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The effects of oral and combined parenteral/oral immunization against an experimental *Escherichia coli* urinary tract infection in mice

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SUMMARY

A double oral immunization (PO/PO) with an outer membrane protein (OMP) from a human uropathogenic strain of *Escherichia coli*, resulted in the partial protection of mice infected per urethrally with the same strain. Complete protection was achieved by immunizing with OMP in Freund's complete adjuvant (FCA), intramuscularly (i.m.), followed by an oral boost (i.m./PO). The PO/PO protocol stimulated mainly local urinary antibody synthesis, particularly IgA, whilst the i.m./PO regimen resulted in the appearance of both serum and urine antibodies. A single dose of OMP, 6 days after infection, rendered the mice resistant to reinfection, in contrast to non-immunized mice, and led to a significant increase in urine, serum and bile IgA anti-OMP levels. Our results confirm previous reports that the urinary tract forms part of the common mucosal immune system and provides further evidence for immunological memory in mucosal immunity. These results also demonstrate that our OMP preparation is a highly effective immunizing antigen, and that such preparations may be suitable as oral vaccines against urinary tract infection in humans.

Keywords oral immunization urinary tract infections mucosal immunity outer membrane proteins

INTRODUCTION

Recent reviews on mucosal immunity (Tomasi *et al.*, 1980; Ganguly & Waldman, 1980) suggest that the genito-urinary tract forms part of the secretory immune system, based in part on the evidence of Mattsby, Manson & Kaijser (1978), who detected specific IgG and IgA antibodies in bronchial lavages of rats experimentally infected with *Escherichia coli* in the urinary bladder and they postulated that these specific IgA antibodies were a result of cell migration, also McDermott & Bienenstock (1979) demonstrated that labelled mesenteric lymph node blast cells migrate into intestinal, genital and respiratory tissues.

In immunoglobulin (Ig) synthesis studies it was observed that IgG was the principal urinary immunoglobulin in an experimental cystitis in rabbits with IgM and IgA playing minor roles (Hand *et al.*, 1970), however, more recently Kuriyama (1979) has shown that in humans IgA synthesis, in relation to other Igs, increases from kidney, bladder to urethra. These findings suggest that successful immunization against urinary tract infections should involve secretory immune system priming. Vaccination against bacterial infections using bacterial surface structures called pili, and

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outer membrane proteins (OMPs) has been reviewed recently by Marx (1980) and clearly the most important criteria for a successful vaccine are its immunogenicity and its ability to stimulate protective antibody production at the site of infection. We have assessed, therefore, the effectiveness of an *E. coli* 06 K13 OMP in preventing an experimental urinary tract infection in mice and in inducing serum and urine anti-OMP antibodies following parenteral/oral or purely oral immunization.

MATERIALS AND METHODS

Animals. Female, Swiss White mice 6–8 weeks old, were obtained from the ICI animal breeding unit.

Organisms. An overnight nutrient broth (Oxoid Ltd., Basingstoke, Hants) subculture of a known human uropathogenic strain of *E. coli* (06:K13:H1) was aliquoted (1 ml) and stored in liquid nitrogen. Aliquots were removed, gradually thawed and pipetted into 100 ml of nutrient broth which was then incubated overnight at 37°C. Bacterial homogeneity was assessed by subculturing 50 μl of the overnight broth cultures on a selection of solid media plates.

Experimental infection. An overnight 100 ml broth culture was centrifuged at 3,000 r/min for 20 min. The cell pellet was then resuspended in 0·9% NaCl to give approximately 1×10^8 colony forming units (cfu). Two hundred microlitres of the suspension was then introduced per urethrally, using a 1 ml syringe with a 23 gauge (1″ × 0·25″) blunt needle (Monoject Ltd., St Louis, Missouri, USA), into fluothane (ICI Ltd.) anaesthetized mice. The infection was allowed to proceed for 6 days. Five per cent glucose was added to the animal's drinking water both before and during the infection.

