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Cross-Reactive Protection against Enterohemorrhagic *Escherichia coli* Infection by Enteropathogenic *Escherichia coli* in a Mouse Model

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Running title: Cross-protection among A/E pathogens in a mouse model

ABSTRACT

Enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *Escherichia coli* are related attaching and effacing (A/E) pathogens. The genes responsible for the A/E pathology are encoded in a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE). Both pathogens share a high degree of homology in the LEE and additional 'O' islands. EHEC prevalence is much lower in EPEC endemic areas. This may be due to the development of antibodies against common EPEC and EHEC antigens. This study investigated the hypothesis that EPEC infections may protect against EHEC infections. We used a mouse model to inoculate BALB/c mice intragastrically, first with EPEC, followed by EHEC (*E. coli* O157:H7). Four control groups received either a non-pathogenic *E. coli* (NPEC) strain followed by EHEC (NPEC/EHEC), alternatively PBS/EHEC, EPEC/PBS or PBS/PBS. Mice were monitored for weight loss and symptoms. EPEC colonized the intestine after challenge and mice developed serum antibodies to intimin and *E. coli* secreted protein B (encoded in the LEE). Prechallenge with an EPEC strain had a protective effect after EHEC infection as few mice developed mild symptoms, from which they recovered. These mice had an increase in body weight similar to control animals and tissue morphology exhibited mild intestinal changes and normal renal histology. All mice that were not pre-challenged with the EPEC strain developed mild to severe symptoms after EHEC infection, weight loss as well as intestinal and renal histopathological changes. These data suggest that EPEC may protect against EHEC infection in this mouse model.

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) is a causative agent of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (17). EHEC is characterized by the presence of Shiga toxins (Stx) as a major virulence factor (26). Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of acute diarrhea among infants living under poor social conditions in developing countries (35). Typical EPEC is characterized by the presence of a virulence plasmid know as the EAF (EPEC adherence factor) (49). The EAF plasmid contains a cluster of genes encoding the bundle-forming pili (Bfp), which is required for localized adherence to epithelial cells (15). In contrast to EHEC, EPEC strains do not produce Stx.

Both pathogens induce characteristic attaching and effacing lesions (A/E) on intestinal enterocytes, characterized by intimate bacterial adhesion, destruction of microvilli and accumulation of polymerized actin in pedestals beneath intimately attached bacteria (24). Bacterial factors required for the formation of the A/E lesion are encoded on a chromosomal pathogenicity island called the Locus of Enterocyte Effacement (LEE) (12), which contains the genes *eae* encoding the adhesin intimin (22), *esc* and *sep* encoding a type III secretion apparatus (12) and genes encoding proteins that are secreted via the type III secretion system including *E. coli* secreted protein A (EspA), EspB, EspD (21) and the receptor for intimin (Tir) (29). Intimate attachment of bacteria is mediated by intimin and its receptor translocated into host cells (29). EspA forms a filamentous organelle that acts as a channel through which bacterial proteins are transported into the eukaryotic cell (30). EspB and EspD form a pore in the membranes of infected cells (19). EPEC and EHEC share a high degree of homology across the 41 genes contained in the LEE (39).

77 Epidemiological surveys regarding the prevalence of A/E pathogens revealed that EHEC
78 infections are mainly present in developed countries and not frequently found in developing
79 countries with the exception of Argentina (35). In Brazil, EPEC prevalence accounted for
80 33% among children younger than two years of age with diarrhea and EHEC isolates were not
81 detected (16, 43). Prevalence of A/E pathogens in Bolivia among children younger than five
82 years of age with diarrhea was 7%, of which 95% corresponded to EPEC and 5% to EHEC
83 isolates (44).
84
85 The low prevalence of EHEC infections in developing countries may be explained by the
86 development of antibodies against common EPEC and EHEC antigens by individuals living
87 in EPEC endemic areas (34, 38). Several studies showed that children and adults develop an
88 immune response against highly immunogenic virulence factors such as intimin and the Esps
89 (7, 34, 38, 47), which are potential targets for vaccine development. In addition, IgA
90 antibodies against intimin, Bfp, EspA and EspB have been detected in colostrum from
91 mothers living in EPEC endemic areas (33, 38), which may provide infants with effective
92 protection against A/E pathogen infections (32).
93
94 This study used an established mouse model (5) to examine the hypothesis that EPEC
95 infection could have a protective effect against subsequent EHEC infection in mice.

MATERIALS AND METHODS

Mice

BALB/c mice were bred in the animal facilities of the Department of Microbiology, Immunology and Glycobiology, Institute of Laboratory Medicine, Lund University. Male mice were used at 8 - 9 weeks of age.

Bacterial strains and cultures

Bacterial strains used in this study are listed in Table 1. The EPEC strain, 73-1 was isolated from the feces of a two-year old boy with diarrhea in La Paz, Bolivia in 2004. The strain was typed for the O serogroup antigen and genotypically characterized for the presence of *bfp, eae, stx1,* and *stx2* genes (2, 18, 25). The strain corresponded to the O127:H6 serotype and was positive for the *bfp* and *eae* genes. The strain was found to be sensitive for ampicillin (Amp^s) and streptomycin (Str^s). Spontaneous ampicillin-resistant derivatives of this strain were developed as previously described (42). In order to enhance the virulence, the resulting strain 73-1 (Amp^r, Str^s) was first intragastrically inoculated into four ampicillin-treated BALB/c mice (1g/l ampicillin, Sigma Aldrich, Stockholm, Sweden, in drinking water 24 h before challenge and throughout the experiment). To confirm the colonization fecal samples were collected 24, 48, 72 and 96 hours after inoculation, plated on Luria broth (LB) agar supplemented with 50 µg/ml ampicillin and analyzed by PCR for the detection of *bfp* (18) and *eae* (25) genes. After 24 hours one mouse presented positive fecal culture for EPEC, this strain was isolated, termed 73-1PB and kept in LB/glycerol (85:15%, v/v) at -80°C for further experiments. After 72 hours all fecal cultures from the four mice were found to be positive for EPEC by PCR. All mice presented some systemic symptoms such as ruffled fur from which they recovered by day 3 to 5.

The EHEC strain (*E. coli* O157:H7), 86-24, was isolated during the Walla Walla Washington State outbreak of HUS and hemorrhagic colitis, in November 1986 (48) and was kindly provided by A.D. O'Brien (Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethseda, MD). This strain was previously genotypically and phenotypically characterized (28). The strain was found to be ampicillin- and streptomycin-sensitive. Spontaneous streptomycin-resistant derivatives of this strain were developed as previously described (42). To enhance virulence the resulting strain 86-24 (Amp^s, Str^r) was first inoculated in three streptomycin-treated BALB/c mice (5g/l streptomycin sulfate; MP Biomedicals, OH, in drinking water 24 h before inoculation and throughout the experiment). *E. coli* O157:H7 was isolated on day 9 from colonic content of a sick mouse and detected by slide agglutination using an *E. coli* O157 latex test kit (Oxoid, Basingstoke, UK). The strain was termed 86-24PB and stored in LB/glycerol (85:15%, v/v) at -80°C until used.

A laboratory *E. coli* strain, Select96^{TM} competent cells (Promega, Madison, WI), was used as a control strain. The strain has a plasmid mediating ampicillin resistance (pcDNA3, Invitrogen, Carlsbad, CA). The strain was genotypically characterized for the presence of *bfp,* *eae,* and *stx2* and found to be negative for these genes. The strain was termed non-pathogenic *E. coli* (NPEC).

