Pneumocystis carinii induces ICAM-1 expression in lung epithelial cells through a TNF-α-mediated mechanism

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Yu, Mariette L., and Andrew H. Limper. Pneumocystis carinii induces ICAM-1 expression in lung epithelial cells through a TNF-α-mediated mechanism. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1103–L1111, 1997.—Inflammatory cell recruitment contributes to respiratory impairment during Pneumocystis carinii pneumonia. We evaluated expression of intercellular adhesion molecule-1 (ICAM-1), a key participant in leukocyte accumulation, in rats with P. carinii pneumonia. Immunostaining for ICAM-1 was most marked on bronchiolar epithelium but was also evident on type II pneumocytes, endothelium, and macrophages. Lung from normal and dexamethasone-treated uninfected animals exhibited markedly less ICAM-1. We hypothesized that P. carinii promoted ICAM-1 expression in epithelium through tumor necrosis factor-α (TNF-α) release from macrophages or that P. carinii directly stimulated ICAM-1 expression. Alveolar macrophages were incubated with P. carinii, and the medium was added to A549 epithelial cells. Treatment of macrophages with P. carinii enhanced A549 ICAM-1, which was inhibited with antibody to TNF-α. To determine whether P. carinii alone also stimulated ICAM-1, A549 cells were cultured with P. carinii, also augmenting ICAM-1. Of note, A549 ICAM-1 expression from P. carinii alone was less than with P. carinii-exposed macrophages. Thus ICAM-1 is enhanced in lung epithelium during P. carinii infection, in part, through TNF-α-mediated mechanisms.

intercellular adhesion molecule-1; macrophage; tumor necrosis factor-α

PNEUMOCYSTIS CARINII pneumonia is an opportunistic fungus causing severe pneumonia in immunocompromised hosts. Despite effective prophylaxis and treatment regimens, this infection remains a major complication of patients with human immunodeficiency virus infection or hematologic or solid malignancies or after organ transplantation (13, 22, 41). Among patients with acquired immunodeficiency syndrome (AIDS), severe P. carinii pneumonia is the most frequent cause of acute respiratory failure requiring admission to the intensive care unit (7, 39). Furthermore, the mortality of P. carinii pneumonia in all patients ranges between 15 and 40% (7, 22, 39). Prior investigations indicate that lung inflammation contributes significantly to respiratory impairment and clinical outcome during P. carinii pneumonia (22, 35). In particular, increased numbers of neutrophils in bronchoalveolar lavage predict poorer oxygenation and survival during P. carinii pneumonia (22). The contention that pulmonary inflammation potentiates lung injury during P. carinii pneumonia is further supported by clinical observations that corticosteroids prevent deterioration and improve outcome in moderate to severe P. carinii pneumonia (28).

The mechanisms regulating lung inflammation during P. carinii pneumonia remain poorly understood. Recent investigations have focused on a family of cell surface adhesion molecules that enable circulating leukocytes to accumulate in areas of lung inflammation (1, 33). Of primary importance is intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin (Ig) supergene family, that, together with its ligands lymphocyte function-associated antigen-1 and macrophage-1 antigen, promotes the firm adherence of leukocytes onto vascular endothelium, thereby permitting transmigration into injured tissues. In addition to vascular endothelium, ICAM-1 is also expressed by epithelial cells, lymphocytes, platelets, monocytes, and macrophages (6, 12, 33). The expression of ICAM-1 is upregulated by proinflammatory cytokines, most notably tumor necrosis factor-α (TNF-α) and γ-interferon (γ-IFN; see Refs. 6, 12, and 24). ICAM-1 has previously been implicated in the inflammatory component of several lung diseases, including tuberculosis, asthma, and sarcoidosis (12, 36, 40). However, the expression of ICAM-1 in P. carinii pneumonia has not yet been evaluated.

A number of investigations indicate that TNF-α expression is augmented during P. carinii pneumonia in humans and animal models (9, 18, 19). Studies from our laboratory have demonstrated that P. carinii stimulates release of TNF-α from macrophages through interaction of the cell wall of the organism with cognate macrophage receptors (29). We hypothesized that this enhanced release of TNF-α would act locally to increase ICAM-1 expression during P. carinii pneumonia. Accordingly, this investigation was undertaken to address the following goals: 1) to determine the extent and cellular localization of ICAM-1 protein expression during P. carinii pneumonia and 2) to determine potential mechanisms by which ICAM-1 expression may be regulated during P. carinii infection, particularly whether alterations in ICAM-1 occur through TNF-α-mediated mechanisms.

