INFECTION AND IMMUNITY, Mar. 2008, p. 1170–1178 Vol. 76, No. 3
0019-9567/08/$08.00+0 doi:10.1128/IAI.01340-07
Copyright © 2008, American Society for Microbiology. All Rights Reserved.

C-Terminal Repeats of *Clostridium difficile* Toxin A Induce Production of Chemokine and Adhesion Molecules in Endothelial Cells and Promote Migration of Leukocytes▽

Chiou-Yueh Yeh,[1] Chun-Nan Lin,[2] Chuan-Fa Chang,[2] Chun-Hung Lin,[2] Huei-Ting Lien,[1] Jen-Yang Chen,[3] and Jean-San Chia[1]*

Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China[1]; Institute of Biological Chemistry and Genomics Research Center, Academic Sinica, Taipei, Taiwan, Republic of China[2]; and National Health Research Institute, Taipei, Taiwan, Republic of China[3]

Received 5 October 2007/Returned for modification 25 November 2007/Accepted 13 December 2007

The C-terminal repeating sequences of *Clostridium difficile* toxin A (designated ARU) are homologous to the carbohydrate-binding domain of streptococcal glucosyltransferases (GTFs) that were recently identified as potent modulins. To test the hypothesis that ARU might exert a similar biological activity on endothelial cells, recombinant ARU (rARU), which was noncytotoxic to cell cultures, was analyzed using human umbilical vein endothelial cells. The rARU could bind directly to endothelial cells in a serum- and calcium-dependent manner and induce the production of interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein 1 in a dose-dependent manner. An oligosaccharide binding assay indicated that rARU, but not GTFC, binds preferentially to Lewis antigens and 3′HSO_3-containing oligosaccharides. Binding of rARU to human endothelial or intestinal cells correlated directly with the expression of Lewis Y antigen. Bound rARU directly activated mitogen-activated protein kinases and the NF-κB signaling pathway in endothelial cells to release biologically active chemokines and adhesion molecules that promoted migration in a transwell assay and the adherence of polymorphonuclear and mononuclear cells to the endothelial cells. These results suggest that ARU may bind to multiple carbohydrate motifs to exert its biological activity on human endothelial cells.

Downloaded from iai.asm.org at NATIONAL TAIWAN UNIV MED LIB on June 17, 2009

Clostridium difficile is an opportunistic pathogen of antibiotic-associated diarrhea and pseudomembranous colitis in humans (17, 25). The pathogen produces two toxins, toxin A (TcdA) and toxin B (TcdB), that belong to a family of large clostridial cytotoxins and function as major virulence factors (18, 38, 45). They transfer the glucosyl moiety from UDP-glucose to the Rho family of small GTPases, thereby inactivating GTPase functions and leading to actin cytoskeleton disaggregation and cytotoxicity (41). TcdA and TcdB also contain the typical AB toxin features, including N-terminal catalytic domains that glycosylate small GTPases (6, 14) and C-terminal binding domains that recognize an undetermined glycoprotein receptor on mammalian cells (8, 39). The N-terminal catalytic domains of TcdA and TcdB exhibit cytopathic effects similar to those of the holoproteins (35); by contrast, the C-terminal domains, which contain repeating sequences, have no toxic effect on Chinese hamster ovary cells (34). The biological activities of these C-terminal domains—other than glycoprotein receptor binding—remain to be determined.

The C termini of large clostridial toxins are composed of clostridial repetitive oligopeptides (CROPs) (42, 43, 48). Individual CROPs consist of 20 to 50 amino acids with a consensus YYF motif that is repeated 14 to 30 times. This C-terminal CROP sequence of large clostridial toxins is also found in proteins of other gram-positive bacteria, such as in the glucan-binding domains of glucosyltransferases (GTFs) in *Streptococcus mutans* and other viridans streptococci and in the choline-binding domain of autolysin in *Streptococcus pneumoniae* (43, 44, 48). Common functional relevance derived from this homology suggests that CROPs might be structurally related to the carbohydrate-binding domains of TcdA/B (43, 44). The minimum carbohydrate motif in TcdA necessary for host cell binding was proposed to be Gal-α1,3-Gal-β1,4-GlcNAc (21, 37). TcdA binds to a variety of cells from different lineages, and such broad binding specificity might be attributed to the interaction of the C-terminal repetitive domain (CRD) with multiple cell surface glycoproteins or glycolipids. TcdA is reported to interact with carbohydrate Lewis antigens I, A, and Y (39). The crystal structure analysis of the CRD of TcdA indicates a binding site for trisaccharide that is fairly open and may accommodate variations at the nonreducing end of the sugar or the addition of α-fucose attached to GlcNAc or galactose to Lewis X antigen and Lewis Y antigen (11, 13). Based on these findings, TcdA may form multivalent interactions with cell surface carbohydrates through the CRD with its multiple binding sites (13).

Proteins containing repeating peptide fragments such as leucine-rich repeats, tetratricopeptide repeats, ankyrin repeats, and hexapeptide repeats are common in eukaryotes and prokaryotes and have a broad range of biological functions (2, 27). Unlike for other repeats, little is known about the biological functions of CROPs. Besides carbohydrate-binding activity, the TcdA CRD has an immune modulating effect when used as an adjuvant or carrier protein for mucosal immunization (4, 31). Nevertheless, conflicting results have been ob-

* Corresponding author. Mailing address: No. 1, Jen Ai Road 1st Section, Room 713, Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China. Phone: 886-2-23123456, ext. 8222. Fax: 886-2-23926238. E-mail address: chiajs@ntu.edu.tw.

▽ Published ahead of print on 26 December 2007.

tained from in vitro studies of the biological activity of the CRD of TcdA alone in different cell culture systems: activation signals were detected in epithelial cells (33), but no activation was observed for monocytes (16). Moreover, the number of carbohydrate-binding motifs recognized by the TcdA CRD is limited, and the relevance of these motifs to cellular activation remains to be demonstrated. Such information is important to clarify the mechanisms of toxin function so that a vaccine that uses the nontoxic CRD can be developed to prevent *C. difficile*- or TcdA-induced colitis (26, 36, 46, 47).

Considering the vascular endothelial cells was one of the cells involved in inflammatory responses (12, 22), we examined here the biological activities of the TcdA CRD, toxin A repeating units, known as ARU (31), by use of a recombinant version (rARU) on human endothelial cells. We also investigated the ability of rARU to bind to different cells and related carbohydrate motifs. *Streptococcus mutans* GTFC is a member of the GTFs responsible for the synthesis of glucan (an α-1,6- and α-1,3-linked glucose polymer). GTFC binds to glucan through its C-terminal repeated domain that is homologous to the CRD of TcdA (43, 44). Our previous study demonstrated that GTFC is a potent modulin for human endothelial cells (50). In this work, it was tested in parallel as a positive control for endothelial activation and also for the purpose of comparison in a carbohydrate-binding screening assay. The results indicate that rARU exerts biological activities on human umbilical vein endothelial cells (HUVECs) that are similar to those of GTFC, but the two proteins have distinct carbohydrate-binding specificities in an in vitro sugar binding assay.

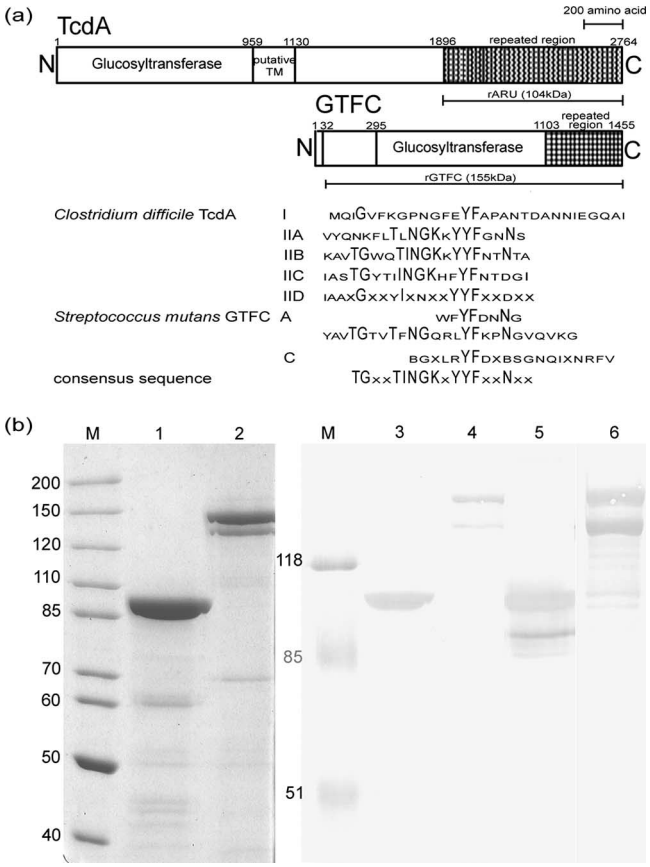

FIG. 1. (a) Alignment of the C-terminal repeats of *C. difficile* TcdA. Schematic representation of positions and amino acid compositions of the repeats in TcdA and *S. mutans* GTFC. TcdA contains 38 repeats, which themselves consist of combinations of five repeated sequences designated class I repeats and the class II A to D repeats. GTFC contains five repeats composed of class IIA and C repeats. A consensus sequence present in all of these repeats is shown. (b) SDS-PAGE analysis with silver staining of purified rARU (lane 1) and rGTFC (lane 2). Western blot analysis of rARU (lanes 3 and 5) and rGTFC (lanes 4 and 6) with anti-His MAb (lanes 3 and 4) and anti-GTFs (lanes 5 and 6), respectively. M, molecular mass markers in kDa.

