Proinflammatory Cytokine, Chemokine, and Cellular Adhesion Molecule Expression during the Acute Phase of Experimental Brain Abscess Development

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Brain abscess represents the infectious disease sequelae associated with the influx of inflammatory cells and activation of resident parenchymal cells in the central nervous system. However, the immune response leading to the establishment of a brain abscess remains poorly defined. In this study, we have characterized cytokine and chemokine expression in an experimental brain abscess model in the rat during the acute stage of abscess development. RNase protection assay revealed the induction of the proinflammatory cytokines interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor-α as early as 1 to 6 hours after *Staphylococcus aureus* exposure. Evaluation of chemokine expression by reverse transcription-polymerase chain reaction demonstrated enhanced levels of the CXC chemokine KC 24 hours after bacterial exposure, which correlated with the appearance of neutrophils in the abscess. In addition, two CC chemokines, monocyte chemoattractant protein-1 and macrophage inflammatory protein-1α were induced within 24 hours after *S. aureus* exposure and preceded the influx of macrophages and lymphocytes into the brain. Analysis of abscess lesions by *in situ* hybridization identified CD11b+ cells as the source of IL-1β in response to *S. aureus.* Both intercellular adhesion molecule-1 and platelet endothelial cell adhesion molecule expression were enhanced on microvessels in *S. aureus* but not sterile bead-implanted tissues at 24 and 48 hours after treatment. These results characterize proinflammatory cytokine and chemokine expression during the early response to *S. aureus* in the brain and provide the foundation to assess the functional significance of these mediators in brain abscess pathogenesis.   *(Am J Pathol 2000, 157:647–658)*

Brain abscess is a focal, intracerebral lesion that begins as a localized area of cerebritis and develops into a collection of pus surrounded by a well-vascularized capsule. The end result of a brain abscess includes the replacement of the abscessed area with a fibrotic scar, loss of brain tissue by surgical removal, or abscess rupture and death. Brain abscesses represent a serious medical problem accounting for approximately 1 in 10,000 hospital admissions in the United States.[1] The leading etiological agents of brain abscess are the streptococcal strains and *Staphylococcus aureus.*[2,3] Our laboratory has developed an experimental brain abscess model in the rat[4] that allows for dissection of the basic cellular and immunological mechanisms involved in the progression and resolution of brain abscesses.

The rat brain abscess model closely mimics human disease in that the abscess progresses through a series of well-defined stages. In the rat, each stage is defined on the basis of predominant cell types and the histological appearance of the abscess.[4] In the human, these stages have been identified using CT scanning techniques.[1] The early stage, or early cerebritis, occurs from days 1 to 3 and is typified by neutrophil accumulation, tissue necrosis, and edema. The intermediate, or late cerebritis stage, occurs from days 4 to 9 and is associated with a predominant macrophage and lymphocyte infiltrate. The final, or capsule, stage occurs from day 10 onward and is associated with the formation of a well-vascularized abscess wall that sequesters the lesion and protects the surrounding normal brain parenchyma. Myofibroblasts are found associated with the developing abscess wall at this stage and plasma cells predominate the lesion. Currently, it is not clear what cells are responsible for mediating the immunopathological response to *S. aureus* in the brain.

Chemokines represent a family of low molecular weight chemotactic cytokines, classified into groups based on the presence and position of conserved cysteine residues.[5–7] CXC or α chemokines function as neutrophil and lymphocyte chemoattractants and prototypical family members include IL-8, KC (rodent homologue of growth related oncogene-α), interferon-inducible protein-10 kd (IP-10), and monokine induced by interferon γ (MIG). CC or β chemokines are chemotactic for monocytes and T cells and include such members as macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant

proteins (MCPs) 1–5, and regulated on activation T cell expressed and secreted (RANTES). Chemokines play an important role in recruiting leukocytes into areas of active inflammation. They are synthesized locally at sites of inflammation and establish a concentration gradient to which target cell populations migrate. Several studies have examined the expression of chemokines in response to central nervous system (CNS) insults such as stab wound injury in the brain[8,9] and experimental autoimmune encephalomyelitis (EAE).[10–12] In these models, KC, IP-10, MCP-1, and MIP-1α are up-regulated after injury. However, the signals responsible for the initial recruitment of inflammatory cells into brain abscesses have not yet been defined.

In this report, we characterize chemokines and cytokines that are expressed immediately after the introduction of *S. aureus* into the brain. Several proinflammatory cytokines, including IL-1α, IL-1β, IL-6, and tumor necrosis factor-α (TNF-α), were found to be induced. Consistent with the observed neutrophil influx into early abscess lesions, KC expression was enhanced within 24 hours after *S. aureus* exposure. In addition, IP-10, MCP-1, and MIP-1α expression were enhanced in abscesses, preceding the observed influx of macrophages and lymphocytes. *In situ* hybridization revealed that CD11b+ cells produce IL-1β after *S. aureus* exposure. Analysis of adhesion molecule expression revealed enhanced levels of both intercellular adhesion molecule-1 (ICAM-1) and platelet endothelial cell adhesion molecule (PECAM) on vessels associated with *S. aureus* but not sterile bead-implanted tissues. These findings characterize cytokine and chemokine expression in early brain abscess lesions, and provide a basis for future studies to assess the functional significance of individual mediators in abscess pathogenesis.

Materials and Methods

Preparation of S. aureus-*Laden Agarose Beads*

Live *S. aureus* were encapsulated in agarose beads before implantation in the brain as previously described.[4] The use of agarose beads prevents widespread bacterial dissemination or rapid wound sterilization by the host. Briefly, a stock culture of coagulase-positive *S. aureus* obtained from the microbiology laboratory at Dartmouth Hitchcock Medical Center was grown overnight (16 hours) at 37°C in Luria-Bertani broth (Fisher Biotech, Fair Lawn, NJ). A total of 1×10^9 bacteria were added to a solution of 1.4% low melt agarose (type XII, Sigma, St. Louis, MO) at 40°C. The mixture was then added to rapidly swirling heavy mineral oil (Sigma) pre-warmed to 37°C and cooled to 0°C on crushed ice. Beads were washed four times in 1× DPBS (Mediatech Cellgro, Herndon, VA) to remove mineral oil. Beads with dimensions between 50 and 100 μm, as determined by phase contrast microscopy, were used for implantation into the brain. Sterile beads were prepared in the same manner, without bacteria. The bacterial viability or sterility of bead preparations was confirmed by overnight culture in LB medium.

