEC per well and cultured overnight at 37 °C in 5% CO₂. The cells were stimulated with LPS using varying concentrations or durations. Fixation was performed with 1% paraformaldehyde for 10 min. For each washing step, PBS supplemented with 2% heat-inactivated fetal calf serum and 1% Tween 20 was used. After fixation, a blocking step of 5% nonfat dry milk was added. The biotinylated polyclonal ICAM-1 antibody (Pierce Co., Rockford, IL, USA, ImmunoPure NHS-LC-Biotinylation Kit) was used at a dilution of 1:100 (60 min, 37 °C). The plate was washed three times and streptavidin-horseradish-peroxidase was added at a concentration of 0.1 µg/ml for 15 min at room temperature.

Macrophages from bronchoalveolar lavage (BAL) fluids from rat lungs were placed into 96 well plates (5×10^5 cells/well) and stimulated with increasing concentrations of recombinant murine TNFα (5, 10, 20, 50 and 100 ng/ml). The subsequent steps were the same as described above. A similar study was performed, using 5 µg/ml LPS instead of TNFα. The macrophages were stimulated for 2, 4, 6 and 8 h with LPS and subsequently fixed.

Immunohistochemical and immunofluorescent analysis of rat endothelial cells and aortic rings

RPAEC were plated at a density of 1×10^5 per chamber in Lab Tek® chamber slides (Nunc Co., Naperville, IL, USA) and cultured overnight. Cells were stimulated with the indicated concentrations of recombinant murine TNFα (R&D Systems Inc., Minneapolis, MN, USA) and incubated for 4 h at 37 °C. As a control, purified IgG was used. The cells were then washed, fixed for 10 min in cold acetone and incubated with the polyclonal anti-ICAM antibody or the control IgG antibody (10 µg/ml) for 60 min at room temperature. Further processing was performed using the Vectastain biotin-avidin-peroxidase system (Vector Laboratories Inc., Burlingame, CA, USA) or the immunofluorescent secondary anti-rabbit antibody. Rat aortic rings cultured in medium were stimulated with 20 ng TNFα/ml for 4 h at 37 °C, frozen sections prepared and immunofluorescent staining performed as described above.

Anti-ICAM-1 antibody

New Zealand white rabbits of mixed sexes were immunized with 0.5 mg recombinant soluble rat ICAM-1 subcutaneously using Freund's complete adjuvant (Lampire Biological Laboratories Inc., Pipersville, PA, USA). The animals were boosted every 4 weeks until an acceptable titer ($>10^6$) was achieved. The antibody did not demonstrate cross-reactivity with E-selectin, P-selectin or β-integrins (data not shown). When used, mouse anti-ICAM-1 monoclonal antibody 1A29 was a gift from Dr. Masayuki Miyasaka (Osaka University, Osaka, Japan).

Animal model of lung injury

275–300 g male Long-Evans rats (Harlan Industries Inc., Rochester, MI, USA) were anesthetized with ketamine and lung injury was induced by intratracheal instillation of 150 µg LPS (from *E. coli*, serotype 055:B5), in 300 µl PBS via an intratracheal catheter. Animals were sacrificed at given time points. To prepare lung homogenates, the vascular and bronchoalveolar areas of lungs were flushed before en bloc removal of lungs. For immune complex-induced alveolitis, rabbit polyclonal IgG rich in antibody to BSA (anti-BSA) was used [14]. Rabbit IgG anti-BSA (2.5 mg) in a final volume of 300 µl of PBS was instilled via an intratracheal catheter during inspiration. Immediately thereafter, 10 mg BSA was injected intravenously.

Lung homogenates from LPS and IgG immune complex-injured lungs

LPS lung injury was induced according to the protocol described above. The rats were sacrificed at 4 h. Lungs homogenized in PBS containing 10 mM 3-[(3-chloramidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) (Sigma Co., St. Louis, MO, USA), 20 µg/ml aprotinin (Boehringer Co.) 20 µg/ml bestatin (Sigma Co.) [15], 20 µg/ml leupeptin (Boehringer Co.) and 20 µl 0.1 M phenylmethylsulfonyl fluoride (PMSF) (Sigma Co.). The samples were pulse-homogenized with a homogenizer at maximum speed. The same procedure was performed with IgG immune complex-injured lungs (0, 2, 4, 6 and 8 h). The total volume for each lung homogenate was 10 ml.