MATERIALS AND METHODS

Preparation and characterization of endothelial cells, leukocytes, and human intestinal epithelial cells. HUVECs were isolated from human umbilical veins as described previously (15) and maintained in M199 medium with 20% heat-inactivated fetal calf serum (Hi-FCS), 2 mM glutamine, 100 μM sodium pyruvate solution, antibiotics (penicillin, 100 U/ml; streptomycin, 100 U/ml; Cambrex Bio Science), 25 μg/ml sodium heparin, and 25 μg/ml endothelial growth factor supplement (Upstate). All experiments were performed between the second and fifth passage before the cells undergo senescence that may result in a higher level of cytokine production. The identity and purity of the HUVECs were characterized by flow cytometry using anti-CD31 or von Willebrand factor monoclonal antibody (MAb) (50).

HT-29 cells, a human colonic epithelial cell line, and CaCo2 cells, a human ileocecal epithelial cell line, were cultured in RPMI 1640 and Dulbecco's modified Eagle's medium (JRH Biosciences, Inc.) containing 10% Hi-FCS, 2 mM glutamine, and antibiotics, respectively.

All human subjects participating in this study provided informed consent that was reviewed and approved by the institutional review board of the National Taiwan University Hospital. Whole-blood samples isolated from heparinized venous blood of healthy volunteers were layered on Histopaque (Sigma) for separation of mononuclear cells (MNCs) and polymorphonuclear cells (PMNs). Purified cells were suspended in RPMI 1640 medium containing 10% Hi-FCS, 100 μM sodium pyruvate solution, 100 μM minimal essential medium nonessential amino acid solution (Invitrogen), 2 mM L-glutamine, and antibiotics. The identity of the cells was determined by microscopic observation and fluorescence-activated cell sorter (FACS) analysis after staining the cells with anti-CD14 and anti-CD45 MAbs (clones 61D3 and HI30; eBioscience) to identify MNCs and PMNs, respectively.

Preparation and characterization of rARU and rGTFC. Purified His-tagged rARU was kindly provided by David M. Lyerly and Limin Zheng (Techlab, Inc., Blacksburg, VA) (31), and His-tagged rGTFC was purified as described previously (50). These two recombinant proteins were purified to homogeneity by affinity chromatography, and the protein purity was determined by silver staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. rGTFC and rARU migrated at their respective predicted molecular sizes of approximately 155 and 104 kDa (Fig. 1b). The identities of the two recombinant proteins were confirmed by liquid chromatography/tandem mass spectrometry. Possible contamination of the protein preparations with lipopolysaccharide (LPS) was assayed with the E-Toxate *Limulus* test (Sigma). Preparations with detectable amounts of LPS were further processed by polymyxin B agarose column chromatography (Sigma) to remove the contaminating LPS. For Western blot analysis, a monoclonal anti-ARU was also provided by David M. Lyerly and Limin Zheng, and polyclonal anti-GTFC was prepared as described previously (50).

Cell-binding assay. A confluent monolayer of HUVECs, CaCo2 cells, or HT-29 cells was treated with 250 nM rARU at 37°C for 1 h. The rARU-treated and untreated cells were harvested and treated with lysis buffer (150 mM NaCl, 0.5% [wt/vol] NP-40, 50 mM Tris-HCl, 2 mM EDTA, 0.25% sodium deoxycholate, 10 mM Na_3VO_4, pH 7.4) that contained protease inhibitors (2 ng/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 15 ng/ml aprotinin). Total protein concentration was determined by Bradford assay (Bio-Rad). Twenty micrograms of total protein was electrophoresed on 10% SDS-PAGE gels and then subjected to sliver staining or transferred to polyvinylidene difluoride membranes. Membranes were blocked and probed with 1:1,000 MAbs specific for ARU or polyclonal antibodies specific for GTFC at 25°C for 2 h and then with 1:5,000 horseradish peroxidase-conjugated secondary antibodies at 25°C for 2 h. Following washing, immunoreactivity was detected using the ECL enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Downloaded from iai.asm.org at NATIONAL TAIWAN UNIV MED LIB on June 17, 2009

Screening of rARU-binding oligosaccharides with the AlphaScreen assay. The AlphaScreen assay was performed with a PerkinElmer EnVision instrument in triplicate in 384-well white opaque plates with the appropriate assay kits (PerkinElmer) as follows (C.-F. Chang, C.-F. Pan, C.-N. Lin, Y.-L. Wu, Y.-S. E. Cheng, C.-H. Wong, and C.-H. Lin, submitted for publication). The donor beads contained a photosensitizer that generates short-lived singlet oxygen upon irradiation at 680 nm. The singlet oxygen species diffuse only a short distance (~200 nm) before decaying to the ground state. The acceptor beads contained a mixture of chemiluminescent molecules and fluorophores. Upon reaction with singlet oxygen, the chemiluminescent molecules undergo a series of chemical transformations that result in a time-delayed energy transfer to the fluorophores, leading to the emission of an amplified light signal at ~600 nm with very low background.

In the assay, all of the procedures and incubations were carried out in the dark because both beads are light sensitive. Biotin-polyacrylamide-sugars, antibodies, and donor and acceptor beads were diluted with the assay buffer (50 mM HEPES, 50 mM EDTA, 0.1% bovine serum albumin, pH 7.5) to an appropriate concentration. An anti-six-His tag antibody (Abcam, Ltd.) and acceptor beads were incubated (as the acceptor mixture) (PerkinElmer Life Sciences) in the assay buffer for 1 h at 25°C before use. Biotin-polyacrylamide-sugars (GlycoTech), donor beads, and rGTFC or rARU were added separately to the wells of microtiter plates (384-well format) and incubated at 25°C for 1 h. An aliquot of the acceptor mixture was then added to the wells, and the incubation was continued at 25°C for another 2 h. Data were obtained by the fluorescence readout of the EnVision instrument and processed using the AlphaScreen program.

Detection of cytokines and chemokines with ELISA. For activation studies, rARU at different concentrations was added to confluent HUVECs (10^5 cells per well) in M199 medium with 0, 2, 5, or 10% Hi-FCS. All experiments were performed in the presence of polymyxin B (40 μg/ml; Sigma). After treatment, the culture supernatants were collected at different times for quantification of cytokines or chemokines by sandwich enzyme-linked immunosorbent assay (ELISA) using purified proteins as standards (R & D Systems). All experiments were conducted in triplicate, and data are expressed as ng/ml of interleukin-6 (IL-6) or IL-8 released by 1×10^5 cells per well and are shown as the means ± standard deviations (SD).

For inhibition of cellular activation, monolayer HUVECs were preincubated with 5 mM EDTA, 5 mM EDTA plus 10 mM $CaCl_2$, RGD (ARG-GLY-ASP) synthetic peptide (100 μg/ml), or rARU plus monoclonal anti-ARU. In all cases, the treatments were diluted in M199 medium supplemented with 10% FCS. The cells were incubated for 1 h at 37°C before activation with rARU. The culture supernatants after 24 h of rARU stimulation were collected, and secreted IL-8 and IL-6 were quantitated by sandwich ELISA as described above.

RNA isolation and RT-PCR. After stimulation with rARU, total RNA was isolated from confluent HUVECs (5×10^6 cells) by guanidine isothiocyanate-phenol-chloroform extraction. Primers specific for IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), and GAPDH were selected based on the published human cDNA sequences. The oligonucleotide primers used and reverse transcription (RT)-PCR procedures were described previously (50).