OMP preparation. This was prepared by the method of Poxton (1979), modified slightly. Bacterial cell pellets from 10 × 100 ml, 24 h broth cultures were agitated, pooled, washed twice in phosphate-buffered saline (PBS, 50 mM phosphate, 0·9% NaCl) and incubated in 10 ml PBS containing 10 mM ethylene diamine tetra-acetic acid (EDTA, Sigma Ltd., Poole, Dorset) for 30 min at 45°C. The cell suspension was then ultrasonicated (M.S.E. Instruments, Crawley, Sussex), for 60 s at half speed, then centrifuged at 6000 r/min for 20 min. The supernatant (10 ml), containing the outer membranes, was then mixed with an equal volume of 8% Triton X-100 (BDH Chemicals Ltd., Poole, Dorset) in PBS at room temperature for 30 min on a shaker followed by ultra centrifugation at 30,000 r/min for 15 min. Four per cent Triton X-100 in PBS (10 ml), containing 10 mM EDTA, was then added to the deposit and the incubation and extraction procedure, described above, repeated. The Triton X-100/EDTA insoluble material was resuspended and dialysed against PBS (2 l × 2) and the protein concentration, measured by the method of Lowry *et al.* (1951), adjusted to 10 mg/ml.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) revealed a single protein band of approximately 40,000 daltons. Amino acid analysis of acid/heat hydrolysed OMP samples, revealed that the protein contained a relatively low content of hydrophobic amino acids but was relatively rich in negatively charged polar amino acids, glutamic acid and aspartic acid. The calculated molecular weight was 42,925.

Mouse toxicity tests, by the intraperitoneal injection of OMP (1 mg/0·5 ml) in PBS, were negative for all OMP preparations showing that there was little or no contaminating lipopolysaccharide.

ELISA technique. This was performed by the method of Voller, Bidwell & Bartlett (1976). One hundred and fifty microlitres of the OMP (10 μg/ml) in 0·05M carbonate buffer (pH 9·6) was incubated in the wells of microtitre plates (Becton Dickinson, Wembley, Middlesex) at 4°C, overnight. The plates were then washed in PBS containing 0·05% Tween 20 (PBS/T). One hundred microlitres of each serum, diluted 1/100 in PBS/T; urine, diluted 1/2; bile, diluted 1/100 (eight per group) and doubly diluted serum standard, (all in duplicate) were incubated in each of three plates for 2 h at room temperature in a moist chamber.

The plates were washed, and 100 μl of either goat anti-mouse IgG, goat anti-mouse IgM or goat anti-mouse IgA antisera (all obtained from Meloy Labs., Springfield, New Jersey, USA) diluted

1/1,000 in PBS/T, was added. After incubating and washing, 100 μl of a rabbit anti-goat IgG serum conjugated to horseradish peroxidase (Nordic Ltd., Maidenhead, Berkshire), diluted 1/3,000 in PBS/T was added to the wells of each plate. After incubation and washing, 100 μl of freshly prepared substrate (orthophenylene diamine dihydrochloride, from Sigma Ltd., 0·4 mg/ml, and urea peroxide from BDH Ltd., 0·2 mg/ml, in distilled water) was added to the wells. The reaction was stopped after 30 min with 25 μl 25% H_2SO_4. The plates were read in a Multiskan plate reader (Flow Labs., Irvine, Scotland) at 492 nm and the results expressed as a percentage of the hyperimmune standard serum by interpolation from a graph constructed from the mean optical densities of the standard serum dilutions from 100% (1/25) down to 0·78% (1/3,200). The specificity of the three anti-mouse reagents was checked in an ELISA using mouse myeloma cell culture supernatants, diluted from 100 μg/ml to 0·78 μg/ml in pH 9·6 0·05M carbonate buffer, to sensitize the microtitre plates. The MOPC 31C (IgG), MOPC 104 E (IgM), and MOPC 315 (IgA) myelomas used were detected only when the correct antiserum was applied and no cross-reaction was observed.

Immunizations. i.m./PO regimen: mice were immunized intramuscularly (i.m.) 30 days prior to infection with OMP (1 mg/0·1 ml) in Freund's complete adjuvant (FCA), followed by an oral (PO) boost, 7 days before infection with OMP (0·5 mg/0·5 ml) in PBS.

