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Inhibitory effect of probiotic Escherichia coli *strain Nissle 1917 on adhesion to and invasion of intestinal epithelial cells by adherent–invasive* E. coli *strains isolated from patients with Crohn's disease*

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SUMMARY

Background: Pathogenic adherent–invasive *Escherichia coli* have been isolated from ileal lesions of Crohn's disease.

Aim: To investigate the non-pathogenic *E. coli* strain Nissle 1917 (Mutaflor) as possible maintenance therapy in inflammatory bowel disease by testing its ability to prevent adherent–invasive *E. coli* strains from adhering to and invading human intestinal epithelial cells *in vitro*.

Methods: Bacterial adhesion to and invasion of intestinal epithelial cells (Intestine-407) were assessed by counting the colony-forming units. The inhibitory effect of *E. coli* Nissle 1917 was determined after co-incubation with adherent–invasive *E. coli* strains or after pre-incubation of the intestinal epithelial cells with this probiotic strain prior to infection with adherent–invasive *E. coli* strains.

Results: Strain Nissle 1917 exhibited dose- and time-dependent adherence to intestinal epithelial cells and inhibited the adhesion and invasion of various adherent–invasive *E. coli* strains. In co-infection experiments, the inhibitory effect on adherent–invasive *E. coli* adhesion reached 78–99.9%. Pre-incubation of intestinal epithelial cells with strain Nissle 1917 reduced adherent–invasive *E. coli* adhesion by 97.2–99.9%. The inhibitory effect on adherent–invasive *E. coli* invasion paralleled that on adhesion.

Conclusion: As strong and significant inhibitory effects on adherent–invasive *E. coli* adhesion and invasion were observed in co-infection and pre-infection experiments, *E. coli* Nissle 1917 could be efficient for preventive or curative probiotic therapy in patients with Crohn's disease.

INTRODUCTION

Crohn's disease is an inflammatory bowel disease of unknown aetiology (for a review, see Podolsky[1]). Some characteristic pathological elements of Crohn's disease, including aphthous ulcers of the mucosa, mural abscesses and macrophage and epithelioid cell granulomas, also occur in well-recognized infectious diseases, such as shigellosis, salmonellosis and *Yersinia* enterocolitis, in which invasiveness is an essential virulence factor of the bacteria involved.[2] The identification of mutations of the nucleotide-binding oligomerization domain 2 (NOD2)/caspase-recruitment domain 15 (CARD15) encoding gene in patients with Crohn's disease suggests a link between the innate immune response to invasive bacteria and the development of

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Crohn's disease.[3, 4] NOD2 is a cytosolic receptor responding to the presence of bacterial components, such as muramyl dipeptide.[5, 6] Other findings have also linked adherent *Escherichia coli* with Crohn's disease. It has been shown that intestinal epithelial cells in colonic biopsies from Crohn's disease patients over-express the major histocompatibility complex class I-related molecule MICA, and that the *in vitro* interaction between adherent *E. coli* and human intestinal epithelial cells leads to the over-expression of MICA. MICA plays a critical role in the innate immune response as it is a ligand for the activating natural killer (NK) cell receptor NKG2D activating receptor expressed on most γδ T cells, CD8+ αβ T cells and natural killer cells.[7]

The onset of inflammation in inflammatory bowel disease may be associated with an imbalance in the intestinal microflora, with a relative predominance of aggressive bacteria and an insufficient amount of protective species. We have previously reported that early and chronic ileal lesions of patients with Crohn's disease are abnormally colonized by adherent–invasive *E. coli* (AIEC) strains able to adhere to and invade a wide range of intestinal epithelial cells *in vitro* by a unique mechanism.[8–11] The adherence of AIEC reference strain LF82 was shown to be mediated by type 1 pili, which triggered massive host cell cytoskeletal rearrangements involved in the invasive process.[12] *In vitro* analysis of AIEC interactions with macrophages has indicated that these strains are able to survive and replicate within the phagocytes without inducing cell death. AIEC-infected macrophages secrete large amounts of tumour necrosis factor-α.[13]

The pathogenic features of AIEC strains raise the hypothesis that they may cross the epithelial barrier, evade the immune bactericidal mechanisms and elicit an inflammatory response within the mucosa.[14] Therefore, treatments aimed at the eradication of AIEC or at the prevention of their colonization of the gut may be of high therapeutic value. This hypothesis is supported by the results from various clinical studies which have shown that the administration of antibiotics may be a reasonable treatment approach in inflammatory bowel disease.[15–20] The eradication of these pathogenic strains and their replacement with non-pathogenic bacteria, i.e. probiotic therapy, may provide another option for the prevention of the development of inflammatory bowel disease and/or for the maintenance of remission.

Probiotics are viable micro-organisms usually belonging to the resident microflora. These bacteria are non-pathogenic and contribute to the health and well-being of the host.[21] Probiotics may represent effective tools for the control of the overgrowth of pathogens and, as a result, may control or prevent infections.[22–25] Indeed, numerous *in vitro* and *in vivo* studies performed with various genera of probiotic bacteria have shown that they can interfere with both the growth and virulence mechanisms of a number of pathogens.[26–32] The efficacy of several probiotic bacteria in inflammatory bowel disease has been investigated in clinical trials.[33–40] The non-pathogenic *E. coli* strain Nissle 1917 (Mutaflor), originally isolated during World War I from a soldier who withstood a severe outbreak of diarrhoea affecting his detachment, has been proposed for the maintenance therapy of Crohn's disease[38] and ulcerative colitis.[39, 40] Moreover, it has been reported that the administration of *E. coli* strain Nissle 1917 to newborn infants prevents the colonization of the intestine by microbial pathogens.[41]