Isolation of rat alveolar macrophages

Alveolar macrophages were isolated from BAL fluids of rats as described previously [16]. Anesthetized rats were exsanguinated and a tracheotomy was performed. The lungs were lavaged 3 times with 10 ml of PBS, this fluid then being centrifuged at 1500 RPM for 10 min. The resuspended macrophages were again subjected to centrifugation and finally diluted to achieve a concentration of 1×10^6 cells/ml.

ELISA with lung homogenates

ELISA was performed in a similar way as described earlier [15]. 96 well plates (Nunc. Co.) were coated with polyclonal antibody 2 µg/ml in binding buffer and incubated overnight. All washing steps were performed with PBS/0.2% Tween. A blocking step followed, using PBS containing 1% bovine serum and 0.05% Tween (27°C for 30 min). Standards and samples were loaded and incubated for 1 h at 37°C. The detecting biotinylated antibody was incubated for 1 h at 37°C at a concentration of 1 µg/ml. Streptavidin-horseradish peroxidase (Pierce Co.) was added (0.1 mg/ml, 15 min, 37°C) and plates were developed with *O*-phenylenediamine dihydrochloride. The reaction was stopped by addition of sulfuric acid and the optical density determined at 490 nm.

RNA extraction, reverse transcriptase (RT)-PCR, Northern blot and quantitative analysis of the PCR product

Whole lungs were homogenized, and RNA extracted with RNA solubility buffer (RNAZOL) (Gibco-BRL Co. Inc.) according to previous studies [17]. RNA was purified over oligo-dT cellulose (Invitrogen Inc.). The final amount of mRNA isolated was between 5 and 30 µg. RT-PCR was performed according to the protocol described in the cDNA-cycle kit (Invitrogen Inc.). In the case of whole-lung mRNA, the reaction was performed with oligo-dT primers and with random primers when cDNA was used as the starting material. The reaction was carried out for two 1-h cycles at 42°C with avian myeloblastosis virus (AMV) reverse transcriptase. The final product was precipitated using yeast-tRNA as carrier and resuspended in 30 µl double distilled water.

The PCR amplifications were performed using 1.5 Units Taq polymerase (Gibco Inc.), 50 µM dNTPs (nucleoside triphosphate), 2 mM magnesium chloride and 1 µl of the different dilutions of cDNA templates. The total reaction volume was 50 µl in each MicroAmpTM Reaction Tube (Perkin Elmer Co., Norwalk, CT, USA). All amplification and hybridization reactions were performed in a GeneAmpTM PCR System 9600 (Perkin Elmer Co.). The following PCR primers with the corresponding annealing temperatures were used:

Biotin-5'-ATGGCTTCAACCCGTGCCAGGC-3' and 5'-TCAGG-GAGGCGGGGCTGTAC-3', 64°C

Amplification protocols utilized the following conditions: 30 cycles at 94°C for 30 s, specific annealing temperature for 1 min, and at 72°C for 1 min, followed by a final incubation at 72°C for 10 min. For the

Noerhern blot 12 µg total RNA was electrophoresed in a 1.5% agarose gel containing formaldehyde and transferred to a nylon membrane. The blot was hybridized overnight with 1.5×10^7 cpm (³²P(dCTP)) labeled oligonucleotides using previously described methods [18]. The quantitative analysis of the PCR product required hybridization of the products with oligonucleotides, labelled with tris 2,2 (bipyridine ruthenium (II) chelate (TBR). A bead capture reaction was used and electrochemiluminescence PCR products were quantitated in the QPCR System 5000TM (Perkin Elmer Co.). Hybridization reactions were carried out using the following temperature program: 94°C for 2 min to denature the PCR product and 55°C for 3 min to hybridize the biotinylated strand with the TBR-labeled oligonucleotide. The sequence for the TBR-labeled probes were as follows:

TBR-5'-CAGTGTCTCATTCCCACGGAG-3', 64°C.