MATERIALS AND METHODS

Materials. Mouse monoclonal anti-rat ICAM-1 antibody for immunohistochemistry was obtained from Genzyme (Cambridge, MA), and monoclonal mouse anti-human ICAM-1 antibody used in the enzyme-linked immunosorbent assay (ELISA) was from R & D Systems (Minneapolis, MN). Recombinant human TNF-α was purchased from Genzyme, and type I collagen was from Vitrogen (Palo Alto, CA). Sheep anti-mouse IgG conjugated to β-galactosidase and p-nitrophenyl-β-galactoside were from Gibco-BRL Laboratories (Gaithersburg, MD). A human ICAM-1 cDNA was the kind gift of Dr. Michael Dustin (Washington University, St. Louis, MO; 1040-0605/97 $5.00 Copyright © 1997 the American Physiological Society L1103).
see Ref. 37). Ciprofloxacin was provided by Miles Pharmaceuticals (West Haven, CT).

Preparation of P. carinii. All animal studies were approved by the appropriate institutional animal care and utilization committee. P. carinii pneumonia was induced in Harlan Sprague-Dawley rats by immunosuppression with dexamethasone and transtracheal injection with P. carinii as previously described (2, 21, 31). Specific pathogen-free rats were provided freely with drinking water containing 2 mg/ml dexamethasone, 500 mg/l tracyclicine, and 200,000 U/l nystatin and were fed an 8% protein diet (Teklad, Madison, WI). On a weekly basis, the animals also received 0.45 g/l oral ciprofloxin for 2 consecutive days to further reduce the risk of bacterial infections. After 5 days of immunosuppression, rats were transtracheally inoculated with P. carinii (~500,000 cfu) by homogenizing infected rat lung in a Stomacher microbiological blender (Tekmar, Cincinnati, OH). After tracheal injection, the rats were immunosuppressed for an additional 6–8 wk and were killed, and whole lung lavage was performed with 50 ml of Hanks' balanced salt solution (HBSS). P. carinii were purified from this lavage by differential centrifugation (21). Lavage fluid was centrifuged (400 g for 10 min), and associated P. carinii cysts were identified in the pellet by Diff-Quik staining. The supernatant containing predominantly suspended P. carinii organisms was centrifuged (1,400 g for 30 min), and the pellet was resuspended in 1 ml of HBSS. Duplicate 10-µl aliquots of suspension were spotted onto glass slides and stained with Diff-Quik, and P. carinii were quantified as previously described (2, 21). If other microorganisms were noted in the lavage smear or on microbiological culture, the material was discarded. P. carinii isolates were found to contain <0.125 U/ml of soluble endotoxin using a sensitive Limulus amoebocyte lysate assay as previously described (29).

Immunolocalization of ICAM-1 in P. carinii-infected rat lung. Lung specimens were obtained from moribund rats with well-established P. carinii pneumonia 6–8 wk after inoculation, fixed with 10% phosphate-buffered Formalin in 15 cmH2O pressure, embedded in paraffin, and sectioned (20, 31). Five-micrometer sections were deparaffinized with xylene and graded alcohols and were submitted to immunohistochemical examination by an avidin-biotin-mediated immunoperoxidase method (Vectorstain ABC method; Vector Laboratories). Endogenous peroxidase activity was quenched by 1% normal goat serum. The sections were washed with tris(hydroxymethyl)aminomethane-buffered saline (pH 7.4) containing 0.1% Tween 20. To further quantify the relative expression of ICAM-1 during P. carinii pneumonia, additional lung specimens from P. carinii-infected animals and from dexamethasone-treated control animals without P. carinii were stained, and the staining intensity was scored. The specimens were coded and reviewed in a blinded fashion. Analogous to a previously published immunohistochemical study by Limper et al. (20), each specimen was given a score from zero to four, where zero was no detectable ICAM-1 staining, one was minimally detectable staining, two was weakly positive staining, three was moderate ICAM-1 staining, and four was abundant ICAM-1 staining.