As a first step towards a better understanding of the efficacy of *E. coli* Nissle 1917 in the treatment of patients with inflammatory bowel disease, the present study was designed to examine whether this probiotic strain could inhibit the ability of pathogenic AIEC strains, isolated from patients with Crohn's disease, to adhere to and invade intestinal epithelial cells *in vitro*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Seven AIEC strains, isolated from patients with Crohn's disease, were examined in this study, including the AIEC reference strain LF82.[8, 11] Strain LF82 is naturally resistant to ampicillin.[11] The non-pathogenic *E. coli* strain Nissle 1917, also known as *E. coli* DSM 6601 or Mutaflor, was provided by Ardeypharm (Herdecke, Germany). *E. coli* strain K-12 C600 was used as a non-probiotic control strain and as a non-invasive control in all invasion experiments. All strains were highly sensitive to gentamicin. The minimal bactericidal concentration of gentamicin that reduced the bacterial count by 99.99% was determined in tissue culture medium to be ≤ 1 μg/mL for all strains included in this study.

Mutants of AIEC strains LF9, LF15, LF31, LF65, LF110 and LF134, resistant to rifampicin, were selected on Mueller–Hinton agar containing 300 μg/mL rifampin (Sigma Chemical Company, St Louis, MO, USA). Compared with their wild-type counterparts, all mutants

showed similar adherence to and invasion of Intestine-407 cells.

All *E. coli* strains were grown in Luria–Bertani broth without shaking or on Mueller–Hinton agar plates (Institut Pasteur Production, Marnes-la-Coquette, France) overnight at 37 °C. When required, ampicillin or rifampicin was added to Mueller–Hinton agar at a concentration of 20 μg/mL or 300 μg/mL, respectively.

Cell line and cell culture

The Intestine-407 cell line (ATCC CCL6), derived from human embryonic jejunum and ileum, was purchased from Flow Laboratories (Flow Laboratories Inc., McLean, VA, USA). The cells were maintained in an atmosphere containing 5% CO_2 at 37 °C in Modified Eagle's Medium (Seromed, Biochrom KG, Berlin, Germany) supplemented with 10% (v/v) heat-inactivated foetal calf serum (Seromed), 1% non-essential amino acids (Life Technologies, Cergy-Pontoise, France), 1% L-glutamine (Life Technologies), 100 000 U/L penicillin, 100 mg/L streptomycin, 25 μg/L amphotericin B and 1% Modified Eagle's Medium vitamin solution X-100 (Life Technologies).

Adhesion and invasion assays

Intestine-407 cells were seeded in 24-well tissue culture plates (Polylabo, Strasbourg, France) at 4×10^5 cells per well and grown for 20 h. The cell monolayers were washed twice with phosphate-buffered saline (pH 7.2). Each monolayer was infected in 1 mL of the cell culture medium without antibiotic at a multiplicity of infection (MOI) of either 10 or 100 bacteria per epithelial cell. After a 1–4-h incubation period at 37 °C with 10% CO_2, the infected monolayers were washed three times with phosphate-buffered saline.

To determine the total number of cell-associated bacteria, corresponding to adherent and intracellular bacteria, the cells were lysed with 1% Triton X-100 (Sigma) in deionized water. This concentration of Triton X-100 did not affect bacterial viability for at least 30 min (data not shown). Samples were diluted and plated on to Mueller–Hinton agar plates to determine the number of colony-forming units recovered from the lysed monolayers.

For the measurement of invasion, fresh cell culture medium containing 100 μg/mL gentamicin was added after the infection step to kill extracellular bacteria. The monolayers were then incubated for a further 2 h and the medium was replaced every 30 min by fresh cell culture medium containing 100 μg/mL gentamicin. The eukaryotic cells were then lysed and the bacteria were quantified as described above.

Adhesion and invasion inhibition assays

Two different procedures were used in order to assess the exclusion of AIEC by *E. coli* Nissle 1917 and competition between the two strains. Exclusion was assessed by performing pre-infection experiments, in which Intestine-407 cell monolayers were first incubated with *E. coli* Nissle 1917 (MOI 10 or 100) for 1–4 h at 37 °C. Non-adherent bacteria were then removed by three washes in phosphate-buffered saline, AIEC bacteria (MOI 10 or 100) were added and incubation was continued for a further 3 h. Competition was assessed by performing co-infection experiments, in which *E. coli* Nissle 1917 and each of the AIEC strains tested were added together to the cell monolayer at equivalent MOI (10 or 100). Incubation times from 1 to 4 h were performed. The number of bacteria adhering to or invading the intestinal cells was determined as described above. Platings were performed simultaneously on Luria–Bertani agar plates to determine the total number of bacteria (*E. coli* Nissle 1917 and AIEC bacteria) and on Luria–Bertani plates containing rifampicin or ampicillin to determine the number of AIEC bacteria. The number of *E. coli* Nissle 1917 bacteria was determined by subtracting the number of colony-forming units recovered on Luria–Bertani plates containing rifampicin from the total number of colony-forming units recovered on Luria–Bertani plates without antibiotic. For each assay, three to five experiments were performed in duplicate.

Epithelial cell detachment and viability

Parallel to each experiment of *E. coli* Nissle 1917 adherence, a duplicate 24-well plate of epithelial cell monolayers was inoculated with bacteria, and assayed as described above. At the end of each incubation period, the monolayers were washed three times with phosphate-buffered saline and trypsinized. After neutralization with fresh culture medium containing 10% foetal calf serum, the cell suspension was removed and diluted. Epithelial cell viability was estimated by the trypan blue dye exclusion assay using a 0.4% trypan

blue solution in phosphate-buffered saline. The cells were counted to determine the number of viable epithelial cells remaining attached to the plates.

