Mucosal Vaccination Increases Endothelial Expression of Mucosal Addressin Cell Adhesion Molecule 1 in the Human Gastrointestinal Tract

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Received 7 July 2003/Returned for modification 2 September 2003/Accepted 3 November 2003

Homing of leukocytes to various tissues is dependent on the interaction between homing receptors on leukocytes and their ligands, addressins, on endothelial cells. Mucosal immunization results in homing of antigen-specific lymphocytes back to the mucosa where they first encountered the antigen. However, it is unknown whether this homing of antigen-specific cells is mediated by an altered endothelial addressin expression after vaccination. Using different immunization routes with an oral cholera vaccine, we show that the endothelial expression of mucosal addressin cell adhesion molecule 1 (MAdCAM-1) is increased in the gastric and upper small intestinal mucosae after immunization through various local routes in the upper gastrointestinal tract. In contrast, rectal immunization did not influence the levels of MAdCAM-1 in the gastric or duodenal mucosa. Furthermore, we show that MAdCAM-1 can be induced on human endothelial cells by tumor necrosis factor alpha (TNF-α) and gamma interferon. The vaccine component cholera toxin B subunit (CTB) increased MAdCAM-1 expression on endothelial cells in cultured human gastric explants, an effect that seemed to be mediated by TNF-α. In conclusion, MAdCAM-1 expression is increased in the upper gastrointestinal tract after local immunizations with a vaccine containing CTB. This strongly suggests the involvement of MAdCAM-1 in the preferential homing of mucosal lymphocytes to their original site of activation.

Lymphocyte trafficking through secondary lymphoid and nonlymphoid tissues serves both to ensure contact of naive and memory cells with antigen and to distribute effector lymphocytes to their target tissues. Lymphocyte migration is far from random but varies with lymphocyte lineage and activation stage. In addition, memory and effector lymphocytes tend to home back to their original site of activation (4, 19, 32). This organ-specific lymphocyte homing is dependent on the expression of tissue-specific adhesion molecules on both the circulating lymphocytes and the specialized endothelial cells, i.e., high endothelial venules, in the tissue, through which most lymphocyte extravasation takes place. Homing to the gastrointestinal mucosa is mediated by lymphocyte expression of integrin α4β7, which binds to the mucosal addressin cell adhesion molecule 1 (MAdCAM-1) (1, 12). MAdCAM-1 is selectively expressed by endothelial cells in the gastrointestinal tract and in mesenteric lymph nodes, with expression seen on high endothelial venules in Peyer’s patches as well as on small venules in the lamina propria in gastric and intestinal mucosae (2, 5). The expression of MAdCAM-1 on mouse endothelial cells has been shown to be upregulated by tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) (21, 23, 31). However, whether these cytokines also are involved in the regulation of MAdCAM-1 expression on human endothelial cells is not known.

In addition to adhesion molecules, chemokines and their ligands are important for the homing of lymphocytes. Recent studies have demonstrated that the chemokine receptor CCR9 is specifically expressed on a subset of gut-homing T cells expressing α4β7 and that its ligand thymus-expressed chemokine (TECK) is produced in the small intestine (16, 37). In addition, human immunoglobulin A (IgA)-secreting B cells from most organs express CCR10 and migrate toward its ligand mucosa-associated epithelial chemokine (MEC) (17). Endothelial cells in many inflamed tissues increase their expression of vascular cellular adhesion molecule 1 (VCAM-1), which contributes to recruitment of lymphocytes expressing integrin α4β1 (6). We and others have previously shown that circulating B and T cells activated by mucosal immunization or infection carry the mucosal homing receptor α4β7 (14, 15, 27, 29, 30, 34) and home back to mucosal tissues. During the last few years it has also become evident that there is a certain degree of compartmentalization within the mucosal immune system, in the sense that effector B cells home to the specific part of the gut where they first encountered the antigen (7, 8, 26). These regional immune responses cannot be explained only by α4β7 expression, since virtually all circulating B cells induced by oral, rectal, or nasal antigen delivery express α4β7 (27). The mechanisms behind the compartmentalization of mucosal immune responses are not well characterized but could involve differences in chemokine production (16) or differential regulation of endothelial adhesion molecules. In this study, we examined whether local or distant mucosal immunizations could influence the endothelial expression of MAdCAM-1, VCAM-1, and E-selectin in the upper gastrointestinal tract. Furthermore, the role of TNF-α and IFN-γ in the regulation of MAdCAM-1 expression on human endothelial cells was evaluated.
MATERIALS AND METHODS

Subjects. This study was approved by the Human Research Ethical Committee of the Medical Faculty, Göteborg University, and the Medical Products Agency and was performed after informed consent was given by all of the volunteers.

