

Mononuclear Leukocytes Preferentially Bind via CD44 to Hyaluronan on Human Intestinal Mucosal Smooth Muscle Cells after Virus Infection or Treatment with Poly(I·C)*

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Pathological changes in inflammatory bowel disease include an increase in intestinal mucosal mononuclear leukocytes and hyperplasia of the muscularis mucosae smooth muscle cells (M-SMCs). Because virus infections have correlated with disease flare, we tested the response of cultured M-SMCs to respiratory syncytial virus, measles virus, and the viral analogue, poly(I·C). Adhesion of U937 cells and peripheral blood mononuclear cells was used to measure the leukocyte-interactive potential of M-SMCs. Untreated M-SMCs, only minimally adhesive for leukocytes, bound U937 cells after treatment with respiratory syncytial virus or measles virus. Mononuclear leukocytes also bound to poly(I·C)-treated M-SMCs. Although both vascular cell adhesion molecule-1 mRNA and protein increased 3–4-fold in poly(I·C)-treated M-SMC cultures, U937 cell adhesion was not blocked by an anti-vascular cell adhesion molecule-1 monoclonal antibody. However, hyaluronidase digestion of poly(I·C)- or virus-treated M-SMCs dramatically reduced leukocyte adhesion (~75%). Fluorophore-assisted carbohydrate electrophoresis demonstrated a ~3-fold increase in surface-bound hyaluronan on poly(I·C)-treated M-SMCs compared with untreated controls. In addition, pretreatment of mononuclear cells with a blocking anti-CD44 antibody, greatly decreased adhesion to poly(I·C)-treated M-SMCs. Recognition of this virus-induced hyaluronan/CD44 mechanism of mesenchymal cell/leukocyte interaction introduces a new avenue in the research of gut inflammation.

The origin of inflammatory bowel disease (IBD)¹ is multifactorial, where environmental and microbiological factors initiate and perpetuate an immune response in the intestine of genet-

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¹ The abbreviations used are: IBD, inflammatory bowel disease; M-SMC, mucosal smooth muscle cell; RSV, respiratory syncytial virus; MV, measles virus; poly(I·C), polyinosinic acid:polycytidylic acid; TNF- α , tumor necrosis factor- α ; BSS, balanced salt solution; FBS, fetal bovine serum; PBML, peripheral blood mononuclear leukocyte; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intracellular adhesion molecule-1; HLA-DR, human leukocyte antigen-DR; IFN, interferon; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T expressed and secreted; IL, interleukin; FITC, fluorescein isothiocyanate; SMC, smooth muscle cell.

ically susceptible individuals, which results in the clinical manifestations of Crohn's disease and ulcerative colitis. Recently, several IBD susceptibility genes have been identified (1, 2), supporting the genetic predisposition component of this theory. Immune phenomena involved in this disease have also been investigated extensively and have underscored the differences between the responses of normal and affected individuals (3, 4). However, much less is known about how microbial agents affect the disease process.

Speculations that viruses may be involved in the pathogenesis of IBD have been advanced for some time due to the clinical association of respiratory virus infections with subsequent IBD flare-up. In an extensive study, Kangro *et al.* (5) reported that 40% of disease flares in a population of susceptible individuals were temporally associated with documented viral respiratory infections. Other groups have demonstrated a higher incidence of measles virus particles in the resected colon tissue of patients with Crohn's disease as compared with tissue from patients who did not have IBD (6, 7). Very recently, Montgomery *et al.* (8), in a prospective study of over 7000 patients, found that the combination of measles and mumps infections in the same year of childhood is significantly associated with subsequent IBD. In a separate study, the simultaneous presence of DNA from Herpesvirus 6 and Epstein-Barr virus was detected more frequently in ulcerative colitis (76%) than in Crohn's disease or control tissues (9). Farmer *et al.* (10) reported a similar association between cytomegalovirus and ulcerative colitis.

Under normal circumstances, colonic mucosal tissue (lamina propria) contains a population of leukocytes, including T- and B-lymphocytes, plasma cells, histiocytes, and mast cells, which are scattered in a network of collagen fibers and smooth muscle cell bundles (11). These leukocytes arrive to the area via the regularly distributed capillaries in the lamina propria. They serve a surveillance function in the tissue, providing immune protection against the luminal contents of the colon. Mucosal lymphocytes may reenter the bloodstream, presumably via the lymphatic vessels located in close proximity to the muscularis mucosae, and are free to recirculate through blood and lymphoid organs until a specific antigenic challenge recalls them to an affected area (12).

In IBD, the mucosal immune cell population increases dramatically, and the infiltrate is predominantly mononuclear leukocytes. Further, a hyperplastic thickening of the juxtaposed muscularis mucosae also occurs (13). This suggests that interactions between leukocytes and mesenchymal smooth muscle cells are important in the development of IBD. We (14) have recently shown that colonic mesenchymal cells proliferate in response to leukocyte-derived inflammatory cytokines. Increasingly, however, investigators are finding evidence for bi-

directional interaction and communication between smooth muscle cells and immune cells within tissues, events that can play a role not only in IBD (15, 16), but in other chronic inflammatory diseases (17). Therefore, we investigated the impact that virus infection can have on one of the early events in the colon's inflammatory process, namely leukocyte interaction with mucosal smooth muscle cells (M-SMCs) via leukocyte adhesion molecules.

Our data indicate that virus infection dramatically increases the level of mononuclear leukocyte adhesion to M-SMCs through a distinctly different mechanism of interaction from that involved in leukocyte adhesion after treating M-SMCs with inflammatory cytokines (18). The data indicate that respiratory syncytial virus (RSV), measles virus and the viral mimic, poly(I-C), up-regulate leukocyte adhesion primarily through a novel mechanism involving hyaluronan interaction with CD44, a cell surface hyaluronan-binding protein expressed on many leukocytes.

MATERIALS AND METHODS

Cell Isolation and Culture—M-SMCs were isolated from human colon specimens (14) obtained within 1 h after resection, which were provided by the Surgical Pathology Department of the Cleveland Clinic Foundation. Briefly, the mucosal layer (lamina propria) of each colon was removed and cut into strips, washed in 50 ml of Hanks' BSS containing 0.15% dithiothreitol (w/v) for 30 min, washed three times in 100 ml of Hanks' BSS containing 1 mM EDTA for 1 h each, and Hanks' BSS alone for at least 2 h with a 100 ml/wash changed every 30 min. The tissue samples were then minced and digested overnight in 100 ml of Hanks' BSS containing collagenase and DNase (0.1 mg/ml each), penicillin (250 units/ml), streptomycin (250 µg/ml), and fungizone (0.625 µg/ml). The liberated cells were filtered from the undigested debris with a tissue screen, cultured in DMEM/F-12 medium supplemented with 10% FBS (Bio-Whittaker, Walkersville, MD) and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone), and by incubation at 37 °C, in a 5% CO₂, humidified environment. One 75-cm² flask, containing 15 ml of medium, was seeded with the cells obtained from an ~125-cm² area of original tissue. Two to 3 days after plating, the non-adherent cells were washed away, and the culture fluid replenished. When cell cultures were confluent (~10 days), they were split at a 1:3 ratio. Cultured M-SMCs obtained by this method routinely stain positively for α-smooth muscle cell actin (antibody from Sigma/Aldrich). M-SMC cultures were used in the first through fourth passages.

