FEMS Microbiology Immunology 47 (1989) 229–236
Published by Elsevier

FIM 00034

Oral vaccination of rats with live avirulent *Salmonella* derivatives expressing adhesive fimbrial antigens of uropathogenic *Escherichia coli*

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Received 14 June 1988
Accepted 9 October 1988

Key words: Avirulent *Salmonella*; S-Fimbriae; Uropathogenic *E. coli*

1. SUMMARY

The avirulent *Salmonella typhimurium* F885 was transformed with a plasmid carrying the cloned S fimbriae genes of a uropathogenic *Escherichia coli.* The resulting transformant (F885–1) produced efficiently *E. coli* S fimbriae and was used for live oral vaccination of rats. For comparison rats were immunized subcutaneously with isolated S fimbriae. Both routes of vaccination resulted in a significant IgG antibody response to S fimbriae. In addition live oral vaccination induced a serum IgA response against S fimbriae. After transurethral infection of rats with a S fimbriae producing *E. coli* a 10-fold reduction of bacterial counts in the kidney was observed in rats orally vaccinated with F885–1 as compared to unvaccinated controls. This study suggests that the avirulent *Salmonella* F885 may be used as a fimbrial antigen carrier for oral vaccination against renal infections.

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2. INTRODUCTION

Escherichia coli is the causative agent in more than 80% of urinary tract infections. Uropathogenic strains of *E. coli* generally exhibit characteristic virulence factors which enable the bacteria to adhere to urinary tract epithelial cells, to resist the bactericidal action of serum and to damage eukaryotic cells by means of hemolysin [1]. Adherence to uroepithelial cells — the initial step in the infection process — is mediated by microbial adhesins which very often represent minor components of fimbrial structures [2,3]. Depending on their receptor structures, the adhesins are subdivided into P, S, M, and X types [4,5]. The receptor for P fimbriae is the D-α-Gal-(1–4)-β-D-Gal moiety of the P blood group antigens [6]. P fimbriae are associated with the majority of uropathogenic strains. S fimbrial adhesins (Sfa) which interact with sialyl-(2–3) galactoside structures on human erythrocytes [7], are also present on uropathogenic strains and to a larger extent on strains isolated from cases of meningitis in the new-born [4]. M fimbriae require blood group M-specific determinants of glycophorin-A as receptor [8]. The so-

called X adhesins recognize an unidentified receptor on human red blood cells. In addition, the majority of *E. coli* isolates from different sources exhibit type I fimbriae which are able to recognize mannose-containing receptor structures [9].

In parenteral immunization experiments [10–12] it has been shown that anti-fimbrial antibodies afforded protection against experimental ascending pyelonephritis in rats and primates.

This raises the question whether the production of anti-fimbrial antibodies could also be induced by oral vaccines which have several advantages over parenteral vaccines, including the ease of administration and lack of adverse side-reactions. It has been shown earlier [13,14] that efficient oral immunization is achieved with some avirulent *Salmonella* given as live vaccine. These avirulent *Salmonellae* stimulate the production of intestinal and serum antibodies against surface antigens after oral application in mice due to their ability to colonize the small intestine over a certain period without producing any systemic infection [14]. If these avirulent strains are able to express adhesive *E. coli* fimbriae it remains to be determined whether they also can induce antibodies against these fimbriae.

The avirulent *S. typhimurium* F885 used here is a *Salmonella* hybrid expressing the *E. coli* 08 antigens instead of the *Salmonella* factors 04, 5, 12 [14] and has proved to be a strong immunogen in oral vaccination of mice. As fimbrial antigen, we have used the S fimbriae of *E. coli* 536 [15]. The respective genetic determinants termed 'sfa' have been cloned into the vector pBR322, resulting in the recombinant plasmid pANN 801–4 [15], which we used for transformation experiments with *Salmonella* F885.