For inoculation of mice, the EPEC, EHEC and NPEC strains were grown overnight at 37°C in LB broth supplemented with 50 µg/ml ampicillin or 50 µg/ml streptomycin, as appropriate, and harvested by centrifugation. The pellet was washed in sterile phosphate buffer saline (PBS, pH 7,4, Medicago AB, Uppsala, Sweden) and resuspended in a solution of 20% (w/v) sucrose and 10% (w/v) $NaHCO_3$ in sterile water.

146

147 **Infection protocol**

148 Mice were divided into five groups and each group received two inoculations (Table 2) at two
149 different time points (Fig. 1).

150

151 For the first inoculation, mice were treated with ampicillin 24 h prior to inoculation, as
152 described above, to reduce the commensal flora, thereby facilitating ampicillin-resistant
153 strains (EPEC and NPEC) to colonize the gastrointestinal tract. Mice were fasted for 16 h
154 before inoculation. Mice were anesthesized with isoflurane (Forene; Abbott, Wiesbaden,
155 Germany) and 100 µl of a bacterial suspension at 10^9 CFU/ml, or sterile PBS, was
156 administered intragastrically through a soft polyethylene catheter (0,61 mm OD; 0.28 mm ID;
157 Clay Adams, Parsippany, NJ) (28). After inoculation the catheter was removed and food was
158 provided ad libitum. During the course of infection, mice were monitored three to five times
159 per day. The ampicillin treatment was discontinued after 7 days, to enable recovery of the
160 commensal flora.

161

162 In order to facilitate the clearing of bacteria from the first inoculation and to improve
163 colonization of the EHEC strain, mice were treated with streptomycin from day 16 after the
164 first inoculation until the end of the experiment (Fig. 1). Before the second inoculation, mice
165 were fasted for 16 h. Under isoflurane anesthesia, 100 µl of a bacterial suspension at 10^9
166 CFU/ml, or sterile PBS, was administered intragastrically using a soft polyethylene catheter.
167 Ten days after the second inoculation or when mice presented evident signs of disease,
168 infected and control mice were sacrificed by cervical dislocation under isoflurane anesthesia,
169 tissues were collected for histological examination, and a final disease score was given to

each mouse as previously described (5) (Table 3). Symptom score 3 depicts the most severe clinical findings as spontaneous death did not occur.

An attempt was made to perform the first inoculation without antibiotic treatment but bacteria from the first challenge were not able to colonize efficiently. A second attempt was made to treat mice with streptomycin alone using a streptomycin-resistant EPEC strain followed by the streptomycin-resistant EHEC strain. Under continuous streptomycin treatment EPEC bacteria colonized the intestine persistently and were shed in the feces up to 50 days after infection. Challenge with the streptomycin-resistant EHEC strain was performed at day 40, but under these conditions EHEC bacteria were unable to colonize. For this reason all experiments were carried out, as described above, using first ampicillin treatment for the ampicillin-resistant EPEC or NPEC strains followed by streptomycin treatment for the streptomycin-resistant EHEC strain. BALB/c mice were chosen for this study due to their high susceptibility to EHEC infection. Inoculation with EHEC under streptomycin treatment, without prior treatment with PBS or other bacterial strains, causes terminal illness in 100% of infected mice. All experiments were approved by the Animal Ethics Committee of Lund University.

Confirmation of colonization and bacterial shedding

To confirm the colonization of EPEC and NPEC strains, fecal samples were collected from day 1 to 3 after the first inoculation. To monitor bacterial shedding and to confirm clearance of bacteria after the first inoculation, fecal samples were also collected from day 7 to 20 (as per Fig. 1). Samples were plated on LB agar supplemented with 50 µg/ml ampicillin (for culture of both strains) and tested by PCR for the presence of *bfp* and *eae* genes (for detection of EPEC).

Samples collected from the EPEC/EHEC, NPEC/EHEC and EPEC/PBS groups (as per Table 2) taken on days 1 to 3 were positive for the EPEC or NPEC strains. In the EPEC/EHEC group EPEC were cleared spontaneously (even before streptomycin-treatment was initiated) in 5/11 mice 6 - 8 days before the second inoculation, the remainder (n = 6) cleared the EPEC infection within 2 days after the start of streptomycin treatment. All mice in the NPEC/EHEC group cleared the NPEC infection before streptomycin-treatment was given, 8 days before the second inoculation. In the EPEC/PBS group all mice were positive for EPEC 8 days after inoculation with EPEC and cleared the EPEC infection within 3 days after the start of streptomycin treatment.

To confirm the colonization of EHEC, fecal samples were collected on days 1, 2 and 7 after the second inoculation, plated on LB agar supplemented with 50 μg/ml streptomycin and tested for the presence of the O157 serogroup by slide agglutination. Samples from the EPEC/EHEC, NPEC/EHEC and PBS/EHEC groups were positive for the EHEC strain at the three time points after the second inoculation.

Weight measurement

Body weight changes were calculated as a percentage of the initial body weight. In the five groups weight was taken one day before the second inoculation, to account for initial body weight before fasting, and on a daily basis afterwards.

Antibody detection in serum samples

Blood samples were collected 6 days before and 14 days after the first inoculation from the saphenous vein of mice in the EPEC/EHEC group (n = 11), PBS/EHEC (n = 6), and PBS/PBS

220 group (n = 4) using capillary tubes for collection of serum (Sarstedt, Nümbrecht, Germany).
221 Serum samples were stored at -20°C until analyzed.
222
223 The presence of IgM antibodies against EspB was detected by ELISA as previously described
224 (45). Briefly, plates were coated with rabbit-anti EspB (45) 1/2000 overnight at 4°C, wells
225 were washed in PBS/Tween (Medicago, Uppsala, Sweden), blocked for 2 h with bovine
226 serum albumin (Sigma Aldrich, Stockholm, Sweden), washed and incubated for 1 h with
227 recombinant His-tagged EspB (45) (50ng/well). After washing, wells were incubated with
228 serum samples 1/50 for 2 h at 37°C. Wells were washed and incubated with peroxidase-
229 conjugated goat anti-mouse IgM for 1.5 h (1/500 , Sigma Aldrich). After washing, wells were
230 incubated for 30 min in the dark with an OPD solution (Dako, Glostrup, Denmark),
231 resuspended in deionized water with the addition of H_2O_2 30%) and the reaction was
232 terminated with 0.5 M H_2SO_4. Absorbance was measured at OD_{490nm}. Changes in serum
233 antibody levels were calculated as the percent increase/decrease compared to the initial
234 absorbance values (before the first inoculation).
235
236 **Isolation of EPEC and EHEC secreted proteins**
237 EPEC (73-1PB) and EHEC (86-24PB) secreted proteins were isolated according to a
238 previously published protocol (21) with modifications as follows. Bacteria were grown in
239 DMEM low glucose (GIBCO, Paisley, UK) to an OD_{600nm} of 1.0. Bacteria were pelleted by
240 centrifugation at 10000 x g for 10 min after which the supernatant was collected.
241 Phenylmethylsulfonyl fluoride (50 µg/ml, Sigma Aldrich) and EDTA (0,5 µM, Merck,
242 Darmstadt, Germany) were added to the supernatant which was passed through a 0.2 µm filter
243 (Pall Corp., Ann Arbor, MI) and concentrated 330 times using IVD ultracel - 10K (Millipore,
244 Carrigtwohill, Ireland).