Detection of cell surface expression of cell adhesion molecules. The expression of adhesion molecules ICAM-1 (CD54), E-selectin (CD-62E), and VCAM-1 (CD106) were determined by whole-cell-surface ELISA (9). After stimulation by rARU for a period of 6, 9, 16, or 24 h, the HUVEC monolayer in 24-well plates was fixed with 2% paraformaldehyde at room temperature for 1 h and then stained with MAbs (e-Bioscience) specific for CD54 (clone HA58), CD62E (clone CTB202), or CD106 (clone STA) at 25°C for 2 h. The intensity of the bound antibody staining on the cell surface was determined after exposure to horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G antibody (1:3,000; Cappel) and the addition of a tetramethylbenzidine substrate (Clinical Science Products Inc.). All experiments were conducted in triplicate and results are shown as the mean optical densities ± SD.

In parallel experiments, the expression of the adhesion molecules or cell surface Lewis Y antigen was confirmed by indirect immunofluorescence followed by FACS (Becton Dickinson). The cells were stimulated with rARU for 9 h and harvested by treating with 0.005% trypsin-0.002% EDTA solution. Following fixation and washing, the cells were stained with a MAb specific for the cell adhesion molecule of interest and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. Washed cells were resuspended in phosphate-buffered saline containing 1% paraformaldehyde and applied to a FACS analyzer. HUVECs treated with FITC-conjugated secondary antibody alone served as a control for background fluorescence. Surface-expressed Lewis Y antigen was stained with anti-Lewis Y antigen MAb (clone F3; Abcam, Ltd., United Kingdom).

Leukocyte migration assay. Chemotaxis of leukocytes was performed using Transwell inserts (Corning Inc.) in 24-well plates. Confluent HUVECs on 12-mm glass coverslips were treated with 250 nM rARU or 40 nM rGTFC in the presence of FCS for 6 h at 37°C. Subsequently, 10^6 PMNs or MNCs in a total volume of 100 μl were added to the Transwell inserts and incubated at 37°C for 2 h. Leukocytes that had migrated and adhered to the HUVECs on the coverslips in the bottom chambers were fixed in 2% paraformaldehyde and then reacted with MAb specific for CD45 or CD14, followed by FITC-conjugated secondary antibodies. The numbers of transmigrated PMNs and MNCs were counted from five high-resolution fields under a fluorescence microscope, and results are shown as the means ± SD.

Detection of cellular signaling pathway using inhibitors. Nuclear translocation of nuclear factor-κB (NF-κB) in HUVECs after rARU activation was detected by immunostaining. Confluent HUVECs on 12-mm glass coverslips were treated with 250 nM rARU for 1 h at 37°C, washed carefully with ice-cold phosphate-buffered saline, and then permeabilized with 1% (wt/vol) Triton X-100 for 15 min at 25°C. The permeabilized cells were incubated with polyclonal antibodies specific for NF-κB p65 (clone c-20; Santa Cruz Biotechnology) at 25°C for 1 h and then examined by fluorescence microscopy after incubation with FITC-conjugated secondary antibodies (Zymed Laboratories). The number of NF-κB nucleus-translocated cells was counted from 10 high-resolution fields under the fluorescence microscope, and data are shown as the means ± SD.

For inhibition of cellular signaling, HUVECs were preincubated in culture medium containing specific inhibitors of protein kinase C (Ro-31-8220, 1 or 10 μM; Sigma), phosphoinositol 3 kinase (LY294002, 2 or 20 μM; Sigma); protein tyrosine kinase (genistein, 25 or 50 μM; Sigma), extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein (MAP) kinase (PD98059, 25 or 50 μM; Cashmere Biotech), p38 MAP kinase (SB203580, 1 or 5 μM; Cashmere Biotech), Jun N-terminal protein kinase MAP kinase (SP600125, 1 or 5 μM; Cashmere Biotech), and NF-κB (pyrrolidine dithiocarbamate [PDTC], 25 or 50 μM; Sigma) for 1 h before activation by rARU. Pretreatment with these inhibitors alone did not affect the cell viability or the release of IL-6 and IL-8, indicating that the observed stimulation was not caused by nonspecific cytopathic effects.

Statistical analysis. Data are given as the means ± SD and analyzed using the two-tailed Student's *t* test to compare the mean levels of cytokine secretion and adhesion molecule expression following a particular treatment, and differences with *P* values of less than 0.05 were considered statistically significant.

RESULTS

Homology between GTFC and ARU/TcdA and cross-reactivity of antibodies. Recently we demonstrated that GTFC of *S. mutans* could bind directly to and activate HUVECs (50). Sequence alignment between GTFC and ARU of TcdA identified the consensus sequence of the multiple repeats to be TINGKxYYFx (Fig. 1a). These repeated sequences are also located at the C termini of other streptococcal GTFs and glucan-binding proteins (44, 48). A polyclonal antibody against the synthetic peptide TIDGKKYYFN (found in the repeated units of ARU) cross-reacts with glucan-binding proteins of *S. mutans* (49). Polyclonal rabbit immunoglobulin G raised against rGTFC could also recognize rARU on ELISA (data not shown) and Western blots, although the reactivity of rARU was weaker than that of rGTFC (Fig. 1b, compare lanes 5 and 6), suggesting that in addition to exhibiting conservation of the primary sequence, the carbohydrate-binding domains of GTFC, glucan-binding proteins, and ARU may share structural similarities.

Oligosaccharide-binding specificity of rARU correlates with cell type-binding specificity. Previous studies demonstrated that TcdA binds directly to human intestinal epithelial CaCo2 cells through the CRD (32) and that CRD could directly bind to HT-29 (8). Lewis Y antigen is one of the major carbohydrate motifs recognized by TcdA (39). To test if rARU has the same carbohydrate-binding specificity as TcdA, the binding activity of rARU was tested in different cell types. We found that more rARU bound to endothelial HUVECs and intestinal CaCo2

