Identification of α-Fodrin as a Candidate Autoantigen in Primary Sjögren's Syndrome

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It is unclear whether organ-specific autoantigens are critical for the development of primary Sjögren's syndrome (SS). A 120-kilodalton organ-specific autoantigen was purified from salivary gland tissues of an NFS/*sld* mouse model of human SS. The amino-terminal residues were identical to those of the human cytoskeletal protein α-fodrin. The purified antigen induced proliferative T cell responses and production of interleukin-2 and interferon-γ in vitro. Neonatal immunization with the 120-kilodalton antigen prevented the disease in mice. Sera from patients with SS reacted positively with purified antigen and recombinant human α-fodrin protein, whereas those from patients with systemic lupus erythematosus and rheumatoid arthritis did not. Thus, the immune response to 120-kilodalton α-fodrin could be important in the initial development of primary SS.

Sjögren's syndrome is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein (RNP) particles SS-A (also called Ro RNA particle) and SS-B (also called La snRNP) (*1*, *2*). However, the pathogenesis of this syndrome remains unclear. We established an animal model of primary SS in NFS/*sld* mutant mice thymectomized 3 days after birth (3d-Tx) (*3*). The T cell receptor $V_\beta 8$ gene is preferentially expressed in these inflammatory lesions from the onset of disease, and high concentrations of salivary duct autoantibodies of immunoglobulin G (IgG) type were detected in sera from mice with autoimmune lesions (*3*). The 3d-Tx NFS/*sld* mice did not develop any other autoimmune lesions. To identify an organ-specific antigen, we used protein immunoblot analysis to detect a salivary gland autoantigen reactive with affinity-purified IgG from sera of 3d-Tx NFS/*sld* mice; no reactivity was detected in tissue homogenates from the lung, liver, heart, kidney, pancreas, spleen, or brain (Fig. 1A) (*4*). Moreover, no reactivity was detected in tissue homogenates from the salivary glands in other mouse strains such as BALB/c and C3H/He. We purified the salivary gland autoantigen from tissue homogenates by fast protein liquid chromatography (FPLC). The fraction containing autoantigen activity was purified further by ion exchange high-performance liquid chromatography (HPLC). Autoantigen activity was recovered in fractions 27 and 28 (Fig. 1B). Protein immunoblot analysis of these fractions revealed a major band of 120 kD (Fig. 1B, inset) (*4*).

To identify the 120-kD salivary gland protein, we electroblotted the band onto an Immobilon membrane and directly sequenced it with an Applied Biosystems 477A Protein Sequencer. The bands had a single NH_2-terminal sequence. This sequence was identical to the NH_2-terminal sequence of human α-fodrin (nonerythroid α-spectrin) (*5*): RQKLEDSYRFQFFQRDAEEL (*6*).

Proliferative T cell responses of spleen cells from 3d-Tx NFS/*sld* mice to the identified 120-kD antigen developed spontaneously at 4 weeks of age, consistent with the onset of the autoimmune lesions in the salivary gland (Fig. 1C) (*7*). This response increased at 8 and 12 weeks of age, then declined by week 16. In contrast, no response was detected with control antigens (fraction 40, lysozyme, and α-amylase). The spontaneous development of a proliferative T cell response to the 120-kD antigen is consistent with endogenous priming. We tested the 120-kD antigen-reactive T cells for additional properties to confirm the specific T cell responses. Splenic T cells from 8-week-old 3d-Tx NFS/*sld* mice challenged with the 120-kD antigen produced interleukin-2 (IL-2) and interferon-γ (IFN-γ) (Fig. 1D) (*8*). Thus, a potentially pathogenic T helper type 1 (T_H1) T cell population may be spontaneously primed to the 120-kD salivary gland autoantigen early in the development of autoimmune lesions in the salivary glands of this mouse model.