P. carinii induction of ICAM-1 expression in lung epithelial cells. P. carinii induces alveolar macrophages (AMs) to release TNF-α, a potent stimulant of ICAM-1 expression (29). To determine whether P. carinii-induced macrophage release of TNF-α caused enhanced ICAM-1 expression by lung epithelial cells, AMs were cultured with P. carinii. Subsequently, the conditioned medium was removed and plated on A549 lung cells, and cell-surface ICAM-1 expression was determined after further overnight incubation. Specifically, uninfected Harlan Sprague-Dawley rats were killed, and whole lung lavage was performed with 50 ml of HBSS. Such lavages contain >95% AMs (8). Macrophages were plated in 24-well plates (100,000/well) and were incubated overnight in the presence of P. carinii in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 2 mM glutamine, 10,000 U penicillin/l, 1 µg streptomycin/ml, and 25 µg amphotericin/ml at 37°C. The conditioned media were removed, clarified by centrifugation (12,000 g for 5 min), and added to confluent monolayers of A549 lung epithelial cells (CCL-185; American Type Culture Collection, Rockville, MD), a cell line that has proven useful for examination of P. carinii-epithelial interactions (11, 21). The next day, ICAM-1 expression on the A549 cells was evaluated by cell-based ELISA (24). To study the role of TNF-α and other cytokines in mediating epithelial ICAM-1 expression, conditioned media were treated with TNF-α antiserum, antibody to γ-IFN, or nonimmune rabbit serum or nonimmune mouse IgG (1:40 dilution of rabbit sera or 50 µg/ml of mouse IgG) for 30 min before and throughout in the subsequent incubation on A549 cells.

It remained possible that P. carinii might also directly stimulate epithelial expression of ICAM-1. To study this, A549 cells were plated in 96-well plates (100,000/well) and were cultured overnight. P. carinii were added to the A549 monolayers at the indicated concentrations and were incubated for an additional 24 h. As a positive control of A549 cell ICAM-1 induction, parallel wells were incubated with media alone in the presence of 5,000 U/ml TNF-α. To further investigate the role of cytokines in mediating stimulation of ICAM-1 in A549 cells cultured with P. carinii, organisms were treated with TNF-α antiserum or nonimmune serum (1:40 dilution) for 30 min before and throughout the subsequent incubation on A549 cells.

Evaluation of cell surface ICAM-1 on A549 cells. After incubation of A549 cells with macrophage-conditioned media or with P. carinii alone, cell surface ICAM-1 was quantified using a cell-based ICAM-1 ELISA (24). A549 cell monolayers were washed with tris(hydroxymethyl)maminemethane-buffered saline containing 0.5% bovine serum albumin (BSA), 1 mM CaCl2, and 1 mM MgCl2 and were fixed in 1% paraformaldehyde for 15 min at 25°C. Next, the cells were blocked with 2% BSA for 1 h at 37°C. After being washed, the cells were sequentially incubated with monoclonal anti-human ICAM-1 antibody (100 µl of a 10 µg/ml stock solution) at 37°C for 1 h, followed by a secondary sheep anti-mouse IgG F(ab')2, conjugated to β-D-galactosidase (1:200 dilution) over an additional hour. Bound antibodies were detected with 1 mg/ml p-nitrophenyl-β-D-galactoside in 50 mM phosphate buffer with 1.5 mM MgCl2 (pH 7.2) reacting for 15 min at 37°C, and absorbances were determined at 405 nm. Unless otherwise stated, the amount of basal ICAM-1 expressed by unstimu-
lated A549 cells was subtracted from each sample to correct for background expression of ICAM-1.