Downloaded from iai.asm.org at NATIONAL TAIWAN UNIV MED LIB on June 17, 2009

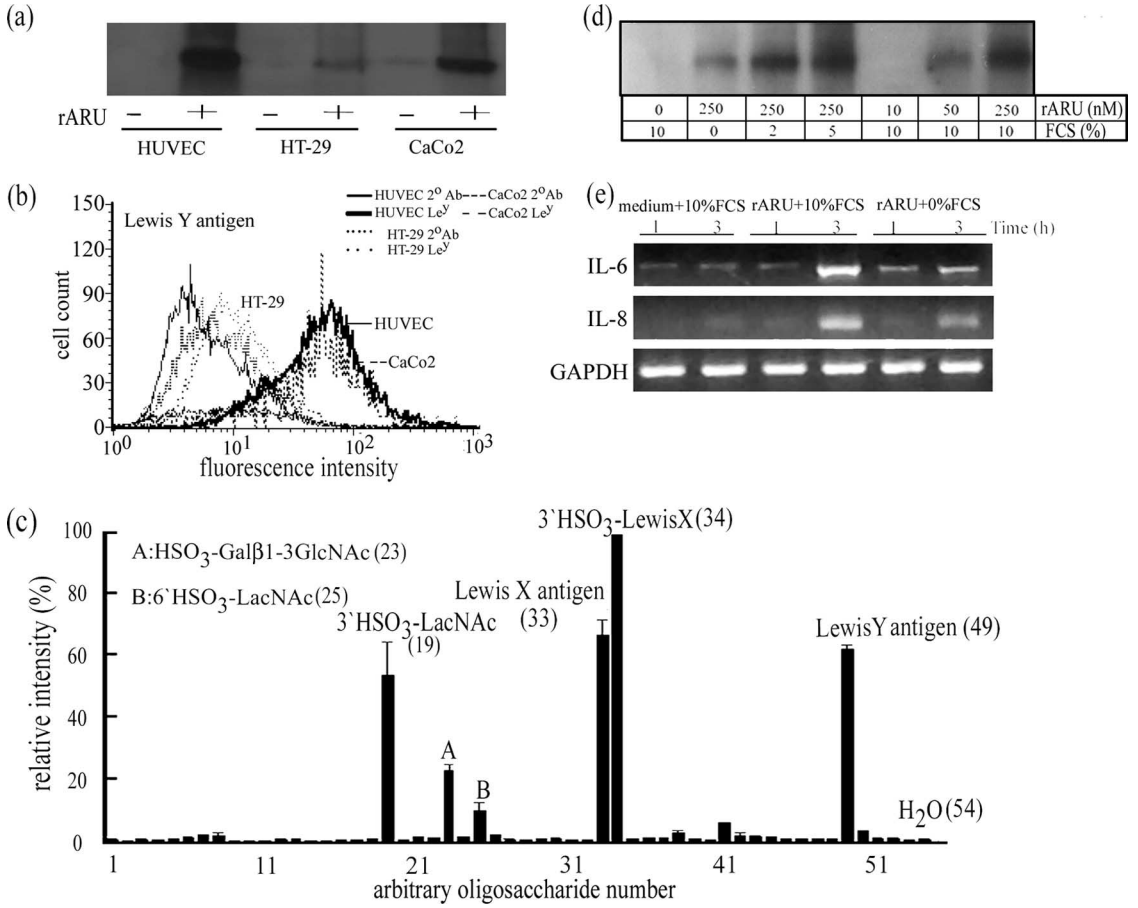

FIG. 2. Identification of oligosaccharides that bind rARU and their correlation with cell binding capacity of rARU, and the serum-enhanced rARU binding and activation of HUVECs. (a) The cell binding assay was performed with 250 nM rARU. The ARU bound to HUVECs, HT-29 cells, and CaCo2 cells was determined by Western blotting. (b) Detection of surface-expressed Lewis Y antigen (Le^y) on HUVECs, HT-29 cells, and CaCo2 cells by FACS analysis. The cells stained with secondary antibodies (2°Ab) were used as the negative control. (c) Sugar binding specificity of rARU is indicated by fluorescence intensity relative to 3′HSO_3-Lewis X (as 100%). The *x* axis indicates the arbitrary number of each oligosaccharide in the panel of 53 oligosaccharides The sugars that produced positive signals are labeled and the sugar numbers are indicated in parentheses. (d) Dose-dependent serum enhancement of rARU binding to HUVECs. (e) Serum-enhanced induction of IL-6 and IL-8 mRNA expression in HUVECs at 1 and 3 h. mRNA levels were measured using RT-PCR, and GAPDH was used as an internal control.

cells than to HT-29 cells (Fig. 2a). In addition, coculture of the primary HUVECs or cell lines with 250 nM rARU for 24 h did not induce cytotoxic changes in morphology or cell death (data not shown). Flow cytometric analysis indicated that HUVECs and CaCo2 cells were comparable in surface Lewis Y antigen expression, but expression was significantly lower in HT-29 cells (Fig. 2b). Therefore, the cellular binding of rARU, similar to TcdA, may be mediated via the interaction with Lewis Y antigen. Interestingly, binding of rARU to HUVECs could not be inhibited, even at a high concentration of anti-Lewis Y antigen MAb (molar ratio, 5 to 1) (data not shown), suggesting that multivalent interactions with other carbohydrates or proteins might also exist.

To further investigate the carbohydrate-binding specificities of rARU and to search for additional minimum ARU-binding carbohydrate motifs, we screened a panel of 53 oligosaccharides for ARU binding using a modified AlphaScreen assay. The assay was developed previously to characterize protein-carbohydrate interactions and glycostructures by in-solution proximity binding with photosensitizers (C.-H. Lin et al., submitted). In the assay, sugars were immobilized on donor beads, and a protein of interest (e.g., rARU) was fixed on the acceptor beads. A light signal is generated when a donor bead and an acceptor bead are brought into proximity (see Materials and Methods for details).

3′HSO_3-Lewis X antigen exhibited the strongest binding specificities for rARU (defined as 100% intensity), as shown in Fig. 2c. Additional rARU-binding oligosaccharides (listed in order of decreasing specificities) included Lewis X antigen, Lewis Y antigen, 3′HSO_3-LacNAc, 3′HSO_3-Galβ1-3GlcNAcβ, and 6′HSO_3-LacNAc, with relative specificities of 67.5% ± 4.6%, 63.1% ± 1.3%, 54.4% ± 10.7%, 23.5% ± 2%, and 10.5% ± 2.7%, respectively. Interestingly, when studied in parallel, streptococcal GTFC did not bind to any of these oligosaccharides, albeit the homologous repeated domain is located at the C terminus (data not shown). These results indicate that in addition to the previous reported binding to Lewis X and Y antigens, the ARU domain of TcdA binds preferentially to 3′HSO_3-containing oligosaccharides. Notably, 3′HSO_3-Lewis X antigen had higher specificities than Lewis X

Downloaded from iai.asm.org at NATIONAL TAIWAN UNIV MED LIB on June 17, 2009

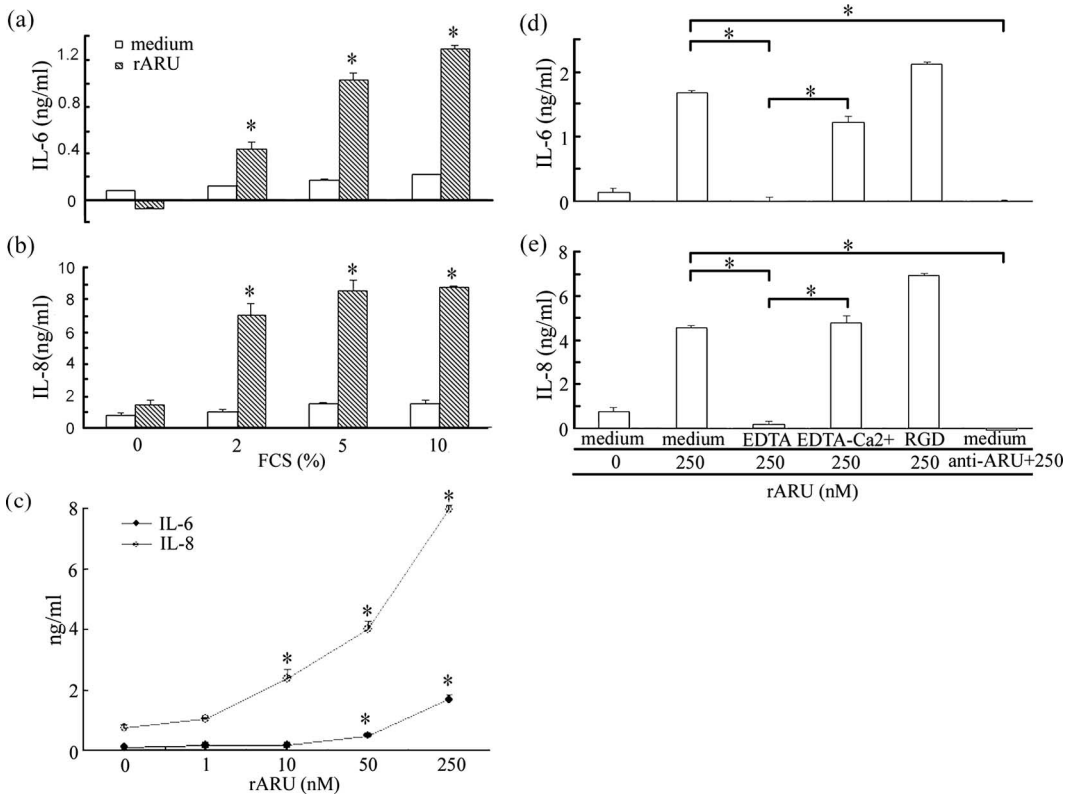

FIG. 3. IL-6 and IL-8 production in rARU-activated cells. (a and b) Serum-enhanced induction of IL-6 (a) and IL-8 (b) production. (c) Dose dependence of IL-6 and IL-8 production in rARU-activated HUVECs. *, P value of <0.05 relative to unstimulated cells. (d and e) Calcium dependence of rARU activation of IL-8 and IL-6 production in HUVECs. The cells were preincubated with medium, EDTA, EDTA plus $CaCl_2$, RGD peptide, or rARU pretreated with anti-ARU at 37°C for 1 h. The cells were then treated with or without 250 nM rARU for 24 h. The release of IL-8 and IL-6 in rARU-activated cell supernatants was quantitated by ELISA. *, P value of <0.05 relative to rARU-stimulated cells.

antigen, suggesting that an additional sulfate substituted at the C3 hydroxyl of galactose may enhance binding.