3. MATERIALS AND METHODS

3.1. Strains

The strains used have previously been described [14,15]. *E. coli* 536 is a uropathogenic wild type strain with the antigen 06 : K15 : H31 and produces S fimbriae (Sfa^+), type I fimbriae and fimbriae which show similarities to P fimbriae but which lack any receptor specificity (Prf, *P related fimbriae*; Hacker et al., in preparation). The S fimbriae-producing *E. coli* K-12/pANN 801–4 was obtained by transformation of *E. coli* K-12 strain HB101 with the plasmid pANN801–4, which carries a 8.0 *K*b DNA fragment encoding the fimbriae of *E. coli* 536 [15]. *Salmonella typhimurium* F885 is a his^+ hybrid from a cross of the *E. coli* 08 donor Hfr59 and a *S. thyphimurium* recipient (his^-). The hybrid exhibits the *E. coli* 08 antigens instead of *Salmonella* 04, 5, 12 due to the introduction of the his-linked donor rfb genes determining the 0 antigen biosynthesis. The bacteria were grown on LB medium or agar. McConkey agar was used for reisolation of bacteria from rat organs.

3.2. Genetic procedures

Plasmid DNA was isolated from *E. coli* K-12/pANN801–4 as described previously [15]. For transformation of bacteria with the plasmid pANN801–4, a modified procedure as described by Lederberg and Cohen [16] was applied.

3.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of fimbrial subunits

Isolated fimbriae were disintegrated and run on slab gels as described previously [17].

3.4. Isolation of fimbriae

In order to avoid any contamination with the 06-specific lipopolysaccharide, the S fimbriae used for vaccination and as test antigen were isolated from the recombinant *E. coli* clone K-12/pANN801–4 instead of from *E. coli* 536. The fimbriae were isolated and purified by gradient ultracentrifugation as described previously [18]. Fimbriae of other strains used in SDS-PAGE were crude preparations without further purification.

3.5. Animal infection model

Female rats (Han Wist) weighing 180–200 g at the beginning of the experiment were used. They were kept in an air-conditioned animal house, fed with Altromin and water ad libitum. The rats were infected with 1.5 ml of an *E. coli* 536 suspension (5×10^7 cfu/ml) given via the urethra into the bladder. The bacteria were harvested from overnight culture into PBS, centrifuged, suspended

again in PBS and adjusted to the desired concentration.

3.6. Immunization procedures

For oral immunization, rats were given 5 ml of a bacterial suspension (10^9 cfu/ml) in PBS. In some experiments oral dosing was repeated on the next day or completed by an intraperitoneal injection of 2 ml of live bacteria two weeks after the first oral dose. Another group of rats received 7 subcutaneous (s.c.) injections of isolated S fimbrial proteins within a period of 10 days (250 μg protein per kg per day).

3.7. Recovery and enumeration of vaccine bacteria from organs

At specified times after oral dosing, rats were killed and the small intestines and spleens were removed. The intestines were rinsed with 20 ml ice-cold saline in order to remove fecal contents and unadsorbed bacteria. The washed small intestines and the spleens were homogenized in ice-cold saline with an MSE homogenizer. Aliquots of the homogenates were spread on XLD agar plates which were incubated for 18 h at 37 °C.

3.8. Infection studies

Rats were challenged with *E. coli* 536 via the urethra one week after one oral dose of F885–1. In comparative studies, rats were infected 10 days after the last s.c. injection of isolated fimbriae. The rats were sacrificed 7 days after the infection and the kidneys were removed. Viable counts of bacteria were made from kidney homogenates on McConkey agar plates.

3.9. Determination of antibodies

Blood was obtained by cardiac puncture at different intervals after immunization. The sera were stored at −20 °C. The antibody-content was measured by a chemiluminescence assay [19], which can briefly be summarized as follows:

Polystyrol plastic balls (Precision plastic balls, Chicago) were coated with S fimbrial protein isolated from *E. coli* K-12/pANN801–4. Goat anti-rat IgG (heavy and light chain specific), and IgA (alpha chain specific) (Cappel, Organon Teknika F.R.G.) were labeled with luminol (Sigma, Munich, F.R.G.). The plastic balls were incubated for 60 min at room temperature with rat serum. The serum was diluted 1 : 20 in PBS 4N Tween. Unbound rat antibodies were removed by washings in Tween and then incubated for 90 min with labeled anti-rat antibodies, which were diluted 1 : 1000 in PBS 4N Tween. After removal of unbound antibodies, saline was added and the chemiluminescence activity was determined in a luminometer (Berthold, F.R.G.) after addition of H_2O_2. Antibody activity was expressed as relative light units. Sera of non-immunized rats served as controls (day 0 in Fig. 2). Each group of rats consisted of 10 animals. Antibody activities were determined in duplicate.