Analysis of steady-state ICAM-1 mRNA content in A549 cells stimulated with P. carinii. Northern hybridizations were performed to additionally determine whether P. carinii-induced changes in ICAM-1 mRNA expression in A549 cells. A549 cells were plated in six-well plates (5 × 10⁶ cells/well) and were grown to confluence at 37°C over 48 h. Subsequently, the cells were incubated with P. carinii at the indicated concentrations overnight. Total RNA was extracted from the cells with the use of a monophasic solution of phenol and guanidine isothiocyanate followed by chloroform extraction and isopropyl alcohol precipitation (Trizol reagent; Gibco-BRL). Equal amounts of total RNA from each condition (20 µg) were loaded and were separated by electrophoresis through a 1.2% agarose gel in the presence of 2.2 M formaldehyde. Equal loading of the RNA was verified by ethidium bromide staining of 18S and 28S rRNA, and the separated RNA was transferred to nitrocellulose membranes and was prehybridized (ExpressHyb; Clonetech Laboratories). A radiolabeled ICAM-1 probe was generated from human ICAM-1 cDNA in the pAprM8 plasmid (37). This plasmid was digested with Hind III and Pst I to release an 800-bp fragment that was separated on agarose and was labeled with [α-32P]dCTP (NEN) by a random-primer method (Rediprime; Amersham). The radiolabeled probe was added to hybridization solution (2 × 10⁶ counts·min⁻¹·ml⁻¹) and was incubated with the membranes for 2 h at 68°C. After hybridization, the membranes were washed with 2× salt-sodium citrate solution (SSC; 1× solution contained 150 mM NaCl and 15 mM sodium citrate at pH 7.0) with 0.05% sodium dodecyl sulfate (SDS) at room temperature for 40 min followed by 0.1× SSC with 0.1% SDS solution at 50°C for 40 min. The blots were visualized by autoradiography. To further verify equal RNA loading, the membranes were stripped in 2× SSC with 0.1% SDS solution at 50°C for 40 min and were rehybridized with a 32P-labeled probe complementary to the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene product. Scanning densitometry of the resulting autoradiographic images was performed, and results were expressed as ICAM-1-to-GAPDH ratios.

Statistical methods. Data are expressed as means ± SE. Differences between multiple data groups were first assessed using the one-way analysis of variance. Differences between experimental data groups were analyzed using the two-tailed Student's t-test for normal parameters and the Mann-Whitney U-test for nonparametric data. P < 0.05 was defined to represent a statistically significant difference.

RESULTS

Immunohistochemical localization of ICAM-1 during P. carinii pneumonia. In all rats studied, markedly enhanced ICAM-1 was detected compared with normal lung (Fig. 1). Lung tissues infected with P. carinii revealed intense staining for ICAM-1 on bronchiolar epithelial cells (Fig. 1A), proliferative type II pneumocytes (Fig. 1D), and vascular endothelial cells (Fig. 1C). Additionally, ICAM-1 was detected in smooth muscle cells surrounding airways (Fig. 1A) and blood vessels (Fig. 1C) and on AMs (Fig. 1D) in these infected animals. To confirm the specificity of the antibody staining for ICAM-1, serial sections were incubated with an identical concentration of nonimmune mouse IgG that failed to demonstrate any specific reactivity (Fig. 1B). For comparison, normal uninfected rat lungs were also stained to detect basal tissue expression of ICAM-1 protein. In normal rat lungs, ICAM-1 was detected in epithelial cells but in markedly less quantities than in infected rats (Fig. 1E). In addition, markedly less ICAM-1 staining was detected in endothelial cells and in AMs compared with tissues obtained from rats with P. carinii pneumonia. Thus ICAM-1 is present in enhanced quantities in specific lung cell populations during P. carinii pneumonia in infected rats.

As an additional control, ICAM-1 staining was also assessed in lung tissues obtained from rats that were treated with a corticosteroid but that also received trimethoprim and sulfamethoxazole to prevent development of P. carinii pneumonia. Prior studies indicate that corticosteroids may suppress ICAM-1 expression (38). The pattern of ICAM-1 staining in these dexamethasone-treated controls was similar, although less intense, than that observed in uninfected normal lungs, with minimal ICAM-1 being present in epithelial cells, endothelial cells, and AMs. To further quantify the relative expression of ICAM-1 during P. carinii pneumonia, specimens from P. carinii-infected animals and from dexamethasone-treated control animals without P. carinii were stained in parallel, coded, and scored as previously described (20). With the use of this method, rat lungs with P. carinii pneumonia had a mean ICAM-1 staining score of 2.83 ± 0.31, whereas dexamethasone-immunosuppressed rats without P. carinii pneumonia had a mean score of 0.50 ± 0.29 (P = 0.0008). Thus ICAM-1 expression is also enhanced in the lungs of P. carinii-infected animals compared with dexamethasone-treated rats without P. carinii pneumonia.