Immunoblotting to detect antibodies to EPEC and EHEC secreted proteins

Secreted proteins from EPEC or EHEC (approximately 30 μg in each well) were run on a 10 % Tris-HCL gel (Bio-Rad, Hercules, CA) and transferred to a PVDF membrane (Bio-Rad). Proteins were detected using goat anti-intimin 1:500 (a gift from A.D. O'Brien), rabbit anti-EspB 1:5000 (27) or serum (1:500) from mice. The sera were collected before inoculation with EPEC as well as 14 days later. Bound antibodies were identified with polyclonal rabbit anti-goat Ig:HRP (1:500, Dako) polyclonal goat anti-rabbit Ig:HRP (1:1000, Dako) or goat anti-mouse IgG-peroxidase (1:500, Sigma Aldrich), respectively, and visualized using ECL plus (GE Healthcare, Buckinghamshire, UK).

Histopathological analysis

Proximal and distal colon samples as well as kidneys were collected at the end of the experiment (ten days after the second inoculation or when evident signs of disease were observed after the second inoculation). Samples were fixed overnight in 4% paraformaldehyde (Sigma-Aldrich) and embedded in paraffin. Tissue sections (3 μm) were stained with hematoxylin-eosin (Merck) for kidneys, and periodic acid-Schiff for intestines. Stained tissue sections were then examined under an Axiostar Zeiss microscope, mounted with an Axiocam MRc5 camera (Carl Zeiss, Göttingen, Germany). AxioVision AC software version 4.4 (Carl Zeiss) was used for image processing. Samples were coded and examined in a blinded fashion. The degree of pathological findings was defined as mild, moderate or severe.

Statistical analysis

Differences between the experimental groups regarding disease score, body weight changes and antibody levels were assessed by the Mann-Whitney *U* test. A P value ≤ 0.05 was considered significant. SPSS software version 11 (SPSS, Chicago, IL) was used for the statistical analyses.

RESULTS

Clinical signs of disease in the different inoculation groups

Mice were divided into five groups and received two separate inoculations (Table 2). After the first inoculation mice from the EPEC/EHEC, NPEC/EHEC and EPEC/PBS groups presented mild symptoms such as ruffled fur from which they recovered within 3 to 5 days. Symptoms were not observed in mice from the PBS/EHEC and PBS/PBS groups.

After the second inoculation the different groups were compared with regard to the development of symptoms. All mice that did not receive a previous EPEC infection, i.e. groups NPEC/EHEC and PBS/EHEC, developed mild to severe symptoms after infection with the EHEC strain, and there was no significant difference ($P > 0.05$) between these two groups regarding the symptom score. Mice in the NPEC/EHEC and PBS/EHEC groups exhibited the highest symptom score (as per Table 3). Terminally ill mice were only found in the PBS/EHEC group (4/12, 33.3% of the mice). Although certain mice were severely ill no spontaneous death occurred during the experiment. Mice in the EPEC/EHEC group exhibited a lower symptom score. Mild symptoms occurred in 3/11 (27 %) mice from which they recovered, and 8/11 (73 %) mice in this group did not show any clinical signs of disease. There was no significant difference ($P > 0.05$) regarding symptom score comparing mice in the EPEC/EHEC group with those in the EPEC/PBS or PBS/PBS groups (Fig. 2).

Body weight changes during EHEC infection

Body weight changes were calculated as a percentage of the initial body weight in the five groups during the course of EHEC infection. After the second inoculation, mice in the NPEC/EHEC and PBS/EHEC groups exhibited weight loss particularly during the first two

297 days after inoculation, whereas mice in the EPEC/EHEC, EPEC/PBS and PBS/PBS groups
298 recovered their initial body weight immediately after the initial fasting period. Body weight
299 changes were expressed as a function of time (Fig. 3).
300
301 **Antibody response to EspB in serum samples**
302 Serum antibody levels were assessed comparing individual values before the first inoculation
303 with values 14 days after the first inoculation. At this time point, mice from the PBS/EHEC
304 and PBS/PBS groups had received the same treatment (only PBS) and were therefore merged
305 into one control group (n=10) for comparison with mice in the EPEC/EHEC group (n=11).
306 Antibody levels against EspB were elevated in the EPEC/EHEC group showing a median
307 increase of 12.5% (range: 0 - 52.3%) 14 days after EPEC infection. As expected there was no
308 increase in anti-EspB levels in sera from mice that were treated with PBS. The increased anti-
309 EspB in the EPEC/EHEC group was statistically significant when comparing with groups
310 PBS/EHEC and PBS/PBS together ($P < 0.05$).
311
312 **Cross reactive antibody response to EHEC secreted proteins detected by**
313 **immunoblotting**
314 For the purpose of testing if mice exposed to EPEC developed an antibody response to EHEC
315 secreted proteins the latter were run on a gel and reacted with sera from mice taken before
316 EPEC inoculation and 14 days later. Results showed that sera from 8/11 mice in the
317 EPEC/EHEC group reacted with intimin and 4/11 sera reacted with EHEC secreted protein B
318 (EspB) after inoculation with EPEC and before inoculation with EHEC. A total of nine mice
319 showed an antibody response to EHEC intimin and/or EspB. Bands were detected at
320 approximately 94 kD and 37 kD corresponding to intimin and EspB, respectively (Fig. 4,
321 lanes 1 and 2) (27). No bands were visualized in sera taken from mice from the PBS/EHEC

group (n=2) before inoculation with EHEC, or from the PBS/PBS (n=3) group. Similar results were obtained using an extract of secreted proteins from the EPEC strain (Fig. 4, lanes 3 and 4). As a positive control the EHEC-secreted proteins reacted with rabbit anti-EspB (Fig. 4 lane 5) and goat anti-intimin (Fig. 4 lane 6).

Intestinal and renal pathology in the different inoculation groups

Intestines and kidneys from mice were coded for blind assessment and examined by light microscopy for histopathological lesions which are summarized according to severity in Table 4. Changes consisted of inflammatory infiltrates, lymph node hyperplasia, thickening of the submucosa, edema and goblet cell depletion (Fig. 5). The most severe intestinal changes were found in mice from the NPEC/EHEC and PBS/EHEC groups. Few pathological changes were found in mice from the EPEC/EHEC and EPEC/PBS groups. No alterations in the intestinal structure were noted in the PBS/PBS group.

Renal pathology was mainly demonstrated in mice from the PBS/EHEC group. Pathological changes in this group included tubular cell desquamation, dilated tubular structures, glomerular capillary congestion and occlusion, and red blood cells in tubular lumina. Tubular desquamation and dilated tubuli were also demonstrated in mice from the NPEC/EHEC group. Mice from groups EPEC/EHEC, EPEC/PBS and PBS/PBS did not exhibit renal pathology (Fig. 6 shows EPEC/EHEC and PBS/PBS mice).