To examine the organ specificity of the

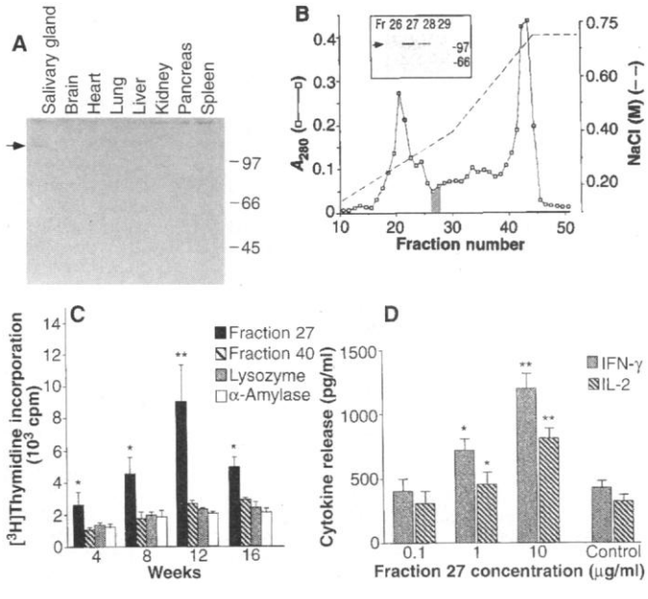

Fig. 1. (**A**) Protein immunoblot analysis of tissue homogenates with serum IgG from 3d-Tx NFS/*sld* mice. The 120-kD antigen reacted with tissue homogenates from the salivary glands, but not from the brain, heart, lung, liver, kidney, pancreas, or spleen (*4*). Molecular size markers are in kilodaltons. (**B**) HPLC fractionation. Absorbance was measured at 280 nm (A_{280}). The purified 120-kD antigen was detected by immunoblot analysis in fractions 27 and 28 (inset). (**C**) Proliferative T cell response to the 120-kD antigen develops spontaneously in 3d-Tx NFS/*sld* mice in a defined chronological order. Antigen-stimulated blastogenesis was measured in spleen cells from 4- to 16-week-old female NFS/*sld* mice. Data are expressed as counts per minute per culture ± SD in triplicate (one asterisk, $P < 0.01$; two asterisks, $P < 0.001$, Student's *t* test) (*7*). (**D**) Detection of IL-2 and IFN-γ by ELISA in culture supernatants of spleen cells from 8-week-old mice 48 hours after challenge with the 120-kD antigen. Data are the mean actual amount (picograms per milliliter) ± SD of triplicate samples [asterisks defined in (C)] (*7*). Control mice were injected with fraction 40 (10 μg/ml).

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120-kD α-fodrin, we immunoblotted various tissue homogenates from 3d-Tx NFS/*sld* mice with a commercially available monoclonal antibody to α-fodrin. More 120-kD α-fodrin was detected in the salivary gland homogenate than in homogenates from other organs (Fig. 2A) (*4*). A very faint 120-kD band in brain, heart, and lung tissue homogenates seems to be a degradation product of intact α-fodrin, because autoimmune lesions were not detected in these organs. In nonthymectomized NFS/*sld* mice, a negligible amount of 120-kD α-fodrin was detected in the salivary gland or in other organs (Fig. 2A). These data suggest that the salivary gland expression of 120-kD α-fodrin may be an initial response in the development of autoimmune lesions in the salivary glands. Polyclonal rabbit antibodies to a synthesized α-fodrin NH_2-terminal peptide reacted with the 120-kD salivary gland antigen in a protein immunoblot (Fig. 2B) (*9*). Immunohistochemical analysis revealed that epithelial duct cells intensely stained for antibodies to synthetic α-fodrin peptide in the salivary glands with autoimmune lesions, but not in salivary glands from nonthymectomized and normal control mice (Fig. 2, C and D) (*10*).