The hybridization reaction contained 2–4 µl of the PCR product, 10–20 pM of TBR-labeled probe and hybridization buffer in a final volume of 50 µl. For the bead capture reaction, 15 µl or 30 µl of a 2 µg/µl 4.5 µm magnetic polystyrene bead suspension (Dynabeads M450, Dynal Co., Great Neck, NY, USA) was added directly in the PCR tube. The capture reaction was carried out for 20 min at 55°C. Thereafter the whole reaction mix was immediately transferred to a separate quick PCR (QPCR) System 5000 tube for measurement of the electrochemiluminescent signal.

Statistical analysis

All experiments were performed in duplicate. Data were analyzed by two-tailed Student's t-test. Data were considered statistically significant if p-values were less than 0.05.

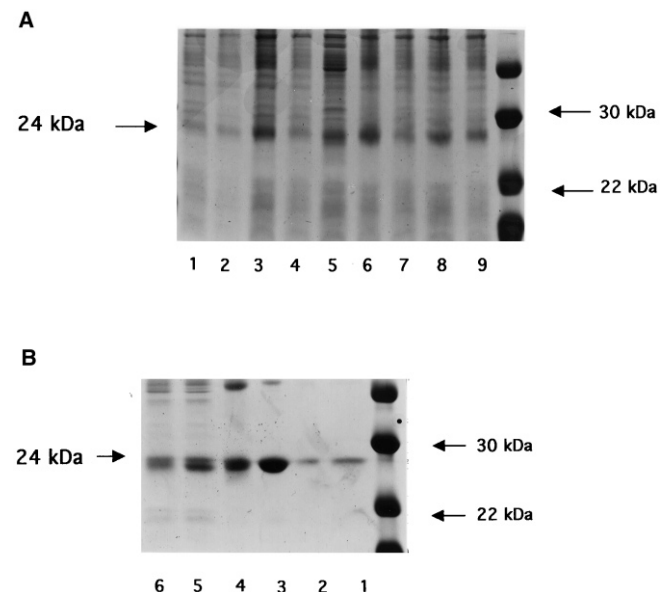


Fig. 1. pLysS bacteria were stimulated and analyzed at different densities (determined by O.D.) for different time periods (h). The soluble products were analyzed on 15% SDS/PAGE (A). The first three lanes were samples from bacteria suspensions with an optical density (O.D.) of 0.7, stimulated for 1 h (lane 1), 2 h (lane 2) and 3 h (lane 3). In lanes 4–6 (O.D. = 0.7) and 7–9 (O.D. = 0.8), stimulation was for 2 or 3 h. There was an increase of protein expression with time. The O.D. of a given sample did not seem to correlate with protein expression. In further studies using 15% SDS/PAGE, products were eluted with three different concentrations of imidazole under nonreducing and reducing conditions (B). The following elution conditions (including imidazole concentrations) were employed: lane 1: 100 mM, nonreducing; lane 2: 100 mM reducing; lane 3: 200 mM, nonreducing; lane 4: 200 mM, reducing; lane 5: 300 mM, nonreducing; lane 6: 300 mM reducing.

Results

Expression of soluble rat ICAM-1 in a bacterial system

A rat ICAM-1 expression vector was created by ligating a 618 bp cDNA containing the ICAM-1 binding CD11a/CD18 sites for (LFA-1) and CD11b/CD18 (Mac-1) into pET and pPBac vectors. This cDNA was about one fourth the full length of ICAM-1 (2680 bp). The chances to obtain a soluble protein were increased with this construct. Sequencing of the construct demonstrated the correct orientation of the insert in the vector. Our 618 bp cDNA construct was totally identical (data not shown) with the same region of the full rat ICAM-1 construct (2680 bp), the sequence of which has already been published [7]. After transformation into *E. coli* pLysS, the cells were stimulated with IPTG (0.4 mM final concentration) for 1, 2 and 3 h. Bacteria were lysed and the fractions analyzed by SDS/PAGE. Maximum expression of protein was found at 3 h (Fig. 1A). Purification of the soluble protein was performed using three different elution concentrations of imidazole (Fig. 1B). As seen in the Coomassie gel, the best yield was with 200 mM imidazole (lanes 3, 4). In bacterial cultures, the final concentration of ICAM-1 was 0.5 mg/liter. The insoluble ICAM-1 was purified and eluted with the same buffers containing 6 M urea. Both proteins were in position at 24 kDa instead of expected 23 kDa position, due to the presence of the polyhistidine tail.