Statistical analysis

The data were analysed by Student's *t*-test. $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Characterization of the interaction of non-pathogenic E. coli *Nissle 1917 with Intestine-407 cells*

The ability of *E. coli* strain Nissle 1917 to adhere to human intestinal epithelial Intestine-407 cells was determined and compared with that of the pathogenic AIEC strain LF82 isolated from an ileal specimen of a patient with Crohn's disease.[9] Epithelial cell monolayers were incubated with bacteria at either MOI 10 or 100 for 1–4 h. The adhesion of *E. coli* Nissle 1917 to Intestine-407 cells increased with time (Figure 1). The adhesion levels of *E. coli* Nissle 1917 were similar to those of pathogenic *E. coli* LF82 at MOI 10 and 100. *E. coli* Nissle 1917 exhibited a dose- and incubation time-dependent ability to adhere to intestinal epithelial cells, comparable with that of AIEC strain LF82. After 4 h of infection at MOI 10, 90% of bacteria of the original inoculum were found to be adherent to the intestinal cells.

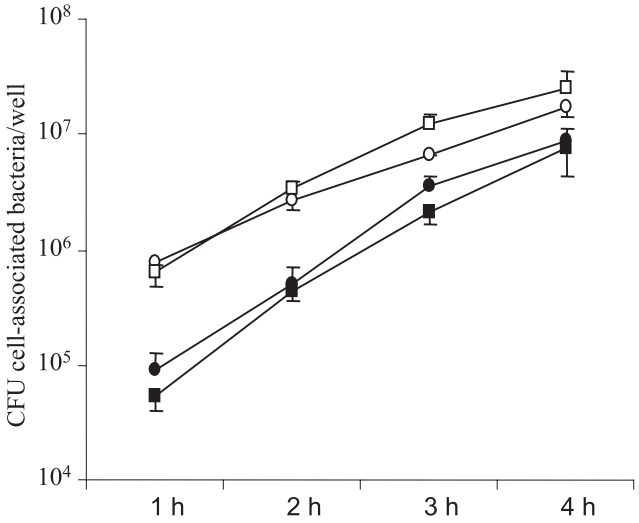

Figure 1. Adhesion of *Escherichia coli* strain Nissle 1917 and adherent–invasive *E. coli* (AIEC) LF82 to cultured Intestine-407 cells in mono-infection experiments. Cell monolayers were infected at a multiplicity of infection (MOI) of 10 or 100 for 1–4 h, washed to remove unbound bacteria and lysed with 1% Triton X-100. The resulting suspensions were plated on to Mueller–Hinton agar plates. Adhesion levels are expressed as the number of colony-forming units (CFU) per well. Data are given as the mean ± S.E.M. of three separate experiments. Symbols: squares, *E. coli* Nissle 1917; circles, AIEC LF82; filled symbols, MOI 10; open symbols, MOI 100.

E. coli Nissle 1917 did not induce any cytotoxic effect, as the number of viable Intestine-407 cells was the same in mono-infected and non-infected control monolayers 1–4 h post-infection at MOI 10 and 100 (data not shown).

Gentamicin protection assays revealed that *E. coli* Nissle 1917 was not able to invade Intestine-407 cells, as its invasion level was very low (0.002%), similar to that of the *E. coli* K-12 C600 negative control strain.

Inhibitory effect of non-pathogenic E. coli *Nissle 1917 on AIEC LF82 adhesion and invasion in co-infection experiments*

The adhesion levels of AIEC LF82 in co-infection experiments with *E. coli* Nissle 1917 are shown in Figure 2. At MOI 10, the number of adherent LF82 bacteria was similar in mono-infection and co-infection experiments with *E. coli* Nissle 1917 up to 3 h (Figure 2a). However, at 4 h of co-infection, the number of adherent LF82 bacteria had decreased significantly ($P < 0.01$), compared with that observed at 3 h of co-infection. Thus, some adherent LF82 bacteria had detached from the epithelial cells, whereas the number of adherent *E. coli* Nissle 1917 bacteria continued to increase (Figure 2c). The increase in adherent *E. coli* Nissle 1917 between 1 and 4 h was similar in mono-infection and co-infection experiments with LF82. At MOI 100, a significantly ($P < 0.05$) lower number of adherent LF82 bacteria was observed at 2 h in co-infection than in mono-infection experiments (Figure 2b). At 4 h of co-infection, the number of adherent LF82 bacteria had decreased compared with that observed at 3 h, indicating that some adherent LF82 bacteria had detached from the epithelial cells. As observed at MOI 10, an increase in adherent *E. coli* Nissle 1917 was found during the course of infection in mono- and co-infection experiments at MOI 100 (Figure 2d).

We have previously reported that a minimum time of 3 h of infection of intestinal cells is necessary for AIEC

