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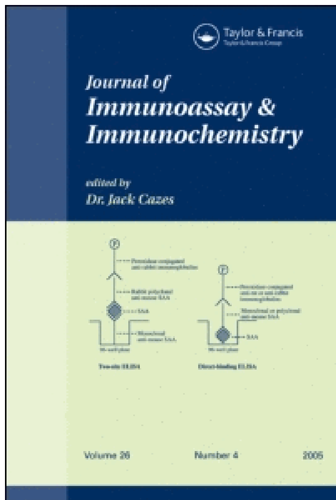

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Application of an Enzyme Immunoassay to Monitor Bacterial Binding and to Measure Inhibition of Binding to Different Types of Solid Surfaces

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APPLICATION OF AN ENZYME IMMUNOASSAY TO MONITOR BACTERIAL BINDING AND TO MEASURE INHIBITION OF BINDING TO DIFFERENT TYPES OF SOLID SURFACES

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ABSTRACT

We describe the application of an enzyme immunoassay (EIA) for detecting bacteria bound to a solid surface. Different *Yersinia enterocolitica* and *Escherichia coli* strains, expressing the YadA protein, type 1 or type P fimbriae were used as models for this study. The assay was used to detect bacteria bound to fixed tissues or to glass slides coated with extracellular matrix molecules (collagen, laminin or fibronectin). *E. coli* specific antiserum (B357, Dakopatts, Glostrup, Denmark) and peroxidase conjugated antiserum (P217) were used to detect all *E. coli* strains used in the study. The bacterial binding could be monitored with a linear detection range between 10^5 and 10^8 bacteria. Most importantly, dose dependent inhibition of bacterial binding by soluble extracellular matrix molecules could be measured. (KEY WORDS: Bacterial adhesion, *E. coli*, Extracellular matrix molecules, Fimbriae, *Yersinia*, YadA)

INTRODUCTION

Adherence of bacteria to a host target is a prerequisite for the initiation of the disease process [1]. Bacterial molecular constituents, in addition to host components, have been implicated in the binding and adhesion [1]. Enteric bacteria produce binding components (adhesins), together with other important molecules such as lipopolysaccharides, flagellae and porins as part of the bacterial outer membrane [1-3]. Because adherence is a key step

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in bacterial virulence [4], much interest has been focused on adherence assays. Both qualitative and quantitative methods to study adhesion of micro-organisms have been used. Quantitative methods are necessary to compare adhesion of different bacterial species or mutants and in inhibition experiments. Measurement of adhesion to cultured epithelial cells is a widely used method to evaluate microbial adhesion [5, 6] .

Quantitation of adhesion has been performed in a number of ways: i) by the direct counting of adherent bacteria per epithelial cell using light microscopy [7-9], ii) by determining the adhering radioactivity using radiolabelled bacteria [10], and iii) by counting colony-forming units after recovery of adherent bacteria [11-13]. Adhesion tests using biological tissue sections as targets have also been developed [14-17]. Alternatively, solid surfaces coated with extracellular matrix molecules have been used as targets in bacterial binding assays [18-21].

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In a recent report [22] we studied the binding of *Yersinia enterocolitica* to fixed tissue sections of human intestine, and for inhibition assays we applied a combination of immunohistochemical staining and enzyme immunoassay (EIA). We felt, however, that this method could be generally applicable to many different bacterial systems, especially in competition experiments, and therefore in the present work we used two species, *Y. enterocolitica* and *E. coli* as model organisms.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial strains used in this study are shown in Table 1. *Y. enterocolitica* strains (YeO3 and YeO3-c) were grown to stationary phase overnight at 37 °C in 10 ml of MedECa (0.1 g of $MgSO_4 \times 7H_2O$, 2 g of citric acid, 10 g of K_2HPO_4, 3.5 g of $NaNH_4HPO_4 \times 4H_2O$, 1 mg vitamin B_1 per litre, containing 0.2% glucose, 0.2% casamino acids, and 2.5 mM $CaCl_2$) [23]. *E. coli* strain C600/pYMS4514 was grown to stationary phase overnight at 37°C in 10 ml MedECa containing 10 µg of chloramphenicol per ml. *E.*

TABLE 1.