AMs infected with P. carinii induce ICAM-1 surface expression on A549 lung epithelial cells. Having observed markedly enhanced quantities of ICAM-1 on lung epithelium during P. carinii infection, we next sought to determine potential mechanisms by which ICAM-1 expression might be mediated. Prior studies indicate that AMs bind and phagocytize P. carinii and are stimulated by the organism to release oxidants, eicosanoids, and TNF-α, a potent stimulant of ICAM-1 expression in the epithelium (8, 15, 29). To investigate whether macrophage interaction with P. carinii causes cytokine release, thereby promoting ICAM-1 expression in epithelial cells, AMs were preincubated with P. carinii, and the resulting conditioned medium was removed and recultured on A549 alveolar epithelial cells (Fig. 2). Conditioned media obtained from cultures of control macrophages incubated in the absence of P. carinii mildly stimulated basal ICAM-1 expression in A549 cells. However, culturing AMs with increasing numbers of P. carinii resulted in significant enhancement in immunoreactive ICAM-1 expression in the A549 epithelial cells. Incubating P. carinii with AMs at a P. carinii-to-AM ratio of 1:1 resulted in 292 ± 107% enhanced expression of ICAM-1 in A549 epithelial cells compared with control macrophage-conditioned media incubated without organisms (P = 0.009). Incubations of P. carinii with macrophages at a P. carinii-to-AM ratio of 5:1 resulted in 199 ± 41% enhanced ICAM-1 expression (P = 0.006 compared with control). These
data indicate that the interaction of P. carinii with AMs enhances ICAM-1 expression on cultured lung epithelial cells.

P. carinii directly promotes enhanced ICAM-1 surface expression on A549 cells. During the life cycle of the organism, P. carinii trophozoites attach to alveolar epithelial cells by intimate approximation and interdigitation of their surfaces with the cell membranes of host epithelium (21). We postulated that the interaction of P. carinii with A549 lung epithelial cells might directly stimulate surface expression of ICAM-1. To examine this, freshly purified P. carinii were incubated overnight on A549 cell monolayers. The next day, the monolayers were washed, and A549 surface ICAM-1 expression was determined by ELISA with a monoclonal antibody specific for human ICAM-1 (Fig. 3). Increased ICAM-1 expression occurred between a P. carinii-to-A549 ratio of 0:1 compared with 1:1, 2.5:1, 5:1, and 10:1. A P. carinii-to-A549 ratio of 1:1 resulted in significantly increased A549 ICAM-1 surface expression (P = 0.022), whereas a P. carinii-to-A549 ratio of 5:1 yielded maximal enhancement of expression (P =...
ICAM-1 surface expression induced by P. carinii was noted, however, that the maximal absolute level of ICAM-1 was not statistically different from the level of ICAM-1 induced by the 5:1 concentration of P. carinii. These data indicate that the interaction of P. carinii to A549 cells exhibited no significant increase in A549 cell ICAM-1 expression (OD = 0.015 ± 0.013 RAU; P = 0.243 compared with control A549 cells without P. carinii). Thus direct adherence of the organisms to the epithelial cell appears to be necessary for the induction of ICAM-1 expression.

We next sought to determine whether P. carinii and conditioned media from P. carinii-stimulated macrophages exert a synergistic effect on ICAM-1 expression by A549 lung epithelial cells. A small additive increase (30.8 ± 2.8%) in A549 cell ICAM-1 expression was observed in A549 cells incubated with both P. carinii-stimulated macrophage-conditioned media and with freshly isolated P. carinii organisms compared with A549 cells incubated with conditioned media alone. This increase, however, was not statistically greater than expected on an additive basis alone. Therefore, a synergistic effect in A549 cell ICAM-1 expression was not observed by these two stimuli. P. carinii increase steady-state ICAM-1 mRNA in cultured A549 lung cells. It was conceivable that the P. carinii preparations might contain small amounts of contaminating rat ICAM-1 that, if transferred into the A549 cell cultures, might be detected with the cell-based ELISA assay. This was considered rather unlikely because the A549 cell monolayers were thoroughly washed before analysis and were examined using a monoclonal antibody specific for the human ICAM-1 expressed by A549 cells. However, to further confirm that P. carinii stimulates the expression of ICAM-1 in lung epithelial cells, we examined steady-state ICAM-1 mRNA expression in A549 cells cultured with increasing numbers of P. carinii using a cDNA probe specific for human ICAM-1 (Fig. 4). The human cDNA probe was hybridized with a 1.3-kb RNA species from A549 cells. Incubation of A549 cells in the presence of P. carinii augmented the levels of steady-state ICAM-1 mRNA at P. carinii-to-A549 ratios of 5:1 and 10:1. This was comparable with the pattern of hybridization observed using RNA obtained from A549 cells incubated with TNF-α, a potent inducer of epithelial ICAM-1 expression. No appreciable ICAM-1 mRNA induction was observed in A549 cells incubated in the absence of P. carinii. Equality of RNA loading under the

