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NOTES

Differential Expression of Interleukin-6, Intracellular Adhesion Molecule 1, and Major Histocompatibility Complex Class II Molecules in Renal Carcinoma Cells Stimulated with S Fimbriae of Uropathogenic *Escherichia coli*

BURKHARD KREFT,[1]* SABINE BOHNET,[2] OLAF CARSTENSEN,[2] JÖRG HACKER,[3] AND REINHARD MARRE[4]

Klinik für Innere Medizin[1] and Institut für Medizinische Mikrobiologie der Medizinischen Universität zu Lübeck,[2] Ratzeburger Allee 160, 2400 Lübeck, Institut für Genetik und Mikrobiologie der Universität Würzburg, Röntgenring 11, 8700 Würzburg,[3] and Institut für Medizinische Mikrobiologie der Universität Ulm, 7900 Ulm,[4] Germany

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The production of interleukin-6, intracellular adhesion molecule 1, and major histocompatibility complex class II molecules by a renal carcinoma cell line (ACHN) in response to S fimbriae of uropathogenic *Escherichia coli* was studied. S fimbriae adhered to ACHN cells and stimulated the production of interleukin-6 and intercellular adhesion molecule 1 but did not affect major histocompatibility complex class II expression by renal carcinoma cells. Our data demonstrate that S fimbriae of *E. coli* display immunomodulating properties on kidney-derived epithelial cells.

Renal parenchymal cells are involved in the pathogenesis of acute pyelonephritis by serving as a morphological substrate for bacterial attachment within the kidney (8). The renal inflammatory response in experimental pyelonephritis is characterized by the presence of neutrophil granulocytes (8) and T lymphocytes of the $CD4^+$ phenotype (18). However, the molecular events leading to the recruitment of immunologically active cells to the kidney in renal infections are poorly understood.

Experimental studies have demonstrated the contribution of bacterial adherence factors, such as fimbriae and adhesion proteins produced by uropathogenic *Escherichia coli* (20), to nephropathogenicity. In addition, S fimbriae of *E. coli* mediate bacterial adherence to tubular cells in culture (21). Because renal tubular cells are capable of expressing cell surface molecules crucial in lymphocyte adhesion and antigen presentation (5, 14) and produce various cytokines (30, 32), we determined the effects of S fimbriae on the expression of interleukin-6 (IL-6), intracellular adhesion molecule 1 (ICAM-1), and major histocompatibility complex (MHC) class II molecules by renal epithelial cells. A renal carcinoma cell line (i.e., ACHN) was used as a model because it is of human origin and renal carcinomas are derived from tubular epithelial cells (29).

ACHN cells (ATCC CRL1611) were cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO, Eggenstein, Germany) supplemented with fetal calf serum (FCS, 10%; GIBCO) and glutamine (2 mM; GIBCO). The porcine renal tubular cell line $LLC\text{-}PK_1$ (ATCC CL101) was maintained in M199 medium (GIBCO) supplemented with 5% FCS. Both cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and split twice a week (1:3 to 1:7).

S fimbriae were purified from the recombinant *E. coli* clone HB 101 pANN 801-4 carrying the S-fimbrial adhesin specific for NeuAca2-3Gal from *E. coli* 536 (10). The recombinant *E. coli* clone K-12 (HB 101) was used instead of the original uropathogenic wild-type strain to avoid any contamination with the O6-specific lipopolysaccharide (LPS). The fimbriae were isolated and purified by gradient ultracentrifugation as described previously (31). Endotoxin contamination was quantified by the *Limulus* amoebocyte lysate assay (Byk-Santec Diagnostics, Dietzenbach, Germany) (7). LPS from *E. coli* O55:B5 was used as a standard. The lower detection limit for this assay was 80 pg/ml. Fimbrial preparations at concentrations of 100 μg/ml contained no detectable endotoxin. Antiserum against S fimbriae was produced in rabbits by intravenous injection of 200 μg of S-fimbrial protein on days 0, 4, 8, 12, and 16.