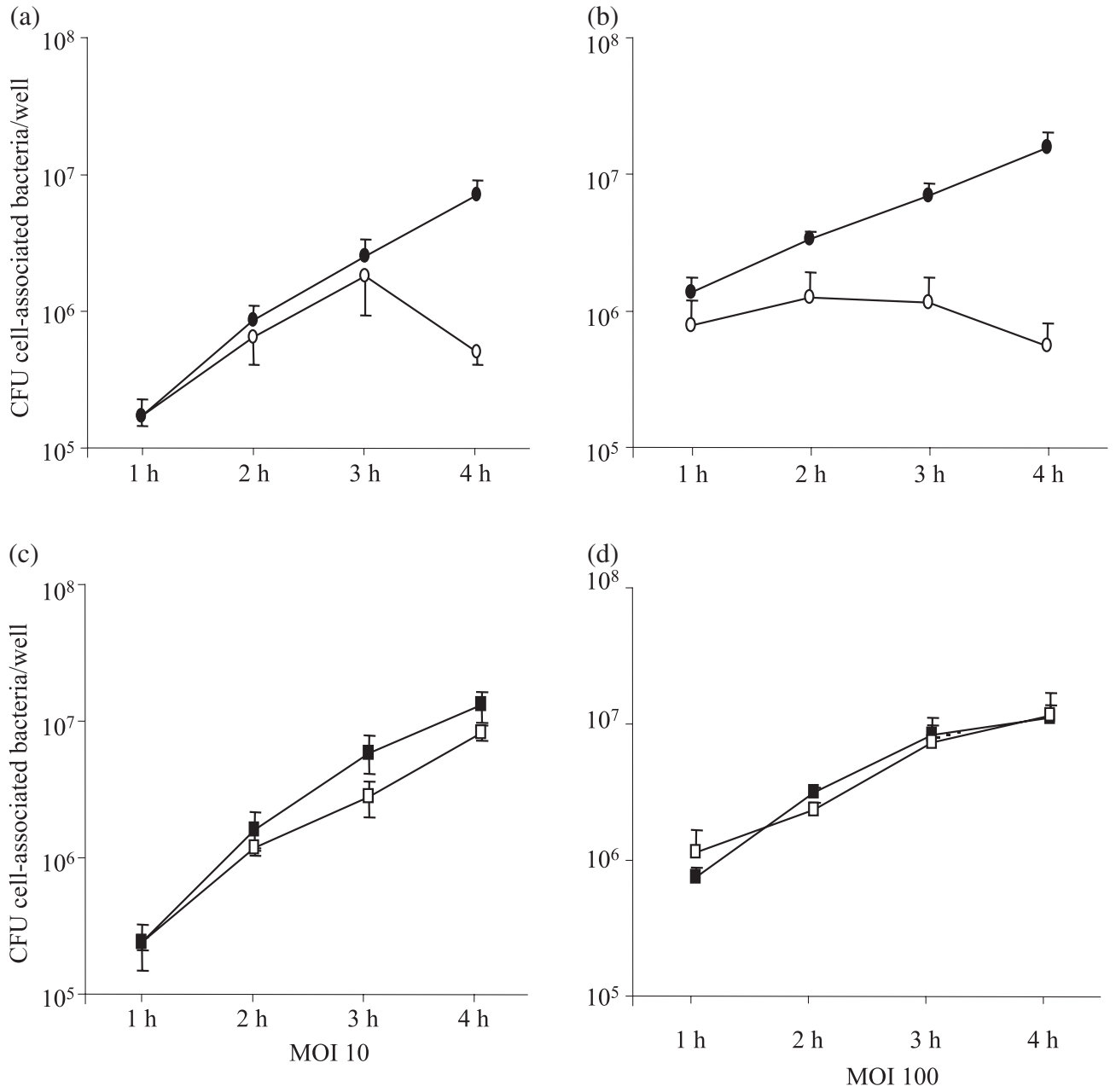

Figure 2. Adhesion of adherent–invasive *Escherichia coli* (AIEC) LF82 (a and b) and *E. coli* Nissle 1917 (c and d) strains to Intestine-407 cells in co-infection experiments. For co-infection experiments, *E. coli* Nissle 1917 and AIEC LF82 were used at the same multiplicity of infection (MOI) (10 or 100). Infections were performed for 1–4 h. As controls, cell monolayers were incubated with either *E. coli* Nissle 1917 or AIEC LF82 alone. Adhesion levels are expressed as the number of colony-forming units (CFU) per well. Data are given as the mean ± S.E.M. of three to five separate experiments. Symbols: squares, *E. coli* Nissle 1917; circles, AIEC LF82; filled symbols, mono-infection; open symbols, co-infection.

strain LF82 to achieve bacterial invasion.[11] Therefore, experiments to quantify LF82 invasion were performed with 3 h and 4 h courses of co-infection. In mono-infection assays, the number of intracellular LF82 bacteria varied slightly with the infection time, but increased sharply with MOI (Figure 3). During co-infection with *E. coli* Nissle 1917, the number of intracellular LF82 bacteria remained constant,

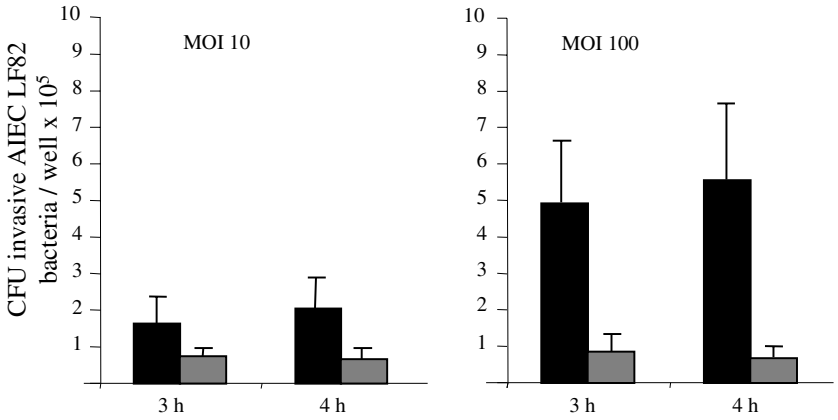

Figure 3. Invasion of adherent–invasive *Escherichia coli* (AIEC) LF82 strain in Intestine-407 cells in co-infection experiments with *E. coli* Nissle 1917 (grey bars), compared with mono-infection experiments with LF82 alone (black bars). For co-infections, *E. coli* Nissle 1917 and AIEC LF82 were tested using the same multiplicity of infection (MOI) (10 or 100). Infections were performed for 3 or 4 h. Invasion levels were determined after gentamicin treatment for one additional hour and expressed as the number of colony-forming units (CFU) per well. Data are given as the mean ± S.E.M. of three to five separate experiments.

irrespective of the infection time or MOI. The difference between the number of intracellular LF82 bacteria in mono-infection and co-infection experiments was significant at MOI 10 ($P < 0.05$) and highly significant at MOI 100 ($P < 0.01$).