PO/PO regimen: mice were immunized PO 30 days prior to infection with OMP (1 mg/0·5 ml) in PBS followed by an oral boost 7 days before infection with OMP (0·5 mg/0·5 ml) in PBS.

PO regimen prior to reinfection: mice were immunized PO 6 days after infection with OMP (0·5 mg/0·5 ml) in PBS.

Serum, urine and in the reinfection studies, bile samples were obtained from all groups of mice at the time of killing as were bladders for bacteriological and, in some experiments, histological/immunofluorescence studies.

ELISA serum standard; 20 mice were immunized i.m. with OMP, as above, and boosted 21 days later intraperitoneally (100 μg/0·5 ml) in PBS. The animals were killed, 4 days after the boost and the sera removed and pooled. Fifty microlitres of the serum, diluted 1/10 in PBS, was placed on acetone fixed smears of overnight broth cultures of *E. coli* (06:K2), (06:K13); *Proteus mirabilis*, *Salmonella typhimurium*, *Shigella flexneri* and *Citrobacter freundii* on glass slides. After 30 min at room temperature the slides were washed three times in PBS, and 50 μl of a rabbit anti-mouse

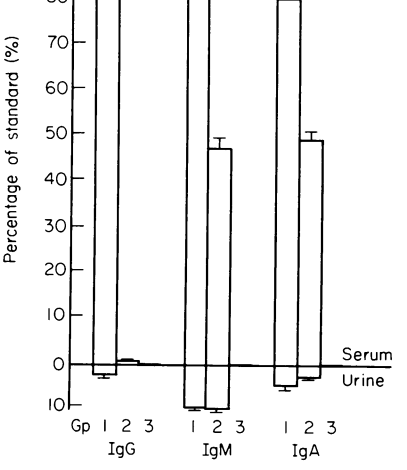

Fig. 1. Results represent the means of eight sera and urine samples tested per group. Error bars show one standard error from the means. Group one mice were immunized i.m./PO, then infected. Group 2 mice were infected only and group 3 mice were controls.

immunoglobulins labelled with fluorescein isothiocyanate (Dako Ig's Ltd., Copenhagen, Denmark), diluted 1/40 in PBS, was layered on to the smears and the slides incubated for 30 min at room temperature before washing in PBS three times and mounting in 50% PBS/glycerol. Only the 06 K13 *E. coli* organisms were stained.

Bacteriological culture of bladders. Bladders were homogenized in 1 ml PBS and diluted in PBS to give a range of neat to 10^{-4}, 50 μl of each dilution was then placed on to nutrient agar plates (Oxoid Ltd) and after overnight incubation at 37°C the number of colonies on each innoculation point was counted at the dilution which yielded discrete colonies. The number of organisms per bladder was then calculated.

Immunohistological study of bladders. In some experiments, bladders were processed by the Sainte-Marie technique (1962) using cold ethanol fixation, 4 μm serial sections were then mounted on glass slides, dewaxed and rehydrated in PBS and 100 μl of either goat anti-mouse IgG, goat anti-mouse IgM or goat anti-mouse IgA all labelled with fluorescein isothiocyanate (Meloy Labs.) and diluted 1/40 in PBS was incubated on the sections for 30 min at room temperature. The slides were then washed in PBS three times, mounted in 50% PBS/glycerol and the number of IgG, IgA and IgM containing plasma cells counted, using a Leitz Orthomat fluorescence microscope. Haematoxylin and eosin stained sections were also prepared in one experiment.

Statistical analysis. A comparison of anti-OMP levels detected, following infection and the various immunization regimens, was made using the Students' *t*-test ($P < 0{\cdot}05$ was considered significant). The chi-squared test was used to compare the number of organisms per bladder in non-immunized and immunized infected groups of mice and all immunizations resulted in highly significant ($P < 0{\cdot}002$) decreases.

RESULTS

Our approach was to first establish a reproducibly protective regimen, and then to compare it with oral immunization.