Adherence of S fimbriae to ACHN cells was determined by an enzyme-linked immunosorbent assay (ELISA) as described by Parkkinen et al. (24), with minor modifications. Briefly, ACHN cells (30,000 cells per well) were cultured in 96-well flat-bottom microtiter plates (Greiner, Nürtingen, Germany) to confluence. The medium was removed, and S fimbriae (0 to 100 μg/ml in DMEM) were added for 4 h at 4°C. Unspecific adherence of S fimbriae to microtiter plates was determined in the absence of ACHN cells in wells coated with bovine serum albumin (BSA, 5%, 20 h). After removing nonadherent fimbriae by three washing steps with phosphate-buffered saline (PBS, pH 7.2), the monolayer was fixed with paraformaldehyde (3.5% in PBS) for 20 min at room temperature. After three washing steps, free binding sites were blocked with 1% BSA-PBS. Microtiter plates were washed three times with PBS, and polyclonal rabbit anti-S-fimbrial antiserum (1:1,000 in 1.0% BSA-PBS) was added for 16 h at 4°C. After three washing steps, peroxidase-

* Corresponding author.

conjugated goat anti-rabbit immunoglobulin G (IgG; 1:3,000 in 0.1% BSA-PBS, 37°C, 1 h; Sigma) was added. The wells were washed three times, and then ABTS (Sigma; 1 mg/ml) plus hydrogen peroxide (0.28 μl of 30% H_2O_2 per ml) in 0.1 M sodium acetate (pH 4.2) was added. The color was allowed to develop for 30 min at room temperature, and the A_{405} of the plates was read with a microreader (Behring EL 311). In control experiments, the binding of the polyclonal anti-S-fimbrial antibody to ACHN cells and that to microtiter plates were determined separately.

For the induction of IL-6, ACHN cells were cultured in 24-well plates (300,000 cells per well; Nunc) to confluence. The medium was removed, and DMEM (1% FCS) containing LPS from *Salmonella abortus equii* (0 to 10 μg/ml; Sigma) or S fimbriae (0 to 100 μg/ml) was added. Supernatants were collected after 0, 1, 6, 24, 48, and 72 h and frozen at −20°C.

IL-6 bioactivity was measured by using the IL-6-dependent B-cell hybridoma B9.9 originally characterized by Aarden et al. (1). B9.9 cells (kindly provided by H. Kirchner, Lübeck, Germany) were maintained in RPMI 1640 (GIBCO) supplemented with FCS (10%). For the IL-6 determination, B9.9 cells were harvested, washed with RPMI 1640, and seeded at a density of 10,000 cells per well into 96-well round-bottom microtiter plates (Greiner) containing serial dilutions of conditioned supernatants of ACHN cells and RPMI 1640 (1% FCS). After 72 h of incubation, cell proliferation was determined by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) (23). The IL-6 concentration of the samples was quantified by comparing the results with the linear portion of a standard curve obtained with human recombinant IL-6 (specific activity, 5×10^7 U/mg; Serva).

In the following discussion of the method used for quantifying the expression of ICAM-1, the differences in the method for MHC class II molecules are shown in parentheses. To quantify the expression of ICAM-1 (MHC class II molecules) on ACHN cells, cells were seeded into 96-well flat-bottom microtiter plates at 30,000 cells per well (10,000 cells per well) for 8 h at 37°C. The medium was removed, and complete DMEM containing LPS, S fimbriae, or gamma interferon (γ-IFN) was added in the concentrations indicated. Stimulation was continued for 20 h (72 h), and ICAM-1 (MHC class II) expression was quantified by using a sandwich ELISA technique (26). Briefly, cells were fixed with paraformaldehyde (1% in PBS), and a monoclonal mouse anti-ICAM-1 antibody (Dianova) (anti-HLA-DR antibody; DAKO PATTS) (1:1,000 in 0.1% PBS-BSA) was added for 1 h (16 h) at 37°C (4°C). Peroxidase-conjugated goat anti-mouse IgG (1:1,000 in 0.1% BSA-PBS; Sigma) was used as developing antibody (37°C, 1 h), and after additional washings, ABTS plus hydrogen peroxide dissolved in 0.1 M sodium acetate was used as a substrate as described above. The tubular cell line LLC-PK_1 served as a negative control in ICAM-1 stimulation experiments (14).