Bacteria And Plasmids Used In This Study

Bacterial strains	Comments	Ref.
Y. enterocolitica:		
6471/76 (YeO3)	serotype O:3, virulence plasmid positive (pYV+), patient isolate. pYV carries the *yadA* gene when grown at 37°C	[29]
6471/76-c (YeO3-c)	pYV- derivative of YeO3	[29]
E. coli :		
C600	*thi thr leu tonA lacY supE*	[30]
HB101	*Δgpt- proA leu6 thi1 lacY1 galK2 ara14 xyl5 mtl1 hsdS phx recA supE44 rpsL*	[31]
C600/pYMS4514	pYMS4514 carries $yadA_{YeO3}$ and $lcrF_{YeO3}$ cloned into pTM100. Expresses YadA when grown at 37° C.	[32]
HB101/pPIL110-75	The $F7_1$ fimbrial gene cluster of *E. coli* AD110 cloned into pJB8	[33]
HB101/pPKL4	Type 1 fimbrial gene cluster of *E. coli* cloned into pBR 322	[34]
HB101/pPIL110-708	The $F7_1$ (FSO) gene cluster of *E. coli* AD110 cloned into pBR 322	[35]

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coli strains C600 and HB101 were grown overnight at 37°C on Luria agar. *E. coli* HB101/pPKL4, HB101/pPIL110-75 and HB101/pPIL110-708 were grown on Luria agar supplemented with 50 μg ampicillin per ml. The plate cultures were then suspended in 10 ml of phosphate buffered salaine, pH 7.4 (PBS). The liquid cultures and suspensions were centrifuged at 3000 rpm for 15 minutes, then the bacteria were resuspended into an appropriate amount of PBS to obtain an optical density of 600 nm (OD_{600})between 0.19 and 0.21. The corresponding bacterial concentration was determined by the dilution plating method. For *Y. enterocolitica* strains and *E. coli* C600 clones it was about $2x10^8$ (cfu) per ml, and for *E. coli* HB101, HB101/pPKL4, HB101/pPIL110-75, and HB101/pPIL110-708 clones it was $1.5x10^8$ cfu per ml. Different concentrations (10^6-10^9 per ml) were prepared from each of the bacterial suspensions. For the inhibition assays, each of the inhibitor solutions was added to 800 μl of one of bacterial suspensions, and the final volume was brought to one ml with PBS.

Proteins used

Collagen type I (C-7774) and IV (C-7521), from human placenta, laminin (L2020), from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma (1 mg per ml in 0.15 M NaCl, 0.05 M Tris solution), fibronectin (F-2006) and Bovine Serum Albumin (BSA, A-8022) were purchased from Sigma Chemical Company (St Louis, MO). Collagen was dissolved into 0.1 M acetic acid and fibronectin into distilled water to a concentration of 1 mg per ml. Further dilutions were made in PBS.

Preparation of frozen sections and coated slides

Small pieces of human colon removed surgically at Turku University Hospital, were immediately packed on ice for transportation to the laboratory, cut into smaller pieces (5-10mm) and frozen in blocks using O.C.T. compound (Miles Inc. Diagnostics Division, Elkhart, IN). Eight-micrometer frozen sections were cut from the blocks and mounted on sterile microscopic slides inside water repellent circles 2 cm in diameter drawn with a PAP pen (peroxidase anti peroxidase pen from Diado Sangyo Co. Ltd., Tokyo Japan). We prepared Lewis rat kidney sections similarly. We fixed the sections for 30 minutes in methanol containing 2% H_2O_2, washed them three times for 5 minutes with PBS, and kept them at -20°C till needed .

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Glass slides were coated using different concentrations (1 ng-100 μg per ml) of collagen type I, type IV or fibronectin by incubating the solutions inside water repellent circles for 16 hours at room temperature. Control circles were coated with BSA, at 30 μg per ml. Circles were washed three times with PBS and used immediately.

Enzyme immunoassay (EIA) detection method

To reduce the background reactivity, we used a two-step blocking procedure. Fixed tissue sections and coated circles were blocked first by immersing the whole slides in PBS-3% BSA for 30 minutes at room temperature. After three washings with PBS, the slides were incubated for 30 minutes at room temperature in a wet chamber, the circles overlaid

with 100 μl of 10% sheep serum in PBS-3% BSA. In addition, the antibody dilutions were also made in 3% BSA-PBS. We did not test systematically other blocking procedures, however, omitting the sheep serum clearly increased the background level. The slides were washed three times, then the circles were overlaid with 100 μl of bacterial suspensions of different concentrations (10^6-10^9 cfu per ml) and incubated under gentle rotation at 60 rpm. Incubation temperatures and times are given for each experiment individually in the results. Very long incubation times were not used since the 100 μl droplets tend to dry even if the incubations take place in moist chambers. The unbound bacteria were poured away and the slides washed three times with PBS. For the *Y. enterocolitica* strains, the circles were overlaid with 100 μl of monoclonal antibody (Moab) A6, specific for the O-antigen of *Y. enterocolitica* serotype 0:3 [24], diluted 1:10 in PBS-3% BSA. For *E. coli* strains, the circles were overlaid with rabbit anti *E. coli* lysate polyspecific antibody (B 357, Dakopatts, Glostrup, Denmark), diluted 1:1000 in PBS-3% BSA. B357 has been produced in rabbits immunized with an aqueous extract of a sonicate of *E. coli* (non-transformed strain K12 C600). After three washings, the *Y. enterocolitica* circles were overlaid with 100 μl of peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dakopatts P260) diluted 1:500 in PBS-3% BSA, and the *E. coli* circles with 100 μl peroxidase-conjugated swine anti-rabbit immunoglobulins (P 217 Dakopatts) diluted 1:1000 in PBS-3% BSA. We experimentally optimized the antibody concentrations to be used for relatively short incubation times (<30 min).