Animals and Generation of Experimental Brain Abscesses

Adult female Lewis rats were obtained from Charles River Laboratories (Wilmington, MA). The animal use protocol has been approved by the Dartmouth College Institutional Animal Care and Use Committee. Anesthesia was provided by an i.p. injection of 60 mg/kg ketamine and 5 mg/kg xylazine on a body weight basis. A 1-cm longitudinal incision was made along the midline between the ear and the eye to expose the frontal sutures. A burr hole was drilled 5 mm caudal and 0.5 mm lateral to the frontal suture. A Hamilton syringe fitted with flexible tubing and a pulled, fine-tipped glass-fired pipet (diameter < 1 mm) was used to deliver beads into the brain parenchyma. A total of 5 μl of beads were placed 5 to 6 mm deep from the external surface of the calvarium to prevent reflux during injection. Using this approach, bacteria were reproducibly deposited into the head of the caudate or frontal lobe white matter. Control animals were implanted with sterile agarose beads. Previous studies have established that implanting sterile beads into the brain induces minimal inflammation and edema[4] (Kielian, unpublished observations). Incisions were closed using sterile wound clips. The mortality rate associated with abscess generation was minimal, with >95% of animals surviving the procedure.

Processing of Tissues for Immunostaining and RNA Analysis

To prepare tissues for immunohistochemistry, animals were perfusion-fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. The brain was removed, postfixed in paraformaldehyde for 30 minutes, and washed in 0.2 mol/L phosphate buffer, pH 7.4, overnight. Tissues were cryoprotected in 30% sucrose and snap-frozen in OCT for immunohistochemistry.

To prepare tissues for RNA isolation, samples of brain tissue containing *S. aureus* or sterile beads were snap-frozen in liquid N_2 and stored at −70°C until RNA extraction was performed.

In Situ *Hybridization (ISH) Probe Labeling*

ISH was performed using digoxigenin (DIG)-labeled riboprobes specific for rat IL-1β and MCP-1. IL-1β and MCP-1 cDNA fragments were amplified by reverse transcription-polymerase chain reaction (RT-PCR) from mRNA isolated from 24-hour abscess tissue using the following primers: IL-1β sense (5′-TTCAGGAAGGCAGTGTCA-3′), IL-1β anti-sense (5′-TCTTTGGGTATTGTTTGG-3′), MCP-1 sense (5′-ATGCAGGTCTCTGTCACGCTTC-3′), and MCP-1 anti-sense (5′-AGTTCTCTGTCATACTGGTCAC-3′). The size of products amplified by the IL-1β and MCP-1 primer pairs were 448 and 445 bp, respectively and verified by sequence analysis. DIG-labeled riboprobes were synthesized from cDNA templates subcloned into the PCR vector pGEM T (Promega, Madison, WI) using the DIG RNA labeling kit (Boehringer Mannheim, Indianapolis, IN) according to the

manufacturer's instructions. The efficiency and intensity of riboprobe labeling was compared with a DIG-labeled RNA standard using dot blot analysis.

ISH

Frozen sections of fixed tissue were mounted onto RNase-free polyL-lysine-treated glass slides and air-dried for 30 minutes at room temperature before use. Tissues were rehydrated in three changes of Tris-buffered saline (TBS), pH 7.4, before proteinase K treatment. Sections were permeabilized with proteinase K (10 μg/ml) in TBS with 2 mmol/L CaCl$_2$ for 15 minutes at 37°C. Slides were rinsed with TBS at 4°C and transferred to a solution of 0.1 mol/L triethanolamine, pH 8.0, for 3 minutes, followed by incubation in 0.5% acetic anhydride for 10 minutes on a stir plate. Following two washes in TBS, tissues were dehydrated through a series of graded alcohols and allowed to air-dry. Before use, DIG-labeled riboprobes (100 ng/ml) were denatured for 5 minutes at 65°C in hybridization buffer (50% deionized formamide, 10% dextran sulfate, 100 μg/ml salmon sperm DNA, 2× standard saline citrate, 0.02% sodium dodecyl sulfate). Riboprobes were added to tissue sections, coverslipped, and incubated overnight (12–16 hours) at 55°C in a humidified chamber. The following day, slides were washed with increasing stringency using standard saline citrate. Next, tissues were treated with blocking reagent (Boehringer Mannheim) for 15 minutes at room temperature before incubation with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1:500 dilution) for 1 hour at room temperature. Following three washes in TBS, slides were developed using a NBT/BCIP substrate (Boehringer Mannheim) at 4°C in the dark. The color reaction was terminated by extensive washes in ddH$_2$O. Tissues were dehydrated through a series of graded alcohols and xylene before coverslipping.

Immunohistochemistry

Frozen sections of fixed tissue were processed for immunohistochemistry using the avidin-peroxidase method as previously described.[13] The following antibodies were used for adhesion molecule analysis: ICAM-1 (TLD-4C9) and PECAM (TLD-3A12; both from Serotec, Raleigh, NC), L-selectin, P-selectin, and vascular cell adhesion molecule-1 (VCAM-1; PharMingen, San Diego, CA), and the isotype control Abs MOPC-21 and UPC10 (mouse IgG$_1$ and IgG$_{2a}$, respectively, Sigma), and rabbit IgG (Vector Laboratories, Burlingame, CA). Astrocytes and monocytes/microglia were identified on the basis of GFAP (Dako, Carpenteria, CA) and CD11b (OX-42, Serotec) staining, respectively. Biotinylated secondary antibodies included anti-mouse IgG, anti-rabbit IgG (both from Vector Laboratories), or anti-hamster IgG (Caltag, South San Francisco, CA). Slides were developed using the substrate 3,3′-diaminobenzidine (DAB).

Cytokine Immunohistochemistry

Cells producing the proinflammatory cytokine IL-1β were identified by immunohistochemical staining as previously

Table 1. Oligonucleotide Primers

Primer	Product size (bp)	Sequence (5′ to 3′)
IP-10 (+)	286	GCTGCTGAGTCTGAGTGG
IP-10 (−)		CTGTGGCGAGTGGCTTCT
KC (+)	253	GCTTGCCTTGACCCTGAA
KC (−)		TGAAACGCATCCACATCG
MIP-2 (+)	286	GTCCTGCTCCTCCTGCTG
MIP-2 (−)		GCCCATGTTCTTCCTTCC
Fractalkine (+)	394	CTGCTGGCTGGTTAGAGG
Fractalkine (−)		TTCACGGCACATCAAAGT
MIP-1α (+)	121	CGCTCTGGAACGAAGTCT
MIP-1α (−)		AGGCTGCTGGTCTCAAAA
MCP-1 (+)	266	ATGAGTCGGCTGGAGAAC
MCP-1 (−)		GTGGAAAAGAGAGTGGAT
RANTES (+)	127	CACTCCCTGCTGCTTTGC
RANTES (−)		CACTTGGCGGTTCCTTCG
β-actin (+)	554	TACAACCTCCTTGCAGCTCC
β-actin (−)		GGATCTTCATGAGGTAGTCTGTC

(+), sense primer; (−), antisense primer; IP-10, gamma interferon inducible protein-10; KC, homolog of growth related oncogene-α; MIP-1α, macrophage inflammatory protein-1α; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated on activation T cell expressed and secreted.

described.[14] Primary mouse anti-rat IL-1β (Serotec) and mouse IgG$_1$ isotype control antibodies (MOPC-21, Sigma) were used to evaluate IL-1β protein expression in *S. aureus*- and sterile bead-implanted tissues. Biotinylated horse anti-mouse IgG (Vector Labs) was used with the ABC peroxidase reagent (Vector Elite ABC kit, Vector Labs) to detect IL-1β protein. Reaction products were developed using a DAB substrate (Vector Labs).