Twenty-three Helicobacter pylori-infected subjects were recruited among patients referred for gastroduodenal endoscopy at the Sahlgrenska University Hospital, Göteborg, Sweden, or by serological screening of healthy blood donors (9). H. pylori infection was diagnosed by positive serology or urea breath test and was confirmed by positive culture of biopsies. None of the volunteers had taken any medication for at least 1 week before the study. In addition, eight uninfected healthy Swedish adult volunteers were recruited. Gastric mucosa was also obtained from four patients undergoing gastrectomy due to gastric adenocarcinoma. Normal gastric mucosa was obtained from sites at least 5 cm from the tumor tissue.

Vaccination protocols and specimen collection. All volunteers received two doses of an oral choler vaccine (Dukoral; SBL Vaccin, Stockholm, Sweden) (11) 2 weeks apart. Each dose of the vaccine consisted of 1 mg of recombinantly produced cholera toxin B subunit (CTB) and 10²⁵ formalin-inactivated Vibrio cholerae bacteria. Eight of the volunteers (two women and six men; mean age, 45 years [range, 36 to 61 years]) were given the vaccine twice intragastrically after 36 h of antisecretory treatment as described previously (28). Briefly, 4 ml of the vaccine was given through the endoscope as droplets on the gastric antral mucosa with the patient in the left-side position.

Another group of H. pylori-infected subjects (four women and four men; mean age, 46 years [range, 33 to 58 years]) were given the vaccine twice in the small intestine as previously described (28). In short, the vaccine was distributed in a volume of 20 ml through the endoscope at the level of the ligament of Treitz, i.e., 30 cm distal of the pyloric sphincter.

Seven additional H. pylori-infected subjects (one woman and six men; mean age, 45 years [range, 21 to 57 years]) were given the vaccine rectally in a volume of 1 ml.

In addition, eight healthy uninfected volunteers (three women and five men; mean age, 28 years [range, 21 to 35 years]) received two doses of the vaccine, mixed in sodium bicarbonate to a final volume of 150 ml, orally.

Gastroduodenal endoscopies were performed before and 1 week after the last immunization of each subject, and biopsies were collected from the antral and duodenal mucosae at both time points from all subjects. Biopsies for immunohistochemical analysis were embedded in O.C.T. compound (Tissue Tek) and frozen in liquid nitrogen.

The putative impact of immunization on the density of blood vessels in the human gastrointestinal mucosa was studied by immunohistochemical detection of von Willebrand factor, as a general endothelial cell marker. The von Willebrand factor-positive blood vessels constituted approximately 2% of the total area of both gastric and duodenal tissue sections and were not influenced by the various immunization routes. In addition, the endothelium of von Willebrand factor expression was similar in the different study groups.

Culture of gastric explants. Gastric mucosal explants were prepared as previously described (18) from resected noncancerous tissue from the antra of patients undergoing gastrectomy. In short, the mucosal layer was cut from underlying muscle layers, and pieces with a diameter of 3 mm were punched out and put in culture dishes with Iscove’s complete medium supplemented with 5% (vol/vol) fetal calf serum and 100 µg of gentamicin per ml. The explants were then stimulated by addition of 200 ng of TNF-α per ml, 500 ng of IFN-γ per ml, 100 or 500 µg of CTB per ml, or the Dukoral vaccine diluted 1:4, 1:10, 1:20, TNF-α at 200 ng/ml; or IFN-γ at 500 ng/ml for 5, 20, or 28 h. After fixation with acetone, the cells were incubated overnight at 4°C with anti-MAdCAM-1 antibody diluted 1:10 in PBS, followed by incubation for 3 h at room temperature with fluorescein isothiocyanate-conjugated Fab(ab)² fragments of rat anti-mouse IgG (Dakopatts, Alvsjö, Sweden). Propidium iodide (Sigma) was added and left briefly for contrast nuclear staining. After mounting, the slides were examined in a Leica microscope.

Statistical analyses. Comparisons of addressin expression before and after vaccination within each study group were made by the Wilcoxon matched pairs test. P values of <0.05 were regarded as indicating significant differences.