Each experiment consisted of 6 to 18 determinations and was repeated three times. Statistical analysis was performed by using the U test of Wilcoxon, Mann, and Whitney (28). Differences were considered significant for P of <0.05. Results are expressed as means and standard deviations. Data of representative experiments are shown.

Initial experiments were designed to investigate the binding of S fimbriae to ACHN cells. By using an ELISA, a dose-dependent adherence of S fimbriae (5 to 100 μg/ml) to this cell line was detected (Fig. 1). A significant binding was observed when 5 μg/ml was used. The binding of S fimbriae to ACHN cells was specific since neuraminidase pretreatment partially reduced S-fimbrial adherence. Pretreatment of unfixed ACHN cells with 0.5 mU of neuraminidase from *Vibrio cholerae* (Merck, Darmstadt, Germany) per ml (100 μl per well) for 45 min at 37°C diminished the degree of S-fimbrial binding by 38%. Four hours of incubation was needed for maximal binding to be demonstrated, although a significant adherence of S fimbriae (50 μg/ml) was already detectable after 15 min of incubation (data not shown). Control experiments revealed a low nonspecific adherence of S fimbriae to microtiter plates and a low binding of polyclonal rabbit anti-S-fimbrial antiserum to ACHN cells (Fig. 1).

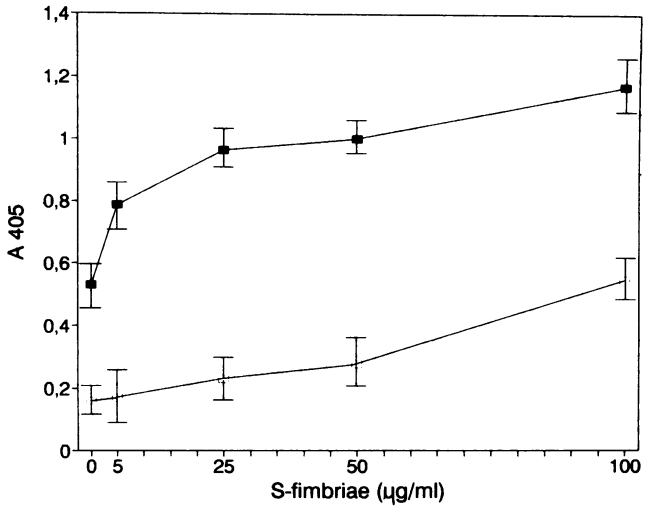

FIG. 1. Adherence of S fimbriae of *E. coli* to ACHN renal carcinoma cells (filled squares) and polystyrol microtiter wells (open squares) as quantified by ELISA. Means ± standard deviations are shown. The unspecific binding of peroxidase-conjugated goat anti-rabbit IgG to ACHN cells was 0.260 ± 0.01, and the unspecific binding of peroxidase-conjugated goat anti-rabbit IgG to polystyrol microtiter plates was 0.15 ± 0.02 in this experiment (data not shown in the figure).

Basal IL-6 secretion of ACHN cells was detected in supernatants of cells maintained in DMEM without stimulation. IL-6 activity in these supernatants became detectable after 6 h of culture and reached a maximal level after 48 h. Enhanced IL-6 activity was found in supernatants from ACHN cells incubated with S fimbriae of *E. coli*. At a concentration of 100 μg/ml, S fimbriae led to an elevated IL-6 activity in ACHN supernatants measurable after 1 and 6 h, and this increase peaked after 48 h (Fig. 2). A similar time course of IL-6 production was found when 10 μg of S fimbriae per ml was used. LPS at 0.1 μg/ml did not increase IL-6 production in ACHN cells after 24 h of incubation, but at 10 μg/ml, it enhanced IL-6 secretion after 48 h (231 ± 57 pg/ml).