RNA Isolation and RT-PCR

Total RNA was isolated from brain tissue using the TriZol reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. Before use in RT-PCR, RNA samples were treated with DNase I (Gibco BRL). RT-PCR was performed using a GeneAmp RNA PCR kit (Perkin Elmer, Foster City, CA) with 100 ng of total input RNA. After reverse transcription, cDNA templates were denatured for 105 seconds at 95°C, then amplified in 35 cycles of annealing (60°C, 30 seconds), extension (60°C, 30 seconds), and denaturing (95°C, 15 seconds). The final step was incubation for 7 minutes at 42°C. Each experimental sample was amplified in parallel using primers specific for β-actin to assess uniformity in the amount of input RNA and gel loading. Primers used for PCR amplification are listed in Table 1.

RNase Protection Assay

Cytokine expression in brain tissue was examined by RNase protection assay (RPA) using the RiboQuant RPA kit (PharMingen). The following multiprobe template sets were used, each specific for rat cytokines: CK-1, which detects transcripts for IL-1α and -1β, TNF-β, IL-3, IL-4, IL-5, IL-6, IL-10, TNF-α, IL-2, and interferon (IFN)-γ; and CK-3, which detects IFN-β, TNF-β, granulocyte-macrophage colony stimulating factor (GM-CSF), transforming growth factor (TGF)-β_1, -β_2, and -β_3, lymphotoxin β (Ltβ),

TNF-α, macrophage migration inhibitory factor (MIF), and IFN-γ. Both template sets contained probes for the housekeeping genes L32 and GAPDH to serve as internal controls for the assay. Probes were synthesized using [α-^{33}P]UTP (New England Nuclear, Boston, MA) resulting in an average specific activity of 1×10^6 cpm/μl. The RNase protection procedure was carried out according to the manufacturer's instructions using 5 to 10 μg per sample of total RNA. Each assay included normal Lewis rat brain RNA and yeast tRNA as controls. Products were resolved on a 6% acrylamide gel, dried, and exposed to film (Kodak BioMax MR, Rochester, NY).

Results

Chemokine Expression Associated with Brain Abscesses in Vivo

The signals responsible for the initial recruitment of inflammatory cells into brain abscesses have not yet been investigated. The predominant cell type present within early abscess lesions are neutrophils, whereas macrophages begin to accumulate at day 4 after bacterial exposure.[4] We examined the expression of several CXC and CC chemokines to define those that may play a role in the recruitment of neutrophils and macrophages into brain abscess lesions. Consistent with the observed neutrophil influx into early abscess lesions, KC expression was enhanced in animals implanted with *S. aureus*-containing beads at 24 hours, with levels remaining elevated up to 4 days (Figure 1A). This finding suggests that KC may be important for neutrophil recruitment into brain abscesses *in vivo.* Similarly, the levels of IP-10, a CXC chemoattractant, were up-regulated in the brains of animals receiving *S. aureus* beads within 24 hours (Figure 1A). This increase in IP-10 expression preceded the appearance of macrophages and lymphocytes into the abscess.

In addition, we have examined the expression of three monocyte-attracting CC chemokines, including MIP-1α, MCP-1, and RANTES, by RT-PCR. As shown in Figure 1B, MCP-1 and MIP-1α were elevated within 24 hours after *S. aureus* exposure. This increase in CC chemokine expression precedes the first detectable accumulation of macrophages in the abscess, which occurs at days 4 and 5.[4] At time points before 24 hours, we found no significant differences in the levels of chemokine expression between *S. aureus*- and sterile bead-implanted tissues (data not shown). However, chemokine induction in response to sterile beads was transient, with levels beginning to decline by 24 hours compared to the continued expression of MCP-1, MIP-1α, KC, and IP-10 in *S. aureus*-implanted tissues. There was no significant difference in the amount of MIP-2, RANTES, or fractalkine expression between *S. aureus*- and sterile bead-implanted tissues (data not shown). Importantly, we were not able to detect any of the chemokines examined here in normal Lewis rat brain (data not shown). These results suggest that KC, IP-10, MIP-1α, and MCP-1 participate in inflammatory cell recruitment into experimental brain ab-
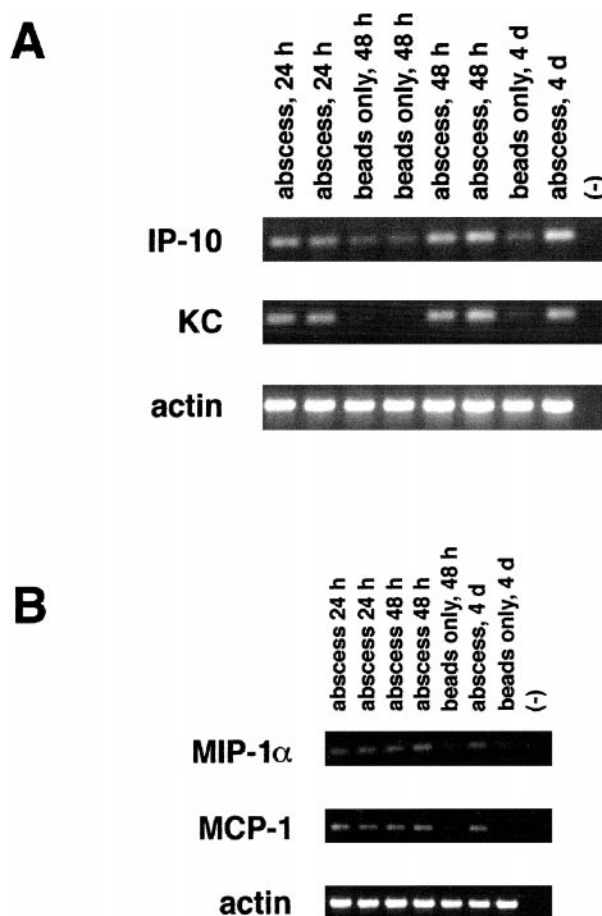

Figure 1. CXC and CC chemokine expression are up-regulated in acute experimental brain abscess. Adult Lewis rats were implanted with *S. aureus*-containing or sterile beads. Tissues were collected from the lesion site of individual animals at the indicated time points for RNA extraction and RT-PCR. A total of 100 ng of DNase I-treated RNA was added to each RT reaction. A total of 35 cycles of PCR were conducted using primers specific for either the CXC chemokines KC and IP-10 (**A**), or the CC chemokines MIP-1α and MCP-1 (**B**). Amplification using primers specific for β-actin was included to account for uniformity in the amount of input RNA and gel loading. Primer controls are denoted by ($-$). "Abscess" refers to animals receiving *S. aureus*-containing agarose beads compared to those implanted with sterile beads.

scesses. In turn, newly recruited neutrophils and macrophages can amplify the immune response through the synthesis of proinflammatory cytokines and chemokines.