Thus, the presence of *E. coli* Nissle 1917 in co-infection experiments limited the number of intracellular LF82 bacteria.

Inhibitory effect of non-pathogenic E. coli *Nissle 1917 on AIEC LF82 adhesion and invasion in pre-infection experiments*

Pre-incubation of Intestine-407 cell monolayers with *E. coli* Nissle 1917 prior to infection with *E. coli* LF82 prevented the adhesion of LF82 in a time- and dose-dependent manner (Figure 4a). At MOI 10, a significantly ($P < 0.05$) lower number of adherent LF82 bacteria was observed after a 3 h pre-incubation period of the cells with *E. coli* Nissle 1917 in comparison with that observed without pre-incubation. This difference was even more significant after a 4 h pre-incubation period with *E. coli* Nissle 1917 ($P < 0.001$). At MOI 100, in comparison with MOI 10, a significantly ($P < 0.01$) lower number of adherent LF82 bacteria was observed after 1 h of pre-incubation with *E. coli* Nissle 1917.

The inhibition of LF82 invasion paralleled the inhibition of adhesion (Figure 4b). At MOI 10, the number of intracellular bacteria was significantly ($P < 0.05$) lower when the cells were pre-incubated for 4 h with *E. coli* Nissle 1917 compared with that observed with control cells. At MOI 100, a significantly ($P < 0.01$) lower number of intracellular LF82 bacteria was observed when the intestinal epithelial cells were pre-incubated for 2–4 h with *E. coli* Nissle 1917.

Comparison of E. coli *Nissle 1917 and* E. coli *K-12 C600 inhibitory effects on AIEC LF82 adhesion and invasion*

In order to assess the specificity of the *E. coli* Nissle 1917-mediated inhibition of AIEC LF82 adhesion, this effect was compared with that of the non-pathogenic *E. coli* K-12 C600 strain (Table 1). Co-infection experiments of LF82 with K-12 C600 showed a significant ($P < 0.05$), albeit transient, inhibitory effect on LF82 adhesion at MOI 10 and 100 after 3 h of co-infection (53% and 45%, respectively). However, after 4 h of co-infection, inhibition of only 8% and 13% was observed at MOI 10 and 100, respectively. In contrast with co-infection experiments with *E. coli* Nissle 1917, which induced a strong time- and dose-dependent inhibitory effect on LF82 adhesion, reaching 96%, the inhibitory effect of K-12 C600 on LF82 adhesion was not time and dose dependent.

The number of intracellular LF82 bacteria decreased slightly during co-infection with K-12 C600. The maximum percentage inhibition observed after 4 h of co-infection at MOI 100 was 40%. In contrast, co-infection with *E. coli* Nissle 1917 induced a strong

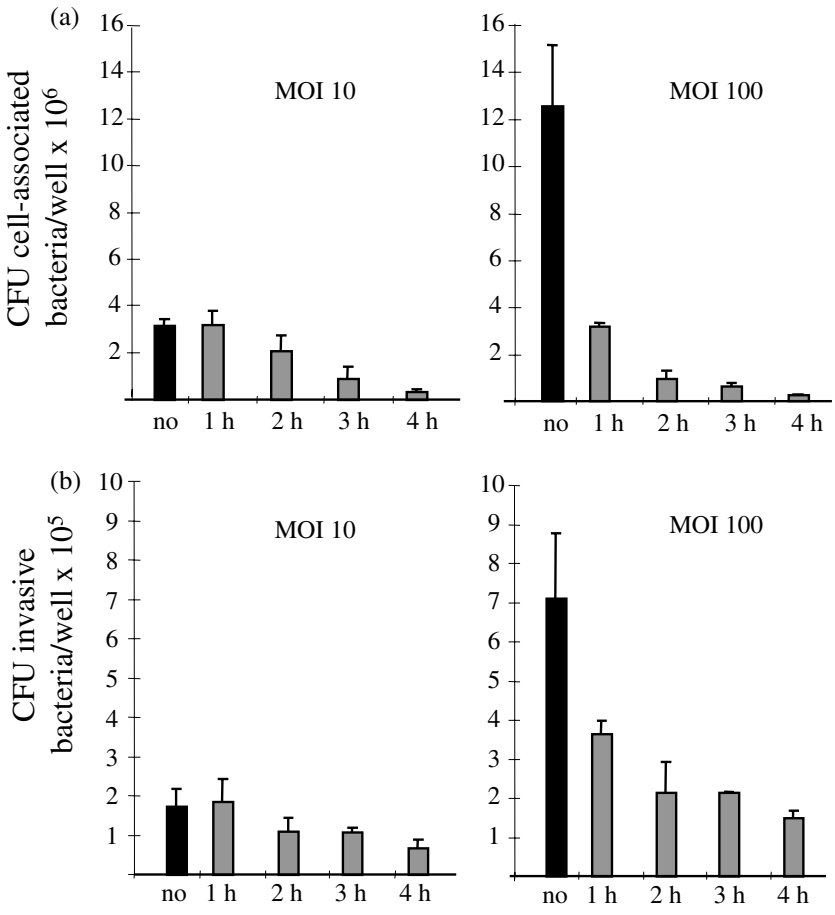

Figure 4. Adhesion to (a) and invasion of (b) Intestine-407 cells by adherent–invasive *Escherichia coli* (AIEC) LF82 after pre-incubation of the cell monolayers with *E. coli* Nissle 1917. Pre-incubations of cell monolayers were performed for 1–4 h (grey bars). As positive controls, the adhesion levels of AIEC LF82 to untreated epithelial cells were determined (black bars). After pre-incubation of the cell monolayers with *E. coli* Nissle 1917, infections with AIEC LF82 were performed for 3 h. A similar multiplicity of infection (MOI) was used for *E. coli* Nissle 1917 and AIEC LF82. Invasion levels were determined after gentamicin treatment for one additional hour and expressed as the number of colony-forming units (CFU) per well. Data are given as the mean ± S.E.M. of three to five separate experiments.

time- and dose-dependent inhibition of LF82 invasion, reaching 88%.