DISCUSSION

Due to the severity of disease clinical trials using wild-type *E. coli* O157:H7 strains cannot be performed on human volunteers. A mouse model has been developed (5, 51) which mimics certain aspects of severe human *E. coli* O157:H7 infection such as severe systemic and neurological symptoms as well as pronounced pathology of the gastrointestinal tract and kidney. Mice develop marked tubular damage as well as decreased renal function and thrombocytopenia resembling certain aspects of human HUS (5). As it has been hypothesized that EPEC infection may confer immunity against EHEC infection in endemic areas (37, 38) we tested this in the mouse model. Pre-challenge with an EPEC strain protected mice from the symptoms and pathology associated with EHEC infection. The degree of homology between these two A/E pathogens suggests that a protective immune response may occur. Indeed, results show that mice developed antibodies to intimin and EspB after the EPEC infection indicating an immune response to EPEC virulence factors. An antibody response was also mounted to EHEC intimin and EspB even before inoculation with EHEC, suggesting cross-reactivity. Due to the similarity between the strains there may be multiple protective mechanisms conferring cross-reactive immunity between EPEC and EHEC pathogens.

EPEC and EHEC share a high degree of homology across the genes encoded in the LEE pathogenicity island (39). The LEE represents only one such island. In addition, comparison of 177 'O' islands showed that 69 islands shared more than 90% nucleotide homology between EHEC O157:H7 (EDL933) and EPEC O127:H6 (2348/69) (46). Moreover, phylogenetic analysis suggested that EHEC O157:H7 may have evolved from EPEC strains that acquired phage-encoded Stx (13, 52) or that the strains developed in parallel acquiring similar virulence factors (41).

368

369 A protective effect among A/E pathogens was suggested in a study in which challenge of
370 rabbits with a Shiga toxin-producing RDEC-1 strain possessing truncated intimin, with
371 retained immunogenicity, had a protective effect when the same rabbits were subsequently
372 challenged with the wild-type strain (1). Antibodies to intimin have been detected during and
373 after EPEC or EHEC infections (11, 27, 31). It has been suggested that these antibodies could
374 confer protection against subsequent EPEC infection. This could, however, not be confirmed
375 in a homologous rechallenge setting in which human volunteers were first challenged with
376 EPEC strains (wild-type *E. coli* O127:H6 or its corresponding isogenic Δ*eae* mutant) and then
377 rechallenged with the wild-type strain (11). No correlation was found between anti-intimin
378 antibodies and severity of disease although volunteers pre-challenged with the wild-type
379 strain developed fewer symptoms than those pre-challenged with the Δ*eae* mutant. In the
380 same study heterologous rechallenge with EPEC strains also failed to induce preventive
381 immunity determined by the clinical end-point of diarrhea in human volunteers. The reduction
382 in severity of symptoms after homologous challenge was thus attributed to an antibody
383 response to O antigens. These studies indicate that protection was serotype-specific. The
384 protective effect demonstrated here would, most probably, not have been mediated by an
385 acquired immune response to lipopolysacharide (LPS), since the EPEC and EHEC strains
386 studied do not share the same LPS serogroup.

387

388 Mice in the NPEC/EHEC group exhibited milder renal pathology compared to mice from the
389 PBS/EHEC group after EHEC infection. This may indicate that a previous challenge with a
390 non-pathogenic *E. coli* strain may have some degree of protective effect although not to the
391 same extent as the protective effect mediated by a previous challenge with an EPEC strain.
392 We speculate that a possible protective effect might be partly explained by the “endotoxin

393 tolerance" mechanism in which a previous challenge with LPS could invoke insensitivity
394 upon a second challenge as a response from the organism to regulate excessive inflammation
395 that may be deleterious (3). This phenomenon could last for hours to days, after which a
396 typical proinflammatory condition would recur upon endotoxin stimulation (3, 4), suggesting
397 that such an effect would have to last for several days after the first challenge to play a role in
398 the present mouse model. We have shown that bacteria from the first challenge were
399 eradicated at least 4 to 8 days before the second bacterial infection. In particular the NPEC
400 strain was eradicated 8 days before the second inoculation. We can thus not exclude the
401 possibility that endotoxin tolerance, or the presence of other shared *E. coli* surface antigens,
402 could have had a minor impact regarding protection from the severity of EHEC-induced
403 pathology.
404
405 Before EHEC infection, mice were treated with streptomycin to remove EPEC bacteria from
406 the gut. Interestingly, while developing the infection protocol, we observed that, when EPEC
407 bacteria colonized the gut persistently EHEC bacteria were incapable of colonizing the gut
408 and mice did not develop any symptoms. This phenomenon could also play a protective role
409 in individuals living in EPEC endemic areas against EHEC infection. The mild
410 histopathological changes found in the intestines of mice in the EPEC/PBS group may explain
411 why mice in the EPEC/EHEC group also had mild intestinal histopathological changes, this
412 may be residual EPEC-mediated damage not related to EHEC infection.
413
414 Most human EPEC strains, including the one used in this study, express intimin type α, while
415 intimin γ is mainly associated with EHEC serotypes including O157:H7 (36). Intestinal tissue
416 tropism may be determined in part by the intimin type (14, 50). Studies using a prototype
417 EPEC strain showed adhesion to proximal and distal human small intestine and follicle-

418 associated epithelium (FAE) of Peyer's patches but showed limited adhesion to human
419 colonic samples (40). It is believed that EHEC binds FAE and villi of the terminal ileal region
420 (9, 40) and subsequently colonizes the human colon. As described above, while developing
421 the present infection protocol, when EPEC bacteria colonized the gut of mice persistently,
422 EHEC bacteria were unable to colonize. This may indicate that EPEC and EHEC compete for
423 common loci of colonization. The infection protocol used in this study included the use of
424 antibiotics to ensure that EPEC bacteria from the first challenge were eradicated before the
425 second challenge with EHEC, and therefore tissue tropism of both strains determining
426 colonization sites most probably did not play a role regarding the protective effect observed.
427 We believe that the protective effect was mediated by a humoral immune response to
428 common EPEC and EHEC antigens.
429
430 The development of antibodies in sera, saliva, colostrum and breast milk against Esps proteins
431 during EPEC infection has been reported before (7, 33, 34, 37, 38, 47) and is consistent with
432 our findings. Younger children have a higher propensity to symptomatic EPEC infection (35,
433 49) most probably due to the fact that they have not developed a sufficient immune response.
434 Breast-feeding has a protective effect against EPEC infection and breast milk contains
435 antibodies capable of preventing EPEC adherence (6, 8). In the present study we showed that
436 mice developed immunity against intimin and EspB, and the protection observed may be
437 mediated by a complex immune response against a wider variety of factors. Nonetheless,
438 EspB was shown to be important in mediating diarrhea in human volunteers who developed
439 an antibody response to a wild-type EPEC strain. Volunteers who ingested an Δe*spB* mutant
440 strain developed fewer symptoms (47).
441

Animal studies in mice and piglets have suggested the use of intimin vaccines (10, 23). The results of this study may be of relevance for the development of live vaccines against EHEC infection, based on its closely related A/E pathogen, namely EPEC bacteria with attenuated virulence. Furthermore, the use of live vaccines would promote an efficient immune response against a broad range of known virulence determinants as well as a number of, as yet, unidentified virulence factors shared by both EHEC and EPEC, in contrast to purified vaccines based on only one or two virulence factors.

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TABLE 1. Bacterial *E. coli* strains used in this study

Strain	Parental strain	Category	Serotype	*bfp*	*eae*	*stx2*	Antibiotic resistance
73-1PB	73-1[a]	EPEC	O127:H6	+	+	-	Amp^r, Str^s
86-24PB	86-24[a]	EHEC	O157:H7	-	+	+	Amp^s, Str^r
Select96™[b]		Non-pathogenic *E. coli* (NPEC)		-	-	-	Amp^r, Str^s

[a] The parental strains were sensitive for ampicillin and streptomycin. [b], Select96™ competent cells (Promega).