4. RESULTS

4.1. Characterization of fimbriated strains

The avirulent *Salmonella* F885 was transformed with the recombinant plasmid pANN801

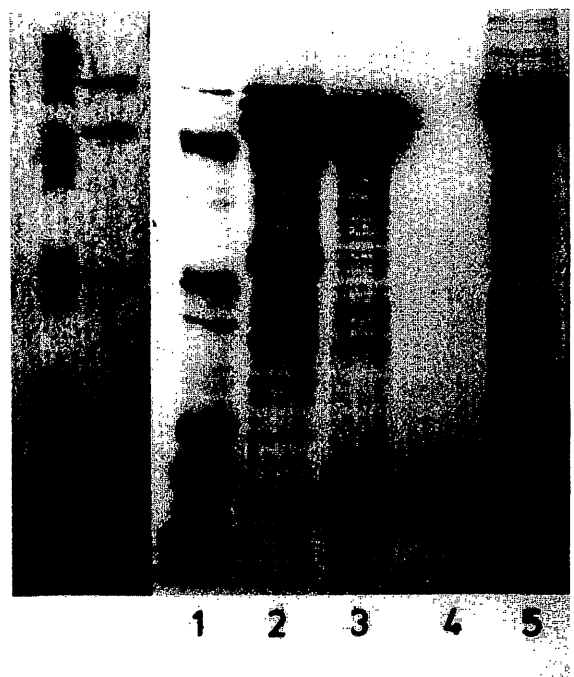

Fig. 1. SDS-PAGE of fimbrial subunits from different strains. Lanes: (1) M_r marker proteins; (2) *Salmonella* F885; (3) *Salmonella*, F885–1; (4) *E. coli* HB101/pANN801–4; (5) *E. coli* 536.

–4 isolated from *E. coli* K-12/pANN801–4. The resulting transformant F885–1, like the wild type *E. coli* 536, produced hemagglutination with bovine and human red blood cells and was agglutinable with antiserum specific for S fimbriae of *E. coli* 536 [20].

As demonstrated with SDS-PAGE of disintegrated fimbriae (see Fig. 1), the transformant F885–1 produces fimbrial sub-units identical in size (16.5 kDa) with those of *E. coli* 536 and of *E. coli* K-12/pANN801–4. The fimbriae of *E. coli* K-12/pANN801–4 were highly purified by CsCl gradient centrifugation and were used for s.c. vaccination and as test antigen for detecting S fimbriae antibodies.

The other fimbrial preparations were crude extracts still contaminated with various surface protein components. Thus the protein bands of about 50000 kDa in lanes 2, 3 and 5 (Fig. 1) are flagellin proteins of *Salmonella* F885 and *E. coli* 536, respectively. The 22 kDa protein in lane 5 represents the protein subunits of the non-hemagglutinating P related fimbriae (Prf) which are also associated with *E. coli* 536 [15].

Table 1

Salmonella colonization (mean log cfu/g of tissue ± standard deviation) of the intestines 1, 2, 6, 13, and 20 days after per oral administration of *Salmonella typhimurium* F 885–1

Salmonella colonization on day				
1	2	6	13	20
3.97 ± 0.5 (5/5) *	5.01 ± 0.2 (5/5)	3.97 ± 0.7 (5/5)	1.41 ± 0.4 (5/5)	1.16 ± 0.5 (5/5)

* number of culture positive animals/number of animals tested.

4.2. Persistence of recombinant Salmonella strains in the small intestine

It has been shown that the ability of the F885 bacteria to persist for nearly 20 days in the small intestine of mice after oral application is decisive for the immunogenic efficiency. We therefore examined how long F885–1 bacteria can persist in