Pre-incubation of Intestine-407 cells with *E. coli* K-12 C600 for 3–4 h prior to infection with LF82 did not result in a stronger or more specific inhibition of LF82 adhesion and invasion (Table 1). Indeed, the highest inhibitory effect (49%) on LF82 adhesion was observed after pre-incubation for 3 h at MOI 100, whereas the inhibitory effect after 4 h was only 28%. Small inhibitory effects of K-12 C600 on LF82 invasion were also observed, reaching a maximum of 30% after 4 h of pre-incubation of the cells at MOI 100. Under the same conditions, a strong and significant (79%, $P < 0.01$) inhibitory effect was observed when the cells were pre-incubated with *E. coli* Nissle 1917. Thus, in contrast with the inhibitory effects obtained with *E. coli* Nissle 1917, reaching 98% on LF82 adhesion and 79% on LF82 invasion, no time- and dose-dependent decrease in LF82 adhesion and invasion was observed in pre-infection experiments using *E. coli* K-12 C600.

Inhibitory effect of E. coli *Nissle 1917 on the adhesion and invasion of various AIEC strains isolated from patients with Crohn's disease*

The bacterial adhesion of various AIEC strains to Intestine-407 cell monolayers was prevented for all the strains tested by co-infection or pre-incubation of the cells with *E. coli* Nissle 1917 (Table 2). In co-infection experiments, inhibitory effects on AIEC adhesion of 69.2–99.9% and 78.0–99.9% were observed at MOI 10 and 100, respectively. Pre-incubation experiments with *E. coli* Nissle 1917 also prevented the bacterial adhesion of all the AIEC strains tested. Inhibitory effects of 92.5–99.9% and 92.9–99.9% were observed at MOI 10 and 100, respectively. All inhibitory effects were highly significant ($P < 0.01$).

AIEC invasion was also reduced in the presence of *E. coli* Nissle 1917 (Table 2). In co-infection experiments, inhibitory effects of 29.6–87.2% were observed after 4 h of infection at MOI 10, and of 62.0–95.9% at MOI 100 ($P < 0.01$). At MOI 10, pre-incubation of the

Table 1. Comparison of the inhibitory effects of *Escherichia coli* Nissle 1917 and *E. coli* K-12 C600 on the ability of adherent–invasive *E. coli* (AIEC) strain LF82 to adhere to and invade Intestine-407 cells in co-infection and pre-infection experiments

		Co-infection experiments*				Pre-infection experiments†			
		Inhibition of cell association‡ after co-infection for		Inhibition of cell invasion§ after co-infection for		Inhibition of cell association‡ after pre-infection for		Inhibition of cell invasion§ after pre-infection for	
Strain	MOI	3 h (%)	4 h (%)	3 h (%)	4 h (%)	3 h (%)	4 h (%)	3 h (%)	4 h (%)
Nissle 1917	10	40 ± 17¶	91 ± 2††	37 ± 17	66 ± 5††	70 ± 20¶	89 ± 6**	36 ± 10¶	63 ± 3**
K-12 C600	10	53 ± 17¶	8 ± 7	10 ± 6	34 ± 14¶	20 ± 10	21 ± 7¶	20 ± 8¶	15 ± 6
Nissle 1917	100	72 ± 19**	96 ± 2††	65 ± 19**	88 ± 5††	95 ± 1††	98 ± 1††	68 ± 7**	79 ± 2**
K-12 C600	100	45 ± 16¶	13 ± 6	18 ± 17	40 ± 11¶	49 ± 12¶	28 ± 16	22 ± 13	30 ± 4¶

* Intestine-407 epithelial cell monolayers were co-infected for 3 or 4 h with both *E. coli* Nissle 1917 and *E. coli* LF82 at a multiplicity of infection (MOI) of 10 or 100 bacteria per cell.

† Intestine-407 cell monolayers were pre-incubated for 3 or 4 h with *E. coli* Nissle 1917 at MOI 10 or 100 bacteria per cell. The monolayers were then washed three times with sterile phosphate-buffered saline. Fresh cell culture medium was added. Then, infection experiments with *E. coli* LF82 were performed for 3 h at MOI 10 if pre-incubation with *E. coli* Nissle 1917 had been performed at MOI 10, or at MOI 100 if pre-incubation with *E. coli* Nissle 1917 had been conducted at MOI 100.

‡ Inhibition (%) = [(cell-associated LF82 bacteria in infection experiments with LF82 alone − cell-associated LF82 bacteria in co-infection or pre-infection experiments with *E. coli* Nissle 1917 or *E. coli* K-12 C600 as competitor strain)/cell-associated LF82 bacteria in infection experiments with LF82 alone] × 100. Results are the means of three separate experiments.

§ Inhibition (%) = [(intracellular LF82 bacteria in infection experiments with LF82 alone − intracellular LF82 bacteria in co-infection or pre-infection experiments with *E. coli* Nissle 1917 or *E. coli* K-12 C600 as competitor strain)/intracellular LF82 bacteria in infection experiments with LF82 alone] × 100. Results are the means of three separate experiments.

¶ $P < 0.05$.