Expression of soluble ICAM-1 by insect cells

Using the 618 bp PCR product and the pPBac vector, a transfer vector was created. In contrast to a previous study

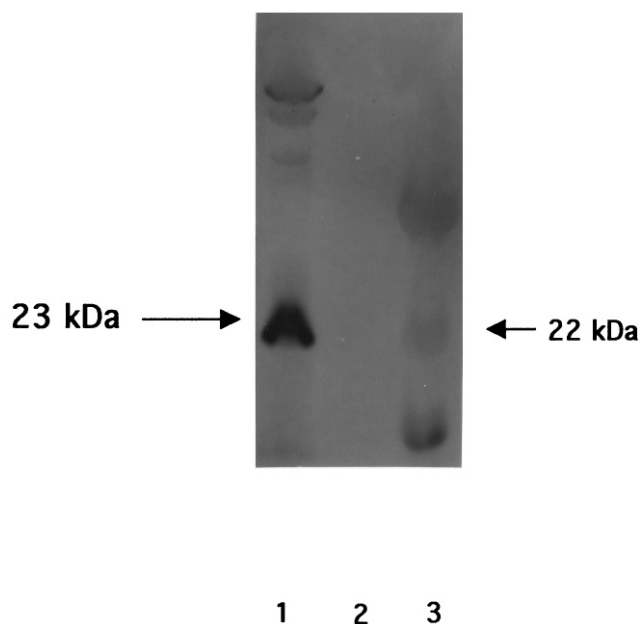


Fig. 2. Western blot analysis using polyclonal anti-ICAM-1 antibody. Using 15% SDS/PAGE, lysed, infected insect cells products were transblotted and Western analysis performed. Lane 1 shows protein expression of ICAM-1 by baculovirus at the 23 kDa position; lane 2 fails to show protein expression in material obtained from uninfected cells; lane 3 contains the low molecular weight marker.

with human ICAM-1 in which several constructs were found to be expressed [19], only one construct was expressed by the baculovirus expression system in this study. The main objective was aimed at developing a polyclonal antibody rather than a functionally active protein. Western blot analysis of cell products obtained using recombinant virus or a wildtype virus (control) demonstrated the expressed protein to be of the correct size (Fig. 2, lane 1). The control virus did not yield a product detected by Western blot analysis (Fig. 3, lane 2). Low molecular weight markers are shown in lane 3. The protein was purified by dialyzing and using an affinity column loaded with the antibody to ICAM-1. As described elsewhere, the insect cell system is very effective in generating large quantities of proteins [19,20] along with a functionally active protein [21]. We achieved 2 mg soluble ICAM-1/liter using these methods. This material was employed for the generation of rabbit polyclonal antibody.

Quantitation of ICAM-1 expression in vitro

Western blot analysis was performed to determine if the polyclonal antibody recognized the same protein as the monoclonal 1A29 anti-ICAM-1 antibody [15]. Using whole lung homogenates from 4 h inflamed rat lungs as a source for full length rat ICAM-1, both antibodies recognized the same