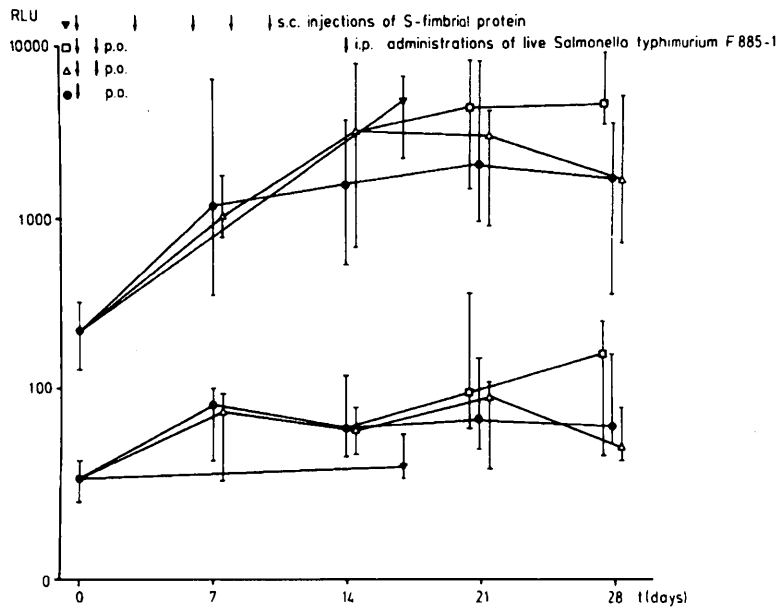

Fig. 2. Antifimbrial antibodies in rat serum (median of 10 animals, range) determined by the chemiluminescence method and expressed as relative light units (RLU). Arrows indicate vaccination. The upper group of curves represent IgG antibody activities, the lower group of curves represent IgA antibody response. ●———● one oral administration: △———△ two oral administrations; □———□ two oral and one intraperitoneal administration of a suspension of *Salmonella typhimurium* F885–1, ▼———▼ s.c. injections of S-fimbrial protein. The values of normal rat serum (median of 10 animals, range) are given at time zero.

the rat small intestine after oral dosing. Five rats were sacrificed on day 1, 2, 6, 13, and 20 after oral application of one dose consisting of 5×10^9 bacteria of F885–1 and the number of organisms associated with the gut epithelium was determined. The results (Table 1) show that there is obviously a steady decline in the number of bacteria after day 2 but organisms were still detected on day 20 after oral dosing. All animals tested had *Salmonella* positive gut cultures. The spleens of the animals were, if at all, generally less colonized with vaccine bacteria. None of the orally vaccinated rats died.

4.3. Anti S fimbriae antibody response

The results of antibody determinations are presented in Fig. 2. One oral dose of *Salmonella* F885–1 led to a 5–10-fold increase of serum anti-fimbrial antibodies of the IgG class during the first week, indicating that S fimbriae were also produced in vivo. The antibody levels reached a peak 3 weeks after oral administration and then declined slightly. In addition, a two-fold increase of the level of anti-fimbrial IgA antibodies was observed ($P < 0.05$). Repeated oral immunization stimulated the IgG antibody production further but did not affect the IgA antibody response. An additional intraperitoneal injection after two oral doses led to a further increase of IgG antibodies and provoked a slight but not significant stimulus of IgA antibody production. S fimbrial protein given subcutaneously seven times within 10 days caused a relatively greater increase of IgG antibodies as compared to the effects of oral vaccination. However, in contrast to orally administered vaccine no serum IgA antibody response could be detected after s.c. administration of fimbrial proteins.

4.4. Influence of vaccination on the growth rate of E. coli *536 in the kidney*

The experimental infection of untreated rats via the urethra with *E. coli* 536 led on day 7 p.i. to renal bacterial counts of more than 20000 cfu/g of kidney (Fig. 3). Five percent of the infected animals died.

After active subcutaneous immunization with fimbrial proteins, the renal bacterial counts of the

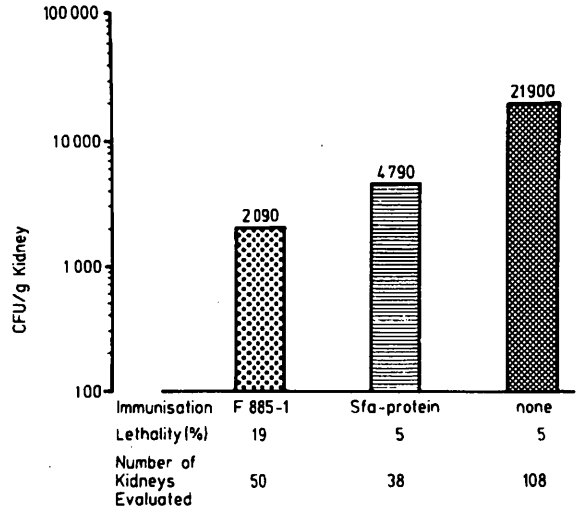

Fig. 3. Nephropathogenicity in a rat pyelonephritis model of the S fimbriae-producing *E. coli* strain 536 after different immunization procedures. Bacterial counts/g of renal tissue were determined one week after transurethral infection. Statistical evaluation: Significant differences ($P < 0.05$) between the animals immunized with F885–1 versus the non-immunized group.