Proinflammatory Cytokines Are Expressed during the Acute Phase of Experimental Brain Abscesses in Vivo

To define the array of cytokines produced during the early phase of brain abscess development, RNase protection assays (RPA) were performed on total RNA isolated from *S. aureus*- and sterile bead-implanted tissues. Figure 2A shows the proinflammatory cytokines that are expressed within 24 hours after *S. aureus* exposure. These include IL-1α, IL-1β, IL-6, and TNF-α. We also found elevated levels of TGF-β1 and lymphotoxin β (Ltβ) in abscess tissues at all time points examined (Figure 2B). During the acute phase of abscess induction, we did
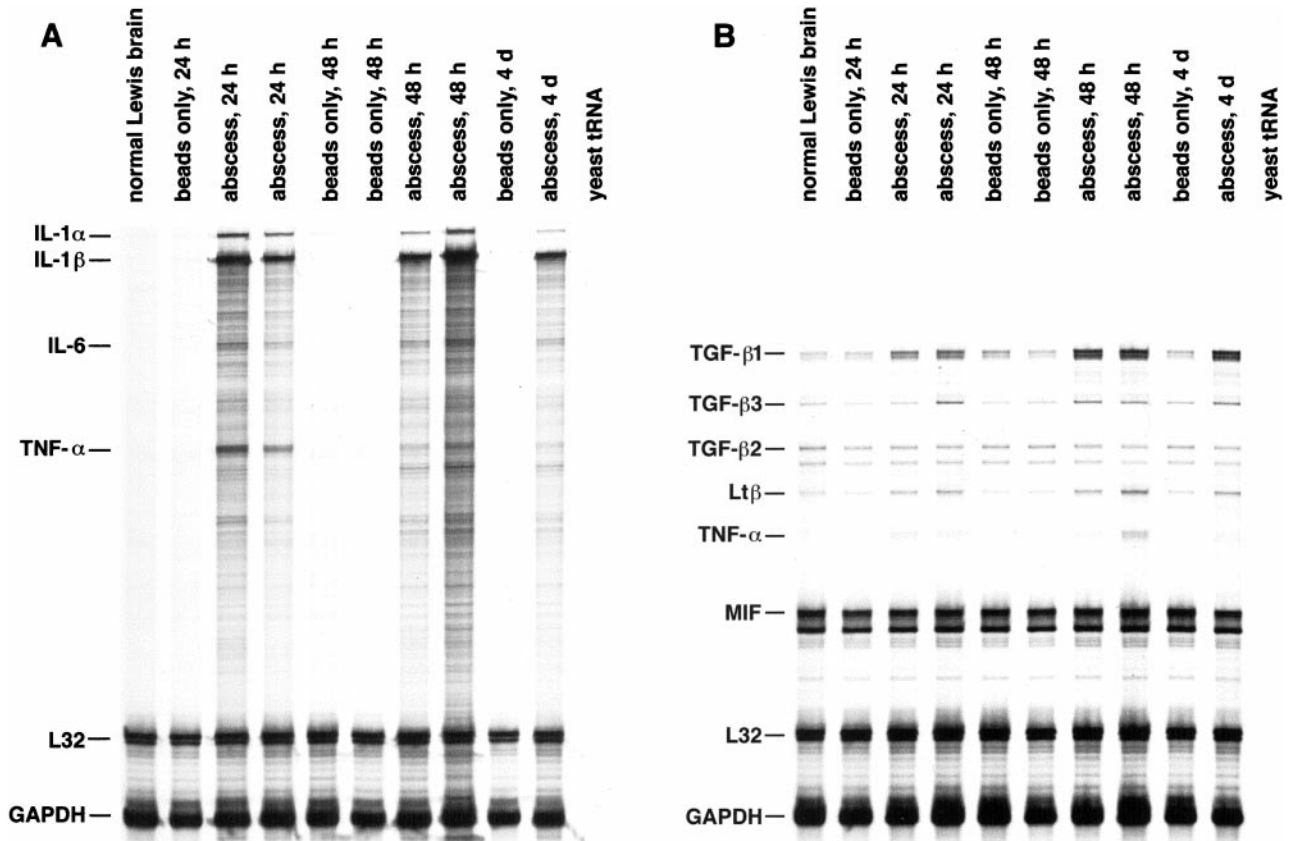
Figure 2. Proinflammatory cytokines are expressed during the acute phase of experimental brain abscess. Adult Lewis rats were implanted with *S. aureus*-containing or sterile beads. Tissues were collected from the lesion site of individual animals at the indicated time points for RNA extraction. A total of 10 μg of RNA from *S. aureus*- or sterile bead-implanted tissues was used in RPA with ^{33}P-labeled riboprobe templates. Controls included yeast tRNA and normal Lewis rat brain RNA. The housekeeping genes L32 and GAPDH serve as internal controls for the assay. **A:** RPA of *S. aureus*- or sterile bead-implanted tissues with the rat CK1 probe set. This probe set detects mRNAs for IL-1α and -1β, TNF-τ and -β, IL-2, -3, -4, -5, -6, and -10, and IFN-γ. **B:** RPA of *S. aureus* or sterile bead implanted tissues with the rat CK3 probe set. This probe set detects mRNAs for TNF-α and β, GM-CSF, IFN-β and -γ, TGF-$β_1$, -$β_2$, and -$β_3$, Ltβ, and MIF. The identity of RNase-protected bands in the experimental samples are determined by plotting a standard curve of the migration distance *versus* log nucleotide length of the undigested probes (not shown). The size of experimental mRNAs are extrapolated from this standard curve and are denoted at the left.

not observe the expression of any T cell-derived cyto-kines, such as IL-2 and IFN-γ. This finding is consistent with the absence of a significant lymphocytic infiltrate early in abscess pathogenesis. *S. aureus*- and sterile bead-implanted tissues and normal Lewis rat brain were found to express equivalent amounts of mRNA for TGF-$β_2$, TGF-$β_3$, and MIF (Figure 2B). Collectively, these find-ings reveal activation of a potent proinflammatory re-sponse to *S. aureus* in the brain.