U937 cells, originally derived from a human histiocytic lymphoma, were procured from the American Type Culture Collection (Rockville, MD). The cells were grown in suspension culture in RPMI medium containing 5% FBS and routinely subcultured at a 1:5 ratio (~2 × 10⁶ cells/ml) three times per week.

Infection of M-SMCs with RSV and Measles Virus—Infectious isolates of RSV and measles virus were obtained from the Clinical Virology Laboratory at the Cleveland Clinic Foundation. Each virus was grown in clinical indicator cell lines (HEP-2 cells for RSV and primary rhesus monkey kidney cells for measles virus), and aliquots of supernatant fluids from these cells were passed directly to the confluent cultures of M-SMCs at the concentrations specified in the figures. The cultures were incubated at 37 °C for the times indicated. Infection of smooth muscle cells was confirmed on acetone-fixed coverslips from parallel cultures by fluorescence immunohistochemistry. Coverslips were treated with blends of monoclonal antibodies against RSV or measles virus (Chemicon, Temecula, CA) at a 1:200 dilution, washed and treated with a FITC-conjugated goat anti-mouse Ig at 1:200, then counterstained with Evan's Blue dye (0.2%) to identify the cell layer, and observed with a fluorescence microscope.

Separation of Human Leukocytes—Total mononuclear cells were separated from heparinized peripheral blood (100 units heparin/ml) by centrifugation on Ficoll-Hypaque density gradients (19). The isolated peripheral blood mononuclear leukocytes (PBMLs) were resuspended in RPMI 1640 supplemented with 5% FBS (25–50 × 10⁶ cells/ml) in a Teflon beaker to prevent attachment during labeling. Viability of the PBMLs was always greater than 95%, as determined by trypan blue dye exclusion.

Neutrophils in the pellet of the Ficoll-Hypaque gradient were further purified according to the method of Stossel *et al.* (20) using sedimentation in a dextran gradient and hypotonic lysis of residual erythrocytes.

Cells isolated by this procedure were routinely greater than 95% neutrophils, as estimated by differential counting.

Assay for Leukocyte Adhesion to M-SMCs—Adhesion of U937 cells to M-SMCs was measured as described previously (21). Briefly, M-SMCs were plated into 24-well plates in their appropriate medium (~2.5 × 10⁴ cells/well in 0.5 ml) 3–5 days before the assay, and grown to confluence. Unless otherwise noted in the figure legends, treatment of M-SMCs with poly(I-C) (10 µg/ml), TNF-α (1 ng/ml), or live virus was done 18–24 h before assay. On the day of the assay, U937 cells or normal human monocytes (up to 70 × 10⁶ cells/ml) were labeled for 90 min at 37 °C with 100 µCi of ⁵¹Cr as sodium chromate (NEN Life Science Products) in 1 ml of culture medium. The labeled cells were washed three times with culture medium, counted on a hemacytometer, and resuspended to 10⁶ viable cells/0.5 ml of culture medium. Incubation medium was aspirated from M-SMCs, and 10⁶ labeled leukocytes were added per well. The binding phase of the assay was done at 4 °C for 1 h. Subsequently, the wells were washed three times with cold medium. The cells were lysed with 1% Triton X-100, and an aliquot removed for quantitation of radiolabel. The number of U937 cells or monocytes bound per well was calculated from the initial specific activity (cpm/cell). Spontaneous release of chromium from the monocyte cells in control incubations without M-SMCs was typically less than 5%.

Antibody Blocking of Leukocyte Adhesion—M-SMCs and leukocytes were prepared for the adhesion assay as described above, with the additional step of treating either the M-SMCs with a blocking monoclonal anti-VCAM-1 antibody (10 µg/ml) or the leukocytes with a blocking monoclonal anti-CD44 antibody (from clone A3D8) at the concentrations indicated in the figure legends. The antibody-treated cells and their untreated controls were incubated at 4 °C, 1 h before continuing with the assay. The number of leukocytes bound was determined as above.

M-SMC Expression of VCAM-1 and ICAM-1 Protein—M-SMCs were plated in 48-well plates and grown to confluence (3–4 days). The cells were treated as described in the figure legends, and cell surface protein expression of VCAM-1 and ICAM-1 determined as described previously (22). Briefly, at the time of the assay, the incubation medium was removed and the cells rinsed with DMEM/F-12 containing 2% FBS. Intact anti-human VCAM-1 antibody (Genzyme, Boston, MA), or anti-human ICAM-1 antibody (Novacastra), or anti-human HLA-DR antibody as an isotype-matched (IgG1) control antibody were added at a concentration of 5 µg/ml in wash medium (100 µl/well). The plates were then incubated at 4 °C for 1 h. After washing the wells three times with cold medium, biotin-conjugated, affinity-purified F(ab')₂ fragments from goat anti-mouse IgG + IgM (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was added to each well at a dilution of 1:1000 in wash medium (100 µl/well). The plate was incubated at 4 °C for 30 min. After washing three times with cold medium, a 1:80 dilution of ¹²⁵I-streptavidin (Amersham Pharmacia Biotech) solution added to each well (100 µl/well), followed by incubation at 4 °C for 15 min. Subsequently, the wells were washed four times with cold medium, the cells lysed with 1% Triton X-100, and an aliquot removed for radiolabel quantitation.