TABLE 2. Inoculation groups in this study

Group number	Number of mice	First inoculation	Second inoculation	Group
1	11	EPEC	EHEC	EPEC/EHEC
2	6	NPEC	EHEC	NPEC/EHEC
3	12	PBS	EHEC	PBS/EHEC
4	7	EPEC	PBS	EPEC/PBS
5	7	PBS	PBS	PBS/PBS

669 **TABLE 3. Symptom score**

Score	Characterization	Clinical signs
0	No clinical signs	-
1	Mild clinical signs	Ruffled fur.
2	Moderate clinical signs	Ruffled fur plus, lethargy, hunched posture, decreased activity.
3	Severe clinical signs	Paresis, paralysis, tremor, shivers, ataxia, terminally ill mice, severe weight loss (>20%).

TABLE 4. Histopathological findings in the different inoculation groups[a]

Pathological finding	EPEC/EHEC			NPEC/EHEC			PBS/EHEC			EPEC/PBS		
	Mild	Moderate	Severe	Mild	Moderate	Severe	Mild	Moderate	Severe	Mild	Moderate	Severe
Intestines		**N=5**			**N=6**			**N=6**			**N=7**	
Inflammatory infiltrates	3	-	-	2	2	-	4	1	-	1	-	-
Lymph node hyperplasia	-	-	-	-	5	-	-	1	-	2	-	-
Crypt hyperplasia	-	-	-	1	1	-	-	-	-	-	-	-
Goblet cell depletion	1	-	-	2	-	-	3	-	1	-	-	-
Thickening of the submucosa	1	-	-	-	4	-	3	-	-	-	-	-
Shrunken interstitial space	-	-	-	-	1	-	1	-	-	-	-	-
Edema	-	-	-	3	-	-	-	-	-	-	-	-
Kidneys		**N=10**			**N=6**			**N=10**			**N=7**	
Tubular desquamation	-	-	-	4	-	-	4	1	1	-	-	-
Glomerular congestion	-	-	-	-	-	-	1	1	-	-	-	-
RBCs in the tubular lumen	-	-	-	-	-	-	-	1	-	-	-	-

Tissues were obtained at the end of the experiment (ten days after the second inoculation or when evident signs of disease were observed after the second inoculation. [a]; Tissues from mice in the PBS/PBS group (n = 7 intestines and 7 kidneys) did not exhibit any histopathological changes. RBCs: red blood cells.

FIGURE LEGENDS

FIG. 1. A schematic presentation of the infection protocol. Mice were initially inoculated with the EPEC or NPEC strains followed 20-22 days later by a second inoculation with the EHEC strain. Antibiotics in drinking water, ampicillin and streptomycin, were used to enhance colonization of each strain but also to eradicate the first strain before the second inoculation. [a], 24 h before inoculation. [b], 16 h before inoculation. [c], See Table 2 for the different inoculation groups. [d], Blood samples were also collected 6 days before the first inoculation.

FIG. 2. Symptom score in mice. A final symptom score was assigned to each mouse after the second inoculation as per Table 3. A median value was calculated for each group (horizontal line). The highest symptom score was found in mice from the NPEC/EHEC and PBS/EHEC groups and there was no significant difference between these two groups. Comparison of symptoms in mice from the EPEC/EHEC group with those in the EPEC/PBS and PBS/PBS groups did not show a significant difference. Significant differences between groups are depicted as *: $P < 0.01$.

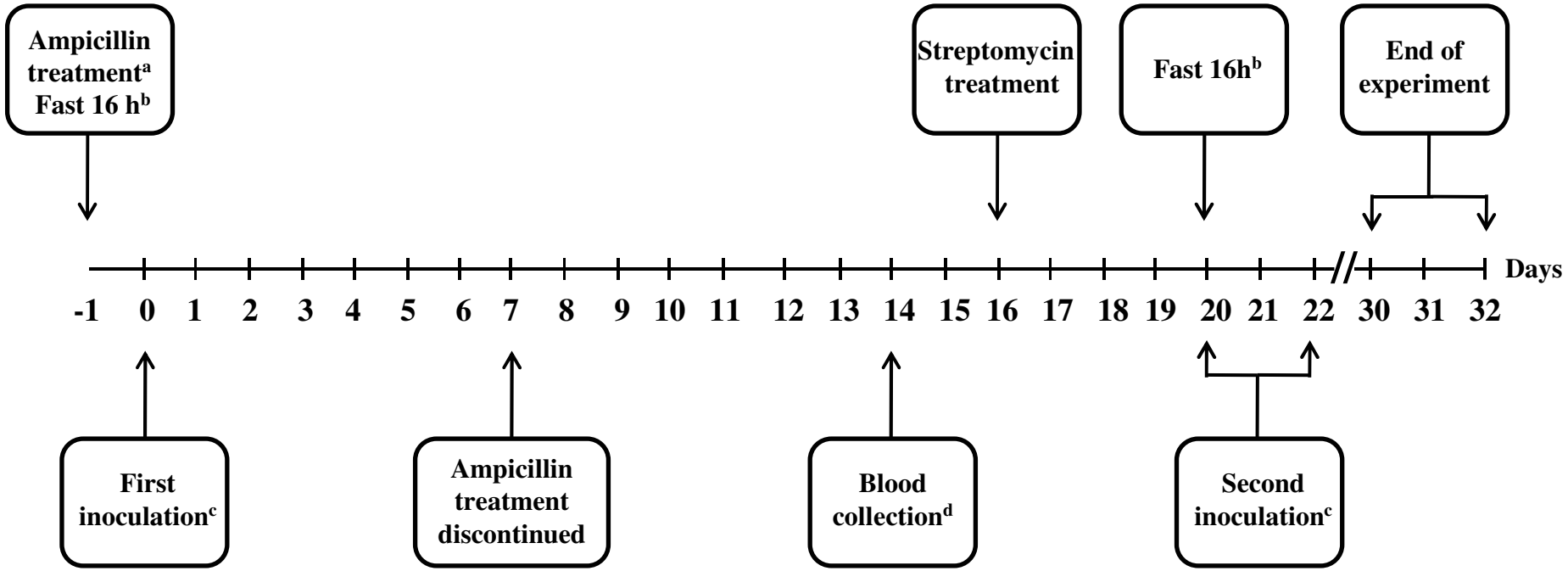
FIG. 3. Body weight changes during EHEC infection. Body weight changes were monitored during 11 days starting one day before the second inoculation. Over the course of infection, mice from the NPEC/EHEC and PBS/EHEC groups exhibited weight loss and towards the end of the observation period regained the initial body weight. Mice from the EPEC/EHEC, EPEC/PBS and PBS/PBS groups recovered initial body weight after the fasting period and exhibited an increase in body weight. Symbols at each time point represent the average value for each group. Significant differences ($P < 0.01$) in body weight changes were

found when comparing the following groups: EPEC/EHEC with NPEC/EHEC; EPEC/EHEC with PBS/EHEC; NPEC/EHEC with PBS/PBS; NPEC/EHEC with EPEC/PBS; PBS/EHEC with PBS/PBS; PBS/EHEC with EPEC/PBS; and $P < 0.05$ for PBS/PBS with EPEC/PBS. No significant differences ($P > 0.05$) in body weight changes were found when comparing the following groups: EPEC/EHEC with EPEC/PBS; EPEC/EHEC with PBS/PBS; NPEC/EHEC with PBS/EHEC.