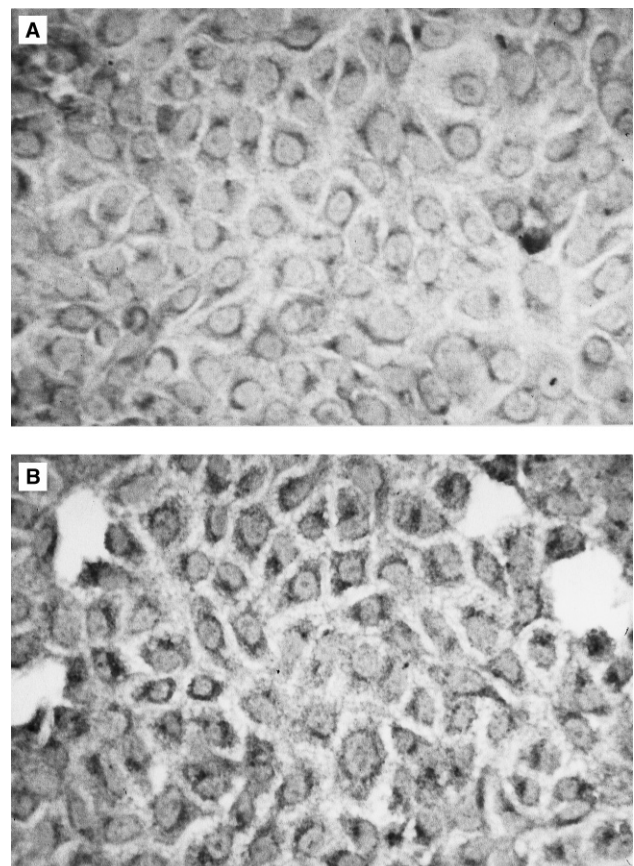


Fig. 3. Immunohistochemical staining of unstimulated RPAEC. Staining for constitutive ICAM-1 expression was visible (A); with stimulation by TNF α (50 ng/ml), ICAM-1 expression increased (B).

protein with the size of 97 kDa (data not shown). This size is consistent with previously published data [22], with a band of 91–97 kDa position. For immunostaining of RPAEC, cells were stimulated for 4 h in the presence or absence of murine TNF α (50 ng/ml). In Fig. 3A in which the newly prepared rabbit anti-ICAM-1 IgG was used, the staining of non-stimulated cells was found, apparently detecting constitutive ICAM-1. In Fig. 3B more intense staining for ICAM-1 was detected. Quantitation of these differences in ICAM-1 content is described below.

Immunofluorescence staining was performed on frozen sections of rat aortic rings which were stimulated *in vitro* with TNF α (20 ng/ml for 4 h). Fig. 4 shows the staining of a stimulated aortic ring for ICAM-1. The endothelial surface stained intensely, but, there was also some staining of smooth muscle cells. ICAM-1 expression on vascular smooth cells has been described previously [23]. Control (non-stimulated) rings did not show evidence of fluorescence (data not shown). When LPS was used to stimulate RPAEC, there was a significant increase (50%) in ICAM-1 content, reaching a plateau after 6 h (Fig. 5A). Using different LPS concentrations, ICAM-1 expression on RPAEC was maximal at the LPS concentration of 10 μ g/ml (Fig. 5B).

Monolayers of alveolar macrophages from normal rat lungs were prepared using 96 well plates and stimulated with increasing concentrations of TNF α (0–100 ng/ml). The time course for LPS-induced expression of ICAM-1 on alveolar macrophages showed a plateau at 2 h (70% increase) (Fig. 6A). Maximal stimulation (40% increase) in ICAM-1 expression was reached at 10 ng TNF α /ml (Fig. 6B).

Quantitation of ICAM-1 mRNA and protein expression *in vivo*

Homogenates from IgG immune complex-injured lungs were evaluated by Northern blot analysis and with quantitative PCR at 0, 2, 4, 6 and 8 h after initiation of the inflammatory reactions. As expected, by Northern blot analysis low constitutive expression of ICAM-1 mRNA was found in normal lungs, with a 12-fold upregulation 4 h after initiation

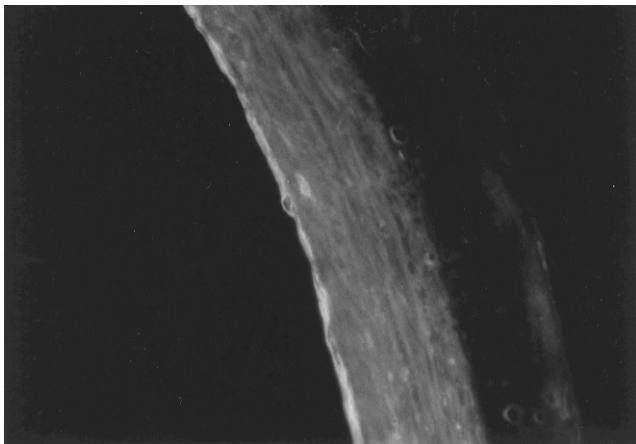


Fig. 4. Immunofluorescence staining of TNF α -stimulated rat aortic ring, showing expression of ICAM-1 on endothelial and smooth muscle cells.