After three washings, the circles were overlaid for 10 minutes (depending on colour production) at room temperature with 100 μl of EIA substrate solution freshly supplemented with 10 μl of 30% H_2O_2 per 15 ml of substrate solution. The EIA substrate solution contained 3 mg per ml of 1,2-phenylenediamine dissolved into citrate buffer (4,97 g citric acidxH_2O+9,90 g Na_2HPO_4x$2H_2O$ dissolved into total of one litre distilled water). From each circle, 75 μl of the substrate solution was pipetted into EIA plate wells (Dynatech Immulon,Roskilde, Denmark), and the reaction was stopped with 125 μl of 1M HCl. The coloured product was recorded using a Labsystems Multiskan Plus

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spectrophotometer at 492 nm. For each bacterial dose and experimental setting a minimum of four parallel sections or coated circles was used.

Non-specific binding to sections or coated circles was controlled in several ways: i) to control the non-specific binding of antibodies bacteria were omitted, ii) to control bacterial endogenous peroxidase activity, both antibodies were omitted, iii) to control non-specific binding of the conjugate, the primary antibodies were omitted. These controls showed that the background activity was always due to non-specific binding of the conjugate, and that there was no bacterial endogenous peroxidase activity present (data not shown).

RESULTS AND DISCUSSION

For measuring bacterial adhesion to a multitude of solid targets, three major approaches have been used. i) radiolabelled bacteria [10], ii) direct microscopic counting [7, 9, 25, 26], and iii) counting colony forming units [11, 13]. We previously used light microscopy in detecting localized bacterial binding on fixed tissue sections, [22]. The above techniques were not readily applicable for inhibition experiments. Thus we developed an EIA method which is based on immunological labelling of bound bacteria with peroxidase and detecting the bound label by colour production. In general, EIA methods have been widely used in a great number of microbiological applications [27], and the basic methodology is thoroughly tested. Usually the target is immobilized on a solid surface, such as a microtiter plate well; tissue sections have not, however, commonly been used as immobilized antigen.

To evaluate the general usefulness of the EIA detection method, we selected three known and well-documented adhesins for our studies (type 1 fimbriae, P fimbriae and the YadA protein) [19-22]. We performed a set of binding experiments with bacteria expressing these adhesins to confirm that the EIA method is applicable as an alternative way of studying bacterial binding and inhibition of binding to tissue sections or to extracellular matrix molecules.

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All three adhesins have previously been shown to bind to tissue sections. P-fimbriated *E. coli* HB101/pPIL110-75 adheres to tissue sections of rat kidney [21], and HB101/pPIL110-75 and another *E. coli* strain, HB101/pPIL110-708, which has a deletion in the *pilA* gene [35], the major subunit of P-fimbriae, adhere strongly to immobilized fibronectin. *E. coli* HB101/pPKL4, expressing type 1 fimbriae binds to immobilised laminin [20]. Finally, *E. coli* C600/pYMS4514, as well as YeO3, expressing the *Yersinia* adhesin YadA, bind to tissue sections and to immobilized collagen, laminin and fibronectin [9,18, 19, 22]

Application of commercial antisera for the detection of *E. coli*

First, we confirmed that the *E. coli* specific antiserum (B357) along with the conjugated antiserum (P217) could be used to detect all the *E. coli* strains irrespective of the binding phenotype of the bacteria. To this end, different numbers of *E. coli* C600, C600/pYMS4514, HB101/pPKL4 and HB101 were fixed on glass slides, and the B357-P217 combination was used for detection as described in Materials and Methods. All the strains gave dose-dependent absorbance readings similar to those previously obtained with glass-fixed *Y. enterocolitica* using Moab A6-P260 combination [22] (data not shown).