FIG. 4. Cross-reactive antibody response to EHEC and EPEC secreted proteins in mouse sera. EHEC secreted proteins blotted with mouse serum from before (lane 1) and after (lane 2) EPEC inoculation showing development of antibodies reacting with EHEC intimin and EspB. EPEC secreted proteins blotted with mouse serum from before (lane 3) and after (lane 4) EPEC inoculation showing development of antibodies to EPEC intimin and EspB. EHEC secreted proteins reacted with anti-EspB at 37 kDa (lane 5) and anti-intimin at 94 kDa (lane 6). All lanes were run on the same gel.

FIG. 5. Intestinal pathology in mice after the second inoculation. Distal colons of mice from the EPEC/EHEC, NPEC/EHEC and PBS/EHEC groups (panels **A**, **B**, and **C,** respectively) showing inflammatory infiltrates and thickening of the submucosa (arrows). **D.** Distal colon from a mouse in the NPEC/EHEC group showing lymph node hyperplasia. **E.** Distal colon from a mouse in the PBS/EHEC group showing goblet cell depletion. See inset for magnification. **F.** Proximal colon from a mouse in the PBS/EHEC group showing shrunken interstitial space (arrow) and interstitial infiltrates (arrowhead). **G.** Distal colon from a mouse in the EPEC/PBS group showing normal histology. Magnification x400 **H.** Distal colon from a mouse in the PBS/PBS group showing normal histology. Magnification of all panels except panel G: x100.

FIG. 6. Renal pathology in mice after the second inoculation. Panels A and B are taken from mice in the NPEC/EHEC group. **A.** Renal cortex showing tubular desquamation (arrow). **B.** Dilated tubuli in the renal cortex are demonstrated (arrow)**.** Panels C, D, E and F were obtained from mice in the PBS/EHEC group. **C.** Renal cortex showing red blood cells in tubuli (arrow). Panels D, E and F show massive tubular desquamation as tubular structures are denuded of cells. Glomerular capillary congestion and occlusion are demonstrated (see arrow in panel D showing congestion and in panel F showing occlusion; see arrowhead in panel C showing occlusion). Renal specimens taken from mice in the EPEC/EHEC and PBS/PBS groups showed normal histology (panels G and H, respectively). Magnification ×400.



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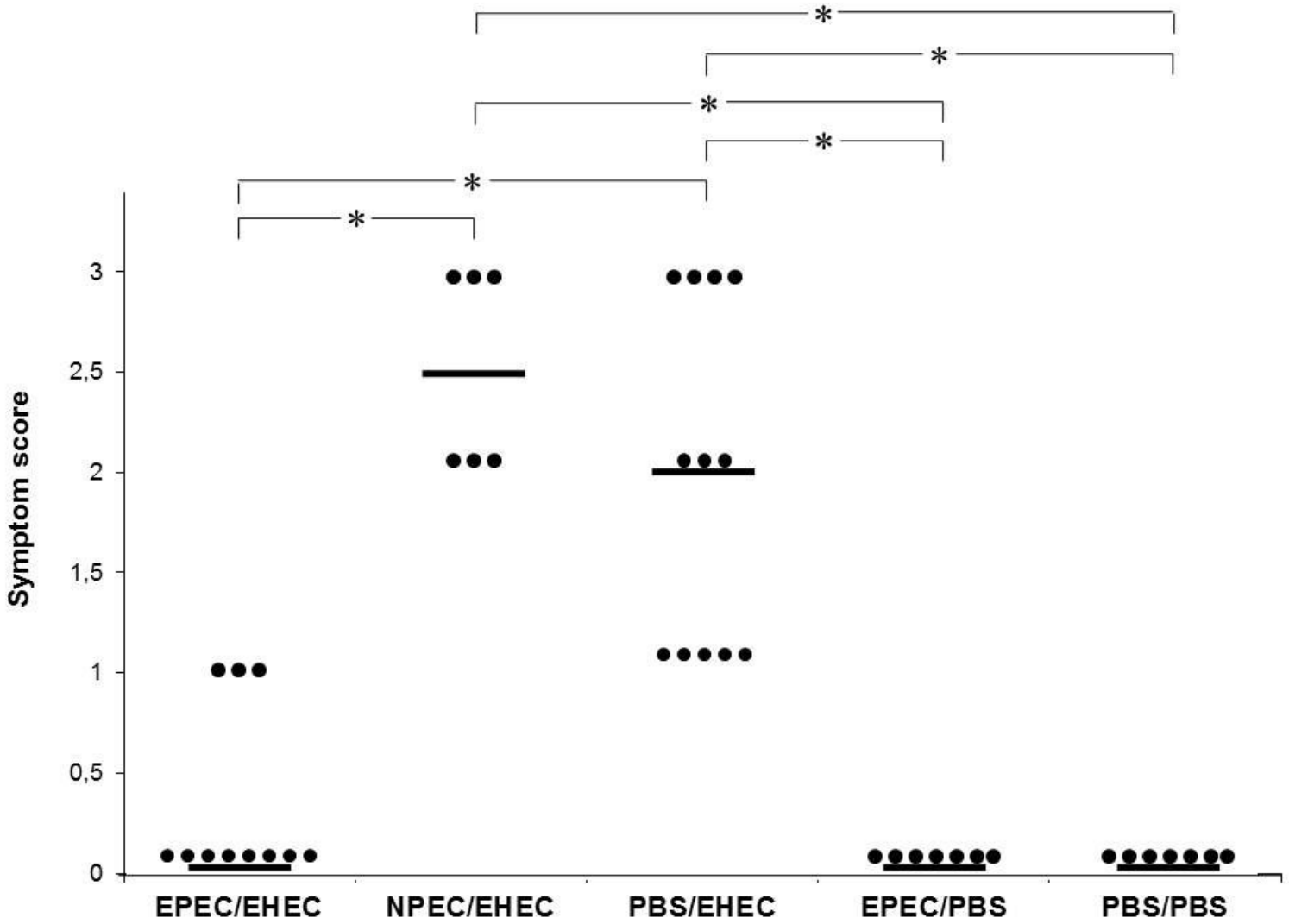