ICAM-1 was expressed constitutively by ACHN cells and was upregulated by γ-IFN, with 8 U/ml being the lowest dose of γ-IFN causing a significant increase (data not shown). Purified S fimbriae (100 μg/ml) also stimulated ICAM-1 expression (Table 1). When S fimbriae (100 μg/ml) were coincubated with γ-IFN (100 U/ml), an additive effect was detectable. The ICAM-1-enhancing capacity of S fimbriae was in the range of 110 to 115% of baseline ELISA values and did not exceed 125% of the absorbance of unstimulated control ACHN cells. LPS at various concentrations (0.1, 1, and 10 μg/ml) had no effect on the expression

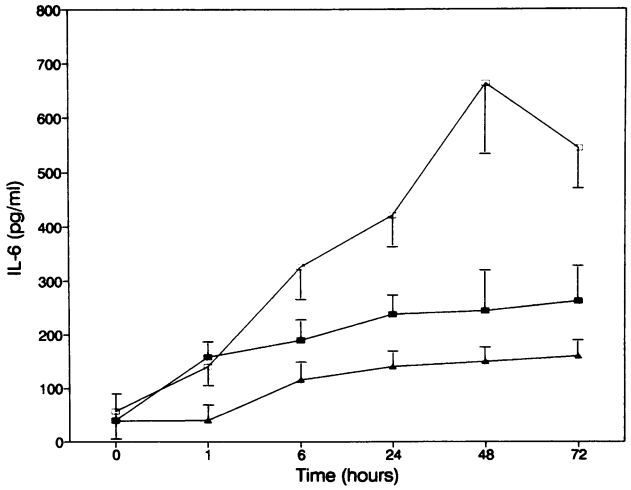

FIG. 2. Production of IL-6 in ACHN renal carcinoma cells without (filled triangles) and after stimulation with 10 (filled squares) and 100 (open squares) μg of S fimbriae of *E. coli* per ml. IL-6 was quantified by the B9.9 proliferation assay (1). Means ± standard deviations are shown.

of ICAM-1 and did not alter ICAM-1 expression induced by γ-IFN. S fimbriae at a concentration of 100 μg/ml did not induce MHC class II (HLA-DR antigen) expression. However, γ-IFN (125 to 250 U/ml) induced MHC class II expression on ACHN cells in a dose-dependent manner. Coincubation of γ-IFN and S fimbriae did not further enhance MHC class II expression compared with stimulation by γ-IFN alone.

Different fimbriae have been identified in *E. coli* causing urinary tract infections. S fimbriae recognize glycoproteins terminating with α-sialyl-acid-2-3-β-galactosamine (15, 25). Experimental data indicate that this type of fimbria is an important virulence factor in renal infections caused by *E. coli* (20). By using an ELISA, we have demonstrated that S fimbriae of *E. coli* adhere to renal carcinoma cells (ACHN) in vitro. This method has been used previously to quantify the adherence of *E. coli* (21) and *Enterococcus faecalis* (17) to tubular epithelial cells (LLC-PK_1). Our data complete recent findings reporting specific adherence of S fimbriae to human endothelial cells (24) and are in line with the marked adherence of *E. coli* strains expressing S fimbriae to human epithelial carcinoma cells (HEp-2) (16).

TABLE 1. Effects of S fimbriae of *E. coli* and γ-IFN on expression of ICAM-1 on ACHN renal carcinoma cells[a]

Stimulus (concn)	n[b]	ICAM-1 expression (A_{405} [mean ± SD])
None	11	0.186 ± 0.018
γ-IFN (100 U/ml)	18	0.360 ± 0.028[c]
S fimbriae (100 μg/ml)	12	0.203 ± 0.016[c]
γ-IFN (100 U/ml) + S fimbriae (100 μg/ml)	12	0.383 ± 0.033[d]

[a] ACHN renal carcinoma cells were incubated with γ-IFN and S fimbriae of *E. coli* for 24 h, and the ELISA detecting ICAM-1 surface expression (26) was performed as described in Material and Methods.
[b] n, number of determinations in representative experiments.
[c] $P < 0.05$ versus control by the U test of Wilcoxon, Mann, and Whitney (28).
[d] $P < 0.05$ versus ACHN cells stimulated with γ-IFN (100 U/ml).