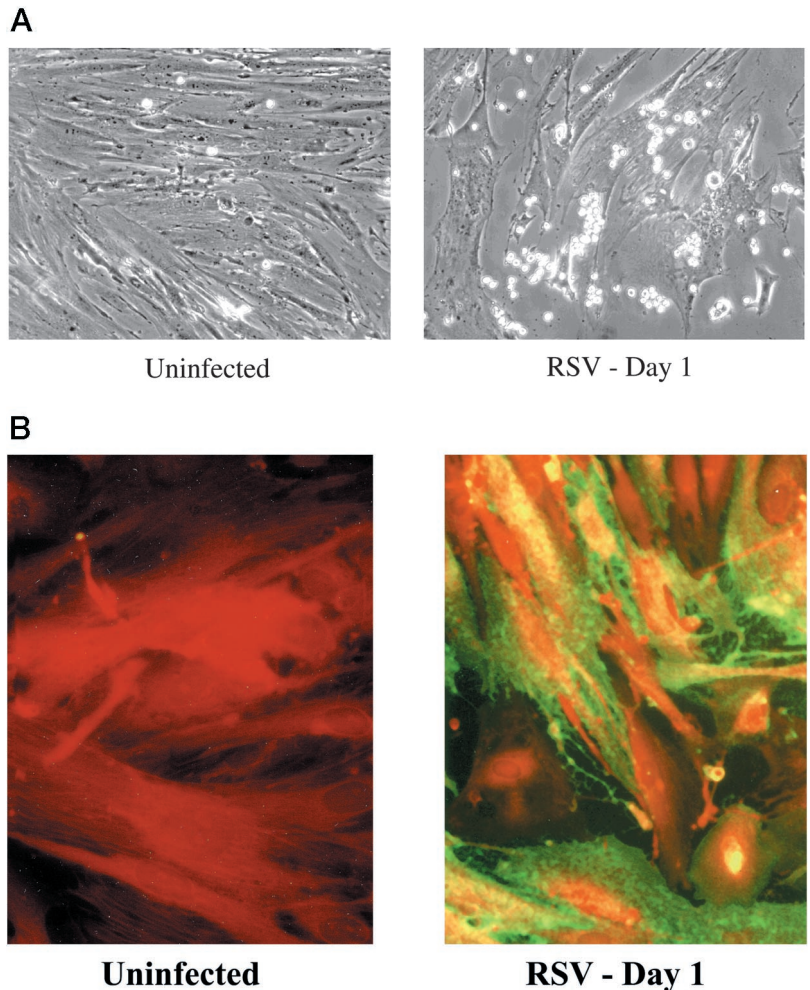
Northern Analysis—Total cellular RNA was isolated as described previously (14) from confluent M-SMCs grown in 75-cm² area flasks. Briefly, M-SMCs were lysed at ~5 × 10⁵ cells/ml with RNazol B (Tel-Test Inc., Friendswood, TX); 10% (v/v) chloroform was added. The samples were centrifuged at 12,000 × g for 15 min, and the aqueous layer recovered. RNA in the aqueous layer was precipitated in 50% isopropanol, and the precipitate pelleted by centrifugation at 12,000 × g for 10 min. The resulting RNA pellets were washed several times in 75% EtOH before dissolving in 0.1% diethyl pyrocarbonate-treated water. RNA samples (10 µg) were denatured with deionized glyoxal, size-fractionated by electrophoresis in 1% agarose, and transferred to a nylon membrane (GeneScreen) by electroblotting. Membranes were air-dried, UV light-cross-linked, and hybridized with a [³²P]dCTP-labeled, full-length probe for VCAM-1 RNA (23). The blots were then stripped and rehybridized by the same procedure using a probe for glyceraldehyde-3-phosphate dehydrogenase mRNA, to determine if sample loading was equivalent.

Hyaluronan Synthesis—Relative levels of hyaluronan synthesis by cultures of M-SMCs were determined by fluorophore-assisted carbohydrate electrophoresis as described in detail by Calabro *et al.*^{2,3} Briefly,

² Calabro, A., Benavides, M., Tammi, M., Hascall, V. C., and Midura, R. J. (1999) *Glycobiology*, in press.

³ Calabro, A., Hascall, V. C., and Midura, R. J. (1999) *Glycobiology*, in press.

FIG. 1. Effect of RSV infection on U937 cell adhesion to M-SMC. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without RSV (100 $\mu\text{l}/\text{cm}^2$ of supernatant collected from RSV-infected BHK cells) for 18 h at 37 °C. *A*, U937 cell adhesion assay was done as described under "Materials and Methods" and observed by phase contrast microscopy (magnification, $\times 100$). U937 cells appear as bright spheres on top of the M-SMCs, which are attached to the culture plate. *B*, coverslips taken from replicate wells prior to the adhesion assay were fixed, immunofluorescently labeled with RSV-specific antibodies and secondary FITC-conjugated antibody, counterstained (described under "Materials and Methods"), and observed by fluorescence microscopy (magnification, $\times 400$).



confluent M-SMC cultures were incubated with minimal essential medium containing 2% FBS, with or without poly(I-C) or TNF- α as described in the figure legends, and incubated for 18 h at 37 °C. The culture fluid and cell layer samples were then collected individually, and treated with proteinase K (0.125 mg/ml) and incubated for 2 h, 60 °C. An additional 50- μl aliquot containing 0.125 mg of enzyme was added to each sample and the incubation continued for 2 h. Proteinase K was inactivated by heating at 100 °C for 10 min. The samples were concentrated to 300 μl in a Speed-Vac centrifuge, and the glycosaminoglycans were precipitated by adding EtOH to 75%, followed by incubation at -20 °C, overnight. Each precipitate was collected by centrifugation and dissolved in 0.1 M ammonium acetate (0.1 ml) containing 0.0005% phenol red, pH 7. Hyaluronidase SD from *Streptococcus dysgalctiae* (final concentration 100 milliunits/ml) was added to each sample, followed by incubation for 1 h at 37 °C. Chondroitinase ABC (final concentration: 100 milliunits/ml) was then added, and subsequently incubated at 37 °C for 3 h. EtOH was then added to 90%, followed by incubation for 2 h at -20 °C to remove any remaining insoluble material. After centrifugation, the supernatants were collected and evaporated to dryness.

Hyaluronidase and chondroitinase digestion products were derivatized by addition of 12.5 mM 2-aminoacridone in 85% Me₂SO, 15% acetic acid for 15 min at ambient temperature. An equal volume of 1.25 M sodium cyanoborohydride in ultrapure water was then added and the incubation continued for 18 h at 37 °C. Glycerol was added (final concentration 20%), and the samples stored at 4 °C in the dark until analysis. Aliquots (5 μl) of each sample, along with derivatized disaccharide standards, were electrophoresed (500 V, 80 min) on MONOTM composition gels (Glyko, Novato, CA), in MONOTM gel running buffer, at 4 °C. The gels were visualized with a UV light transilluminator, imaged with a Quantix CCD camera, and the results analyzed using Gel-Pro AnalyzerTM software.

Reagents—All cell culture media, salt solutions, antibiotics, and the HLA-DR antibody were purchased from Life Technologies, Inc. FBS was purchased from Bio-Whittaker, Walkersville, MD. Poly(I-C) was a

product of Amersham Pharmacia Biotech, Uppsala, Sweden. Dithiothreitol and DNase were from Roche Molecular Biochemicals. TNF- α and VCAM-1 antibody were purchased from Genzyme, Boston, MA. Hyaluronidase SD, chondroitinase ABC, and purified hyaluronan fragments are products of Seikagaku America, Ijamsville, MD. GeneScreen membrane and radioisotopes were from NEN Life Science Products, except the ¹²⁵I-streptavidin was from Amersham Pharmacia Biotech. Bovine testicular hyaluronidase and the CD44 blocking antibody (A3D8) were from Sigma. The VCAM-1 cDNA probe was a generous gift of Dr. Walter Newman, Leukosite, Cambridge, MA.

RESULTS

Respiratory Syncytial Virus and Measles Virus Infect M-SMCs and Cause Increased Adhesiveness for Leukocytes—We used RSV and measles virus (MV) as initial agents to test the hypothesis that virus infection of M-SMCs can alter their interactions with leukocytes. The established U937 cell line was used as a model for leukocyte adhesion (21) since this monocytic tumor cell carries the ligand for most of the recognized leukocyte adhesion molecules (26). Photomicrographs (Fig. 1A) show that leukocytes bind in focal areas to M-SMCs 1 day after exposure to RSV, but did not bind to the untreated control cells. Immunofluorescent histochemistry with a specific anti-RSV antibody (Fig. 1B) revealed that nearly all of the M-SMCs were infected. By day 2 syncytia formed, and cell damage was evident; by day 4 the entire monolayer of cells was destroyed (data not shown).