of the reactions (Fig. 7A). A comparison of Northern blot analysis to quantitative PCR is shown in Fig. 7 (A, B). By 6 h of lung injury, ICAM-1 mRNA as assessed by quantitative PCR decreased to baseline levels. Data derived from the two methods of mRNA measurement were reasonably comparable. To determine whole lung ICAM-1 protein content, lungs were homogenized at indicated time points and ELISA performed. The greatest increase in total ICAM-1 protein was demonstrated at 6 h (Fig. 7C), following the earlier increase in mRNA for ICAM-1. As indicated above, at least two different cell sources for lung ICAM-1 have been identified: alveolar macrophages and vascular endothelial cells.

Discussion

Rat ICAM-1 was expressed *in order* to develop an ELISA for measurement of ICAM-1 protein as well as to raise a polyclonal antibody for further direct protein studies. ICAM-1 was expressed *in vitro* in two different systems. In

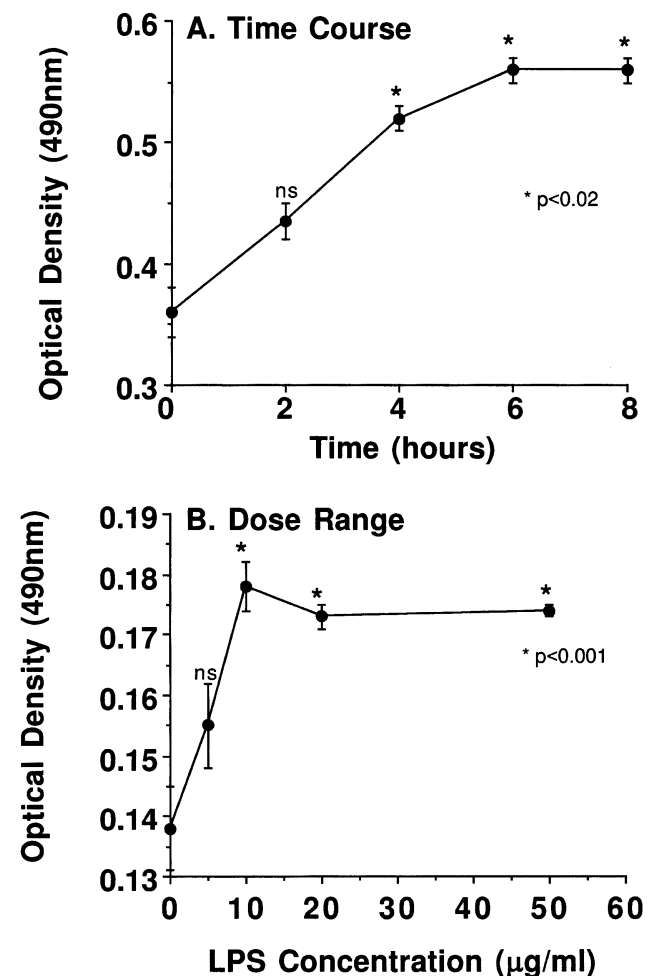


Fig. 5. Time course for ICAM-1 expression in LPS-stimulated (10 μ g/ml) RPAEC (A). Maximum expression was found at 6 h. RPAEC stimulated with increasing concentrations of LPS (0–50 μ g/ml) (B). Maximum expression was found when the concentration of LPS was 10 μ g/ml).