![Fig. 2. P. carinii-stimulated AMs enhance surface ICAM-1 expression on A549 lung epithelial cells. Normal AMs were harvested from rats and were incubated with increasing amounts of P. carinii. The next day, conditioned media were removed, clarified by centrifugation, and recultured with A549 lung cells for an additional 24 h. Surface ICAM-1 on A549 cells was subsequently determined by a cell-based enzyme-linked immunosorbent assay (ELISA). Media conditioned by culturing AMs in the presence of increasing numbers of P. carinii resulted in enhancement of A549 surface ICAM-1 expression. Values are means ± SE from 4 determinations (*P < 0.05 compared with media conditioned with AMs alone). OD, optical density.](image1)

![Fig. 3. P. carinii directly stimulate ICAM-1 surface expression on A549 lung epithelial cells. A549 cells were directly cultured in the presence of increasing numbers of P. carinii. After 24 h, A549 monolayers were washed, and A549 cells surface ICAM-1 was measured by ELISA. Culturing P. carinii with A549 cells directly stimulated expression of ICAM-1 in the A549 lung epithelial cell line. Values are means ± SE from 4 determinations (*P < 0.05 compared with A549 cells cultured in the absence of P. carinii).](image2)
Fig. 5. P. carinii enhance ICAM-1-GAPDH mRNA ratio in A549 lung epithelial cells. Scanning densitometry was performed on nitrocellulose membranes probed for both ICAM-1 and GAPDH, and relative hybridization ratio was determined. In a dose-dependent fashion, P. carinii significantly increased ICAM-1-GAPDH mRNA ratio of A549 cells. Values are means ± SE of scanning densitometry obtained from 3 blots (*P < 0.05 compared with A549 cells cultured in the absence of P. carinii).
significant effect on A549 ICAM-1 expression (P = 0.09, not significantly different from control).

Recent studies further indicate that TNF-α binds to P. carinii and fungal cell wall β-glucans through its lectin-binding domain (30, 32). Such lectin binding occurs through a site distinct from the mammalian TNF-α-receptor binding region, leaving this domain potentially accessible to mediate effects on host cells (30). To test the possibility that TNF-α associated with the surface of P. carinii might be causing the observed increases in A549 cells cultured directly with freshly isolated organisms, P. carinii were pretreated with anti-TNF-α before and throughout the subsequent incubation with A549 cells (Fig. 7). Treatment of P. carinii with anti-TNF-α did not significantly impair subsequent ICAM-1 expression by the A549 cells (P = 0.11, not significantly different from A549 cells stimulated with P. carinii in the absence of anti-TNF-α). Nonimmune serum had minimal effect on A549 cell ICAM-1 expression. These experiments indicate that, although P. carinii induces ICAM-1 expression in cultured lung epithelium, this effect is not significantly mediated by surface-bound TNF-α on the organisms.

**DISCUSSION**

This study demonstrates that ICAM-1, a potent leukocyte cell adhesion molecule, is present in enhanced quantities in the lung during P. carinii pneumonia in a rat model. Increased ICAM-1 protein expression was first observed on bronchial and type II epithelial cells, endothelial cells, smooth muscle cells, and AMs in animals with established P. carinii infection. We have further demonstrated that P. carinii induces enhanced ICAM-1 expression in cultured A549 lung epithelial cells through interaction of the organisms with AMs and through the subsequent release of TNF-α. In addition, direct contact of P. carinii with cultured lung epithelial cells also significantly augments ICAM-1 expression through alternate mechanisms.