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Detection of specific binding of bacteria to fixed tissue sections

The EIA method was then used to monitor the binding of adhesin-expressing bacteria to tissue sections. To obtain statistically-significant results we routinely used at least four parallel sections for each experimental condition. We paid special care when preparing the frozen sections to make sure they were as uniform as possible. Also when pipetting the 100 μl reagent droplets on the sections, we made sure that the droplets spread all over the targets leaving no dry areas.

Different concentrations of *E. coli* C600 and C600/pYMS4514 were incubated on fixed sections of human intestine and detected by EIA (Fig. 1A). As expected, only the YadA-expressing strain, C600/pYMS4514, bound to the sections, whereas the control strain,

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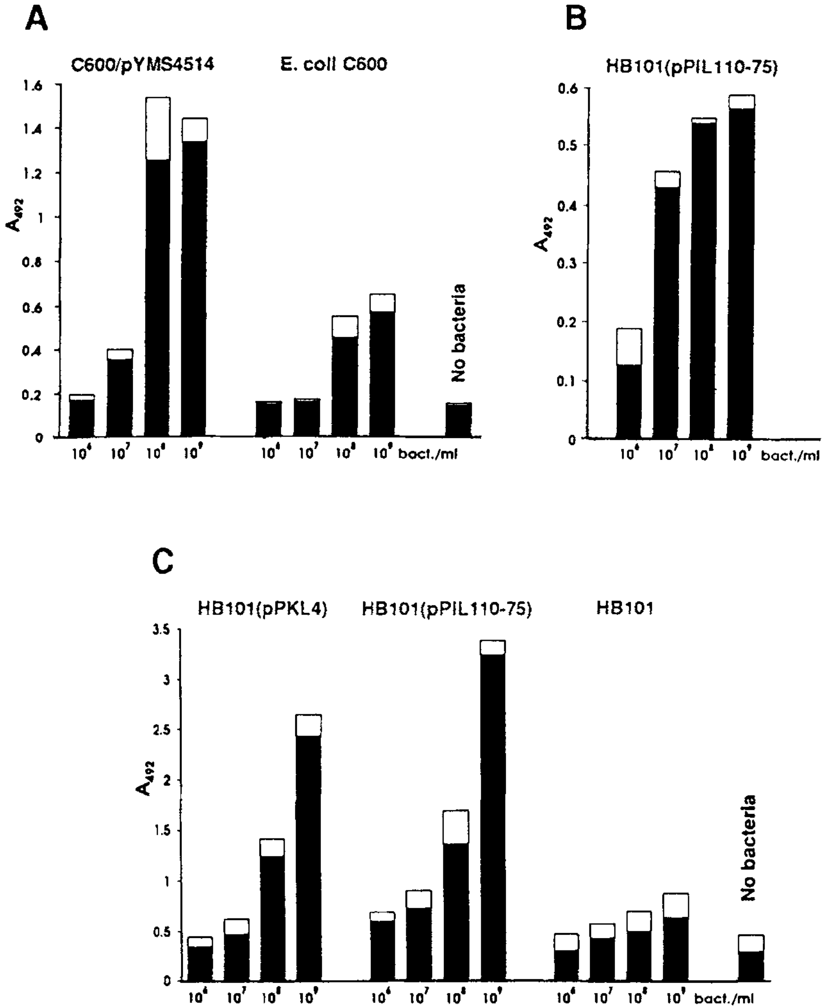

Figure 1. EIA detection of bacterial binding to fixed tissue sections. Panel A, binding of *E. coli* C600 and C600/pYMS4514 to fixed human intestinal tissue sections. Bacteria, primary and secondary antibodies were incubated on the sections for 30 minutes each at 22°C. Panel B and C, binding of *E. coli* HB101, HB101(pPKL4) and HB101(pPIL110-75) to fixed rat kidney tissue sections. The bacterial suspensions were incubated on the sections for one hour at 22°C. Panel B shows the results of HB101(pPIL110-75) using logarithmic scale for both axes, and panel C, using actual absorbance values on y-axis (actual absorbance values are used in all other figures on y-axis), The primary and secondary antibodies were incubated for 30 minutes each at 22°C. Black columns represent the mean values obtained from four parallel sections, and the open columns, corresponding standard deviations. As background control, samples with no bacteria were also measured. In this and other figures, increasing bacterial or inhibitor concentrations are given on the X-axis, and EIA absorbances, on the Y-axis.

C600, did not. In another experiment, *E. coli* strains HB101, HB101/pPKL4, and HB101/pPIL110-75 were incubated on fixed rat kidney sections. EIA absorbance showed that HB101/pPKL4 and HB101/pPIL110-75, expressing type 1 and type P fimbriae respectively bound efficiently to the kidney sections whereas HB101 did not (Fig. 1B). These results are in good agreement with previous results on both YadA [19] and fimbriae-expressing *E. coli* strains [20, 21], and demonstrate for the first time that HB101 (pPKL4) binds to rat kidney.