** $P < 0.01$.

†† $P < 0.001$.

intestinal epithelial cells with *E. coli* Nissle 1917 was more efficient in preventing AIEC invasion than was co-infection. Significant ($P < 0.01$) inhibitory effects of 74.2–98.3% and 57.9–94.8% were observed at MOI 10 and 100, respectively.

DISCUSSION

The eradication of bacteria involved in the pathogenesis of inflammatory bowel disease and their permanent replacement with non-pathogenic, probiotic bacteria may provide a new option for the treatment of inflammatory bowel disease and for the maintenance of remission. As the adhesion of pathogenic bacteria to intestinal epithelial cells is an important prerequisite for their colonization and the establishment of the pathogenesis, the inhibition of their ability to adhere could decrease intestinal colonization, prevent bacterial invasion and subsequently modify the process of pathogenicity. Recent clinical studies have suggested a benefit of the administration of probiotics as maintenance therapy in inflammatory bowel disease and in the prevention of pouchitis.[35–37, 39, 40, 42, 43] However, this approach remains controversial, because the mechanisms whereby probiotic bacterial strains antagonize pathogenic gastrointestinal micro-organisms or exert beneficial effects in the host *in vivo* have yet to be defined. As *E. coli* Nissle 1917 has been shown to be effective as maintenance therapy in Crohn's disease[38] and ulcerative colitis,[39, 40] we investigated whether this non-pathogenic *E. coli* strain could counteract the ability of pathogenic AIEC strains, isolated from patients with Crohn's disease, to adhere to and invade intestinal epithelial cells.

Bacterial adhesion to intestinal cells has been postulated to be a desirable prerequisite for probiotic strains.[44] Thus, many probiotics possess the ability to adhere to intestinal epithelial cells.[45–48] In this study, we have shown that *E. coli* Nissle 1917 is able to adhere to the human intestinal epithelial cell line Intestine-407 in a dose- and time-dependent manner. This strain can be regarded as strongly adherent, as 90% of the original inoculum was found to adhere to the intestinal cells after 4 h of infection. Interestingly, although high numbers of *E. coli* Nissle 1917 bacteria bind to the epithelial cells, no cell cytotoxicity is induced. Its strong

Table 2. Inhibition of adhesion and invasion of various adherent–invasive *Escherichia coli* (AIEC) strains by probiotic *E. coli* Nissle 1917

		Cell-associated bacteria* (% inhibition)			Intracellular bacteria† (% inhibition)		
Strain	MOI	Alone‡	During co-infection§	After pre-incubation¶	Alone‡	During co-infection§	After pre-incubation¶
LF9	10	109.60 ± 24.54	7.96 ± 6.90 (92.7)	0.04 ± 0.01 (99.9)	1.34 ± 0.17	0.94 ± 0.56 (29.6)	0.03 ± 0.01 (97.9)
LF15	10	230.00 ± 104.00	14.02 ± 13.98 (93.9)	0.76 ± 0.29 (99.4)	1.03 ± 0.20	0.54 ± 0.21 (48.2)	0.23 ± 0.08 (77.8)
LF31	10	169.00 ± 67.00	31.05 ± 30.95 (81.6)	1.20 ± 0.38 (98.7)	0.90 ± 0.29	0.42 ± 0.23 (53.0)	0.08 ± 0.02 (90.8)
LF65	10	148.00 ± 41.76	0.12 ± 0.04 (99.9)	0.05 ± 0.04 (99.9)	3.23 ± 2.26	0.41 ± 0.26 (87.2)	0.02 ± 0.01 (98.3)
LF110	10	159.00 ± 17.00	49.00 ± 0.80 (69.2)	5.47 ± 0.71 (92.5)	0.50 ± 0.12	0.21 ± 0.06 (59.4)	0.04 ± 0.02 (74.2)
LF134	10	109.14 ± 38.30	6.58 ± 5.04 (94.0)	0.13 ± 0.05 (99.8)	3.77 ± 2.17	2.31 ± 1.13 (38.7)	0.08 ± 0.04 (92.6)
LF9	100	347.00 ± 99.96	3.97 ± 2.60 (98.9)	0.49 ± 0.20 (99.8)	2.14 ± 0.74	0.81 ± 0.32 (62.0)	0.60 ± 0.21 (57.9)
LF15	100	306.66 ± 35.41	27.87 ± 17.63 (90.9)	8.38 ± 0.25 (97.2)	3.26 ± 0.71	0.63 ± 0.32 (80.6)	0.57 ± 0.16 (84.3)
LF31	100	376.00 ± 84.13	50.60 ± 15.65 (86.5)	7.70 ± 2.03 (97.2)	1.33 ± 0.52	0.47 ± 0.18 (64.7)	0.20 ± 0.08 (90.7)
LF65	100	374.00 ± 85.51	0.34 ± 0.08 (99.9)	0.08 ± 0.04 (99.9)	9.28 ± 5.49	0.38 ± 0.12 (95.9)	0.21 ± 0.10 (94.8)
LF110	100	273.33 ± 51.33	60.27 ± 8.77 (78.0)	15.90 ± 2.67 (92.9)	0.93 ± 0.39	0.11 ± 0.01 (87.9)	0.13 ± 0.03 (92.5)
LF134	100	214.50 ± 35.82	17.28 ± 6.22 (91.9)	0.30 ± 0.05 (99.8)	6.39 ± 3.96	1.24 ± 0.63 (80.5)	0.12 ± 0.04 (94.0)