MV, which infects cells more slowly, was also tested. Fig. 2A shows that leukocytes bound in focal areas to M-SMCs 1 day after infection with MV, but did not bind to untreated control cells. The number of focal areas of adhesion was infrequent, as

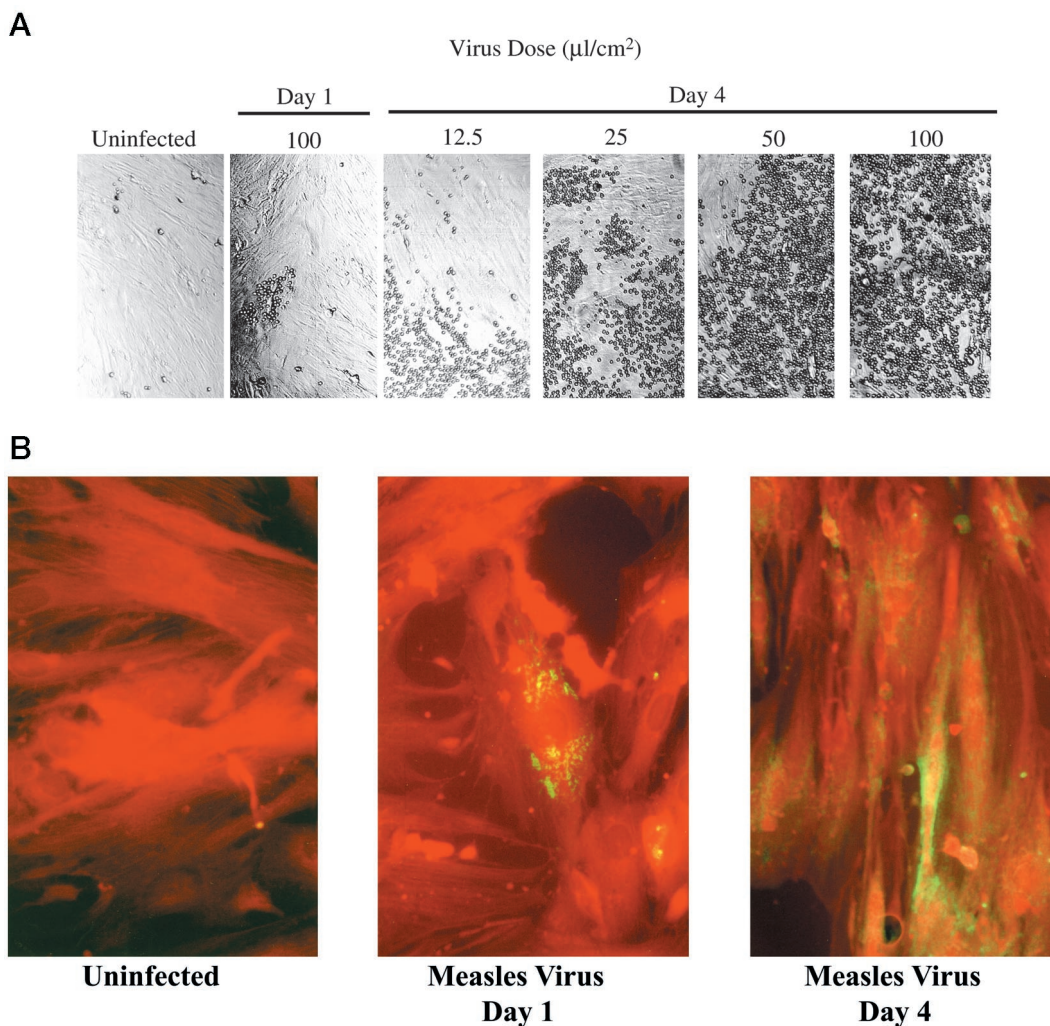


FIG. 2. Effect of measles virus infection on U937 cell adhesion to M-SMC. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without measles virus (at indicated concentrations) for 1 day or 4 days at 37 °C. *A*, the U937 cell adhesion assay was done as described under “Materials and Methods,” and observed by light microscopy (magnification, $\times 40$). U937 cells appear as *dark spheres* on top of the M-SMCs, which are attached to the culture plate. *B*, coverslips taken from replicate wells treated with medium or supernatant containing measles virus ($100 \mu\text{l}/\text{cm}^2$) for 1 day or 4 days were fixed, immunofluorescently labeled with measles virus-specific antibody and secondary FITC-conjugated antibody, counterstained (described under “Materials and Methods”), and observed by fluorescence microscopy (magnification, $\times 400$).

was the number of infected M-SMCs at this time point, as determined by immunohistochemistry with a MV-specific antibody (Fig. 2*B*). Each foci consisted of one or two M-SMCs with an adherent cluster of U937 cells. After 4 days of virus exposure, patches of infected M-SMCs were evident (Fig. 2*B*); leukocyte adhesion to the cell monolayer increased dramatically and in correlation to the original virus dose (Fig. 2*A*). Therefore, M-SMCs are susceptible to infection with RSV and MV, and virus infection alters their interaction with leukocytes.

Poly(I-C) Induces M-SMC Adhesiveness for Mononuclear Leukocytes—We next used an accepted viral mimic model (27), poly(I-C) (synthetic double-stranded RNA), to minimize the effects of infection efficiency and cell destruction inherent in live virus experiments. Fig. 3 demonstrates that treatment of M-SMCs with poly(I-C) (optimal concentration: $10 \mu\text{g}/\text{ml}$; optimal time: 18 h; data not shown) results in an ~ 8 -fold increase in adhesion of U937 cells and a ~ 12 -fold increase in adhesion of PBMLs compared with unstimulated controls. Conversely, adhesion of neutrophils to M-SMCs was only minimally affected by the poly(I-C) treatment, suggesting that the adhesion mechanism is specific for mononuclear leukocytes.

While double-stranded RNA is widely known as an inducer of interferons for many cell types, the ability of poly(I-C) to in-

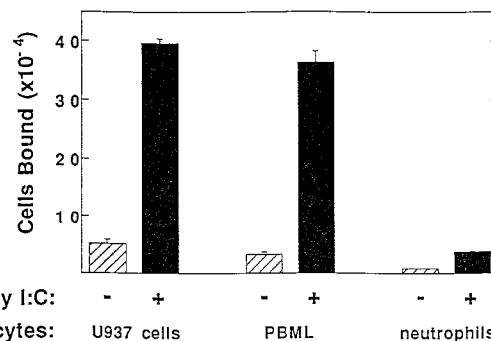


FIG. 3. Effect of poly(I-C) on adhesion of U937 cells, normal human PBMLs, and neutrophils to M-SMCs. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without poly(I-C) ($10 \mu\text{g}/\text{ml}$) for 18 h, 37 °C. Adhesion of U937 cells, PBMLs, and neutrophils was quantitated as described under “Materials and Methods.” (Values are the mean of triplicate wells \pm S.E.)

crease adhesiveness of M-SMCs for leukocytes appears to be direct, since IFN- α (100 units/ml) and IFN- γ (100 units/ml) were unable to induce leukocyte adhesion under identical culture conditions (data not shown).