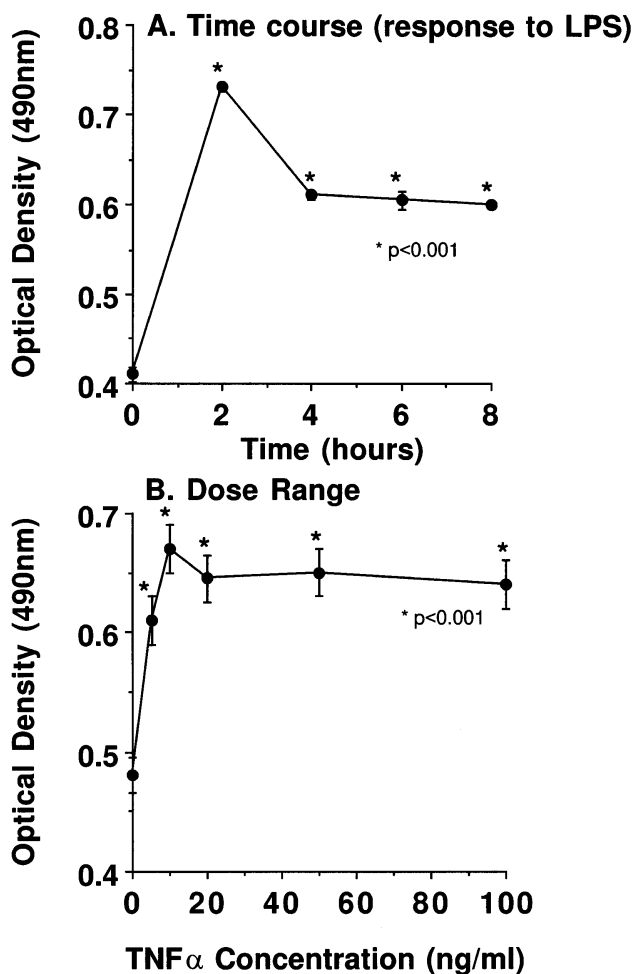


Fig. 6. Expression of ICAM-1 in rat alveolar macrophages. Maximum expression was reached after 2 h (A). Using a dose range of TNF α with the end-point of 2 h, maximal expression of ICAM-1 occurred at a dose of TNF α of 10 ng/ml (B).

comparing the bacterial pET and the baculovirus expression systems, the latter revealed a higher yield and purity but was much more time-consuming. The baculovirus system, however, may have advantages over the pET system with regard to the function of the expressed protein due to post-translational modifications of the protein in the insect cells. Successful purification of a membrane-bound protein begins with selection of an appropriate detergent and establishment of the solubilization conditions [24]. We found that CHAPS was best detergent-suited for our purposes because it was milder than a detergent like SDS, which tended to activate proteases, resulting in cleavage of ICAM-1. To prevent proteolysis, a mixture of bestatin, leupeptin, aprotinin and PMSF was used. This technique of protein extraction from lung tissue has become a common procedure and has been repeatedly used [18]. When ICAM-1 content was evaluated in whole lung homogenates, in the absence of this mixture of inhibitors, Western blots consistently showed an ICAM-1 species considerably below the expected 97 kDa position (data not shown).

Endothelial (RPAEC) ICAM-1 expression in vitro was assessed in RPAEC. Following LPS stimulation (5 μ g/ml)