Kinetics of Proinflammatory Cytokine Induction after S. aureus Exposure

To define the early kinetics of proinflammatory cytokine induction in response to *S. aureus,* RNA was isolated from *S. aureus*- or sterile bead-implanted tissues from 1 to 24 hours after treatment and evaluated by RPA. As shown in Figure 3, the induction of IL-1α and β was immediate, with detectable levels observed as early as 1 hour after *S. aureus* exposure. The amount of IL-1α and β detected in *S. aureus*-implanted tissues progressively increased with time and still remained strong at 4 days (Figures 3 and 2A). Sterile beads induced a transient increase in

IL-1α and -1β that was first evident 3 hours after treat-ment. However, the amount of IL-1α and -1β detected in sterile bead-implanted tissues was significantly less than that observed after *S. aureus* exposure, and the response was short-lived in the former, with expression disappear-ing within 18 hours (Figure 3). Both TNF-α and IL-6 were induced within 3 to 6 hours after *S. aureus* exposure with levels remaining elevated until 4 days (Figures 3 and 2A). As expected, normal Lewis rat brain did not express any of these proinflammatory cytokines (Figure 3). The imme-diate induction of IL-1α and β, TNF-α, and IL-6 suggest that these mediators play an important role in the early response to *S. aureus* in the brain.

Identification of Cell Types Producing IL-1β in Early Brain Abscess Lesions

We have established by RPA that *S. aureus* leads to the induction of proinflammatory cytokine and chemokine ex-pression in the brain. In an attempt to identify the cellular source(s) of IL-1β, ISH and immunohistochemical stain-ing of *S. aureus*-implanted tissues were conducted. IL-1β was selected because of its rapid induction and high
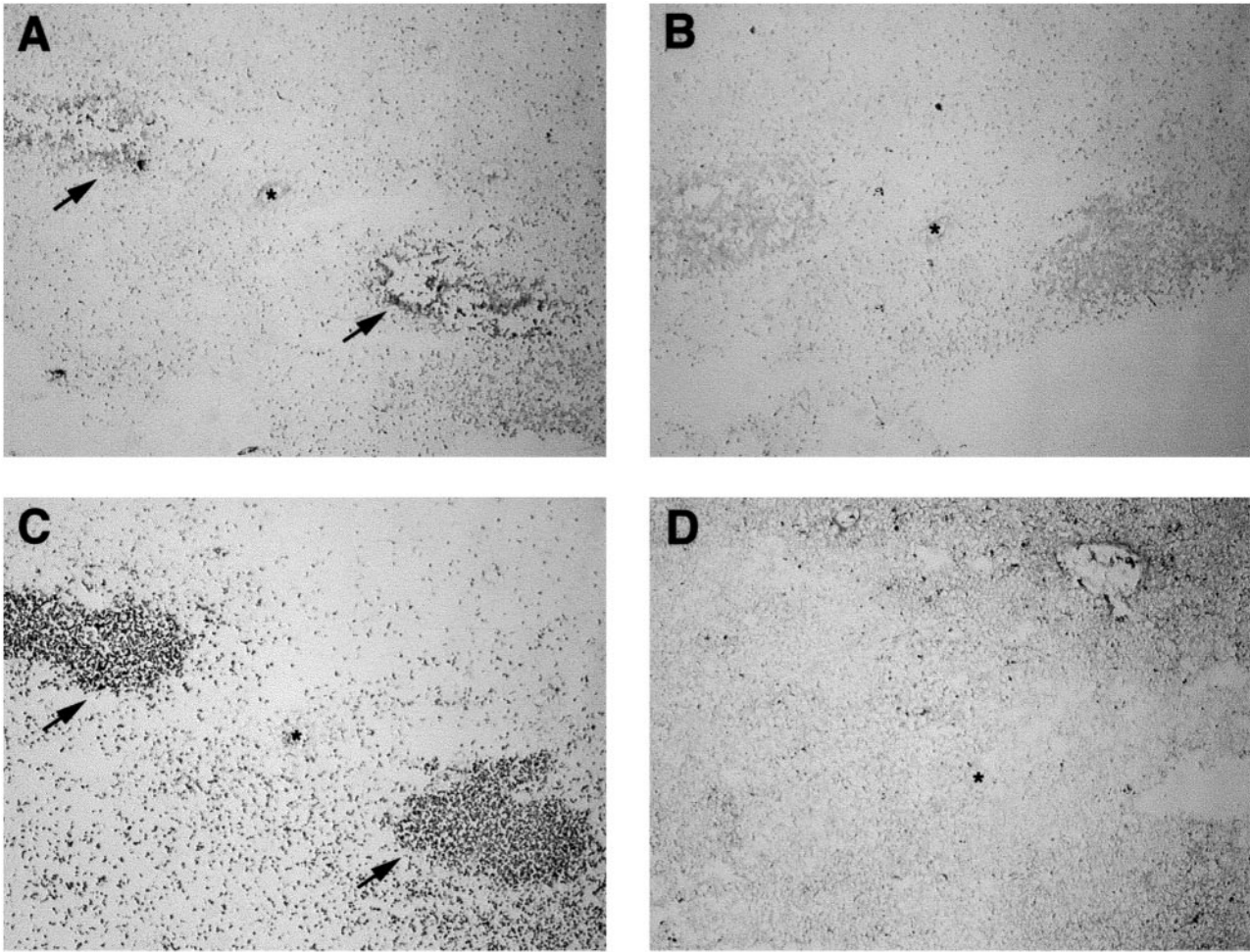
Figure 4. IL-1β-producing cells colocalize with CD11b (OX-42)-positive cells in brain abscess lesions. Adult Lewis rats were implanted with *S. aureus*-containing beads and abscess lesions collected at 24 hours for *in situ* hybridization (ISH) and immunohistochemical (IHC) analysis. Serial sections (5 μm) of a 24-hour abscess lesion were subjected to ISH analysis with IL-1β anti-sense (**A**) or IL-1β sense (**B**) DIG-labeled riboprobes, or IHC staining with antibodies specific for rat CD11b (OX-42; **C**) or GFAP (**D**). **Asterisks** denote the lumen of a small vessel in each serial section. **Arrowheads** in **A** and **C** denote IL-1β+ and CD11b+ cells, respectively. Original magnification, ×22.5.

levels of mRNA expression based on RPA analysis. As shown in Figure 4, IL-1β-positive cells were found to colocalize with CD11b (OX-42) staining, suggesting that they are of the monocyte/macrophage lineage (ie, resident microglia and infiltrating monocytes) or infiltrating polymorphonuclear leukocytes (PMNs). Specificity was confirmed by the inability of an IL-1β sense riboprobe to react with abscess tissue (Figure 4B). MCP-1 was not detected in abscess lesions by ISH, possibly a result of the decreased sensitivity of this method compared to RT-PCR (data not shown). To confirm the presence of IL-1β protein within the developing abscess, tissues were stained with an IL-1β-specific antibody. As shown in Figure 5, numerous IL-1β-positive cells were associated with the area of active inflammation in *S. aureus*-implanted

tissues at both 24 and 48 hours. In contrast, no IL-1β-positive cells were observed in tissues receiving sterile beads (Figure 5). These results indicate that IL-1β mRNA and protein are abundantly expressed within early abscess lesions by CD11b-positive cells.