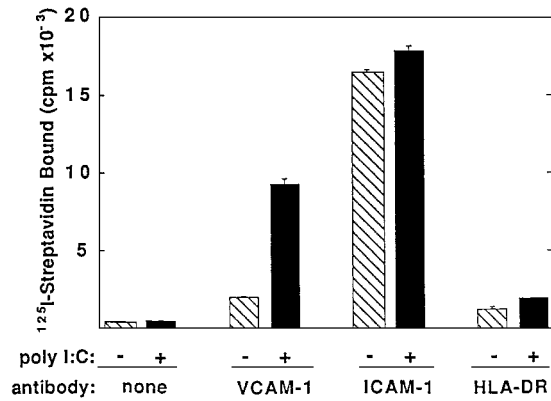


FIG. 4. Effect of poly(I:C) on M-SMC expression of cell surface VCAM-1, ICAM-1, and HLA-DR. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without poly(I:C) (10 μ g/ml) for 18 h, 37 $^{\circ}$ C. Binding of VCAM-1, ICAM-1, and HLA-DR (antibody isotype control) antibodies was quantitated as described under "Materials and Methods." (Values are the mean of triplicate wells \pm S.E.)

Poly(I:C) Treatment of M-SMCs Increases VCAM-1 mRNA and Protein, but Leukocyte Adhesion Is Not VCAM-1-mediated—Smooth muscle cells from a variety of organs are known to express VCAM-1 and ICAM-1 as a result of cytokine stimulation (17, 28, 29), and poly(I:C) has also been shown to induce VCAM-1 and ICAM-1 on endothelial cells (30, 31). Therefore, we investigated the effect of poly(I:C) on cell surface expression of VCAM-1 and ICAM-1 on M-SMCs. Fig. 4 shows typical radioimmune assays to assess levels of ICAM-1, VCAM-1, and HLA-DR (control) on M-SMCs. Untreated cultures express a high level of ICAM-1, a low but measurable level of VCAM-1, and a low level of HLA-DR (Fig. 4). Stimulation with poly(I:C) does not significantly alter the constitutive levels of ICAM-1 protein, or increase HLA-DR expression, but does increase VCAM-1 surface protein \sim 4-fold.

Northern analyses demonstrate a time-dependent increase in VCAM-1 mRNA expression after poly(I:C) treatment of M-SMCs (Fig. 5). Untreated M-SMCs express very low levels of VCAM-1 mRNA. After adding poly(I:C), the levels increase dramatically through the first 8 h and then decrease during the next 8 h interval. However, they remain above base line even after 24 h. Levels of expression of mRNA for glyceraldehyde-3-phosphate dehydrogenase were not altered by poly(I:C) treatment at any time point. Treatment of M-SMCs with TNF- α also showed up-regulation of VCAM-1 mRNA while IFN- γ and IFN- α had little or no effect.

We next used a monoclonal antibody that specifically blocks VCAM-1 binding to its ligand to determine if mononuclear leukocyte adhesion to poly(I:C)-treated M-SMCs is mediated by VCAM-1 (Fig. 6). As a positive control, M-SMCs were treated with TNF- α (optimum concentration: 1 ng/ml; data not shown), which induces VCAM-1-mediated leukocyte adhesion (18). Adhesion in this case was completely blocked by treatment with the VCAM-1 antibody (10 μ g/ml; added 30 min before and during the leukocyte adhesion step). Conversely, the antibody did not block adhesion of U937 cells to M-SMCs treated with poly(I:C). This is a surprising result since poly(I:C) greatly increased VCAM-1 protein expression as indicated above (Fig. 4).

Poly(I:C)-treated M-SMCs Bind Leukocytes through Hyaluronan—Lazaar *et al.* (17) have shown that endogenously expressed hyaluronan on airway smooth muscle cells can participate in adhesion of activated leukocytes. Although the mononuclear leukocytes (PBMLs and U937 cells) used in our studies are not activated, we nevertheless investigated

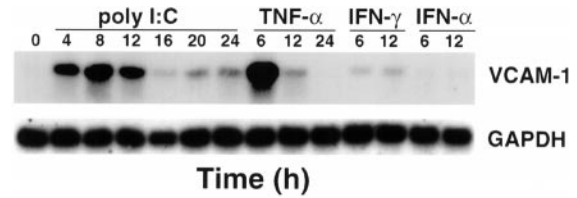


FIG. 5. Effect of poly(I:C), TNF- α , IFN- γ , and IFN- α on the level of VCAM-1 mRNA. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without poly(I:C) (10 μ g/ml), TNF- α (1 ng/ml), IFN- γ (500 units/ml), or IFN- α (500 units/ml) for the indicated times at 37 $^{\circ}$ C. Total cellular RNA was isolated and Northern analyses done as described under "Materials and Methods."

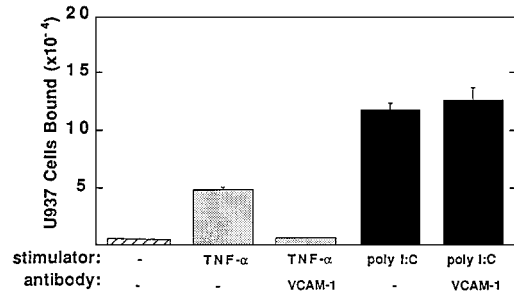


FIG. 6. Effect of anti-VCAM-1 antibody on TNF- α and poly(I:C)-induced U937 cell adhesion. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without TNF- α (1 ng/ml) or poly(I:C) (10 μ g/ml) for 18 h, 37 $^{\circ}$ C. Each medium was aspirated, and a blocking monoclonal antibody directed to VCAM-1 (10 μ g/ml) or control medium was added to the cells followed by incubation for 30 min, 37 $^{\circ}$ C. U937 cell adhesion was measured as described under "Materials and Methods." (Values are the mean of triplicate wells \pm S.E.)

whether hyaluronan was involved in the poly(I:C)-induced adhesion of mononuclear leukocytes by M-SMCs. Replicate cultures of M-SMCs were untreated, treated with poly(I:C), or treated with TNF- α for 18 h. They were then treated for 10 min with medium alone or medium containing 100 units (final concentration 200 units/ml) bovine testicular hyaluronidase, an enzyme that cleaves hyaluronan, before determining adhesion of U937 cells. Hyaluronidase treatment actually increased leukocyte adhesion significantly to untreated or to TNF- α -stimulated M-SMCs (Fig. 7). Conversely, hyaluronidase treatment greatly reduced (\sim 75% decrease) adhesion to poly(I:C)-treated cultures. In other experiments, streptococcal hyaluronidase (100 milliunits/ml), a more specific enzyme for digesting hyaluronan, was equally as effective as bovine hyaluronidase in abrogating leukocyte adhesion (bovine, \sim 75% versus streptococcal, \sim 71% reduction in poly(I:C)-induced adhesion). These results strongly suggest that removal of hyaluronan from the surface of untreated or TNF- α -treated M-SMCs exposes additional adhesion sites, whereas in poly(I:C)-stimulated M-SMCs, most of the leukocytes are bound directly to hyaluronan.