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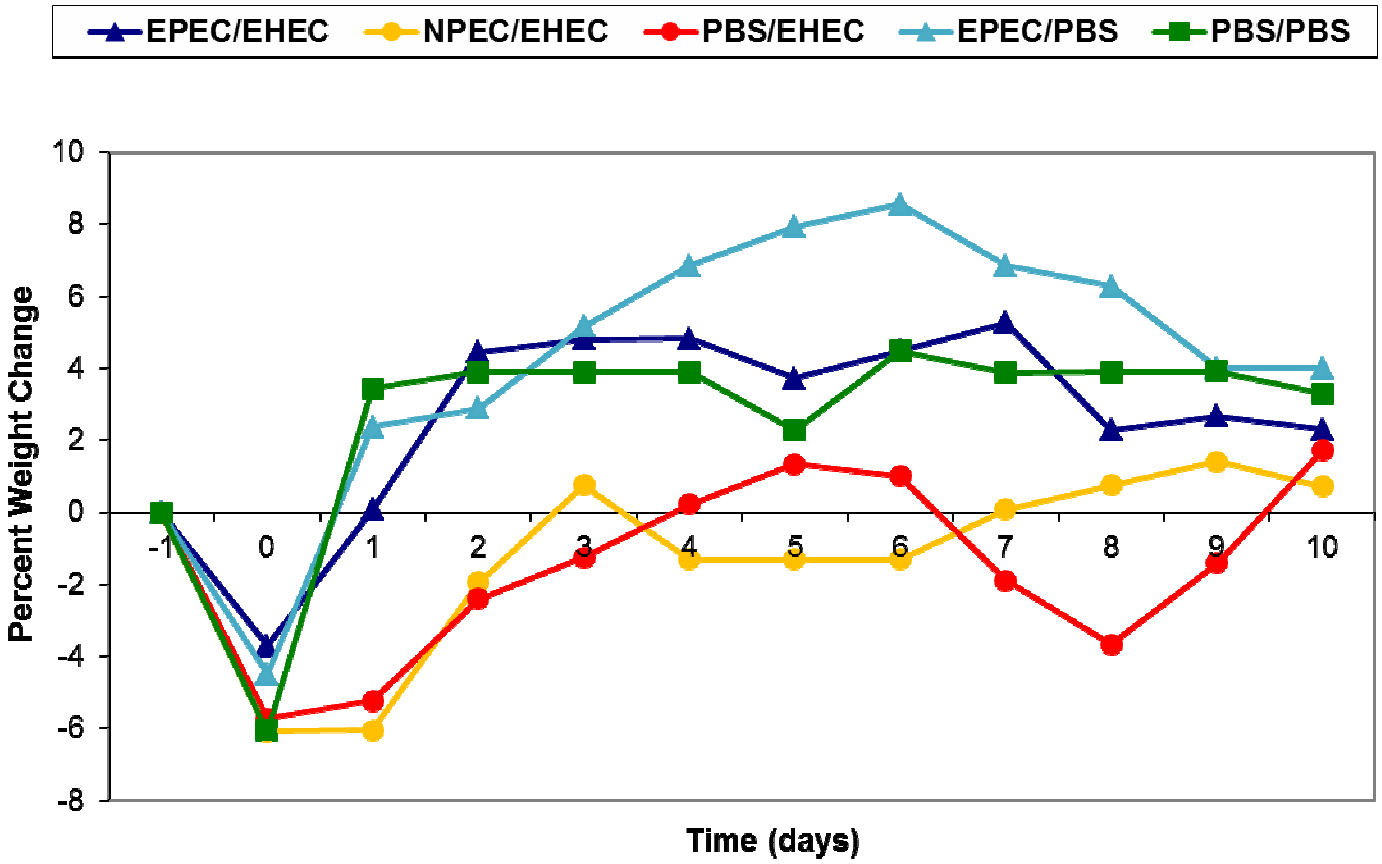

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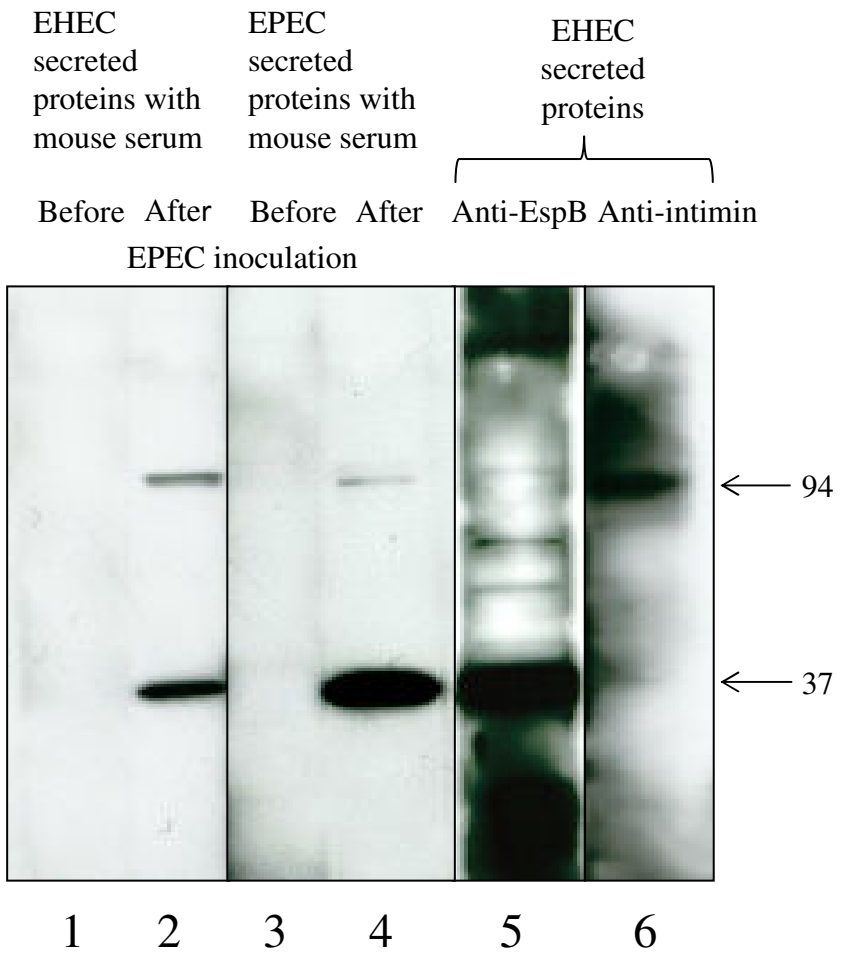