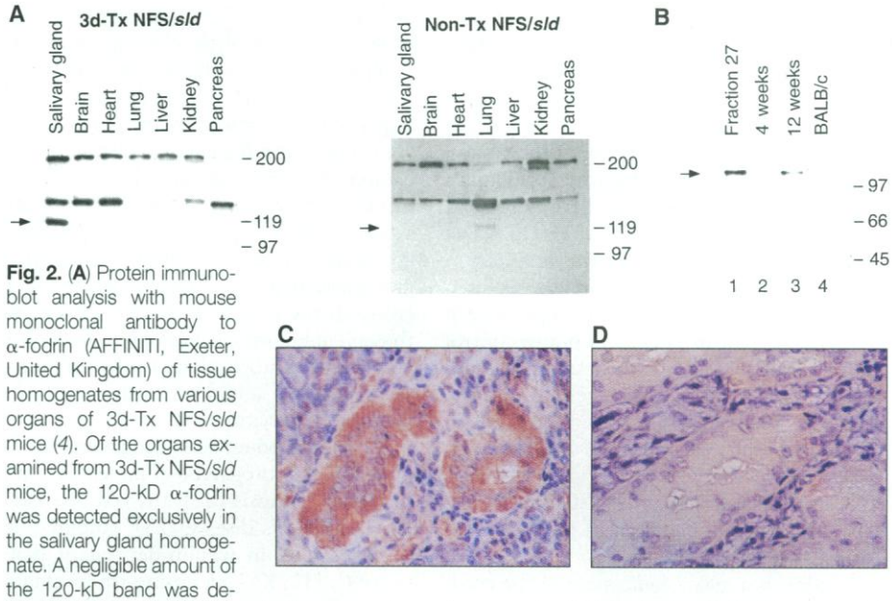

Fig. 2. (**A**) Protein immunoblot analysis with mouse monoclonal antibody to α-fodrin (AFFINITI, Exeter, United Kingdom) of tissue homogenates from various organs of 3d-Tx NFS/*sld* mice (*4*). Of the organs examined from 3d-Tx NFS/*sld* mice, the 120-kD α-fodrin was detected exclusively in the salivary gland homogenate. A negligible amount of the 120-kD band was detected in the salivary gland and other organs in nonthymectomized NFS/*sld* mice. (**B**) Protein immunoblot analysis with polyclonal antibodies to synthetic α-fodrin of the purified 120-kD antigen (lane 1), of salivary gland homogenates from 3d-Tx NFS/*sld* mice at 4 weeks old (lane 2) and at 12 weeks old (lane 3), and from normal BALB/c mice (lane 4). (**C**) Immunohistochemical analysis with polyclonal antibodies to synthetic α-fodrin of salivary gland sections from 3d-Tx NFS/*sld* mice. Epithelial duct cells were intensely stained, especially within the inflammatory lesions. (**D**) Immunoreactivity was absent in salivary glands from unaffected mice. No reactivity was observed in the liver, lung, kidney, heart, pancreas, or spleen from 3d-Tx NFS/*sld* mice nor in control mice (nonthymectomized NFS/*sld*, NFS/+, and BALB/c).

To test the specificity of the 120-kD α-fodrin as the salivary gland autoantigen, we constructed a recombinant α-fodrin fusion protein using the glutathione-S-transferase (GST) fusion system in *Escherichia coli*. JS-1 cDNA (*5*), encoding the NH_2-terminal portion of α-fodrin, was constructed by inserting cDNA (base pairs 1 through 1784) into the Eco RI site of pGEX-2T. Purified recombinant JS-1 protein was determined by examining its reactivity with sera from 3d-Tx NFS/*sld* mice. The reactivity with sera containing antibodies to 120-kD α-fodrin was confirmed by protein immunoblot analysis (Fig. 3A) (*11*). Spleen cells from 3d-Tx mice proliferated to the recombinant JS-1 fusion protein (Fig. 3B). We investigated, on a preliminary basis, whether the intravenous injection of the recombinant α-fodrin protein protects animals against the development of autoimmune lesions and found that intravenous injection of 25 μg of JS-1 inhibited the development of autoimmune conditions including lymphocytic infiltration and autoantibody production to the whole 120-kD molecule (Fig. 3, C and D) (*12*).