In similar experiments using poly(I:C)-stimulated M-SMCs, essentially all the induced leukocyte adhesion is prevented by a combination of hyaluronidase digestion and treatment with the VCAM-1 blocking antibody (Fig. 7, *experiment 2*). This finding, plus the inability of VCAM-1 antibody alone to reduce leukocyte adhesion to poly(I:C)-treated cultures (Fig. 6), suggests that the presence of hyaluronan masks functional VCAM-1 on the surface of the stimulated M-SMCs.

Parallel experiments were done using Ficoll-separated peripheral blood leukocytes to determine if normal mononuclear leukocytes also adhere to poly(I:C)-treated M-SMC. Fig. 8 shows substantial leukocyte adhesion to poly(I:C)-stimulated M-SMCs as compared with unstimulated control cells. Hyaluronidase treatment after the binding phase of the adhesion assay reduced leukocyte adhesion substantially (\sim 76% of in-

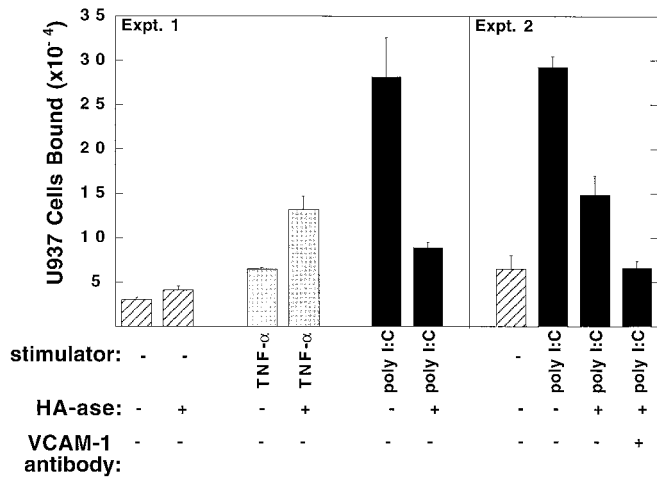


FIG. 7. Effects of hyaluronidase on TNF- α and poly(I-C)-induced U937 cell adhesion. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without TNF- α (1 ng/ml) or poly(I-C) (10 μ g/ml) for 18 h, 37 $^{\circ}$ C. Testicular hyaluronidase (final concentration 200 μ g/ml) was added to indicated culture wells followed by incubation for 10 min, 37 $^{\circ}$ C. All wells were rinsed, and a monoclonal antibody directed to VCAM-1 (10 μ g/ml) or control medium was added to cells followed by incubation for 30 min, 4 $^{\circ}$ C. U937 cell adhesion was measured as described under "Materials and Methods." (Values are the mean of triplicate wells \pm S.E.)

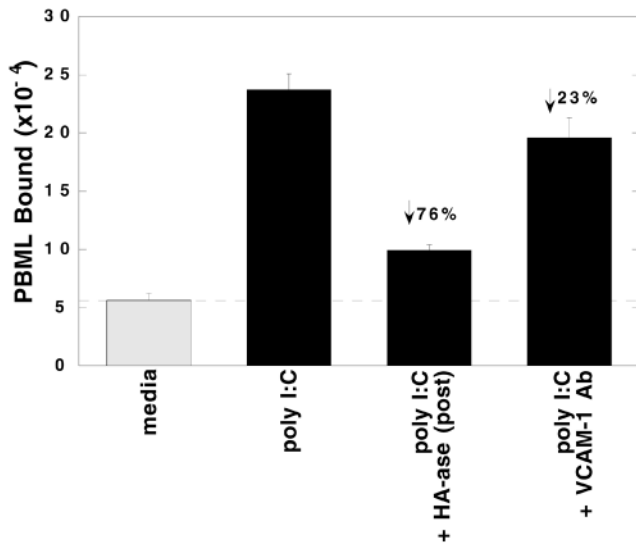


FIG. 8. Effects of hyaluronidase and VCAM-1 blocking antibody on poly(I-C)-induced normal mononuclear leukocyte adhesion. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without poly(I-C) (10 μ g/ml) for 18 h, 37 $^{\circ}$ C. Testicular hyaluronidase (final concentration 200 μ g/ml) was added to indicated culture wells followed by incubation for 10 min, 37 $^{\circ}$ C. All wells were rinsed, and a monoclonal antibody directed to VCAM-1 (10 μ g/ml) or control medium was added to cells followed by incubation for 30 min, 4 $^{\circ}$ C. PBML adhesion was measured as described under "Materials and Methods." (Values are the mean of triplicate wells \pm S.E.)

duced adhesion, \sim 58% of total adhesion). Conversely, VCAM-1 blocking antibody reduced adhesion by only small amount (\sim 23% of induced adhesion, \sim 17% of total adhesion). Thus, normal, unstimulated mononuclear leukocytes also adhere to poly(I-C)-activated M-SMCs primarily by binding to hyaluronan, and VCAM-1, which is present on the cell surface after stimulation (Fig. 4), is only a minor contributor.

M-SMCs Express Increased Matrix-associated Hyaluronan in Response to Poly(I-C) Treatment—Changes in hyaluronan synthesis by M-SMCs after poly(I-C) or TNF- α treatment were assessed by fluorophore-assisted carbohydrate electrophore-

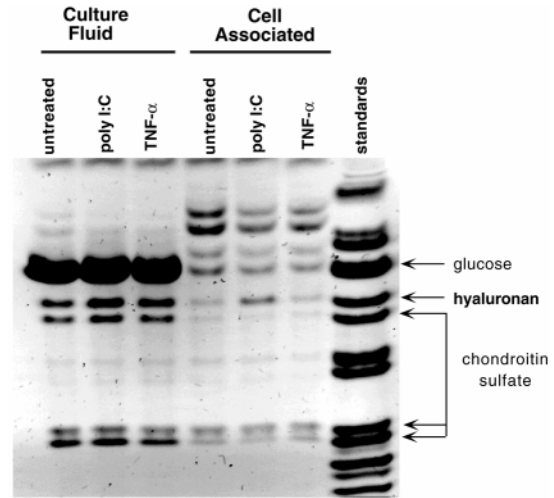


FIG. 9. Effects of poly(I-C) and TNF- α on hyaluronan synthesis by M-SMCs. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without TNF- α (1 ng/ml) or poly(I-C) (10 μ g/ml) for 18 h, 37 $^{\circ}$ C. Cell culture fluids and cell layers were collected, enzymatically processed, 2-aminoacridone-derivatized, and subjected to fluorophore-assisted carbohydrate electrophoresis as described under "Materials and Methods." The band representing the derivatized components of glucose and a hyaluronan disaccharide standard are indicated. Bands indicating chondroitin sulfate digestion products are also noted.

sis.^{2,3} Confluent M-SMC cultures were treated with fresh medium alone, or medium containing poly(I-C) or TNF- α , followed by incubation for 18 h. Each culture fluid and cell layer was collected and enzymatically processed to digest proteins, and glycosaminoglycan molecules were enzymatically digested to their component saccharides. The saccharides were fluorescently labeled as described under "Materials and Methods," and sample aliquots were subjected to electrophoresis, alongside specific markers, on MONOTM composition gels. As shown in Fig. 9, poly(I-C) treatment causes an increase in hyaluronan content in the culture fluid (\sim 2-fold) and more strikingly in the cell layer (\sim 3-fold) when compared with the untreated controls as measured by fluorescence of the disaccharide band derived from hyaluronan. In contrast, TNF- α did not appreciably alter secreted (\sim 1-fold) or cell retained (\sim 1-fold) levels of hyaluronan produced by M-SMCs.