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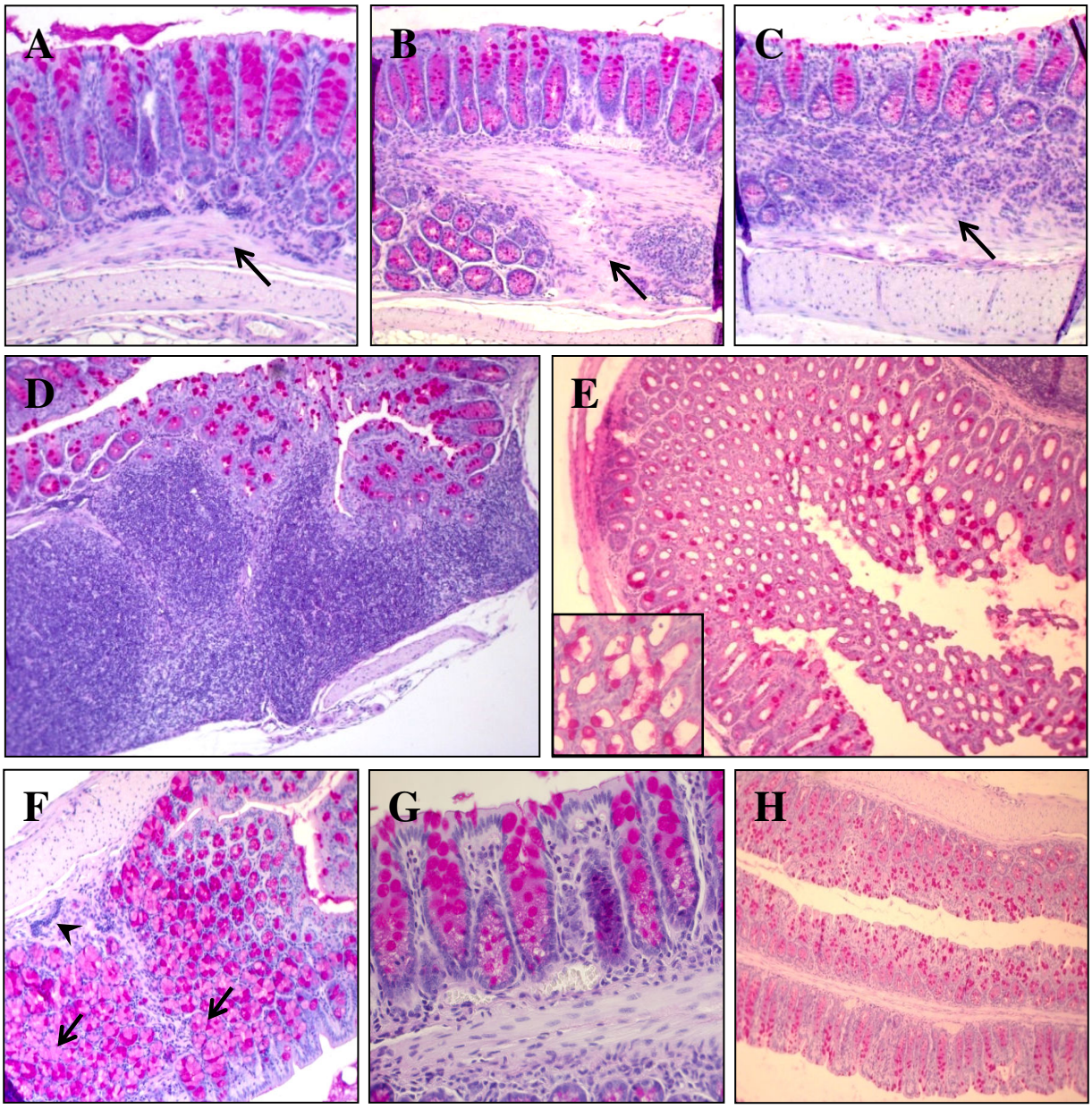

Figure 5. Intestinal pathology in mice after the second inoculation. Distal colons of mice from the EPEC/EHEC, NPEC/EHEC and PBS/EHEC groups (panels **A**, **B**, and **C,** respectively) showing inflammatory infiltrates and thickening of the submucosa (arrows). **D.** Distal colon from a mouse in the NPEC/EHEC group showing lymph node hyperplasia. **E.** Distal colon from a mouse in the PBS/EHEC group showing goblet cell depletion. See inset for magnification. **F.** Proximal colon from a mouse in the PBS/EHEC group showing shrunken interstitial space (arrow) and interstitial infiltrates (arrowhead). **G.** Distal colon from a mouse in the EPEC/PBS group showing normal histology. Magnification x400 **H.** Distal colon from a mouse in the PBS/PBS group showing normal histology. Magnification of all panels except panel G: x100.

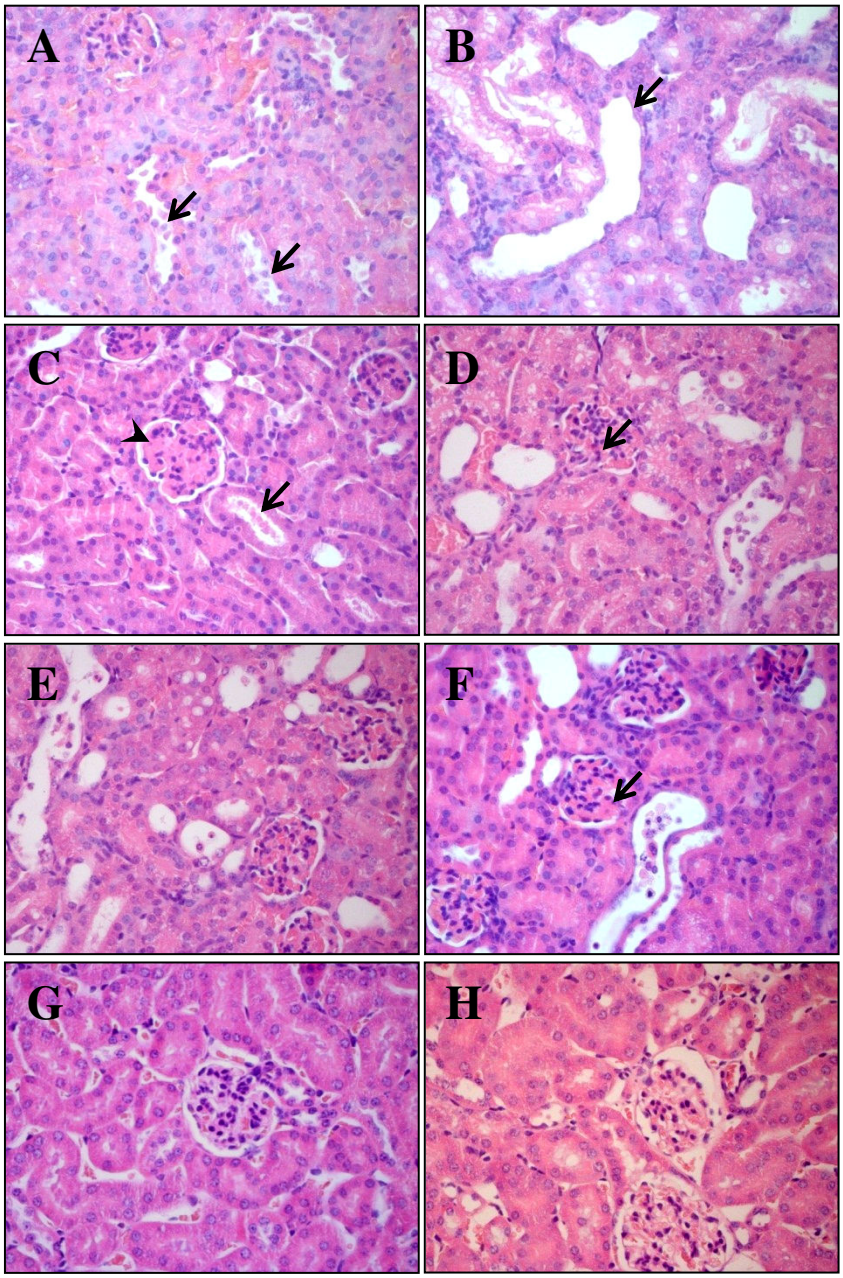

Figure 6. Renal pathology in mice after the second inoculation. Panels A and B are taken from mice in the NPEC/EHEC group. **A.** Renal cortex showing tubular desquamation (arrow). **B.** Dilated tubuli in the renal cortex are demonstrated (arrow)**.** Panels C, D, E and F were obtained from mice in the PBS/EHEC group. **C.** Renal cortex showing red blood cells in tubuli (arrow). Panels D, E and F show massive tubular desquamation as tubular structures are denuded of cells. Glomerular capillary congestion and occlusion are demonstrated (see arrow in panel D showing congestion and in panel F showing occlusion; see arrowhead in panel C showing occlusion). Renal specimens taken from mice in the EPEC/EHEC and PBS/PBS groups showed normal histology (panels G and H, respectively). Magnification ×400.