Serum enhances binding and activation of HUVECs by rARU. Serum components are essential for the growth and survival of endothelial cells (10). Furthermore, we found that serum enhanced the binding of rARU to activate HUVECs in a dose-dependent manner (Fig. 2d). Direct dose-dependent activation by rARU was demonstrated by upregulated transcription and secretion of IL-6 and IL-8 in stimulated HUVECs (Fig. 2e and 3a, b, and c). Furthermore, the dose dependency analysis indicated that the induction of IL-6 and IL-8 in HUVECs was most prominent starting from a concentration of 10 nM and increasing continuously up to 250 nM rARU (Fig. 3c). A similar serum-enhancing effect for rARU was also observed with MNCs. The release of IL-6 24 h after stimulation by rARU in the presence or absence of serum was 3,409 ± 44 or 1,817 ± 41 pg/ml per 5×10^5 MNCs, respectively. The release levels of IL-8 24 h after stimulation by rARU in the presence and absence of serum were 19,096.6 ± 1,696.7 and 2,001.1 ± 32.64 pg/ml per 5×10^5 MNCs, respectively. These results indicate that serum components play an important role in the biological activation by ARU of cells of different lineages.

A previous report demonstrated that, in contrast to TcdA, rARU alone cannot induce IL-8 release from human monocytes cultured under serum-free conditions (16). However, we noted that induction of both IL-6 and IL-8 transcripts could also be detected 3 h after stimulation by rARU in the absence of serum (Fig. 2e). Therefore, the ARU of TcdA was able to directly activate endothelial cells and MNCs to produce IL-6 and IL-8.

Activation by ARU is calcium dependent. Binding of TcdA to CHO cells through the CRD is calcium dependent (5). To test whether calcium is required for the rARU-induced activation of HUVECs, IL-6 and IL-8 release was monitored in the presence of exogenous EDTA. As shown in Fig. 3d and e, the release of either IL-6 or IL-8 from rARU-activated HUVECs was blocked completely by the addition of EDTA and could be restored by replenishing calcium, indicating that HUVECs were activated by rARU in a calcium-dependent manner. Further attempts to block rARU interaction with HUVECs by the addition of an integrin-inhibitory peptide, RGD, failed to inhibit the production of IL-6 or IL-8.

In addition, the induction of either IL-6 or IL-8 by rARU was not affected by polymyxin B (data not shown), which specifically blocks activation by LPS, a potent stimulator for the release of IL-6 and IL-8 (29). Therefore, the lack of polymyxin B inhibition and inhibition by anti-ARU (Fig. 3d and e) excluded the possibility that the observed stimulatory effect on IL-6 and IL-8 production could be attributed to minor contamination by LPS in the rARU preparation. The stimulatory effect of rARU was abolished after heat inactivation, suggest-

Downloaded from iai.asm.org at NATIONAL TAIWAN UNIV MED LIB on June 17, 2009

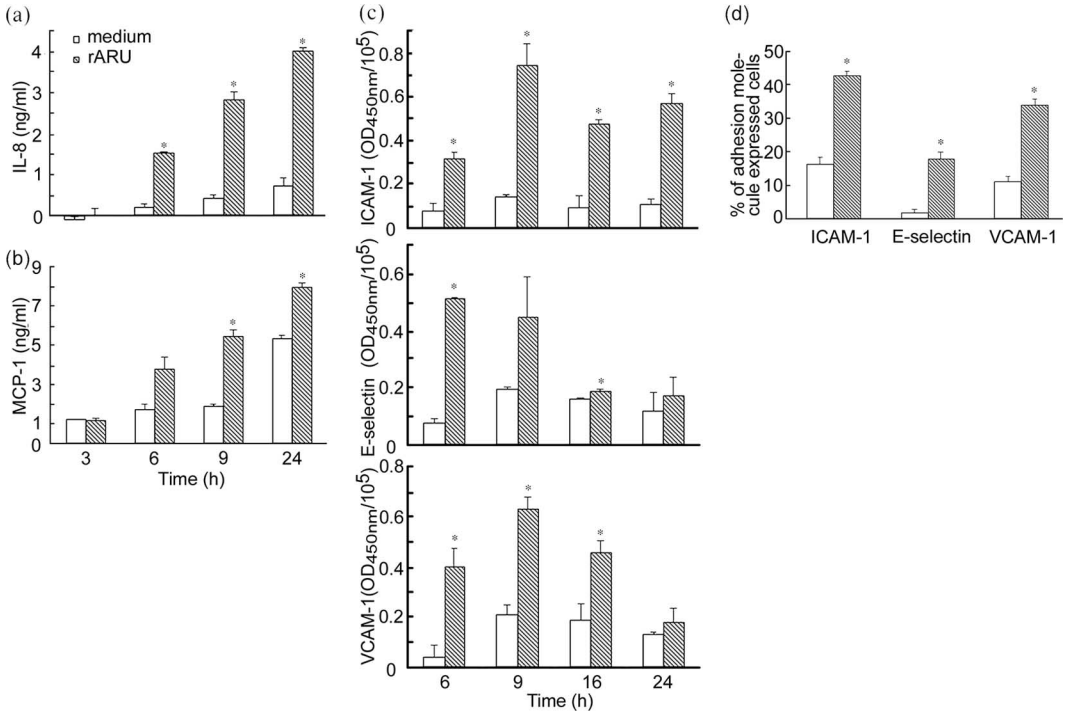

FIG. 4. Kinetics of chemokine and adhesion molecule expression. HUVECs were stimulated with 50 nM rARU. IL-8 (a) or MCP-1 (b) in culture supernatants was detected by ELISA at different times. ICAM-1, E-selectin, and VCAM-1 expression on the cell surface was measured by whole-cell-surface ELISA at the indicated times (c) or detected by FACS analysis at 9 h (d). OD_{450nm}, optical density at 450 nm. *, P value of <0.05 relative to unstimulated cells.

ing that the observed induction of cytokines could not be attributed to the possible contamination of the rARU preparation by LPS-associated lipoprotein.

Kinetics of rARU-induced activation on HUVECs. Following their activation, endothelial cells play a major role in recruiting leukocytes via expression of adhesion molecules and chemokines (28)—mainly IL-8 and MCP-1, key factors for the chemotaxis of neutrophils and monocytes, respectively (20). To further confirm the activation and to monitor the kinetics of rARU-induced activation, chemokine production in culture supernatants and surface expression of adhesion molecules on HUVECs were analyzed following stimulation at a suboptimal concentration of rARU (50 nM) at different times (3, 6, 9, and 24 h poststimulation). Although a time-dependent increase in IL-8 and MCP-1 was observed on the unstimulated HUVEC controls, the addition of rARU increased the amount of the chemokines at 6, 9, and 24 h by 6.82-, 6.47-, and 5.5-fold for IL-8 and 2.2-, 2.9-, and 2.5-fold for MCP-1 relative to what was seen for unstimulated cells (Fig. 4a and b). Further analysis of culture supernatants from HUVECs prepared from different donors showed similar phenomena, and neither tumor necrosis factor alpha nor IL-1β could be detected (data not shown), excluding the presence of possible contaminating monocytes/macrophages in the HUVECs.

The expression of the adhesion molecule ICAM-1 on the cell surface of HUVECs after rARU stimulation followed kinetics similar to those measured for the chemokines and maintained the peak level—first observed at 9 h—for at least 24 h after stimulation with rARU (Fig. 4c). The expression of E-selectin and VCAM-1 was maximal at 6 to 9 h after stimulation but returned gradually to the unstimulated basal level. The expression of the three adhesion molecules on the cell surface 9 h after stimulation was also confirmed by FACS analysis. With rARU stimulation, the percentages of ICAM-1, E-selectin, and VCAM-1 positively stained cells were 42.7% ± 1.3%, 18% ± 2%, and 33.9% ± 1.9%, respectively, and those without stimulation were 16.1% ± 2.4%, 1.9% ± 1.0%, and 11.1% ± 1.5%, respectively. Compared to the expression levels of the untreated cells, the inductions for the expression of ICAM-1, E-selectin, and VCAM-1 on endothelial cells by rARU were 2.7-, 9.7-, and 3-fold, respectively (Fig. 4d).