* Cell-associated bacteria expressed as colony-forming units/well $\times 10^5$, mean ± s.d. of three separate experiments; † Intracellular bacteria expressed as colony-forming units/well $\times 10^4$, mean ± s.d. of three separate experiments; ‡ Adhesion and invasion of AIEC strains in the absence of probiotic *E. coli* Nissle 1917; § Co-infection experiments were performed for 4 h at a similar multiplicity of infection (MOI) for pathogenic AIEC strains and probiotic *E. coli* Nissle 1917; ¶ Pre-infection experiments were performed by pre-incubation for 4 h with probiotic *E. coli* Nissle 1917 prior to infection for 3 h with AIEC strains. The same MOIs were used for AIEC strains and probiotic *E. coli* Nissle 1917.

adhesion to intestinal epithelial cells could enable *E. coli* Nissle 1917 to form *in vivo* a biofilm of non-pathogenic bacteria that may prevent pathogenic micro-organisms from accessing the cell surface. Its strong adhesion could also explain why *E. coli* Nissle 1917 has been reported to be a potent colonizer *in vivo*. Indeed, it has been shown to effectively colonize the intestine of newborn infants within a few days and to remain a component of the developing intestinal microflora for months after the cessation of oral administration.[41]

E. coli Nissle 1917 exerts a strong and specific inhibitory effect on AIEC adhesion to intestinal epithelial cells in both co-infection and pre-incubation experimental models. In co-infection experiments, in which a mixture of *E. coli* Nissle 1917 and AIEC LF82 was used to infect cell monolayers, a strong time- and dose-dependent inhibitory effect on LF82 adhesion, reaching 96%, was observed. Pre-incubation of the epithelial cells with *E. coli* Nissle 1917 prior to infection with *E. coli* LF82 also prevented LF82 adhesion in a dose- and time-dependent manner, and a maximum inhibitory effect of 98% was obtained. The inhibitory effect of *E. coli* Nissle 1917 was not restricted to strain LF82; it was also observed with the other six pathogenic AIEC strains isolated from different patients with Crohn's disease included in this study. Inhibitory effects reaching 92.5–99.9% were obtained. Strikingly, the inhibitory effects observed with *E. coli* Nissle 1917 on AIEC adhesion are similar to or even higher than those reported for *Lactobacilli* or *Bifidobacteria* on the adhesion of various pathogens.[27, 31, 46, 47, 49–51]

The co-infection or pre-incubation of intestinal cells with *E. coli* Nissle 1917 also limited the number of intracellular AIEC. The decrease in the number of intracellular bacteria paralleled the decrease in adherent bacteria. This result is consistent with previous observations, showing that the adhesion step is crucial for AIEC to invade epithelial cells.[11] Overall, pre-incubation of epithelial cells with *E. coli* Nissle 1917 was more effective than co-infection in preventing AIEC invasion. This was mainly observed when a low MOI was used. This finding may be explained by the fact that pre-incubation with a probiotic organism able to adhere to epithelial cells, and thus to form a biofilm on the cell surface layer, may mask cell receptors involved in the internalization of pathogenic bacteria.

In order to assess the specificity of inhibition of AIEC LF82 adhesion by *E. coli* Nissle 1917, its inhibitory effect was compared with that of *E. coli* K-12 C600,

another non-pathogenic strain which has been shown to be unable to inhibit *Salmonella typhimurium* invasion of intestinal epithelial cells.[52] No time- and dose-dependent inhibitory effects of K-12 C600 on LF82 adhesion were observed. Using the higher dose of bacteria and the longest time of co-infection, only 13% inhibition of LF82 adhesion was obtained. Similar to adhesion, no specific inhibitory effects of *E. coli* K-12 C600 on AIEC invasion were observed.

Our study shows that the non-pathogenic *E. coli* strain Nissle 1917 impairs AIEC colonization of intestinal epithelial cells. Competition between *E. coli* Nissle 1917 and AIEC LF82 for adhesion to epithelial cells was observed during co-infection experiments when a similar inoculum of both bacteria was used. The number of adherent *E. coli* Nissle 1917 bacteria was similar during mono-infection or co-infection experiments with LF82, whereas the number of adherent LF82 bacteria decreased after 2–3 h of co-infection in comparison with that observed during mono-infection with LF82. Moreover, when the number of adherent *E. coli* Nissle 1917 bacteria reached approximately 5–10 bacteria per epithelial cell, the number of adherent LF82 bacteria decreased, whereas the number of adherent *E. coli* Nissle 1917 continued to increase. This may indicate that competition for the same receptor may occur between the two strains. In this case, the *E. coli* Nissle 1917 adhesive factor must have a higher affinity than the LF82 adhesive factor to an as yet unidentified receptor. It has been reported previously that *E. coli* Nissle 1917 produces several types of adhesins, including type 1 pili,[53, 54] and we have shown that LF82 synthesizes a type 1 pili variant.[12] Differences in the amino acid composition of the FimH adhesin of type 1 pili, expressed by *E. coli* Nissle 1917 or by AIEC LF82, may be responsible for different affinities to the FimH receptor on the epithelial cell surface.[55, 56]

In conclusion, the present report provides new insights into the mechanism(s) by which *E. coli* Nissle 1917 may exert a protective role against pathogenic *E. coli* colonizing the gut of patients with inflammatory bowel disease. The ability of *E. coli* Nissle 1917 to efficiently colonize the human gastrointestinal tract may be due to its strong adhesion to intestinal epithelial cells, as shown here. As a consequence, this probiotic strain exerts a strong inhibitory effect on both the adhesion and invasion of AIEC strains. This strain may be efficient for preventive and curative probiotic therapy as inhibitory effects were observed when intestinal cells were pre-incubated with *E. coli* Nissle 1917 and when the probiotic was used in co-infection experiments. It remains to be determined to what extent these *in vitro* results correspond to the *in vivo* situation in order to confirm the benefits of administration of *E. coli* Nissle 1917 in the management of inflammatory bowel disease, especially in patients in whom AIEC strains have been isolated.

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