RESULTS

Immunization in the upper gastrointestinal tract increases the endothelial expression of MAdCAM-1 in the gastroduodenal mucosa. The role of endothelial cells in homing of antigen-specific lymphocytes back to their original site of antigen encounter after mucosal immunization is largely unknown. Therefore, an oral choler vaccine consisting of killed vibrios and the nontoxic CTB was given by the oral, gastric, jejunal, or rectal route for evaluation of the impact of local and distant mucosal immunizations on the endothelial expression of MAdCAM-1, VCAM-1, and E-selectin in the gastrointestinal tract.

MAdCAM-1 expression was significantly increased in the duodenal mucosa after upper gastrointestinal tract immunizations, i.e., after jejunal, gastric, or oral vaccination (Fig. 1 and 2E and F).

Jejunal administration of the vaccine increased the MAdCAM-1 expression in the duodena of all of the subjects (eight of eight; P < 0.01), whereas gastric and oral administration increased the duodenal MAdCAM-1 expression in seven out of eight volunteers (P < 0.05). In contrast, the expression of MAdCAM-1 in the duodenal mucosa was not influenced by distant, i.e., rectal, immunization (Fig. 1).

Gastric, jejunal, and oral administration of the vaccine also significantly increased the proportion of MAdCAM-1-positive endothelium in the stomach (P < 0.05) in the majority of the subjects (Fig. 3 and 2C and D). This effect was seen both in individuals with gastric inflammation caused by H. pylori infection and in uninfected subjects. As in the duodenum, rectal immunization had no impact on the gastric MAdCAM-1 levels.

In contrast to MAdCAM-1, E-selectin, which was expressed at low levels in the majority of the biopsies, was not affected by any of the immunization routes. The average stained area was 0.2% in the antrum and 0.08% in the duodenum, both before and after the immunizations. Furthermore, VCAM-1-positive vessels could not be observed before or after immunization by the different immunization routes in any of the volunteers.
TNF-α and IFN-γ upregulate MAdCAM-1 expression on human endothelial cells. Excluding changes in vessel density, the increased MAdCAM-1 expression after local immunization could be due to either a direct effect of vaccine components on the endothelial cells or an indirect effect via different tissue factors such as cytokines. We first assessed whether the vaccine or its components could induce MAdCAM-1 on endothelial cells in cultured human gastric tissue. The whole-cell vaccine as well as CTB alone increased MAdCAM-1 expression on the endothelial cells in the cultured gastric explants (Fig. 4). To directly assess the effect of the vaccine components on endothelial cells, cultured HUVEC were used as an in vitro model of mucosal endothelial cells, since these cells have the ability to express MAdCAM-1 upon stimulation (unpublished data). However, neither the vaccine nor CTB could induce MAdCAM-1 expression on HUVEC, suggesting that the vaccine-induced MAdCAM-1 upregulation in mucosal explants may be an indirect effect mediated by factors produced in the tissue, e.g., cytokines or chemokines. Since MAdCAM-1 expression on mouse endothelium is induced by IFN-γ and TNF-α both in vitro and in vivo (21, 23, 31), we examined whether these cytokines were also involved in the regulation of MAdCAM-1 expression on human endothelial cells.

Indeed, both IFN-γ and TNF-α increased MAdCAM-1 expression on endothelial cells in cultured human stomach explants (Fig. 4), and TNF-α induced de novo MAdCAM-1 expression on cultured HUVEC (Fig. 2B). Enzyme-linked immunosorbent assay analyses of culture medium from stimulated explants showed that CTB induced secretion of TNF-α, with the concentration increasing from <15.6 to 50.2 ± 12.1 pg/ml after 24 h of stimulation. The finding that the CTB effect on MAdCAM-1 expression on endothelial cells in cultured gastric explants could be almost completely blocked by addition of a TNF-α-specific neutralizing antibody further indicates that the CTB effect on MAdCAM-1 expression indeed is mediated by TNF-α. In contrast, CTB did not induce IFN-γ production, probably because the cultured mucosal tissue contained very few IFN-γ-producing cells, e.g., T and natural killer cells.

**DISCUSSION**

Mucosal immunization results in homing of antigen-specific lymphocytes back to the mucosal tissue where they first encountered the antigen. Whereas the impact of immunization on the expression of mucosal homing receptors on circulating lymphocytes has been rather well studied (15, 27), it is unknown whether this homing of specific cells is mediated by an altered endothelial addressin expression after vaccination. In the present study we show that the endothelial expression of MAdCAM-1 is increased in the gastric and upper small intestinal mucosa after immunization by various local routes with an oral cholera vaccine in the upper gastrointestinal tract.