In the three experiments described above, the assay sensitivity was $\geq 10^5$ bacteria. We did not try to increase the sensitivity since bacteria could be detected on both fixed tissue sections and coated circles. Higher sensitivity, which might be needed when the binding target is very small, could be obtained by increasing the antibody incubation times and/or concentrations. However, increasing incubation times and antibody concentrations may also increase background, that is mostly caused by non-specific binding of antibodies.

The EIA-detection method allowed us to monitor bacterial binding to the fixed tissue sections over about a 100-fold range. There was a linear relationship between absorbance values and bacterial concentrations from about 5×10^6 to about 5×10^8 bacteria per ml. The linearity is seen in Fig. 1.B, where both the absorbance values and the bacterial concentrations are plotted on logarithmic scale. It should be noticed, however, that the relationship is not 1:1, i.e., 50 times more bacteria do not give 50-fold increase in absorbance, instead it was 10-20 fold. This inefficiency appears to be inherent in EIA based assays, and there may be many reasons for it. One reason could be that the method includes many reagents used in multiple steps, where the reaction conditions are not always optimal. This, especially with high concentration of antigenic epitopes combined with limiting antibody concentrations, may then lead to the apparent inefficiency. Another plausible reason may be steric hindrance when high bacterial concentrations are used. Bacterial cells binding to closeby receptors will block the antibody binding sites on each other; alternatively, especially with YadA-expressing bacteria, bacterial cells bind to each other with the same result. For this reason when performing EIA-based assays, quantification needs internal standards.

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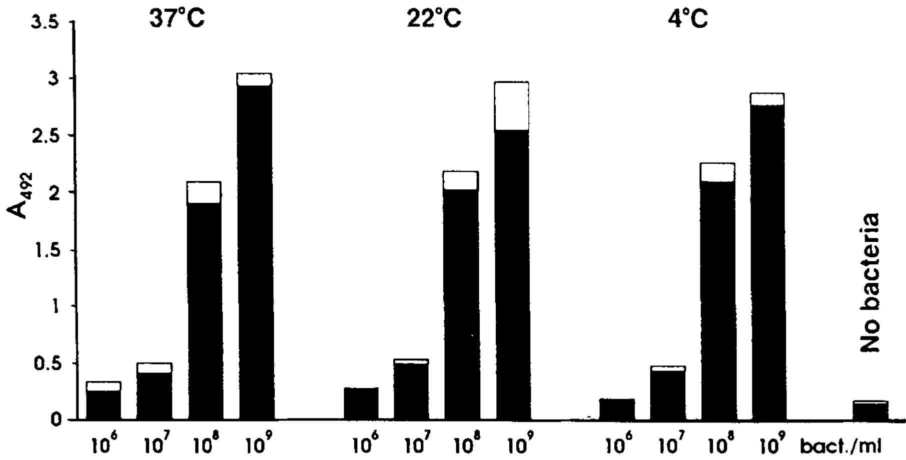

Figure 2 . Effect of temperature on YadA-mediated YeO3 binding to fixed tissue sections of human intestine. Bacteria were incubated for 15 minutes at 4°C, 37°C, and 22°C. Incubation times for the primary and secondary antibodies were 15 minutes each at 22°C.

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Effect of incubation temperature on YadA-mediated binding

We used the EIA method to study the effect of incubation temperature on YadA-mediated binding. Different concentrations of YeO3 and YeO3-c were incubated on fixed and blocked intestinal tissue sections at 4°C, 22°C or 37°C. The EIA absorbances showed that the YadA-mediated binding was not affected by the binding temperature (Fig. 2), suggesting that the affinity of the binding is exceptionally high, especially since the time allowed for the binding was only 15 minutes. YeO3-c showed no significant binding over the background at any temperature (data not shown).

Effect of incubation time on binding

The above results implied that the binding properties of the adhesin determine the binding parameters. To substantiate this we compared the effect of incubation time on the YadA and fimbriae-mediated binding. YeO3, and YeO3-c suspensions ($2x10^8$) were incubated on fixed and blocked intestinal tissue sections for 15, 60 and 120 minutes at