To confirm the disease specificity of the 120-kD α-fodrin and the recombinant JS-1 protein as an autoantigen in primary SS, we examined sera from patients with other autoimmune diseases, including secondary SS (n = 8), systemic lupus erythematosus (SLE, n = 21), and rheumatoid arthritis (RA, n = 14) and sera from normal healthy individuals (n = 15), in addition to sera

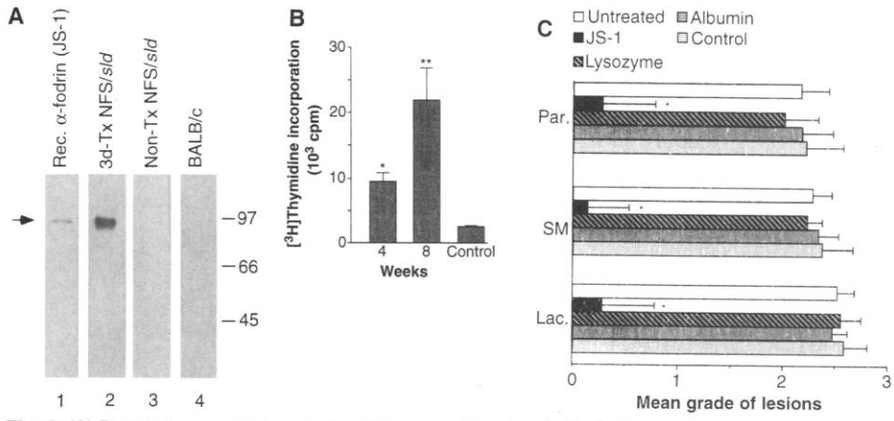

Fig. 3. (**A**) Protein immunoblot analysis of the recombinant α-fodrin fusion protein JS-1 with sera from 3d-Tx NFS/*sld* mice (lane 2), nonthymectomized NFS/*sld* mice (lane 3), and control BALB/c mice (lane 4). Lane 1 shows a Coomassie-stained gel. (**B**) Proliferative T cell response to JS-1 (5 μg/ml) developed at 8 weeks of age. Controls received an equivalent volume of recombinant protein from lysates of *Escherichia coli* carrying the GST gene alone. Data are mean counts per minute per culture ± SD of triplicates samples (one asterisk, $P < 0.01$; two asterisks, $P < 0.001$). (**C**) Preventive effect of intravenous injection of recombinant α-fodrin protein. A 25-μg sample of recombinant JS-1 protein (n = 7), albumin (n = 5), lysozyme (n = 5), or control GST fusion protein (n = 6) was injected intravenously into neonatal 3d-Tx NFS/*sld* mice. Mice with or without treatment were examined histopathologically at 8 weeks and compared with untreated 3d-Tx NFS/*sld* mice (n = 6) (asterisk, $P < 0.01$). Par., parotid gland; SM, submandibular gland; Lac., lacrimal gland. Controls received an equivalent volume of recombinant protein from lysates of *E. coli* carrying the GST gene alone. Mean grade of inflammatory lesions was expressed as described in (*12*). (**D**) Protein immunoblot analysis of the 120-kD α-fodrin with sera from mice with or without treatment. Autoantibody production to the whole 120-kD α-fodrin molecule was clearly inhibited in sera from mice treated with JS-1.

from patients with primary SS (n = 43). Of the 43 patients with primary SS, sera from 41 were positive for both the purified 120-kD antigen and the recombinant JS-1 protein on protein immunoblots (95.35%), whereas sera from all patients with SLE and RA and from normal healthy individuals were negative (Fig. 4, A and B). Sera from secondary SS patients were also positive (five out of eight cases) for this antigen. In addition, the 120-kD α-fodrin was detected in tissue homogenates of lip biopsies from patients with primary SS, but not in control individuals (Fig. 4C). Furthermore, we detected a proliferative T cell response to the purified 120-kD antigen using peripheral blood mononuclear cells (PBMCs) from patients with primary SS (Fig. 4D) (*13*). These data suggest that the 120 kD α-fodrin is a key target autoantigen in the induction of primary SS in humans.