rARU-activated HUVECs induce leukocyte migration and adhesion. To test if rARU-mediated induction of chemokines and adhesion molecules stimulates leukocytes to migrate, a chemotaxis assay was performed. Six hours after rARU or rGTFC stimulation of HUVECs in the lower chamber of a two-chamber unit (with PMNs and MNCs added to the upper chamber), leukocytes that had migrated to the bottom chamber and adhered to HUVECs were counted by fluorescence microscopy following immunostaining for CD45 and CD14 to distinguish PMNs and MNCs, respectively. The numbers of transmigrated PMNs and MNCs onto rARU-activated HUVECs were 151 ± 22 and 140 ± 20, and those onto rGTFC-activated HUVECs were 116 ± 19 and 140 ± 30, respectively (Table 1). In contrast, PMN and MNC migration was much lower (20 ± 8 cells) in unstimulated cells ($P < 0.05$, stimulated versus nonstimulated). These results demonstrate that the chemokines and adhesion molecules induced by either rARU or rGTFC in HUVECs are biologically active in recruiting PMNs and MNCs. The leukocyte migration observed for

Downloaded from iai.asm.org at NATIONAL TAIWAN UNIV MED LIB on June 17, 2009

TABLE 1. Leukocyte transmigration to rARU- or rGTFC-activated HUVECs

Migrated leukocyte	No. of leukocytes migrating to HUVECs activated with[a]:		
	Medium	rARU	rGTFC
PMN	20 ± 8	151 ± 22	116 ± 19
MNC	20 ± 8	140 ± 20	140 ± 30

[a] The numbers of transmigrated leukocytes were counted from five high-resolution fields under a fluorescence microscope, and results are shown as the means ± SD.

the unstimulated wells was probably due to a basal level of expression of chemokines that was sufficient to induce leukocyte migration (24).

Signaling pathway involved in rARU activation of HUVECs. Various inhibitors were used to explore the cellular signaling pathways involved in IL-6 and IL-8 production in HUVECs. As shown in Fig. 5a, IL-6 production in rARU-activated HUVECs was inhibited by PD98059, SB203580, LY294002, Ro-31-8220, genistein, and PDTC but not by SP600125, suggesting that IL-6 secretion requires activation of ERK1/2 and p38 MAP kinase, phosphoinositol 3 kinase, protein kinase C, protein tyrosine kinase, and NF-κB but not activation of Jun N-terminal protein kinase MAP kinase. Similar inhibitory effects were also observed for IL-8 production, except for phosphoinositol 3 kinase inhibition by LY294002. Cytokine and chemokine expression in rARU-activated HUVECs was also significantly inhibited by the NF-κB inhibitor PDTC. In addition, nuclear translocation of NF-κB was also detected in rARU-activated HUVECs, whereas the p65 subunit of NF-κB was retained in the cytoplasms of unstimulated HUVECs (Fig. 5b). The percentages of cells with NF-κB p65 nuclear translocation 60, 90, and 120 min after rARU stimulation were 72% ± 7%, 79% ± 12%, and 86% ± 5% ($P < 0.05$, stimulated versus nonstimulated), respectively. These results suggest that rARU can directly activate MAP kinase or NF-κB and trigger the expression of downstream genes encoding cytokines, chemokines, and adhesion molecules in endothelial cells.

DISCUSSION

Previous studies have indicated that *C. difficile* TcdA and TcdB, in addition to their cytopathic effects, can also trigger cytokine and chemokine production in vitro in human monocytes, neutrophils, and epithelial cells (7, 19, 24). These effects were directly linked to the inflammatory responses triggered in vivo when TcdA was challenged in an experimental model of *C. difficile* enterocolitis (23). In monocytes, IL-8 could be induced by very low concentrations of TcdA (0.1 to 1 nM), and such a response required cellular uptake of the holotoxin. However, others have shown that the CRD or the ARU of TcdA identical to rARU could not exert IL-8 induction activity in monocytes when added at concentrations up to 10 nM (16). Contradictory to that report, we found that the ARU of TcdA could directly activate HUVECs and MNCs to produce cytokine and chemokines. These different results might be due to the use of different cell lines and may also be due to differences in serum conditions. In the absence of serum, a lack of response in monocytes when treated with rARU has been noted (16). Although serum is essential for endothelial cell growth and survival, we found that induction of IL-6 and IL-8 transcripts in HUVECs by rARU was still observable at 3 h after the removal of serum, a period when cells were still viable (Fig. 2e). Therefore, activation by rARU of endothelial cells could still be achieved in the absence of serum components, even though a serum-enhancing effect might contribute to the binding and/or interactions of rARU to MNCs or endothelial cells.

Although ARU and GTFC share a consensus sequence with considerable identity (over 60%), they have different types and numbers of tandem repeats (Fig. 1). The 20-residue ARU repeats are always contiguous, whereas the 33-residue consensus A repeats of GTFC are separated by intervening nonconserved regions of various lengths. This might explain why GTFC did not bind to the carbohydrates that bound rARU. Recently, the crystal structure of the CRD, containing residues 2582 to 2709 of TcdA, was solved (11, 13). This repeated domain folds into a solenoid-like structure that consists of 32 short repeats and 7 long repeats. Structural modeling indicates

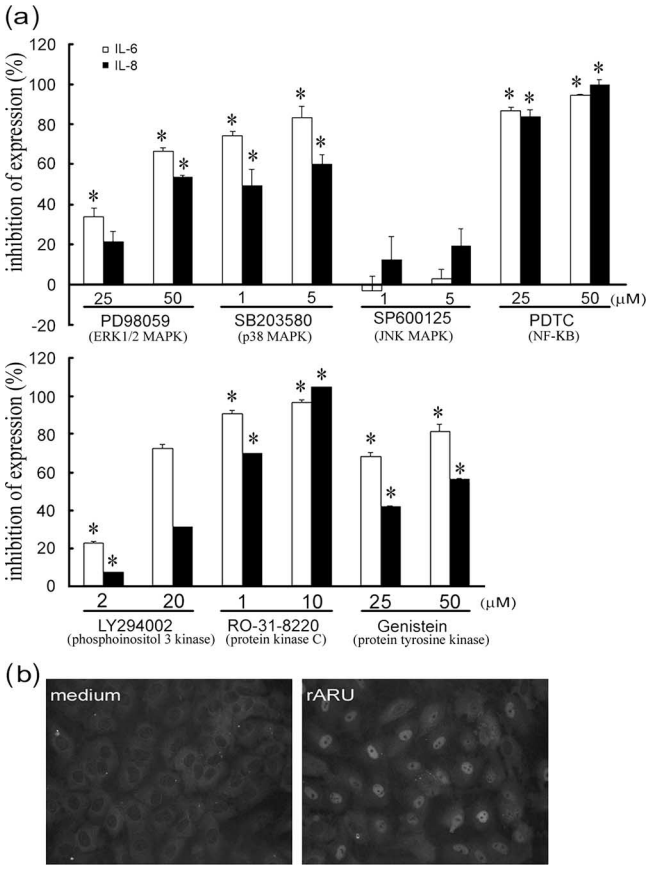

FIG. 5. Inhibition of IL-6 and IL-8 production and nuclear translocation of NF-κB in rARU-stimulated HUVECs. (a) HUVECs were pretreated with different inhibitors for 1 h and then stimulated with 250 nM rARU for 24 h. IL-6 and IL-8 levels in the culture supernatants were determined by ELISA. Abbreviations: JNK, Jun N-terminal kinase; MAPK, MAP kinase. (b) HUVECs, untreated (medium) or stimulated with rARU for 1 h (rARU), were fixed, permeabilized, and stained with an antibody against the p65 subunit of NF-κB. The nuclear translocation of NF-κB was visualized by FITC-labeled secondary antibody. Original magnification, ×400. *, P value of <0.05, rARU-stimulated cells with inhibitor versus without inhibitor.

Downloaded from iai.asm.org at NATIONAL TAIWAN UNIV MED LIB on June 17, 2009

that the binding site at the nonreducing end of the trisaccharide ligand is fairly open and may accommodate variations, such as Gal(α1,3)-, Gal(β1,3)-, or GlcNAc(β1,3)-linked residues. Likewise, the binding pocket has sufficient space to accommodate an additional α-fucose residue attached to GlcNAc or galactose, as found in Lewis X and Y antigens (11, 13).

Comparison of the previously mentioned structural information with the sugar binding specificity of rARU highlights several interesting features in common. First of all, rARU clearly prefers to bind to type II structures, e.g., Gal(β1,4)GlcNAc, as evidenced by the fact that all of the sugars that bound rARU contain the type II backbone, except for 3′HSO_3-Galβ1-3GlcNAcβ. Secondly, the additional presence of a sulfate group enhances the affinity; this is evident in the differences in binding of 3′HSO_3-Lewis X versus that of Lewis X and the binding of 3′HSO_3-LacNAc versus that of LacNAc, which had no detectable binding. The location of the sulfate group is also critical, as demonstrated by the difference between the binding of 3′HSO_3-LacNAc and that of 6′HSO_3-LacNAc. Computational modeling of the TcdA in complex with a trisaccharide ligand [Gal(α1,3)Gal(β1,4)GlcNAc] suggests that replacement of the nonreducing galactose with a sulfate group (e.g., 3′HSO_3-LacNAc) likely creates additional interactions because Lys62 and the backbone amide are in proximity to the sulfate. The interactions are potentially able to compensate for the loss of several hydrogen bond interactions that existed in the nonreducing Gal(α1,3) residue of the trisaccharide ligand.