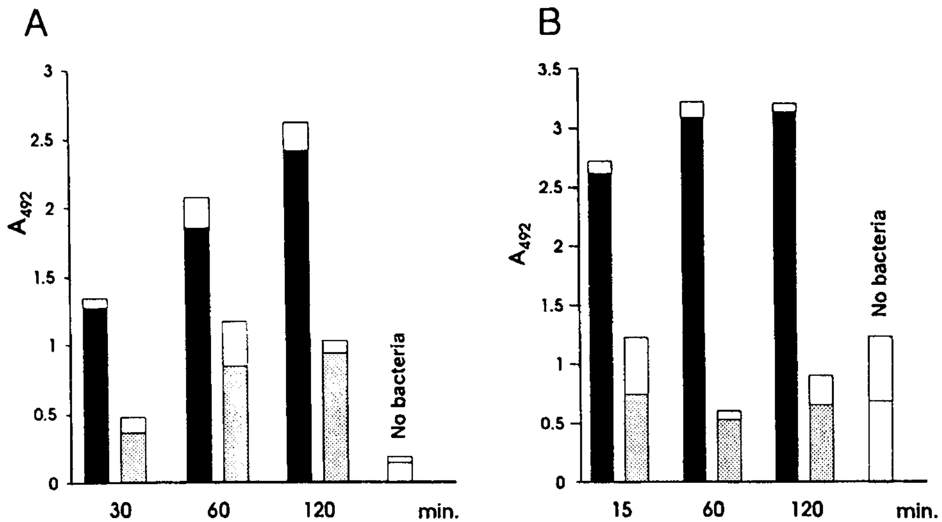

Figure 3. Panel A: effect of incubation time on binding of *E. coli* strains to tissue sections of rat kidney. Black columns, HB101(pPIL110-75); grey columns, HB101; white column, no bacteria. Incubation times for the primary and secondary antibodies were 30 minutes at 22°C. Panel B: effect of incubation time on binding of *Y. enterocolitica* strains to fixed tissue sections of human intestine at 4°C for 15, 60 and 120 minutes. Black columns, YeO3; grey columns, YeO3-c; white column, no bacteria. Incubation times for the primary and secondary antibodies were 15 minutes at 22°CColumns on top represent the standard deviations.

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4°C. Under these conditions, the YadA-mediated binding apparently approached saturation already during the first 15 minutes of incubation; the EIA absorbance was only a little higher with an incubation time of 120 minutes (Fig. 3B). It should be noted, however that at these high readings, the absorbencies are above the linear range of the spectrophotometer. YeO3-c showed no binding even after 120 minutes incubation (Fig. 3B). The fimbriae-mediated binding to fixed and blocked rat kidney sections was tested using HB101/pPIL110-75, and HB101 as control. Fimbriae-mediated binding is much slower than YadA-mediated binding (Fig. 3A); the binding reached saturation only after the first hour of incubation.

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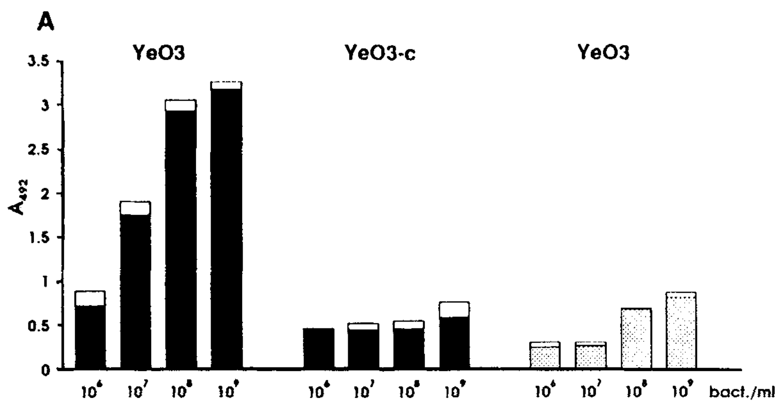