Fodrin is a major component of the cortical cytoskeleton of most eukaryotic cells, and it forms heterodimers aligned in a side-to-side manner composed of an α (240-kD) and a β (235-kD) subunit (*14*). α-Fodrin is an actin-binding protein that is found at the periphery of chromaffin cells and may be involved in secretion (*15*). The stimulation of secretion in parotid acinar cells is associated with dramatic rearrangements of the subplasmalemmal cytoskeleton, particularly of α-fodrin (*16*). Fodrin has binding sites for various proteins including actin (*17*), calmodulin (*18*), and CD45 (*19*). Proteolytic cleavage of α-fodrin resulting from the activation of a neutral calcium-activated protease (calpain) could be responsible for these conformational changes (*20*). The fodrin α subunit is cleaved in association with apoptosis, and the 120-kD fragment is a breakdown product of the fodrin α subunit (*21*). The proteolysis of fodrin during apoptosis may be a consequence of unknown protease activation. Taken together, an increase in the enzymatic activity of proteases through unknown mechanisms is probably involved in the progression of α-fodrin proteolysis during the initial stages in the development of primary SS.

Autoantibodies are produced to the pyruvate dehydrogenase complex in primary biliary cirrhosis (*22*), thyroid peroxidase in Hashimoto's disease (*23*), glutamic acid decarboxylase in insulin-dependent diabetes (*24*), H^+, K^+–adenosine triphosphatase in pernicious anemia (*25*), and RNA polymerase I in SLE and RA (*26*). In SS, autoantibodies are generated to ribonucleoprotein particles SS-A and SS-B (*1*). Among them, disease-specific autoantibodies have only rarely been described. Despite recent findings on the mechanisms of self-tolerance, those leading to pathogenic autoimmunity remain obscure. Autoantigens can stimulate primary T cell proliferative responses (*27*), which suggests that many autoreactive T cells escape elimination and remain in an unresponsive state.

The identification of the salivary gland 120-kD α-fodrin is of relevance in elucidating the crucial role of this autoantigen in the development of primary SS. If the 120-kD α-fodrin is critical for the initiation of exocrinopathy, then preventing or reversing the autoimmune response to it in the salivary and lacrimal glands of susceptible individuals may be an approach for therapy.