Characterization of Adhesion Molecule Expression on Brain Microvascular Endothelial Cells after S. aureus Exposure

Because proinflammatory cytokines such as TNF-α and IL-1 are known to induce adhesion molecule expression on endothelial cells[15–17] and because these cytokines are induced soon after *S. aureus* exposure, we were

Figure 3. *S. aureus* leads to the rapid induction of proinflammatory cytokine gene expression. Adult Lewis rats were implanted with *S. aureus*-containing or sterile beads. Tissues were collected from the lesion site of individual animals at the indicated time points for RNA extraction. A total of 5 μg of RNA from *S. aureus* or sterile bead implanted tissues was used in RPA with ^{33}P-labeled ribroprobe templates. Controls included yeast tRNA and normal Lewis rat brain RNA. The housekeeping genes L32 and GAPDH serve as internal controls for the assay. The identity of RNase-protected bands in the experimental samples are determined by plotting a standard curve of the migration distance *versus* log nucleotide length of the undigested probes (not shown). The size of experimental mRNAs are extrapolated from this standard curve and are denoted at the left.
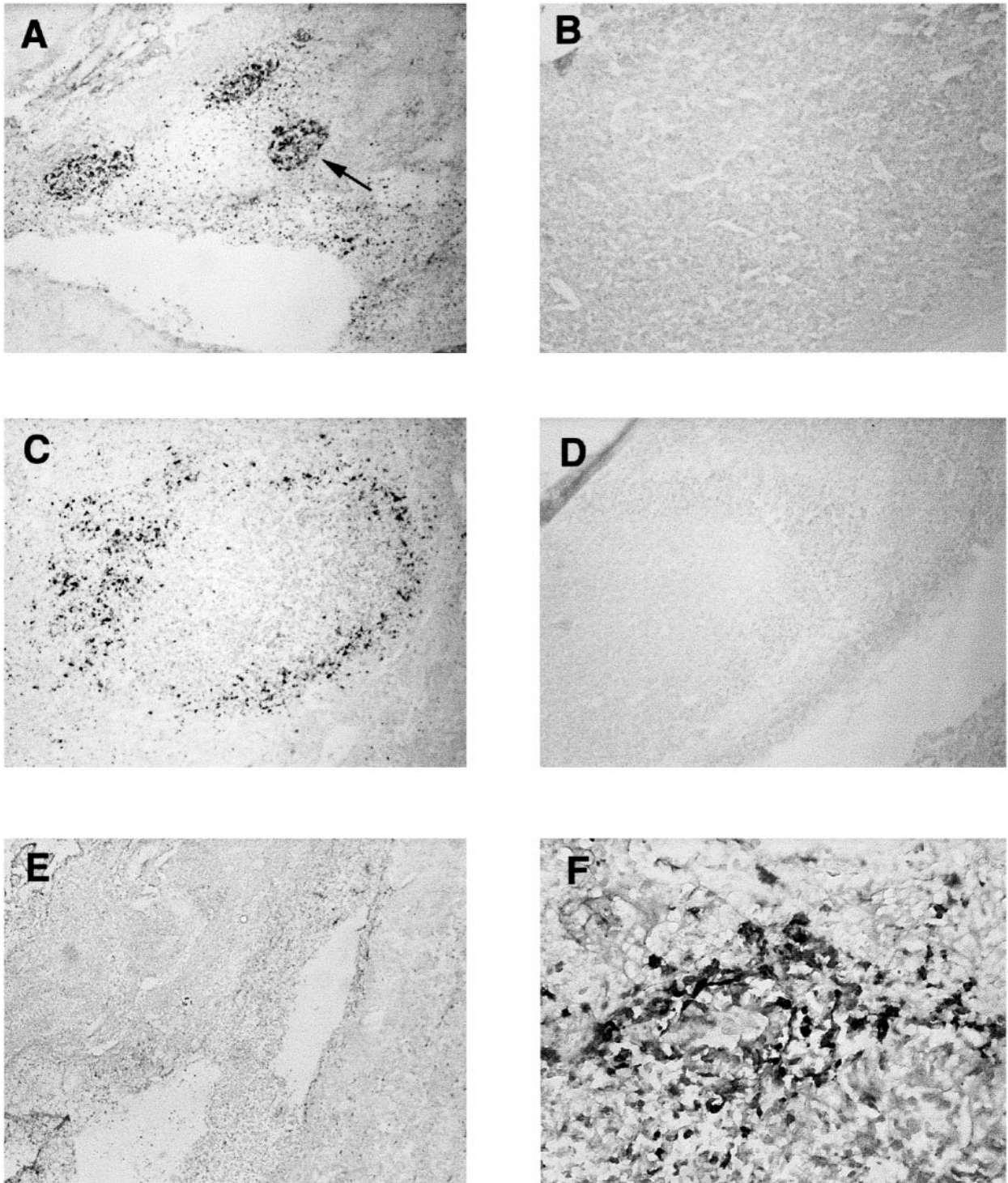
Figure 5. IL-1β protein is specifically expressed within *S. aureus* injected tissues. Adult Lewis rats were implanted with *S. aureus*-containing or sterile beads. Brain sections from animals perfusion-fixed with 4% paraformaldehyde/0.1 mol/L phosphate buffer were stained with antibody specific for IL-1β (**A–D**, **F**) or an isotype-matched control antibody (**E**) for immunohistochemical analysis. **A** and **C:** *S. aureus*-implanted tissues at 24 and 48 hours, respectively, stained with anti-IL-1β. **B** and **D:** Sterile bead-implanted tissues at 24 and 48 hours, respectively, stained with anti-IL-1β. **E:** *S. aureus* implanted tissue at 24 h stained with an isotype-matched control antibody **F:** higher magnification (×100) of IL-1β+ cells within *S. aureus* implanted tissue at 24 hours. The **arrow** in **A** depicts one of the three IL-1β+ foci within the area of active inflammation. Original magnification (**A–E**), ×22.5.