It is likely that the increased MAdCAM-1 expression in the mucosa after immunization is one of the mechanisms controlling the recruitment of vaccine-specific lymphocytes back to the site of antigen encounter. This is further supported by our finding that rectal immunization did not influence the levels of MAdCAM-1 in the gastric or duodenal mucosa. Furthermore, the finding of increased MAdCAM-1 levels only in the mucosa close to the inductive site provides an explanation for the previously demonstrated compartmentalization of mucosal immune responses (7, 8, 26). This notion is also supported by the fact that only jejunal, gastric, and oral immunizations result in specific B-cell migration into the duodenal and gastric mucosa in H. pylori-infected subjects (28), whereas rectal immunization does not (unpublished data). We have previously shown that H. pylori infection per se does not alter the expression of MAdCAM-1 on gastric endothelial cells (28) and the immunization-induced upregulation of MAdCAM-1 in the stomach was independent of H. pylori status. Nevertheless, our previous studies clearly demonstrate that migration of antibody-secreting cells induced by oral immunization into the gastric mucosa is achieved only in H. pylori-infected subjects (20, 28). Therefore, in addition to increased MAdCAM-1 expression, additional signals, such as chemokines, are probably involved in attracting homing lymphocytes to distinct parts of the gastrointestinal mucosa. TECK, for example, is produced almost exclusively in the small intestine (16, 36). In addition, the CXCR3 and CXCR4 ligands have recently been shown to recruit circulating plasmablasts induced by intraperitoneal immunization (10). With regard to H. pylori infection, the IFN-γ-inducible CXCR3 ligands I-TAC, Mig, and IP-10 might well contribute to antibody-secreting cell recruitment to the inflamed gastric mucosa. Indeed, chemokines may even enhance lymphocyte binding to MAdCAM-1 (22, 35). Nevertheless, endothelial expression of MAdCAM-1 is likely a prerequisite for the subsequent action of chemokines in the recruitment of lymphocytes. In the present study we found that the vaccine component CTB as well as the whole vaccine increased MAdCAM-1 expression on endothelial cells in cultured human gastric explants but not on cultured human endothelial cells. We speculated that the vaccine-induced MAdCAM-1 expression could be mediated by tissue factors such as cytokines, which have been shown to induce MAdCAM-1 expression on mouse endothelial cells both in vitro and in vivo.

**DUODENUM**

Jejunal  Gastric  Per oral  Rectal

![Graph showing MAdCAM-1 expression in duodenum before and after immunization](image-url)
FIG. 2. Photomicrographs showing MAdCAM-1 as detected by immunofluorescence (A and B) and by immunohistochemistry (C to F). (A) Lack of MAdCAM-1 expression on unstimulated HUVEC. (B) Induction of MAdCAM-1 expression (fluorescein isothiocyanate staining) on HUVEC after stimulation with TNF-α for 20 h. (C and D) MAdCAM-1-positive blood vessels in the stomach of a subject not infected with *H. pylori* before (C) and 1 week after (D) the last immunization of the same volunteer. (E and F) MAdCAM-1-specific staining in the duodenum of an *H. pylori*-infected subject before (E) and 1 week after (F) the last immunization of the same volunteer. Magnifications ×400 (A and B) and ×200 (C to F).
hand, the CTB–whole-cell vaccine induces Th1 cytokine responses in vivo in humans (25), and these responses might well contribute to upregulation of endothelial MAdCAM-1 expression.

In conclusion, we have shown that mucosal immunization of human volunteers with an oral cholera vaccine containing CTB results in an upregulation of MAdCAM-1 expression close to the inductive site. This strongly supports the involvement of MAdCAM-1 in the preferential homing of mucosal lymphocytes to their original site of activation and suggests that the mucosal homing of antigen-specific lymphocytes after vaccination is dependent on a regionally increased expression of the addressin. Furthermore, our results indicate that TNF-α mediates vaccine-induced MAdCAM-1 expression in the human gastrointestinal mucosa.

ACKNOWLEDGMENTS

We thank all of the volunteers for participating in the study, the staff at the Gastroenterology Unit at Sahlgrenska University Hospital for excellent technical assistance, and Hans Lönnroth for invaluable help with biopsy sampling. We also thank Mikael Innocenti for help with HUVEC culture.

This study was financially supported by the Swedish Research Council, the Swedish Medical Society, and Magnus Bergvalls stiftelse.

REFERENCES


In October 2004, increased expression of MadCAM-1 was observed after vaccination with vaccines that elicit a gut-directed immune response.