The effects of combined parenteral/oral immunization on infection

It can be seen from Fig. 1 that i.m./PO immunized mice (group 1), 6 days after infection, showed very high serum levels and low, but measurable urine levels of all three classes of anti-OMP antibodies. Non-immunized mice (group 2), in contrast, had significantly lower serum levels of IgG, IgM and IgA anti-OMP ($P < 0{\cdot}001$). Urine IgA ($P < 0{\cdot}05$), IgG (not detected) but not IgM anti-OMP levels were also significantly lower. Anti-OMP antibodies were not detected in either the serum or urine of control mice (group 3). Infected bladders normally yielded > 100,000 (1×10^5) organisms, i.m./PO immunized mice however, were completely protected with residual organisms being isolated from only seven of 20 bladders in three experiments. Table 1 shows that both the infection and the protective effects of the i.m./PO regimen are reproducible. Stained sections of

Table 1. Reproducibility of infection and protection afforded by i.m./PO immunization

		Organisms per bladder ± s.d.			
Group	Protocol	Expt. 1a	2b	3c	Mean
1	i.m./PO infected	$3 \pm 5{\cdot}2$	$2 \pm 3{\cdot}0$	$6 \pm 8{\cdot}4$	$3{\cdot}6 \pm 3{\cdot}2$
2	infected only	$5{\cdot}5 \pm 1{\cdot}8 \times 10^5$	$2{\cdot}0 \pm 1{\cdot}2 \times 10^5$	$3{\cdot}2 \pm 1{\cdot}9 \times 10^5$	$3{\cdot}5 \pm 1{\cdot}4 \times 10^5$
3	control	0	0	0	0

Results represent the mean number of viable *E. coli* isolated per bladder from three separate experiments ± 1 standard deviation (s.d.). a = four bladders, b = eight bladders, c = eight bladders per experiment.

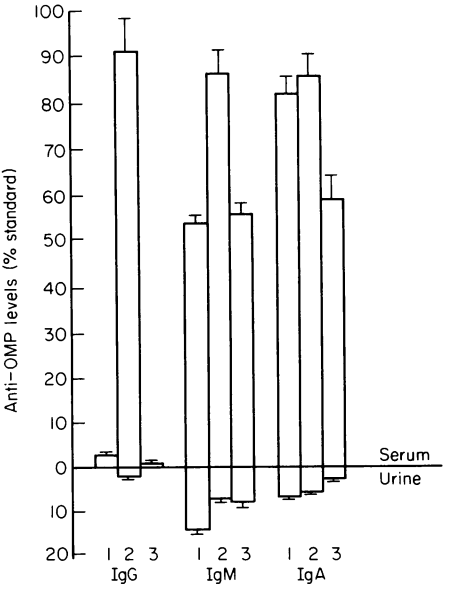

Fig. 2. Results represent the means of eight sera and urine samples tested per group. Error bars show one standard error from the means. Group one mice were immunized PO/PO, group 2 mice were immunized i.m./PO and group 3 mice non-immunized. All groups of mice were infected.

bladders and kidneys from immunized mice revealed a normal histological appearance with no evidence of inflammation as did those from the control group.

Bladders from non-immunized mice, in contrast, demonstrated varying degrees of inflammation. All bladders showed interstitial (predominantly monocytic) infiltration, mucosal hypertrophy, oedema, and thickening of blood vessel walls. The lumen of the bladders contained large aggregates of mainly polymorphonuclear (PMN) cells with many organisms present. Some bladders also showed acute abscess formation with necrosis, extending deep into the submucosa. Two of the four kidneys studied were also affected and showed pathological changes consistent with acute pyelonephritis.

Table 2. Recoverable, viable *E. coli* and plasma cell numbers in bladders from immunized and non-immunized infected mice