TcdA binds to human cells through Lewis X and Y antigens (39) or Lewis-containing glycans containing galactose and sialic acid (32). We provide here confirmatory data that the ARU domain, similar to full-length TcdA, binds strongly to both Lewis X and Lewis Y antigens (Fig. 2c). Our data also indicate that, in addition to Lewis X and Y antigens, the ARU domain also binds preferentially to sulfated carbohydrates such as sulfo-Lewis a, sulfogalactose, or sulfo-*N*-acetylglucosamine. Sulfo-*N*-acetylglucosamine has been implicated in mediating the adhesion of *Helicobacter pylori* to salivary mucin, and this mucin-binding activity can be inhibited by chondroitin sulfate or heparin (30, 40). Heparin and heparin sulfate are sulfated glycans that are also present on endothelial cells and can mediate bacterial adherence (1, 3). However, the addition of heparin sulfate in our system did not inhibit rARU activation of HUVECs (data not shown). Such a difference in the binding activities might also contribute, in part, to the different modes of activation or signaling pathways induced by ARU and GTFC. Unlike the lack of involvement of MAP kinase ERK1/2 in the activation of HUVECs by rGTFC (50), the kinase appears to be involved in the induction of IL-6 by rARU.

In conclusion, our results demonstrate that rARU directly binds to and activates endothelial cells to produce chemokines and adhesion molecules that induce leukocyte migration and adhesion in vitro. Carbohydrate-binding assays indicated that the rARU of TcdA can preferentially recognize 3′HSO_3-containing oligosaccharides of diverse structures. The biological activation of endothelial cells by the TcdA CRD on endothelial cells and the subsequent induction of leukocyte chemotaxis might also be the underlying mechanism that partially accounts for the potential adjuvant effect of this repeat peptide in vivo (4, 31). This TcdA CRD, as the modulin, might also play a key role in the inflammatory response by which the toxin causes tissue damage. Thus, in addition to the role of TcdA as a monoglucosyltransferase, the ability to actively recruit inflammatory cells through the nontoxic receptor portion of TcdA helps to explain the severe inflammation that often accompanies bacterial infection.

ACKNOWLEDGMENTS

We thank David Lyerly and Limin Zheng for providing rARU.

This work was supported in part by the National Health Research Institute (grants NHRI-EX94-9432SI, NHRI-EX95-9432SI, NHRI-EX96-9432SI) and the National Science Council (grants NSC-912320-B002-101, NSC-922320-B002-166, NSC-932320-B002-041, NSC-942320-B002-007, NSC-952320-B002-086-MY3, NSC-963112-B002-031).

REFERENCES

1. **Alston, W. K., D. A. Elliott, M. E. Epstein, V. B. Hatcher, M. Tang, and F. D. Lowy.** 1997. Extracellular matrix heparan sulfate modulates endothelial cell susceptibility to *Staphylococcus aureus*. J. Cell. Physiol. **173:**102–109.
2. **Andrade, M. A., C. Perez-Iratxeta, and C. P. Ponting.** 2001. Protein repeats: structures, functions, and evolution. J. Struct. Biol. **134:**117–131.
3. **Behling-Kelly, E., H. Vonderheid, K. S. Kim, L. B. Corbeil, and C. J. Czuprynski.** 2006. Roles of cellular activation and sulfated glycans in *Haemophilus somnus* adherence to bovine brain microvascular endothelial cells. Infect. Immun. **74:**5311–5318.
4. **Castagliuolo, I., M. Sardina, P. Brun, C. DeRos, C. Mastrotto, L. Lovato, and G. Palu.** 2004. *Clostridium difficile* toxin A carboxyl-terminus peptide lacking ADP-ribosyltransferase activity acts as a mucosal adjuvant. Infect. Immun. **72:**2827–2836.
5. **Demarest, S. J., J. Salbato, M. Elia, J. Zhong, T. Morrow, T. Holland, K. Kline, G. Woodnutt, B. E. Kimmel, and G. Hansen.** 2005. Structural characterization of the cell wall binding domains of *Clostridium difficile* toxins A and B; evidence that Ca2+ plays a role in toxin A cell surface association. J. Mol. Biol. **346:**1197–1206.
6. **Faust, C., B. Ye, and K. P. Song.** 1998. The enzymatic domain of *Clostridium difficile* toxin A is located within its N-terminal region. Biochem. Biophys. Res. Commun. **251:**100–105.
7. **Flegel, W. A., F. Muller, W. Daubener, H. G. Fischer, U. Hadding, and H. Northoff.** 1991. Cytokine response by human monocytes to *Clostridium difficile* toxin A and toxin B. Infect. Immun. **59:**3659–3666.
8. **Frisch, C., R. Gerhard, K. Aktories, F. Hofmann, and I. Just.** 2003. The complete receptor-binding domain of *Clostridium difficile* toxin A is required for endocytosis. Biochem. Biophys. Res. Commun. **300:**706–711.
9. **Gaugler, M. H., V. Vereycken-Holler, C. Squiban, and J. Aigueperse.** 2004. PECAM-1 (CD31) is required for interactions of platelets with endothelial cells after irradiation. J. Thromb. Haemost. **2:**2020–2026.
10. **Grafe, M., G. Steinheider, U. Desaga, C. Warnecke, H. B. Lehmkuhl, V. Regitz-Zagrosek, A. G. Hildebrandt, and E. Fleck.** 1999. Characterization of two distinct mechanisms for induction of apoptosis in human vascular endothelial cells. Clin. Chem. Lab. Med. **37:**505–510.
11. **Greco, A., J. G. Ho, S. J. Lin, M. M. Palcic, M. Rupnik, and K. K. Ng.** 2006. Carbohydrate recognition by *Clostridium difficile* toxin A. Nat. Struct. Mol. Biol. **13:**460–461.
12. **Heidemann, J., W. Domschke, T. Kucharzik, and C. Maaser.** 2006. Intestinal microvascular endothelium and innate immunity in inflammatory bowel disease: a second line of defense? Infect. Immun. **74:**5425–5432.
13. **Ho, J. G., A. Greco, M. Rupnik, and K. K. Ng.** 2005. Crystal structure of receptor-binding C-terminal repeats from *Clostridium difficile* toxin A. Proc. Natl. Acad. Sci. USA **102:**18373–18378.
14. **Hofmann, F., C. Busch, U. Prepens, I. Just, and K. Aktories.** 1997. Localization of the glucosyltransferase activity of *Clostridium difficile* toxin B to the N-terminal part of the holotoxin. J. Biol. Chem. **272:**11074–11078.
15. **Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick.** 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Investig. **52:**2745–2756.
16. **Jefferson, K. K., M. F. Smith, Jr., and D. A. Bobak.** 1999. Roles of intracellular calcium and NF-kappa B in the *Clostridium difficile* toxin A-induced up-regulation and secretion of IL-8 from human monocytes. J. Immunol. **163:**5183–5191.
17. **Johnson, S., and D. N. Gerding.** 1998. *Clostridium difficile*-associated diarrhea. Clin. Infect. Dis. **26:**1027–1034.
18. **Just, I., and R. Gerhard.** 2004. Large clostridial cytotoxins. Rev. Physiol. Biochem. Pharmacol. **152:**23–47.
19. **Kim, J. M., J. Y. Lee, Y. M. Yoon, Y. K. Oh, J. Youn, and Y. J. Kim.** 2006. NF-kappa B activation pathway is essential for the chemokine expression in intestinal epithelial cells stimulated with *Clostridium difficile* toxin A. Scand. J. Immunol. **63:**453–460.