infectious *E. coli* 536 were reduced to less than 5000 cfu/g of kidney. One oral dose of the live *Salmonella* F885–1 had an even more pronounced protective activity as judged by the bacterial counts in the kidney after infection with *E. coli* 536. The lethality, however, which occurred within the first 3 days after transurethral infection, was about 20% in the group of rats vaccinated orally with live F885–1 organisms, indicating that at the given time-point of infection the susceptibility of rats is even enhanced, in some animals, by this immunization procedure.

5. DISCUSSION

It has been demonstrated recently that *E. coli* S fimbrial adhesins contribute to nephropathogenicity in a rat pyelonephritis model [1]. Therefore, S fimbriae were chosen as the basis for vaccines used here for rat immunization studies. We have shown that cloned fimbrial genes of a uropathogenic *E. coli* are efficiently expressed in the avirulent *S. typhimurium* F885–1. The oral administra-

tion of the recombinant *Salmonella* strain F885–1 producing *E. coli* S fimbriae leads to the production of serum IgG and IgA antibodies directed against these fimbriae. An increase in serum IgA antibodies directed against S fimbriae was observed only after oral dosing of a live vaccine but not after parenteral injection of isolated fimbriae. This may be explained by results obtained earlier with F885 in mice [14]. These experiments have shown that the stimulation of serum and intestinal IgA antibodies directed against surface antigens is feasible only by oral administration of bacteria like F885 which are able to colonize the small intestinal epithelium.

Using a similar approach, Stevenson and Manning [21] employed a galactose-epimerase-deficient (gal^-) mutant of *Salmonella typhimurium* as carrier for the cloned *E. coli* K88 antigen, which is a colonization factor of enterotoxigenic *E. coli*. The oral administration of the resulting transformant led to high levels of IgG antibodies directed against the K88 antigen both in serum and in the small intestine of mice.

In a preliminary infection experiment with *E. coli* 536 in rats, the development of bacterial counts in the kidney after immunization by different procedures and of untreated controls was compared. The different ways of immunization applied were successful in stimulating the production of antifimbrial antibodies. As demonstrated in Fig. 2, one oral dose of the live recombinant Sfa^+ *Salmonella* hybrid strain F885–1 is responsible for a 10-fold reduction of renal bacterial counts on day 7 after transurethral infection as compared to results with non-vaccinated controls. Similar results were obtained after multiple sucutaneous injections of fimbrial proteins. Therefore, it seems conceivable that the presence of serum antifimbrial antibodies produced by different immunization procedures in rats exerts a protective effect against urinary tract infections. This has also been shown recently in other animal infection models [10].

Although a live oral vaccine seems to be attractive because of its ease of administration, the increase of lethality after infection with *E. coli* 536 as a side-effect of oral live vaccination indicates that this way of immunization may interfere with the antibacterial host defence mechanisms as has been shown in other studies. Thus, vaccination of mice with a live avirulent *Salmonella* mutant (aro^-) led to marked suppression of the mitogenic responsiveness of spleen cells to *E. coli* and *Salmonella* lipopolysaccharide [22]. Similar effects were observed after vaccination with viable Bacillus Calmette-Guérin [23]. The appearance of suppressor T-cells in the immune response to BCG, possibly induced by a feedback mechanism, may point to the possible role of suppressor cells in turning off the immune response [24] but as a side-effect may increase the infection susceptibility. It will now be necessary to determine to what extent infection susceptibility depends on the time-point of challenge after oral vaccination with *S. typhimurium* F885.

ACKNOWLEDGEMENTS

This study was supported by the DFG (Ma 864/2–2 and Ha 1434/1–5).

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