Group	Protocol	Organisms per bladder ± s.d.	Plasma cells per bladder ± s.d.			
			IgG	IgM	IgA	Total
1	PO/PO infected	$1{\cdot}0 \pm 0{\cdot}1 \times 10^3$	$10{\cdot}1 \pm 1{\cdot}2$	$22{\cdot}5 \pm 2{\cdot}3$	$51{\cdot}2 \pm 4{\cdot}3$	$83{\cdot}8 \pm 7{\cdot}8$
2	i.m./PO infected	$2 \pm 3{\cdot}0$	0 ± 0	$2{\cdot}2 \pm 1{\cdot}3$	$9{\cdot}5 \pm 2{\cdot}1$	$11{\cdot}7 \pm 3{\cdot}4$
3	infected only	$2{\cdot}0 \pm 1{\cdot}2 \times 10^5$	$1{\cdot}1 \pm 1{\cdot}2$	$18{\cdot}8 \pm 1{\cdot}8$	$22{\cdot}1 \pm 3{\cdot}0$	$42{\cdot}0 \pm 6{\cdot}0$
Normal values	—	—	0 ± 0	$2{\cdot}5 \pm 1{\cdot}5$	$7{\cdot}4 \pm 1{\cdot}8$	$9{\cdot}9 \pm 3{\cdot}3$

Results represent the mean number of viable *E. coli* isolated per bladder (mean of eight) ± one standard deviation (s.d.) and the mean number of plasma cells per bladder (mean of four) ± 1 s.d.

A comparison between i.m./PO and PO/PO immunization

Having established that the i.m./PO regimen was protective we compared this to a purely oral regimen without using an adjuvant. Fig. 2 shows that a double oral (PO/PO) regimen (group 1) resulted in a significant increase ($P < 0{\cdot}01$) in serum IgA, urine IgA and IgM anti-OMP levels above those seen in non-immunized mice (group 3).

The i.m./PO regimen (group 2) led to significantly higher serum IgG, IgM ($P < 0{\cdot}001$) but not IgA antibodies above those seen in PO/PO immunized mice. Urine IgA antibody levels were also not significantly different between the two groups however the PO/PO immunized mice showed higher IgM ($P < 0{\cdot}005$) but lower IgG (non-detected) urine anti-OMP levels.

The effects of immunization on the number of recoverable *E. coli* and IgA, IgG and IgM plasma cells per bladder are shown in Table 2. The PO/PP regimen resulted in a substantial two log reduction in the number of organisms per bladder but was not as effective as the i.m./PO regimen. The PO/PO regimen however, stimulated a substantial increase in the number of plasma cells per bladder.

The effects of oral immunization prior to reinfection

It can be seen from Fig. 3 that when 6 day infected mice (group 1) were immunized orally with OMP and reinfected (group 2) it resulted in a significant increase in urine, bile, and serum IgA anti-OMP levels ($P < 0{\cdot}05$) compared to non-immunized reinfected mice (group 3), both prior to, and after reinfection.

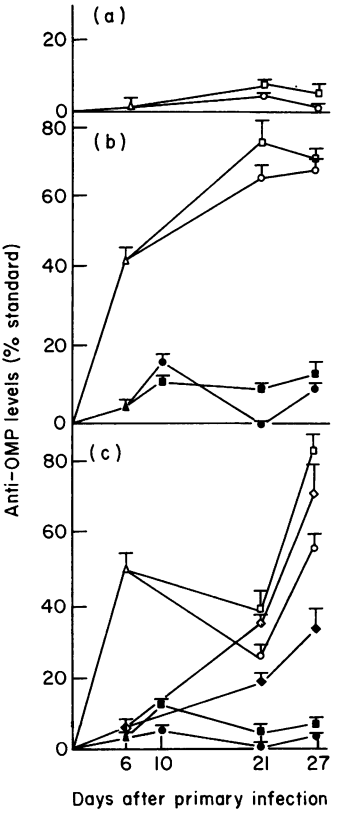

Fig. 3. Results represent the means ± 1 s.d. of eight sera (group 1 = △; group 2 = □; group 3 = ○), urine (group 1 = ▲; group 2 = ■; group 3 = ●) and bile (group 1 = ♦; group 2 = ◊; group 3 = ♦). All groups were infected on day 0. Group 1 mice were killed on day 6. Group 2 mice were immunized orally on day 6, reinfected on day 21 and killed on day 27. Group 3 mice were treated as for group 2 but were not immunized on day 6. (a) IgG; (b) IgM; (c) IgA.