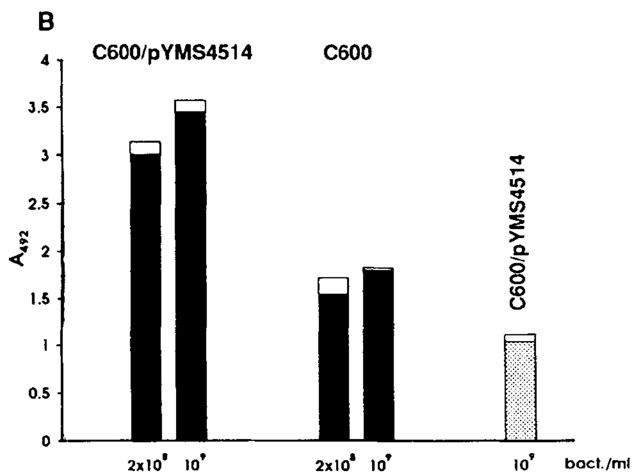

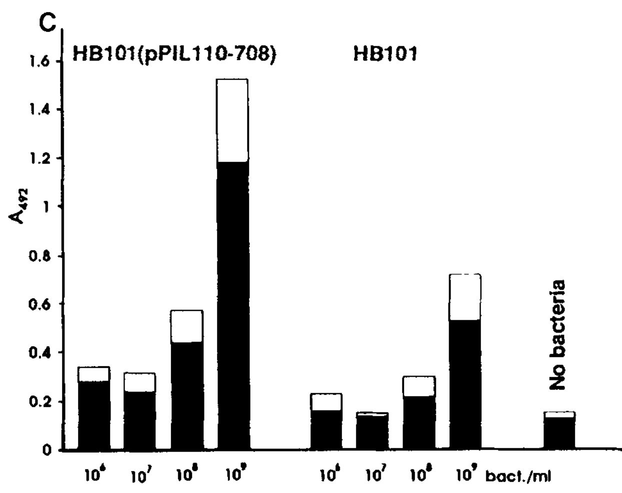

In summary, we found that on fixed tissue sections, YadA-mediated binding is very strong and rapid, whereas fimbriae-mediated binding is much weaker and slower under the condition we studied. For binding experiments with YadA, 15 min incubation time was sufficient whereas with fimbriae, 1 h was needed.

Bacterial adherence to glass slides coated with extracellular matrix molecules.

We then used the EIA method to monitor bacterial binding to glass circles coated with target molecules. To determine a functional coating concentration, adhesive bacteria ($2x10^8$ cfu per ml) were incubated on circles coated with different concentrations of target proteins. Coating concentrations of 10 µg per ml proved to be sufficient for EIA detection (data not shown). Our method could detect YadA-mediated binding to collagen type I coated circles (Fig. 4A), YadA-mediated binding to collagen typeIV coated circles (Fig. 4B), and P fimbriae-mediated binding to fibronectin coated circles (Fig. 4C).

These results are in good agreement with previous findings of YadA- and P-fimbriae-mediated binding to type I and IV collagens and to fibronectin [19, 21, 22], and demonstrate that the EIA method provides an alternative way of studying the binding of bacteria to immobilised extracellular matrix molecules.

In all these experiments, the control circles coated with BSA showed low level dose dependent binding (See Fig. 4A). We do not know the reason, perhaps the BSA preparations are contaminated with minor amounts of extracellular matrix molecules.

Inhibition experiments with extracellular matrix molecules

The EIA method proved most useful in competition experiments. We could detect dose-dependent inhibition of YadA-mediated binding of YeO3 to tissue sections of human

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Figure 4. EIA detection of bacterial adhesion to glass slide circles coated with extracellular matrix molecules. For panels A and B, bacteria were incubated on circles for 30 minutes at 22°C, and incubation times for both primary and secondary antibodies were 15 minutes at 22°C. For panel C, bacteria were incubated on circles for one hour at 22°C, and incubation times for both primary and secondary antibodies were 30 minutes at 22°C. Columns are as in Fig. 1 and binding to BSA coated circles is shown by grey columns. Panel A: binding of YeO3 and YeO3-c to immobilised collagen type I. Panel B: binding of *E. coli* C600 and C600/pYMS4514 to immobilised collagen type IV. Panel C: binding of *E. coli* HB101, HB101(pPIL110-708) to immobilised fibronectin.

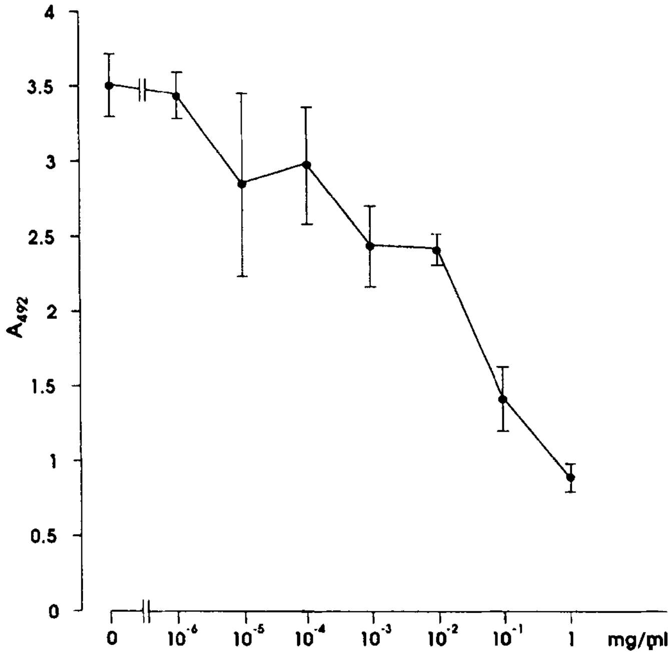

Figure 5. EIA detection of inhibition by collagen type I of YadA-mediated binding to fixed and blocked tissue sections of human intestine. YeO3 ($2x10^8$/ml) was preincubated with different concentrations of collagen type I for 30 minutes at 37°C before incubation on sections for 15 minutes at 4°C. Incubation times for primary and secondary antibodies were 15 min at 22°C. Each point represents the mean of four parallel sections and the vertical bars show the corresponding standard deviations.