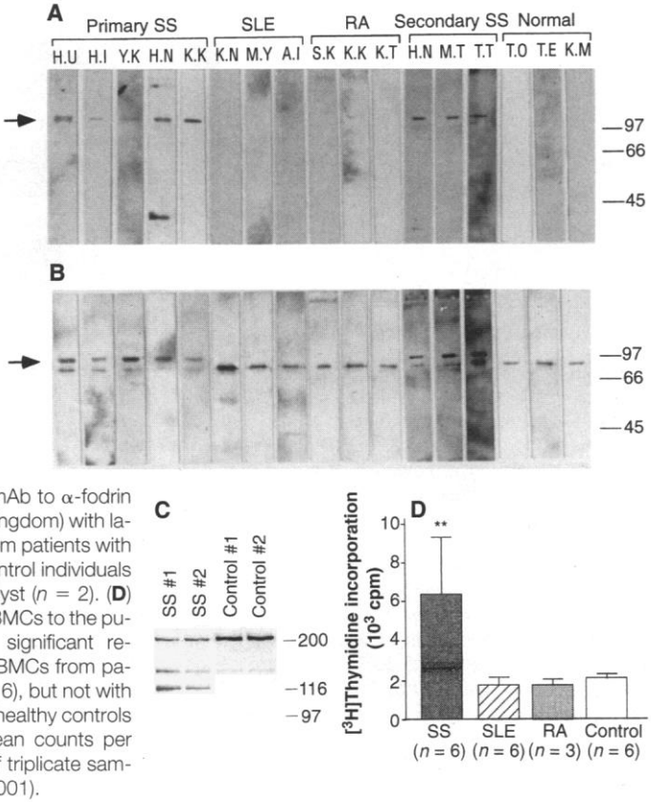

Fig. 4. Protein immunoblot analysis of the 120-kD α-fodrin and recombinant fusion protein JS-1 with sera from patients with various autoimmune diseases. Serum samples were tested in duplicate at a 1:250 dilution. Representative immunoblotting profiles were selected from each group. (**A**) Protein immunoblots of the purified 120-kD α-fodrin. (**B**) Protein immunoblots of JS-1 (70-kD protein is an artifact band of *E. coli* degradation). (**C**) Protein immunoblot analysis of mouse mAb to α-fodrin (AFFINITI, Exeter, United Kingdom) with labial gland homogenates from patients with primary SS (n = 2), and control individuals with labial gland mucous cyst (n = 2). (**D**) Proliferative response of PBMCs to the purified 120-kD antigen. A significant response was detected in PBMCs from patients with primary SS (n = 6), but not with SLE (n = 6), RA (n = 3), or healthy controls (n = 6) (*13*). Data are mean counts per minute per culture ± SD of triplicate samples (two asterisks, $P < 0.001$).

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4. The salivary gland homogenates from 3d-Tx NFS/*sld* mice were prepared in 20 mM tris-HCl buffer (pH 7.2) containing 0.15 M NaCl and proteinase inhibitors (5 mM benzamidine-HCl, 2 mM diisopropyl fluoride, and 2 mM EDTA). The supernatant of the homogenates was fractionated on a Superose 12HR column (Pharmacia) and further purified on a DEAE-Cosmogel column (Nakarai, Japan). SDS-polyacrylamide gel electrophoresis was carried out in 10% gels as described by (*28*).
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6. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
7. Spleen cells were plated at 1 × 10^6 cells per well in 96-well microtiter plates in 200 μl of HL-1 medium (Ventrex) containing 2 mM glutamine and antigen (10 μl/ml). During the last 8 hours of the 72-hour culture period, 1 μCi of [^{3}H]thymidine was added per well, and the incorporated radioactivity was determined.
8. The amounts of IFN-γ and IL-2 produced were measured by enzyme-linked immunosorbent assay (ELISA) (*29*) with IFN-γ–specific monoclonal antibodies (Pharmingen) and mIL-2 ELISA system (Amersham).
9. A synthetic peptide of α-fodrin corresponding to the identified 20–amino acid residues [RQKLEDSYRFQFFQRDAEEL) (*6*)] was produced with an Applied Biosystems model 430A peptide synthesizer. Polyclonal rabbit antibody to the synthetic peptide was developed and affinity purified. Rabbit serum to the synthetic peptide was diluted 1:25 when used for protein immunoblots.
10. For immunohistochemistry, formalin-fixed paraffin-embedded salivary gland sections were deparaffinized in xylen and hydrated in ethanol. Sections were stained with polyclonal rabbit antibody to synthetic α-fodrin peptide diluted 1:500 dilution in 10% (v/v) fetal bovine serum, followed by incubation with biotin-labeled goat serum to rabbit as the linking antibody. After the preparations were rinsed, binding was detected with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Specificity of the staining was confirmed by the observation that incubating polyclonal rabbit antiserum with synthetic peptide completely blocked staining.
11. JS-1 cDNA encoding the human nonerythroid α-fodrin cDNA was constructed by inserting cDNA (base pairs 1 through 1784) into the Eco RI site of plasmid pGEX-2T (Pharmacia). GST fusion protein was expressed and purified with a GST gene fusion system (Pharmacia) as described (*30*). Protein immunoblots were performed and proliferative T cell responses were determined as described in the legend to Fig. 1.

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13. PBMCs from each donor were plated at 5 × 10^5 cells per well in 96-well microtiter plate in 200 μl of RPMI 1640 medium containing penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, and antigen (10 mg/ml) in triplicate. During the last 20 hours of the 72-hour culture period, 1 μCi of [^{3}H]thymidine was added per well. Incorporation was measured by liquid scintillation counting.
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Prevention of Mucosal *Escherichia coli* Infection by FimH-Adhesin–Based Systemic Vaccination

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Virtually all uropathogenic strains of *Escherichia coli*, the primary cause of cystitis, assemble adhesive surface organelles called type 1 pili that contain the FimH adhesin. Sera from animals vaccinated with candidate FimH vaccines inhibited uropathogenic *E. coli* from binding to human bladder cells in vitro. Immunization with FimH reduced in vivo colonization of the bladder mucosa by more than 99 percent in a murine cystitis model, and immunoglobulin G to FimH was detected in urinary samples from protected mice. Furthermore, passive systemic administration of immune sera to FimH also resulted in reduced bladder colonization by uropathogenic *E. coli*. This approach may represent a means of preventing recurrent and acute infections of the urogenital mucosa.