ICAM-1 likely provides a number of key host defense functions during P. carinii pneumonia. Endothelial expression of ICAM-1 promotes the adherence, migration, activation, and subsequent recruitment of mononuclear cells, neutrophils, and T lymphocytes into tissues (1). Consistent with this, perivascular monocytoid infiltrates are typically observed during development of P. carinii pneumonia. Furthermore, patients with severe P. carinii pneumonia document a marked accumulation of neutrophils in the lung (22). Interestingly, T lymphocyte recruitment is also prominent during P. carinii pneumonia. Although CD4 lymphocytes are present in deficient numbers in the lower respiratory tract of patients with P. carinii pneumonia associated with AIDS or malignancy, recent investigations indicate that CD8 lymphocytes are recruited into the lungs during P. carinii infection (5, 27). These CD8 cells, although less effective than CD4 cells in mediating host defense, participate in a weaker fashion in suppressing the establishment of P. carinii pneumonia (5).

We observed augmented ICAM-1 expression on lung epithelial cells during P. carinii pneumonia. It is noteworthy that epithelial surfaces represent key sites controlling the life cycle of P. carinii. Adherence of P. carinii trophozoites to respiratory epithelial cells promotes proliferation of the organism and is a central event in the establishment of lung infection (21, 23). In vitro, ICAM-1 expression on these epithelial cells concomitantly promotes adherence, migration, and activation of recruited macrophages and neutrophils that interact with the organisms and reduce its viability (1, 33). Thus enhanced ICAM-1 with augmentation of host defense cells at the organism-epithelium interface may critically interfere with this essential phase of the P. carinii life cycle.

Our investigations also evaluated potential mechanisms through which epithelial ICAM-1 expression may be augmented during P. carinii infection. AMs release TNF-α when challenged with P. carinii by interaction of β-glucan on the organisms with macrophage glucan receptors, a process that is augmented by vitronectin, fibronectin, and specific anti-Pneumocystis antibodies (29). TNF-α expression is upregulated during P. carinii pneumonia in both animals and humans and is necessary for optimal clearance of infection (9, 18, 19). TNF-α additionally promotes endothelial and epithelial cell ICAM-1 expression (1, 14). As anticipated, in this model system we observed that interaction of P. carinii with AMs promoted increased ICAM-1 expression by A549 lung epithelial cells through release of TNF-α from P. carinii-activated macrophages.

Cultured bronchial, tracheal, and alveolar epithelial cells have all been reported to express ICAM-1 (10, 12, 18). Look and colleagues (24) demonstrated selective γ-IFN responsiveness of bronchial and tracheal epithelial cell lines. In our current study, antibody neutralization of γ-IFN in conditioned media from P. carinii-stimulated macrophages had minimal effect on ICAM-1

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**Fig. 7.** Effect of antibody to TNF-α on A549 ICAM-1 expression induced by direct culture with P. carinii. To evaluate whether surface-associated TNF-α on P. carinii enhanced A549 ICAM-1 expression, organisms were treated with neutralizing antibodies before and throughout subsequent cultures on A549 lung epithelial cells (P. carinii-to-AM ratio of 10:1). Maximal ICAM-1 (100%) was defined as the amount of A549 surface expression observed on A549 cells cultured with P. carinii in the presence of no added neutralizing antibodies. Treatment of P. carinii with anti-TNF-α did not significantly suppress surface expression of ICAM-1 by A549 cells. NI serum had a minimal effect on ICAM-1 expression by A549 cells. Values are means ± SE from 6 determinations.
expression by A549 lung epithelial cells. However, γ-IFN is predominately produced by large T lymphocytes, and minimal amounts of γ-IFN are released from AMs (17). The current findings do not preclude a role for γ-IFN-mediated expression of ICAM-1 in vivo but likely reflect the small amount of γ-IFN released by macrophages during P. carinii infection in the system employed. Indeed, prior investigations suggest that γ-IFN promotes optimal clearance of P. carinii in vitro (9). It has been proposed that lymphocytic destruction in patients with AIDS results in a relative deficiency of γ-IFN during P. carinii pneumonia (4, 27). Such relative deficiency of γ-IFN during P. carinii pneumonia may reduce ICAM-1 expression in the lung, thus hindering complete clearance of infection in these hosts.