Downloaded from iai.asm.org at NATIONAL TAIWAN UNIV MED LIB on June 17, 2009

20. **Krishnaswamy, G., J. Kelley, L. Yerra, J. K. Smith, and D. S. Chi.** 1999. Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease. J. Interferon Cytokine Res. **19:**91–104.
21. **Krivan, H. C., G. F. Clark, D. F. Smith, and T. D. Wilkins.** 1986. Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Galα1-3Galβ1-4GlcNAc. Infect. Immun. **53:** 573–581.
22. **Kurose, I., C. Pothoulakis, J. T. LaMont, D. C. Anderson, J. C. Paulson, M. Miyasaka, R. Wolf, and D. N. Granger.** 1994. Clostridium difficile toxin A-induced microvascular dysfunction. Role of histamine. J. Clin. Investig. **94:**1919–1926.
23. **Lima, A. A., D. M. Lyerly, T. D. Wilkins, D. J. Innes, and R. L. Guerrant.** 1988. Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine in vivo and on cultured cells in vitro. Infect. Immun. **56:**582–588.
24. **Linevsky, J. K., C. Pothoulakis, S. Keates, M. Warny, A. C. Keates, J. T. LaMont, and C. P. Kelly.** 1997. IL-8 release and neutrophil activation by *Clostridium difficile* toxin-exposed human monocytes. Am. J. Physiol. **273:** G1333–G1340.
25. **Lyerly, D. M., H. C. Krivan, and T. D. Wilkins.** 1988. *Clostridium difficile*: its disease and toxins. Clin. Microbiol. Rev. **1:**1–18.
26. **Lyerly, D. M., J. L. Johnson, S. M. Frey, and T. D. Wilkins.** 1990. Vaccination against lethal *Clostridium difficile* enterocolitis with a nontoxic recombinant peptide of toxin A. Curr. Microbiol. **21:**29–32.
27. **Main, E. R., S. E. Jackson, and L. Regan.** 2003. The folding and design of repeat proteins: reaching a consensus. Curr. Opin. Struct. Biol. **13:**482–489.
28. **Muller, W. A.** 2003. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol. **24:**327–334.
29. **Munoz, C., D. Pascual-Salcedo, M. C. Castellanos, A. Alfranca, J. Aragones, A. Vara, J. M. Redondo, and M. O. de Landazuri.** 1996. Pyrrolidine dithiocarbamate inhibits the production of interleukin-6, interleukin-8, and granulocyte-macrophage colony-stimulating factor by human endothelial cells in response to inflammatory mediators: modulation of NF-kappa B and AP-1 transcription factors activity. Blood **88:**3482–3490.
30. **Namavar, F., M. Sparrius, E. C. Veerman, B. J. Appelmelk, and C. M. Vandenbroucke-Grauls.** 1998. Neutrophil-activating protein mediates adhesion of *Helicobacter pylori* to sulfated carbohydrates on high-molecular-weight salivary mucin. Infect. Immun. **66:**444–447.
31. **Pavliakova, D., J. S. Moncrief, D. M. Lyerly, G. Schiffman, D. A. Bryla, J. B. Robbins, and R. Schneerson.** 2000. *Clostridium difficile* recombinant toxin A repeating units as a carrier protein for conjugate vaccines: studies of pneumococcal type 14, *Escherichia coli* K1, and *Shigella flexneri* type 2a polysaccharides in mice. Infect. Immun. **68:**2161–2166.
32. **Perrier, C., N. Sprenger, and B. Corthesy.** 2006. Glycans on secretory component participate in innate protection against mucosal pathogens. J. Biol. Chem. **281:**14280–14287.
33. **Pothoulakis, C., R. J. Gilbert, C. Cladaras, I. Castagliuolo, G. Semenza, Y. Hitti, J. S. Montcrief, J. Linevsky, C. P. Kelly, S. Nikulasson, H. P. Desai, T. D. Wilkins, and J. T. LaMont.** 1996. Rabbit sucrase-isomaltase contains a functional intestinal receptor for *Clostridium difficile* toxin A. J. Clin. Investig. **98:**641–649.
34. **Price, S. B., C. J. Phelps, T. D. Wilkins, and J. L. Johnson.** 1987. Cloning of the carbohydrate-binding portion of the toxin A gene of *Clostridium difficile*. Curr. Microbiol. **16:**55–60.
35. **Rupnik, M., S. Pabst, M. Rupnik, C. von Eichel-Streiber, H. Urlaub, and H. D. Soling.** 2005. Characterization of the cleavage site and function of resulting cleavage fragments after limited proteolysis of *Clostridium difficile* toxin B (TcdB) by host cells. Microbiology **151:**199–208.
36. **Sauerborn, M., P. Leukel, and C. von Eichel-Streiber.** 1997. The C-terminal ligand-binding domain of *Clostridium difficile* toxin A (TcdA) abrogates TcdA-specific binding to cells and prevents mouse lethality. FEMS Microbiol. Lett. **155:**45–54.
37. **Teneberg, S., I. Lonnroth, J. F. Torres Lopez, U. Galili, M. O. Halvarsson, J. Angstrom, and K. A. Karlsson.** 1996. Molecular mimicry in the recognition of glycosphingolipids by Gal alpha 3 Gal beta 4 GlcNAc beta-binding *Clostridium difficile* toxin A, human natural anti alpha-galactosyl IgG and the monoclonal antibody Gal-13: characterization of a binding-active human glycosphingolipid, non-identical with the animal receptor. Glycobiology **6:**599–609.
38. **Thelestam, M., and E. Chaves-Olarte.** 2000. Cytotoxic effects of the *Clostridium difficile* toxins. Curr. Top. Microbiol. Immunol. **250:**85–96.
39. **Tucker, K. D., and T. D. Wilkins.** 1991. Toxin A of *Clostridium difficile* binds to the human carbohydrate antigens I, X, and Y. Infect. Immun. **59:**73–78.
40. **Veerman, E. C., C. M. Bank, F. Namavar, B. J. Appelmelk, J. G. Bolscher, and A. V. Nieuw Amerongen.** 1997. Sulfated glycans on oral mucin as receptors for *Helicobacter pylori*. Glycobiology **7:**737–743.
41. **von Eichel-Streiber, C., P. Boquet, M. Sauerborn, and M. Thelestam.** 1996. Large clostridial cytotoxins—a family of glycosyltransferases modifying small GTP-binding proteins. Trends Microbiol. **4:**375–382.
42. **von Eichel-Streiber, C., R. Laufenberg-Feldmann, S. Sartingen, J. Schulze, and M. Sauerborn.** 1992. Comparative sequence analysis of the *Clostridium difficile* toxins A and B. Mol. Gen. Genet. **233:**260–268.
43. **von Eichel-Streiber, C., and M. Sauerborn.** 1990. *Clostridium difficile* toxin A carries a C-terminal repetitive structure homologous to the carbohydrate binding region of streptococcal glycosyltransferases. Gene **96:**107–113.
44. **von Eichel-Streiber, C., M. Sauerborn, and H. K. Kuramitsu.** 1992. Evidence for a modular structure of the homologous repetitive C-terminal carbohydrate-binding sites of *Clostridium difficile* toxins and *Streptococcus mutans* glucosyltransferases. J. Bacteriol. **174:**6707–6710.
45. **Voth, D. E., and J. D. Ballard.** 2005. *Clostridium difficile* toxins: mechanism of action and role in disease. Clin. Microbiol. Rev. **18:**247–263.
46. **Ward, S. J., G. Douce, G. Dougan, and B. Wren.** 1997. Delivery of non-toxic fragments of *Clostridium difficile* toxin A to the mucosal immune system. Rev. Med. Microbiol. **8:**S34–S36.
47. **Ward, S. J., G. Douce, G. Dougan, and B. W. Wren.** 1999. Local and systemic neutralizing antibody responses induced by intranasal immunization with the nontoxic binding domain of toxin A from *Clostridium difficile*. Infect. Immun. **67:**5124–5132.
48. **Wren, B. W.** 1991. A family of clostridial and streptococcal ligand-binding proteins with conserved C-terminal repeat sequences. Mol. Microbiol. **5:**797–803.
49. **Wren, B. W., R. R. Russell, and S. Tabaqchali.** 1991. Antigenic cross-reactivity and functional inhibition by antibodies to *Clostridium difficile* toxin A, *Streptococcus mutans* glucan-binding protein, and a synthetic peptide. Infect. Immun. **59:**3151–3155.
50. **Yeh, C. Y., J. Y. Chen, and J. S. Chia.** 2006. Glucosyltransferases of viridans group streptococci modulate interleukin-6 and adhesion molecule expression in endothelial cells and augment monocytic cell adherence. Infect. Immun. **74:**1273–1283.

Editor: J. L. Flynn

Downloaded from iai.asm.org at NATIONAL TAIWAN UNIV MED LIB on June 17, 2009