S fimbriae had a dose-dependent effect on IL-6 production by ACHN cells. Similar in its temporal pattern to basal IL-6 production, the peak IL-6 bioactivity in ACHN supernatants after S-fimbrial stimulation was observed after 48 h of incubation. The IL-6-inducing effect of S fimbriae was not due to LPS contamination because the concentration of LPS in this suspension was below the detectable limit (*Limulus* assay) and LPS stimulated IL-6 secretion only at high concentrations. P fimbriae of *E. coli* have been shown to induce IL-6 production in a renal carcinoma cell line, but S fimbriae had no comparable effects on the expression of IL-6 in this study (12). This is in contrast to our results, which showed a marked induction of IL-6 in renal carcinoma cells by S fimbriae. This difference might be explained by cell line specificities or by different properties of the S-fimbrial preparation used. In experimental urinary tract infections in mice, P fimbriae have previously been shown to induce local IL-6 production (19), and renal carcinoma cells produce IL-6 and IL-8 after incubation with *E. coli* expressing type 1 and P fimbriae (2). However, the in vivo contribution of S fimbriae of uropathogenic *E. coli* to the release of cytokines by renal epithelial cells remains to be determined. Given the proinflammatory (9) and chemoattractant effects of IL-6 to immune effector cells (3), IL-6 production by renal tubular cells might contribute to the renal inflammatory response in bacterial pyelonephritis.

In addition, S fimbriae of *E. coli* significantly enhanced ICAM-1 expression by renal carcinoma cells. The elevation of ICAM-1 expression caused by S fimbriae at 100 μg/ml was equivalent to about 10 U of γ-IFN per ml in simultaneous experiments. Harskard et al. demonstrated that γ-IFN increases T-lymphocyte adhesion to human endothelial cells at comparable concentrations of γ-IFN (11). Although this report did not demonstrate ICAM-1 expression on endothelial cells, it demonstrates relevant biological effects of γ-IFN at low concentrations. Interestingly, the elevation of IL-6 and IL-8 production in renal carcinoma cells stimulated with *E. coli* expressing type 1 and P fimbriae (5.2 and 11.1% of controls) was well in the range seen in our experiments on ICAM-1 induction.

In a variety of inflammatory kidney diseases, the expression of ICAM-1 on renal epithelia enhances the interaction between helper T lymphocytes and tubular cells (5, 14). We therefore speculate that the modest effect of S fimbriae on ICAM-1 expression by renal epithelial cells might be of functional relevance with respect to lymphocyte adhesion in renal infections.

S fimbriae of *E. coli* did not consistently induce MHC class II expression on ACHN cells. Therefore, our investigations provide no data that MHC class II expression on renal epithelial cells is directly regulated by S fimbriae of *E. coli*. Renal infections caused by *E. coli* (O6:K13:H1) in rats are associated with increased tubular expression of MHC class II molecules (13). In this model, soluble factors (e.g., γ-IFN) released by kidney-infiltrating T lymphocytes are likely to contribute to the expression of MHC class II molecules on renal parenchymal cells since MHC class II immunostaining was found in both kidneys of rats showing histological and bacteriological evidence of infection only in the contralateral kidney.

The use of a renal carcinoma cell line cannot be viewed as an optimal model for investigation of immunological properties of renal tubular cells in general. However, this approach provides several advantages. By using this human cell line of renal epithelial origin, we can exclude important limitations due to species differences which occur when nonprimate

tubular cells, such as the porcine renal cell line LLC-PK_1 or MDCK cells, are used. Primary cultures of human tubular epithelial cells may represent cell heterogeneity and/or differences between individual preparations. Third, ACHN cells do not produce interferon (27), which might induce MHC class II and ICAM-1 expression in an autocrine manner. In addition, carcinoma cell lines are well accepted in studies on the adherence and invasion of pathogens (6, 22), their molecular interaction with eucaryotic cells (4), and immunological aspects in the host-parasite interaction (2, 12). Finally, renal carcinomas are derived from tubular epithelial cells (29) and therefore are derived from the cell population of interest in our study.

In summary, purified S fimbriae of uropathogenic *E. coli* demonstrate immunomodulating properties, including the induction of IL-6 production and the expression of ICAM-1 by renal epithelial carcinoma cells. These effects may be involved in the recruitment of tissue-infiltrating cells to the kidney in bacterial pyelonephritis.

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