CD44 Is the Major Mononuclear Leukocyte Receptor That Binds to Hyaluronan on Poly(I-C)-treated M-SMCs—We investigated whether CD44, a hyaluronan-binding molecule known to be present on the surface of leukocytes, was present on the U937 cells and whether it was involved in binding of these cells to hyaluronan. Fig. 10A shows the effects of a CD44-specific blocking monoclonal antibody (A3D8) on leukocyte adhesion to poly(I-C)-stimulated M-SMCs. U937 cells, preincubated with the A3D8 antibody at concentrations as low as 10 μ g/ml, were partially blocked from adhering to poly(I-C)-stimulated M-SMCs (\sim 20%), with even greater effects at concentrations up to 25 μ g/ml (\sim 54% reduction).

Fig. 10B confirms that the anti-CD44 antibody also blocks binding of normal peripheral blood mononuclear leukocytes to poly(I-C)-treated M-SMCs. Normal leukocytes pretreated with medium alone or medium containing the A3D8 antibody (20 μ g/ml for 30 min) were incubated with poly(I-C)-stimulated M-SMCs, and the levels of adhesion measured. In addition, a replicate set of the leukocytes was preincubated with purified hyaluronan fragments of $M_r \sim$ 100,000 (250 μ g/ml for 30 min, 4 $^{\circ}$ C) before the adhesion assay. Fig. 10B shows that leukocyte adhesion to poly(I-C)-stimulated M-SMCs increased \sim 5-fold over unstimulated control levels. Pretreating leukocytes with

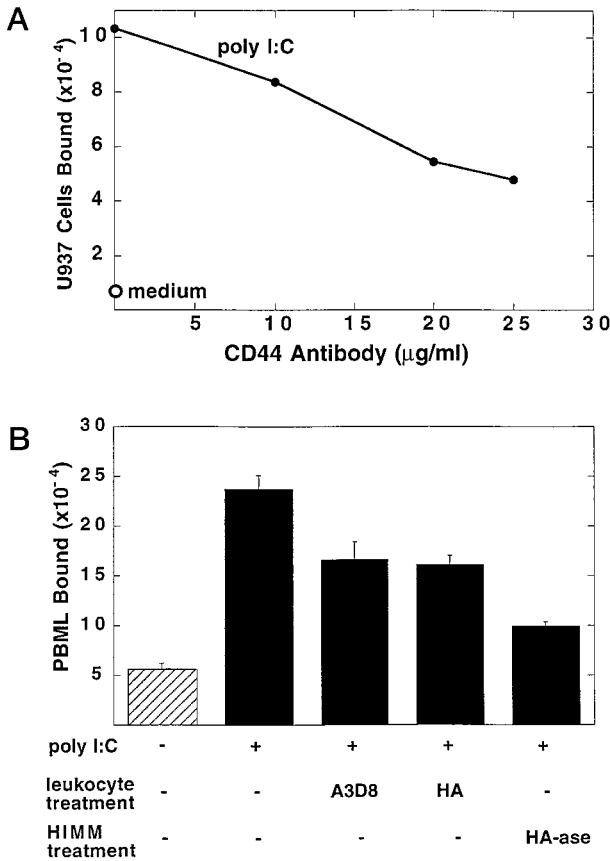


FIG. 10. Effects of anti-CD44 antibody on U937 and normal mononuclear leukocyte adhesion to poly(I:C)-stimulated M-SMCs. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without poly(I:C) (10 μg/ml) for 18 h, 37 °C. **A**, prior to the adhesion assay, aliquots of ⁵¹Cr-labeled U937 cells were treated with the indicated concentrations of A3D8 (anti-CD44 blocking monoclonal antibody), and incubated for 1 h, 4 °C, before addition to the M-SMC cultures. Adhesion was measured as described under "Materials and Methods." (Values are the mean of triplicate wells ± S.E.) **B**, prior to the adhesion assay, aliquots of ⁵¹Cr-labeled PBMLs were treated with A3D8 antibody or with hyaluronan fragments (250 μg/ml) and incubated for 1 h, 4 °C, before addition to the M-SMC cultures. The PBML adhesion assay was done as described under "Materials and Methods," after which time cultures were treated with medium alone or containing hyaluronidase (200 μg/ml) for 5 min at room temperature, washed twice more, and prepared for radioactive counting. (Values are the mean of triplicate wells ± S.E.)

the anti-CD44 antibody reduced specific poly(I:C)-induced adhesion by ~38%, and hyaluronan fragments also reduced leukocyte adhesion to a comparable degree (~41%). However, neither reduced binding as completely as did removal of hyaluronan from the surfaces of the M-SMCs with hyaluronidase (~75%).

Virus-infected M-SMCs Bind Leukocytes through Hyaluronan—To confirm that the poly(I:C)-induced effects on SMC/leukocyte interactions reflected a natural process that could result from virus infection, we tested the effects of hyaluronidase on virus-induced leukocyte adhesion. Cultures of M-SMCs were untreated or infected with RSV (1:500 dilution of stock culture) or poly(I:C) for 24 h. Adhesion of U937 cells was then measured. Following the adhesion step, medium with or without hyaluronidase was added. Both poly(I:C) and RSV induced leukocyte adhesion. Hyaluronidase digestion released essentially all of the bound leukocytes from the RSV-treated cultures and most (~60%) from the poly(I:C)-treated cultures (Fig. 11).

DISCUSSION

M-SMCs are highly susceptible to RSV and MV infections. In addition, these cells, which are normally minimally adhesive

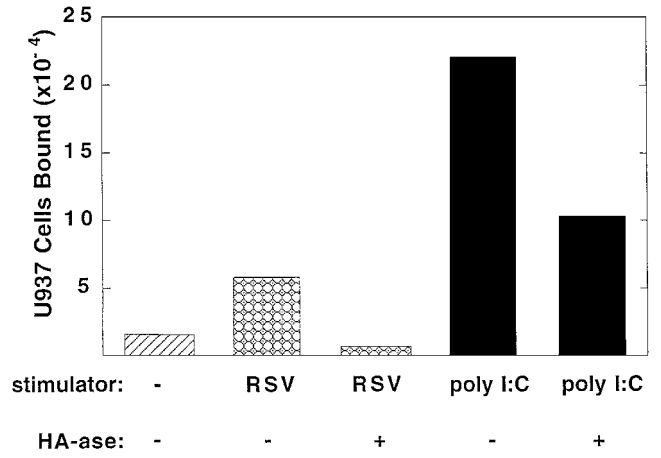


FIG. 11. Effect of hyaluronidase on virus-induced U937 cell adhesion. Confluent M-SMCs were treated with DMEM/F-12 medium containing 10% FBS with or without RSV (50 μl/cm²) or poly(I:C) (10 μg/ml) for 18 h, 37 °C. The U937 cell adhesion assay was done as described under "Materials and Methods," after which time cultures were treated with medium alone or containing hyaluronidase (200 μg/ml) for 5 min at room temperature, washed twice more, and prepared for radioactive counting. (Values are the mean of duplicate wells.)

for leukocytes, bind increased numbers of mononuclear leukocytes after infection with RSV or MV, or treatment with the viral mimic poly(I:C). Hyaluronan on the M-SMCs, interacting with CD44 on the leukocytes, mediates most of the induced adhesion in each of these cases.