Interestingly, our study revealed that purified P. carinii also directly stimulate ICAM-1 by A549 lung epithelial cells. The mechanisms of this direct ICAM-1 stimulation are not yet clear but may involve either surface antigens of the organism or host proteins bound to the microbe that stimulate the epithelial cells. The interaction of P. carinii with alveolar epithelial cells is a complex process involving glycoprotein A (gpA), a mannoside-rich surface protein of P. carinii, as well as host alveolar proteins, including fibronectin, vitronectin, surfactant components, and other host-derived molecules that coat the organisms and promote adherence to the lung epithelium (28, 31, 34). Interestingly, TNF-α also binds to a soluble cell wall fraction of P. carinii and is present on freshly isolated organisms (30, 32). The mechanisms of TNF-α binding to P. carinii are not fully known, but recent investigations demonstrate that TNF-α interacts with fungal cell wall β-glucans through lectin-mediated binding (30). Lectin interactions utilize a region of the TNF-α molecule distinct from that which recognizes mammalian TNF-α receptors (30). Although we postulated that P. carinii-associated TNF-α might mediate enhanced ICAM-1 expression after organism binding to epithelial cells, ICAM-1 expression by A549 cells directly stimulated with freshly isolated P. carinii was not significantly inhibited by TNF-α antiserum, suggesting that alternate mechanisms confer this effect. In additional experiments, antibody to γ-IFN also did not reduce ICAM-1 expression in A549 epithelial cells cultured with P. carinii (data not shown). Further studies are necessary to determine the roles of other native and host proteins in mediating this direct stimulation of epithelial ICAM-1 by P. carinii.

Although the absolute level of ICAM-1 induced in epithelial cells directly by P. carinii was less than that evoked by P. carinii-exposed macrophages, our findings do suggest that both mechanisms may occur during infection. For comparison of the response induced by P. carinii alone to a maximal stimulus, we further included TNF-α in selected experiments in which A549 cells were directly stimulated with P. carinii. The maximal surface ICAM-1 expression resulting from direct P. carinii stimulation ranged between 30 and 50% of that observed with TNF-α stimulation alone (1,000 U/ml). Similarly, we observed that P. carinii stimulated roughly one-third of the ICAM-1 mRNA compared with a maximal stimulation of A549 cells with TNF-α (Fig. 5). Our investigation further indicates that P. carinii directly influence epithelial activation of ICAM-1 expression through mechanisms independent of macrophage-derived TNF-α. Additional studies are required to determine the relative extent to which these mechanisms contribute to lung inflammation in vivo during P. carinii pneumonia.

Although ICAM-1 promotes inflammatory cell recruitment necessary for lung defense, exuberant ICAM-1 expression may also be detrimental to the host. Excessive neutrophils in bronchoalveolar lavage from patients with P. carinii pneumonia correlate with enhanced morbidity, increased gas-exchange abnormalities, and poorer survival during infection (22, 35). Marked ICAM-1 expression in P. carinii infection may exacerbate this deleterious neutrophil recruitment. Furthermore, modulation of pulmonary inflammation with corticosteroids improves survival and reduces the occurrence of respiratory failure in patients with moderate to severe P. carinii pneumonia (28). Corticosteroids are known to reduce TNF-α release from stimulated AMs and to directly suppress ICAM-1 expression (16, 25, 38). Recent investigations further indicate that excess pulmonary neutrophil influx may be selectively inhibited with monoclonal antibodies that recognize ICAM-1 (3, 26, 40). Accordingly, selective modulation of ICAM-1 may also have a potential therapeutic benefit during severe P. carinii pneumonia; however, additional animal and predilential studies are warranted to evaluate the merits of such a strategy.

The mechanisms of lung inflammation that accompany P. carinii infection are only beginning to be defined. This study provides the first evidence that ICAM-1 is upregulated during P. carinii pneumonia. Our data further support a role for macrophage-derived TNF-α in the mediation of increased ICAM-1 expression in cultured lung epithelial cells. In addition, P. carinii organisms also appear to directly augment ICAM-1 protein and mRNA expression by cultured lung cells. Further understanding of lung inflammation during this serious infection should provide additional insights and potential therapeutic strategies for patients with P. carinii pneumonia.

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