Adhesive pili mediate the colonization of mucosal surfaces, allowing bacteria to establish infection (*1*, *2*). Over the years much research has been motivated by the goal of using pili as vaccines to prevent bacterial infections (*3*). However, these efforts have often been thwarted by findings that the major immunodominant component of pilus fibers is often antigenically variable and thus would only offer protection against a limited number of bacterial strains. Pilus-associated adhesins, on the other hand, are typically highly conserved among different strains and species of bacteria (*4*, *5*). However, adhesins are often only minor components of pilus fibers (located at the pilus tips), and as a result, intact whole pili do not elicit a strong antibody response to the adhesin (*6*). The use of expression systems to purify large amounts of adhesin has often failed because most adhesins are proteolytically degraded when expressed as an independent moiety (*7*, *8*). However, the discovery that adhesins could be stabilized in active conformations by specific PapD-like chaperones (*2*, *9*, *10*) makes it possible to purify adhesins in large quantity. We used bacterial cystitis as a model infection to test the hypothesis that bacterial adhesins would prevent microbial attachment and colonization when used as a vaccine.

Cystitis is one of the most common disorders prompting medical evaluation in otherwise healthy women. *Escherichia coli* is the most prevalent pathogen associated with lower urinary tract infections, accounting for >85% of asymptomatic bacteriuria and acute cystitis cases (*11*), as well as >60% of recurrent bouts of cystitis. Recent studies have shown that the incidence of *E. coli* cystitis among women 18 to 40 years old ranges from 0.5 to 0.7 infections per person per year (*12*). Such infections result in 7 to 8 million physician and hospital visits per year at a cost of more than $1 billion (*13*).

In contrast to the extensive studies that have implicated P pili as a critical factor in causing upper urinary tract infections (pyelonephritis) (*14*, *15*), very little is known about the molecular basis of bladder infections. A number of studies have suggested that type 1 pili may play a role in bacterial cystitis by mediating colonization to mannose receptors along the bladder mucosa (*16*). In a murine cystitis model it was shown that colonization of the bladder by clinical isolates of *E. coli* was greatly dependent on growth conditions that favored the expression of type 1 pili (*17*, *18*). In both murine and primate models, expression of P pili was unnecessary and insufficient for bladder colonization (*15*, *17*–*19*). Also, in a murine model, type 1–positive isolates survived in higher numbers and induced a greater neutrophil influx into the urine than isogenic type 1–negative mutants (*20*). Reconstitution of type 1 pili in type 1–negative strains restored virulence similar to that of the wild type. In addition, disease severity was greater in children infected with *E. coli* isolates expressing type 1 pili than type 1–negative isolates of the same serotype, same electrophoretic type, and same P piliated and hemolysin phenotypes.

In light of some of these earlier studies, we obtained 57 urinary tract infection isolates of *E. coli* from multiple clinical centers and found that 52 out of 57 (91%) could be induced to express type 1 pili under optimal growth conditions for type 1 pilus expression (*1*, *2*). In contrast, only 30% of the 14 strains that were tested could be induced to express P pili under optimal growth conditions for P pilus expression (*1*, *2*).

The association of type 1 pili with cystitis led us to investigate whether a receptor is present in human bladder mucosa that is recognized by FimH, the adhesin that confers mannose-specific binding activity to type 1 pili (*21*). In situ binding to human tissues has been used to elucidate the roles in virulence of many different adhesins, including pilus-associated adhesins, in a variety of pathogens including *Helicobacter pylori*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and pyelonephritic *E. coli* (*22*). The NU14 *E. coli* cystitis isolate (*18*) bound avidly to the luminal surface of both mouse (Fig. 1B) and human (Fig. 1D) bladder epithelium. Mannose completely blocked this binding (*23*). A chloramphenicol cassette was recombined into the *fimH* gene in the

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