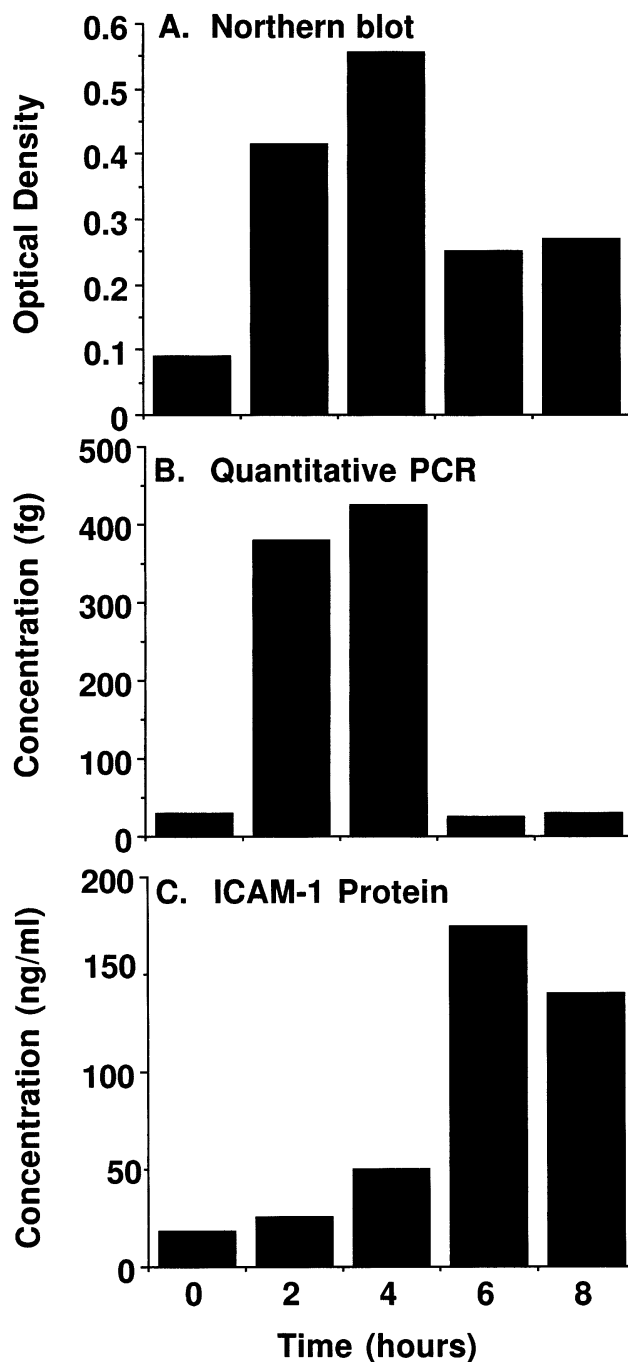


Fig. 7. Northern blot analysis with mRNA for ICAM-1 in a time-course for IgG immune complex-induced lung injury (A). Similar analysis was carried out using quantitative PCR (B). ICAM-1 protein content in whole lung homogenates was measured by ELISA (C).

for 2, 4, 6 and 8 h, a plateau in expression of protein being found after 6 h. In contrast to these in vitro results, in LPS-injured lungs in vivo, ICAM-1 message peaked at 4 h while protein expression was detected at 2 h but persisted for as long as 8 h [15]. Similar results have been obtained with TNF α stimulated human umbilical vein endothelial cells (HUVEC) [13, 25]. In the study by Wertheimer et al. [25] there was an early (2h) peak and followed later by a plateau for ICAM-1 mRNA expression in TNF α -stimulated

HUVEC. We saw a similar pattern of ICAM-1 expression in rat alveolar macrophages, with a plateau after 2 h of stimulation [15]. When canine alveolar macrophages were stimulated in vitro with LPS over 24 h, there was a steady increase of ICAM-1 expression [26]. This result could be due to the difference in LPS concentration used (5 ng/ml versus 5 µg/ml in our studies). At the higher concentration, the macrophages appeared to be maximally stimulated, which may have resulted in earlier down-regulation of the ICAM-1 response.

The quantitative PCR method used in this study correlated well with the data obtained by Northern blot analysis and offers the significant advantage for more accurate quantitation of mRNA levels rather than assessing relative expression levels [27, 28]. The electrochemiluminescence detection of PCR products was time-efficient and highly specific due to the TBR-labeled oligonucleotides and provides a valuable tool for evaluation of mRNA regulation [29, 30].

Interestingly, there was only a slight increase of ICAM-1 expression in the endothelial cell cultures compared to in vivo ICAM-1 upregulation in whole lungs. This could have several explanations: 1) in cell culture, there is no interaction between different cell types, this interaction being required for optimal expression of ICAM-1; 2) apart from endothelial cells or alveolar macrophages, other cells, such as epithelial cells, may contribute to the total production of ICAM-1 in the lung; 3) in cell culture with confluent monolayers, only the apical part of the cell is studied with ELISA techniques, omitting the basolateral area of the cell. In conclusion, soluble rat ICAM-1 was expressed in two different expression systems and a new polyclonal antibody was generated. The cDNA, the recombinantly expressed ICAM-1, and the polyclonal antibody developed represent useful tools in the elucidation of the role of this adhesion molecule. These reagents have permitted a detailed analysis rat ICAM-1 expression in inflammatory processes. Expanding these studies should add additional information in characterizing the role of ICAM-1 expression in lung inflammation.

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