Cell-associated hyaluronan is increased on poly(I:C)-treated M-SMCs compared with untreated control cells. Since hyaluronan is synthesized as very long chains at the cell surface by hyaluronan synthases (HAS1, HAS2, or HAS3) (32), conceivably either inhibition of chain release at the surface or increased incorporation of hyaluronan-binding proteins that stabilize the hyaluronan in the cell matrix may account for the increase in leukocyte adhesion. One of several potential candidates of the latter possibility is the serum protein inter-α-trypsin inhibitor (I-α-I or ITI), which has been described as a stabilizer of hyaluronan pericellular coats (33) on a variety of cells including SMCs (34). Consistent with our findings, several reports have observed increased hyaluronan to be a marker of other inflammatory conditions (35).

Previous reports have demonstrated that viral agents up-regulate VCAM-1 mRNA and protein (30, 36) in endothelial cells and mesenchymal tumor cells (37), a finding we have confirmed in M-SMCs. Interestingly, however, in our studies VCAM-1 function appears to be masked by hyaluronan, but can be restored by removing hyaluronan with hyaluronidase. Adhesion molecules not only have the ability to direct leukocyte traffic, but are also reported to be able to activate the bound leukocyte, which can then lead to cytokine (38), chemokine (39), and protease (40) production. The consequences of two up-regulated molecules, hyaluronan and VCAM-1, acting in temporal or conditional sequence on leukocytes through CD44 and VLA-4 (the ligand for VCAM-1), may be important in the inflammatory process. We have found that viral agents can change not only the number of interactions that M-SMCs can have with leukocytes, but also alter the kinds of recognition molecules available to them.

While others have shown that activated leukocytes can adhere to unstimulated airway smooth muscle cells (17) and endothelial cells (41), or to cytokine-treated small vessel endothelial cells (42) by a hyaluronan-mediated mechanism, we believe this to be the first report showing viral induction of this mechanism. The M-SMC reaction to virus appears to be unique, since none of the other biologic stimulators we em-

ployed (TNF- α (shown); IL-1 β , IL-4, IL-6, IFN- α , IFN- γ , transforming growth factor- β , lipopolysaccharide, thrombin, or phorbol ester (data not shown)) up-regulated hyaluronan-mediated leukocyte adhesion to M-SMCs, although some up-regulate the VCAM-1-mediated pathway, and most are active regulators of endothelial leukocyte adhesion molecules.

CD44 has been reported to be the major receptor for hyaluronan (43). Most circulating leukocytes, including lymphocytes and monocytes, are known to display CD44 on their surface. However, leukocyte activation (44, 45) and subsequent activation of CD44 (46) are currently thought to be necessary for hyaluronan binding by this receptor. A surprising finding of our work is that large numbers of unstimulated, normal leukocytes can bind to virus-induced hyaluronan via their CD44 receptors. Since untreated M-SMCs constitutively express measurable amounts of hyaluronan on their surface, but are not adhesive for leukocytes, we speculate that either a critical mass of hyaluronan must be reached before CD44 binding can occur, or more likely, that virus-induced hyaluronan is presented differently on the M-SMC surface. Conceivably, co-expression, or increased incorporation of one or more of the hyaluronan-binding proteins could cause hyaluronan to be presented in a configuration more conducive to engaging CD44. Hyaladherin molecules such as aggrecan, versican, hyaluronectin, or the protein produced by TNF-stimulated gene-6 (TSG-6) (47, 48) as well as smooth muscle cell-expressed CD44 may play a role in such a process. We can detect CD44 on M-SMCs, as has been shown on SMCs from other tissue sources (17, 49), but its expression in our system is not regulated by viral agents.⁴ Correlations of the hyaluronan binding molecules TSG-6 and the serum molecule, I- α -I, have already been made with certain types of inflammation (50, 51), findings that are potentially pertinent to bowel inflammation as well.

Recent reports have underscored the potential importance of the CD44 receptor/hyaluronan interaction to inflammation. De Grendele *et al.* (45) have demonstrated the requirement for activated T-cell-associated CD44 for extravasation into inflammatory sites, and Brocke *et al.* (52) have shown that CD44 antibodies can help block secondary leukocyte recruitment in central nervous system inflammation and experimental encephalomyelitis. Peripheral blood T-cells are activated by specific ligation of CD44 and produce increased IL-2 levels (53, 54), and similarly treated monocytes release higher levels of IL-1 and TNF- α than untreated controls (54, 55). Macrophage binding to hyaluronan has recently been shown to up-regulate IL-12, as well as the chemokines RANTES and MIP-1 α and MIP-1 β (39). Since viral agent-induced hyaluronan on M-SMCs binds non-activated leukocytes through CD44, subsequent activation seems a likely outcome.

The response of M-SMCs to viral agents appears to be a normal physiological response. We have used over 50 different human isolates of M-SMCs in the course of these studies, and no matter what the source, IBD or not, inflamed tissue or not, we have never isolated M-SMCs that are unresponsive to poly(I-C) in the parameters we have described in this report, although the magnitude of the response does vary among cell isolates. Indeed, this mechanism is not unique to smooth muscle cells of the colon. SMCs isolated from vascular (mesenteric artery) and airway (bronchus) sources also exhibit hyaluronan-mediated leukocyte adhesion in response to poly(I-C).⁴ Clearly this mechanism has important implications for other chronic inflammatory conditions. Asthma, for which a hyaluronan role has already been postulated (17), is known to be exacerbated by respiratory virus infection (56, 57). In models of atherosclerosis

and restenosis injury, up-regulated hyaluronan and SMC-expressed CD44 (49) have been observed. Data supporting an underlying viral association (24, 58) with these two pathological conditions have also been published.

We have presented data that show a novel, virus-induced mechanism of leukocyte recruitment/interaction with mucosal smooth muscle cells, which may, in susceptible individuals, contribute to the chronic inflammation of IBD. Interestingly, the extraintestinal manifestations of IBD, which are exhibited in 30–50% of patients, occur in hyaluronan-rich tissues (*e.g.* joint, skin, eye) (25), leading us to speculate that pre-exposure of inflammatory leukocytes to virus-induced hyaluronan may have a role in directing recirculating leukocytes inappropriately to other hyaluronan-rich sites, in a manner similar to the endothelial cell-directed model put forth by Salmi and Jalkanen (12). The fact that viral agents may induce M-SMC recruitment of leukocytes via a hyaluronan/CD44 mechanism introduces a new direction for investigating the pathogenesis of IBD.

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