interested in examining adhesion molecule expression in *S. aureus*- and sterile bead-implanted tissues. The activation-induced adhesion molecules ICAM-1, P-selectin, and VCAM were evaluated, as were PECAM and the leukocyte activation marker L-selectin. As shown in Figure 6, *S. aureus* induced PECAM expression on brain vascular endothelial cells at 24 and 48 hours, whereas vessels associated with sterile bead-implanted tissues
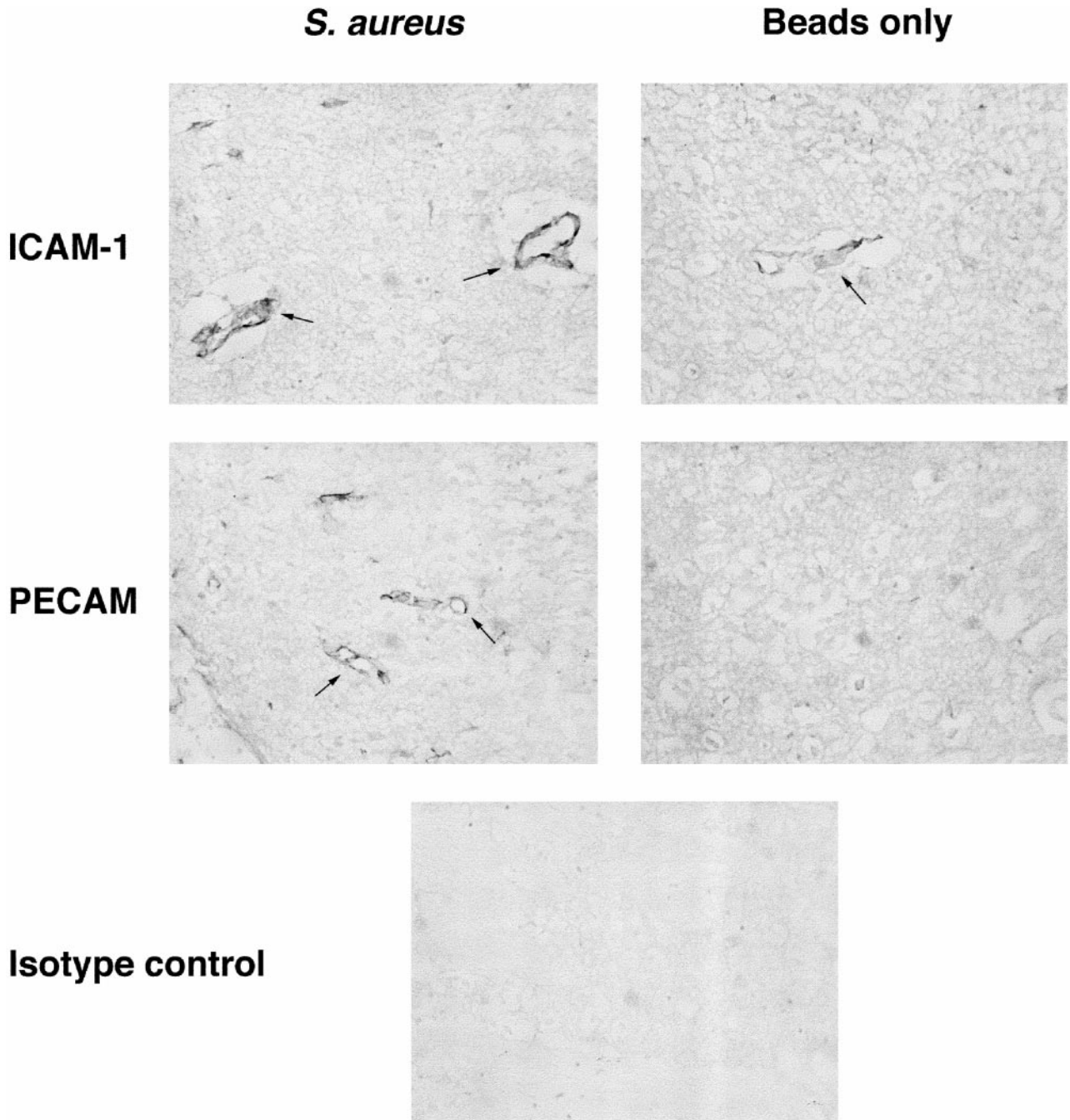
S. aureus   Beads only

ICAM-1

PECAM

Isotype control

Figure 6. Expression of both PECAM and ICAM-1 is up-regulated on vascular endothelium in early brain abscess lesions. Adult Lewis rats were implanted with *S. aureus*-containing or sterile beads. Brain sections from animals perfusion-fixed with 4% paraformaldehyde/0.1 mol/L phosphate buffer were stained with antibodies specific for PECAM or ICAM-1 or an isotype-matched control antibody for immunohistochemical analysis. Images shown represent tissues collected at 48 hours after implantation of *S. aureus* or sterile beads. **Arrows** delineate vascular endothelium expressing PECAM or ICAM-1. Original magnification, ×100.

were negative. Small vessels in both *S. aureus*- and sterile bead-implanted tissues were found to express detectable levels of ICAM-1 (Figure 6), however, the distribution of ICAM-1-positive vessels was distinct. ICAM-1-positive vessels in *S. aureus*-implanted tissues were distributed diffusely throughout the injected hemisphere, compared to the restriction of ICAM-1-positive vessels associated with sterile beads to the vicinity of the stab wound. Both PECAM and ICAM-1 are important adhesion molecules for the extravasation of neutrophils and monocytes into

tissues,[17,18] the predominant cell types found infiltrating early brain abscess lesions. Vessels in both *S. aureus*- and sterile bead-implanted tissues were negative for VCAM and P-selectin during the period examined, and L-selectin expression was not detected on infiltrating leukocytes in either tissue (data not shown). These results reveal the induction/up-regulation of PECAM and ICAM-1 expression on brain vascular endothelial cells in response to *S. aureus,* and suggest that they are critical to the evolving pathological process.

Discussion

Identification of the cytokines and chemokines expressed during the acute phase of brain abscess pathogenesis has not previously been investigated. In this paper, we have characterized a number of cytokines and chemokines that are induced immediately after the introduction of *S. aureus* into the brain.

Within 24 hours after *S. aureus* implantation, KC, IP-10, MIP-1α, and MCP-1 expression were elevated compared to animals implanted with sterile agarose beads. We found that the introduction of sterile beads into the brain induced transient chemokine expression, which began to decline around 24 hours. This effect may be a consequence of direct damage to the blood-brain barrier during bead administration. In contrast, chemokine levels in *S. aureus*-implanted brain remained elevated up to 4 days, suggesting the rapid and persistent establishment of a strong chemotactic gradient that parallels the continued presence of bacteria and their by-products. It appears as if the sustained production of chemokines is required for leukocyte entry into tissues because, although sterile beads induce transient chemokine expression, there is no accumulation of inflammatory cells in these tissues[4] (Kielian, unpublished observations). The chemokines we have detected in our brain abscess model most likely serve as signals for recruitment of neutrophils and monocytes, cells which are important for effective bacterial neutralization and eventual abscess resolution. Chemokines could also serve to facilitate the phagocytic function of glial cells, aiding in the destruction of *S. aureus* and eliminating debris from the abscess site. In addition to serving as chemoattractants, chemokines can activate target cell populations by inducing a rapid and transient calcium flux. It is possible that chemokines induced in response to *S. aureus* serve to regulate mediator production in responding cell populations in the brain, although this remains to be determined.