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intestine by collagen type I (Fig. 5). We also measured the inhibition of binding to glass-immobilized extracellular matrix molecules. Using, for instance, collagen type I immoblized on glass slides as a target and two different concentrations, 10^7 and 10^8 per ml, of YeO3 (Fig. 6A). Again, there was clear dose-dependent inhibition of binding, similar to the inhibition of binding to tisssue sections of human intestine (Fig. 5), suggesting that collagen is an important receptor for YadA in the intestine.

Inhibition of YadA-mediated binding was further studied using *E. coli* C600 and C600/pYMS4514. As YadA binds both collagen and laminin, we attempted to inhibit the binding to immobilized collagen type IV by both. Binding was inhibited by collagen but not

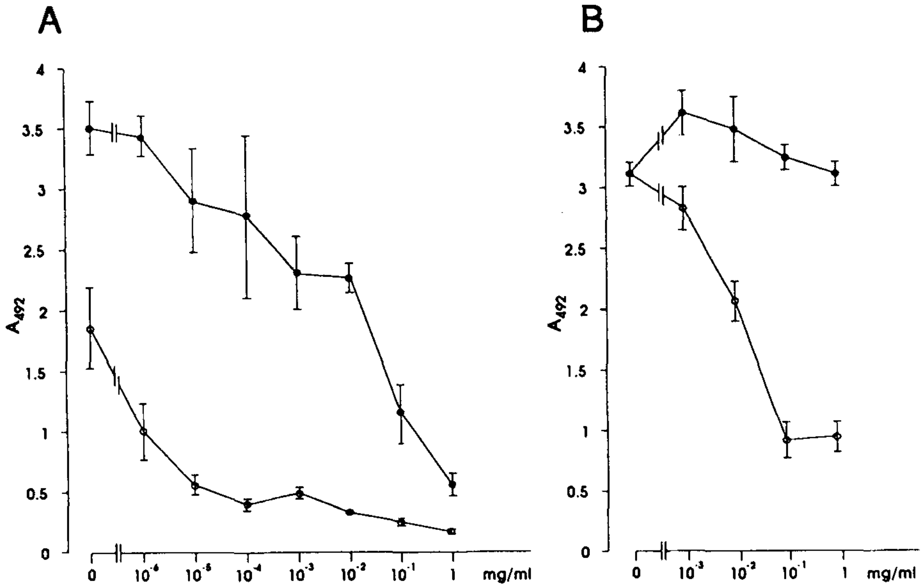

Figure 6. EIA detection of inhibition of YadA-mediated binding to coated circles. Panel A: YeO3 (open circles, 10^7, filled circles, 10^8 bacteria per ml) was preincubated for 30 minutes at 37°C with different concentrations of collagen type I before incubation on coated circles for 30 minutes at 22°C. Panel B: C600/pYMS4514 ($2x10^8$/ml) was preincubated for 30 minutes at 37°C with soluble collagen IV (open circles) or soluble laminin (filled circles) at different concentrations before incubation on collagen type IV coated circles for 30 minutes at 22°C. Incubation times for the primary and secondary antibodies were 30 minutes.

by laminin (Fig 6B), indicating that the collagen and laminin binding domains of YadA are discrete.

CONCLUSION

The new enzyme immunoassay method is a rapid and reliable technique for measuring bacterial adherence to fixed tissue sections and to immobilized extracellular matrix molecules on glass slides. The advantages of the technique are i) it is reliable, evidenced by small standard deviations and by highly significant binding inhibition experiments. ii) it is rapid; one experiment can be performed in six hours. iii) it is flexible, allowing

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monitoring of bacteria bound both to non-uniform targets, such as fixed sections, and to coated surfaces.

A potential drawback is the requirement for a primary antibody specific for the bacterial species under investigation. Borén and coworkers [28] used digoxigenin-labelled *Helicobacter pylori* bacteria to study binding to gastric tissues. This labelling system coupled with detection using enzyme labelled anti-digoxigenin antibodies could also be a good choice with bacteria where no bacteria-specific antibodies are available. It must be kept in mind, however, that the labelling procedure may affect the bacterial adhesin *per se* and interfere with the binding. This does not occur with specific antibodies that are added after the bacteria are bound to their targets.

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