Table 3. Recoverable *E. coli* from the bladders of primary and reinfected mice

Group	Protocol	Organisms per bladder ± s.d.
1	Infected	$3{\cdot}2 \pm 1{\cdot}9 \times 10^5$
2	Infected/PO/infected	0 ± 0
3	Infected/infected	$1{\cdot}2 \pm 0{\cdot}2 \times 10^3$

Results represent the mean number of viable *E. coli* isolated per bladder (mean of eight) ± one standard deviation (s.d.).

IgG anti-OMP was not detected in either the urine or bile of group 1, 2 or 3 mice but was detected in very low levels in sera from all three groups.

IgM antibody levels were significantly elevated in the urine ($P < 0{\cdot}005$), but not serum, from immunized compared to non-immunized reinfected mice prior to, but not after reinfection. IgM anti-OMP was not detected in any of the bile samples tested. It can also be seen that serum and bile IgA, in both the immunized and non-immunized groups, increased dramatically upon reinfection.

Table 3 shows that the primary infected mice developed a certain degree of resistance to the organism resulting in a 2 log reduction in the number of recoverable *E. coli* per bladder, however, oral immunization after the primary infection resulted in complete protection.

DISCUSSION

Our OMP resembles the matrix protein Ib described by DiRienzo, Nakamura & Inouye (1978). Other *E. coli* antigens which could be used as vaccines have various disadvantages. The enterobacterial common antigen (ECA) gives rise to a predominantly IgM response in rabbits immunized with whole *E. coli* (Rinno, Golecki & Mayer, 1980), which is probably not protective, and Svanborg-Eden & Svennerholm (1978) described how *E. coli* lipopolysaccharide, but not capsular polysaccharide, antibodies can prevent adhesion of *E. coli* to human uroepithelial cells *in vitro*. Attempts to prevent *in vivo* bacterial adhesion, mediated in the case of uropathogenic *E. coli* by pili (Svanborg-Eden & Hanson, 1978), have been made in humans using isolated pili vaccines against *Neisseria gonorrhoea* and enteropathogenic *E. coli* with promising results (Marx 1980), however epithelial cell adherence by non-piliated strains of *E. coli* has been described (Van den Bosch *et al.*, 1980). Our immunization studies, using the OMP, confirm the findings of Pierce, Sack & Sircar (1977) who showed that parenteral priming followed by an oral boost induces both systemic and local antibody synthesis of a highly protective nature. The fact that the i.m./PO regimen was more protective than the PO/PO schedule was probably due to the presence of serum derived antibodies in addition to locally synthesized IgM and IgA antibodies, at the time of infection, thus preventing adhesion more effectively. The PO/PO regimen however led to a larger increase in bladder plasma cell numbers, particularly IgA plasma cells, and higher urine IgA and IgM antibody levels than the i.m./PO regimen.

Oral immunization 6 days after infection, however, conferred complete protection against reinfection probably by virtue of the significantly enhanced IgA antibody levels in the bile, serum, and more importantly the urine, of these animals prior to reinfection. The high serum levels of IgA anti-OMP found after oral immunization may also be important for protection since it is known that IgA antibodies can be selectively transported into mucosal secretions from the serum (Virella, Montgomery & Lemaitre-Coelho, 1978) and vice versa (Nagura, Nakane & Brown, 1979). The finding by Jackson *et al.* (1978) and by Orlans *et al.* (1978) that polymeric IgA is transported from blood to bile, via the liver, by virtue of secretory component on hepatocytes and biliary duct epithelial cells in the rat prompted us to study the effects of oral immunization on biliary anti-OMP levels. Following primary and then reinfection, biliary IgA anti-OMP levels reflected those seen in

the serum and also showed an anamnestic response which was increased two-fold by oral immunization after the primary infection. These results confirm those of a recent study in rats (Andrew & Hall, 1982) which demonstrated that immunological memory in secretory immunity does occur, is mediated by circulating lymphocytes and can be monitored by measuring biliary antibody levels.

From our studies, therefore, oral immunization with outer membrane proteins would appear to be effective in treating urinary tract infections. It is also evident that the use of an oral adjuvant such as muramyl dipeptide (Chedid *et al.*, 1976) would be of great value in potentiating specific local immune responses during treatment or prophylaxis, by oral immunization.

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