Pyogenic bacteria, such as *S. aureus,* elicit a potent innate immune response typified by the production of proinflammatory cytokines such as IL-1α, IL-1β, TNF-α, and IL-6.[19,20] Indeed, we have detected these proinflammatory cytokines in the rat brain immediately after *S. aureus* exposure. These cytokines can also exert pleiotropic effects on cells within the CNS. For example, both IL-1β and TNF-α act as potent stimulators of cytokine and chemokine expression for both astrocytes and microglia.[21–24] TNF-α and IL-1 can also stimulate IL-6 production by astrocytes which, in turn, acts in an autocrine matter to potentiate IL-6 release.[25] Due to this complex regulation of proinflammatory mediators, it is not unreasonable to predict that if not tightly controlled, amplification of the immune response would result in damage to normal host tissue. Indeed, in addition to bacterial neutralization, the immune response induced by *S. aureus* leads to the destruction of a significant portion of normal brain tissue in proximity to the bacteria. Evidence in support of this is demonstrated by several studies revealing the detrimental effects of IL-1β, TNF-α, and IL-6 on the CNS. For example, in concert with IFN-γ, TNF-γ activates murine microglia to produce toxic reactive nitrogen intermediates.[26,27] Exposure of the CNS vasculature to IL-1β and

TNF-α leads to increased adhesion and migration of inflammatory cells into the CNS and alterations in vascular permeability which may persist for several weeks.[28,29] TNF-α has been shown to indirectly mediate neuronal cell injury[30] and is toxic to oligodendrocytes.[31,32] Finally, overexpression of IL-6 in the CNS can lead to several detrimental conditions including reactive gliosis, neurodegeneration, breakdown of the blood-brain barrier, angiogenesis, and chronic expression of C3.[33,34] Studies are ongoing to directly assess the importance of individual proinflammatory cytokines in the initiation and progression of brain abscess using *in vivo* Ab neutralization experiments. Nevertheless, it is likely that a complex interaction exists between IL-1α, β, TNF-α, and IL-6, bacterial neutralization, and destruction of normal brain parenchyma.

Previous studies have described chemokine receptor expression on those cell types associated with brain abscesses *in vivo*.[35] For example, monocytes express CCR5 whose natural ligands include MIP-1α, MIP-1β, and RANTES.[6,7] Microglia[36–38] and astrocytes[39,40] have also been shown to express CCR5, which may contribute, in part, to the activation of these cells in areas immediately surrounding the developing abscess. One characteristic of chemokines is their promiscuity in terms of their ability to bind multiple receptors. In addition to CCR5, monocytes and microglia express CCR1 which also binds MIP-1α with high affinity. Both microglia and monocytes express CCR2, the only known receptor for MCP-1. We do not currently know whether CCR1, CCR2, and/or CCR5 are important for monocyte recruitment into evolving brain abscesses. Receptors for the proinflammatory cytokines TNF-α and IL-1 have been shown to be constitutively expressed on some neurons and glial cells in the rat brain.[41] Based on this pattern of receptor expression, it is intriguing to consider what effect IL-1 and TNF-α might exert on neurons in our brain abscess model. In this case, receptor expression may be detrimental, leading to neuronal cell death via signaling through TNFR. In addition to glial and neuronal cells, TNFR is expressed by monocytes/macrophages which are found infiltrating abscess lesions.[42,43] Activation of monocytes/macrophages via TNF-α-dependent signaling leads to the synthesis of nitric oxide[44] and cytokine and chemokine mediators[45–47] which can amplify the immune response. In the CNS, some neurons and astrocytes express IL-6R. However, the amount of IL-6R on astrocytes is not sufficient by itself to deliver an activation signal, and requires the presence of a soluble form of the IL-6R generated by shedding of the membrane-bound receptor or by mRNA alternative splicing.[25,48] Interestingly, astrocytes and sympathetic neurons can produce IL-6 and may respond to it in an autocrine/paracrine manner if sIL-6R is present,[25,48,49] a mechanism for amplifying IL-6 expression in the CNS. We are actively investigating proinflammatory cytokine receptor expression to identify which cell populations are competent to respond to the mediators detected in early brain abscess lesions.

The cell types responsible for proinflammatory cytokine and chemokine production in experimental brain abscesses remain to be defined. Their rapid induction

suggests that the response is driven by resident CNS glia or cells associated with the ensuing inflammatory infiltrate (ie, neutrophils and/or monocytes/macrophages). Results from ISH analysis revealed that CD11b+ cells are the source of IL-1β within early brain abscesses. This population includes resident microglia and infiltrating monocytes and neutrophils. In agreement with our findings, it has been shown that human microglia can produce IL-1β,[50,51] and *S. aureus* has been shown to directly stimulate IL-1β production by neonatal rat microglia *in vitro*.[24] We have preliminary data (Kielian, unpublished observations) which demonstrates that *S. aureus* can directly induce neonatal mouse microglia and astrocytes to produce numerous proinflammatory cytokines and chemokines including IL-1β, TNF-α, IL-6, MCP-1, MIP-1α, MIP-1β, MIP-2, RANTES, and MIP-1α. Studies are ongoing to further identify the cell types within developing brain abscesses responsible for mediator production.

The question remains, what is the impetus driving abscess formation and can the progression of the abscess and immunopathological destruction of surrounding normal brain tissue be minimized by altering the production of proinflammatory cytokines? The chronic overexpression of proinflammatory cytokines initially induced in response to *S. aureus* may itself have a direct detrimental effect on the surrounding normal brain parenchyma, ultimately leading to tissue destruction. The uncontrolled amplification of the immune response to *S. aureus* can be explained by the autocrine/paracrine action of proinflammatory cytokines on resident glial cell populations in the brain. For example, IL-1 could stimulate astrocyte activation and production of TNF-α and IL-6,[21,52–54] whereas activated microglia produce IL-1 and TNF-α.[50,51] It is possible that a tissue toxic microenvironment might evolve from the robust production of proinflammatory cytokines immediately following exposure to *S. aureus*, as shown in this study. This overproduction of proinflammatory cytokines could result in a detrimental or cytocidal bystander effect, leading to the damage of surrounding normal brain parenchyma. Thus, the process of neural tissue necrosis may be underway before the arrival of large numbers of neutrophils. By controlling the amount of proinflammatory cytokine production in response to *S. aureus*, this could lead to bacterial neutralization while minimizing tissue destruction due to the chronic overactive immune response.

In summary, the results presented in this report establish the expression of several proinflammatory cytokines and chemokines within early brain abscess lesions which may participate in cell activation and amplification of the immune response initiated by *S. aureus*. Characterization of inflammatory mediators produced in response to *S. aureus* in the brain may lead to novel therapies for the clinical management of brain abscesses.

Acknowledgments

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