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In Vivo Properties of a Cloned K88 Adherence Antigen Determinant

GORDON DOUGAN,[1]* RICHARD SELLWOOD,[2] DUNCAN MASKELL,[1] KATHY SWEENEY,[1] FOO Y. LIEW,[1] JULIAN BEESLEY,[1] AND CARLOS HORMAECHE[3]

Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS,[1] Institute for Research on Animal Diseases, Compton Newbury, Berkshire, CR3 3R5,[2] and Microbiology Division, Department of Pathology, University of Cambridge, Cambridge, CB2 1QP,[3] United Kingdom

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An *Escherichia coli* strain of serotype O9:K36:H19 harboring the K88 recombinant plasmid pMK005 was able to efficiently colonize the small bowel of young piglets after oral infection. The strain expressed K88 antigen in vivo, and bacteria were detected in close association with the surface of the intestinal villi. Mice infected orally or intravenously with attenuated *Salmonella typhimurium* SL3261 harboring pMK005 were well protected against subsequent challenge with the highly virulent *S. typhimurium* SL1344. Anti-K88 antibodies were detected in the serum of mice immunized with SL3261(pMK005).

The K88 fimbrial antigen is an adhesin associated with a high percentage of enterotoxigenic *Escherichia coli* strains isolated from neonatal piglets with diarrhea (3, 4). In a series of experiments, Smith and co-workers demonstrated that the acquisition of plasmids encoding the genetic determinant for K88 fimbriae by a nonpathogenic *E. coli* O9:K36:H19 strain, referred to here as WBL1212, enabled the strain to adhere to enterocytes lining the small bowel of young piglets, thus allowing the strain to colonize this region of the intestine (16, 17). The K88 determinant is normally located on plasmids at least 70 kilobase pairs in size (15). It is not known whether these plasmids encode factors in addition to the K88 determinant which are required to promote the efficient colonization of the small bowel of piglets or whether the K88 determinant itself is all that is required. This can be assessed by using chimeric plasmids containing DNA sequences encoding only genes involved in K88 expression and vector DNA sequences. The results of such experiments are reported in this paper. Plasmid pMK005 is a pBR322-based recombinant plasmid containing a DNA sequence of 4.3 kilobase pairs encoding five cistrons required for the expression of the K88 fimbriae on the surface of *E. coli* cells (2, 7, 8, 14). These cistrons account for most of the cloned DNA sequences on pMK005 (8, 11). pMK005 was introduced into a nalidixic acid-resistant derivative of the nonpathogenic strain WBL1212 used by Smith and co-workers. WBL1212(pMK005) expressed levels of K88 fimbriae similar to those observed with *E. coli* K-12 strains harboring pMK005. WBL1212 and WBL1212(pMK005) bacterial cells were then assayed for their ability to bind in vitro to isolated porcine small bowel brush border cells in the presence of 0.1 M D-mannose as described previously (7). Only WBL1212(pMK005) bacterial cells were able to bind to the brush border enterocytes. These two bacterial strains were then used to orally infect neonatal piglets, and the levels of colonization of the small bowel by the strains were enumerated.

Oral infection of piglets with strains WBL1212 and WBL1212(pMK005). Genetically susceptible piglets (5) born to susceptible sows were allowed to suck the dam for 34 to 48 h and were then transferred either to open pens within a security unit or to plastic gnotobiotic isolators. The piglets were fed on Carnation milk for 48 h and were infected with 10^8 CFU of either WBL1212 or WBL1212(pMK005). Piglets were maintained on Carnation milk ad libitum throughout the experiment. Serial 10-fold dilutions of contents of the stomach, the upper, middle, and lower small intestines, and the large intestine were made in phosphate-buffered saline as described previously (17; R. Sellwood, Ph.D. thesis, Reading University, Reading, England, 1983), and the mean number of organisms per milliliter was estimated by the method of Miles and Misra (10) with sheep blood agar plates containing no antibiotics, 10 μg of nalidixic acid per ml, 50 μg of ampicillin per ml, or both drugs. Samples of tissue were also taken and placed in liquid nitrogen for fluorescent antibody tests.

Table 1 shows the results of a typical infection experiment involving six piglets which were orally infected with 10^8 CFU of strain WBL1212(pMK005) and two piglets which were infected with the parental strain. The two piglets infected with WBL1212 and two of those infected with WBL1212(pMK005) were necropsied at 18 h postinfection. The remaining four piglets were necropsied at 36 h postinfection. Numbers were determined as described by Smith and Linggood (17). Pigs were killed by intracardiac injection of pentobarbitone sodium. The abdomen was opened quickly, and ligatures were applied to close off the duodenum close to the stomach, to the ileo-cecal junction, and to 1-m sections of the upper, middle, and lower intestines. The whole alimentary tract was removed and unravelled. The contents of the stomach, the ligatured regions of the upper, middle, and lower intestines, and the cecum were removed, and their content of WBL1212 or WBL1212(pMK005) was estimated by the method of Miles and Misra (10). At 18 h postinfection the number of viable *E. coli* found in the small bowel of the piglets challenged with WBL1212(pMK005) was higher than the number detected in the intestines of animals challenged with WBL1212. In the four piglets necropsied at 36 h postinfection, the number of WBL1212 (pMK005) had risen to very high levels per milliliter of intestinal contents. Interestingly, these four piglets began to develop clinical symptoms of diarrhea at about 24 h postinfection and were dehydrated at 36 h postinfection. Wild-type adhesive and enterotoxigenic *E. coli* cells were not isolated from the intestinal contents of these piglets, indicating that a concurrent naturally acquired infection was not the cause of diarrhea. Control piglets from the same litter and housed in an adjacent pen were unaffected.

* Corresponding author.

TABLE 1. Colonization of the intestinal tract of susceptible piglets infected with *E. coli* strains WBL1212(pMK005) and WBL1212

Strain	Time after infection (h)	Piglet	Log_{10} CFU/ml[a]				
			Stomach	Small intestine			Cecum
				Upper	Middle	Lower	
WBL1212(pMK005)	18	a	5.05	6.97	6.48	7.38	8.53
		b	4.00	4.60	4.11	5.56	ND[b]
	36	c	7.16	7.66	7.78	8.47	9.22
		d	6.65	6.95	8.58	9.16	9.43
		e	8.26	7.84	7.40	8.10	8.68
		f	6.89	8.43	8.48	6.97	8.76
WBL1212	18	g	<2.00	3.36	4.74	4.58	8.38
		h	<2.00	2.85	4.87	3.81	8.81

[a] Numbers of nalidixic acid-resistant *E. coli* detected at particular regions of the intestines of individual piglets. Individual piglets infected with WBL1212(pMK005) are listed as a through f. Individual piglets infected with WBL1212 are listed as g and h.
[b] ND, Not determined.

In a further experiment designed to assess the duration of colonization by WBL1212(pMK005), eight piglets housed in pairs in gnotobiotic isolators were infected with 10^8 CFU of WBL1212(pMK005). Pairs of piglets were necropsied at 18, 42, 66, and 162 h postinfection, and the numbers of WBL1212(pMK005) bacteria in the intestinal contents were counted. WBL1212(pMK005) could be detected in all regions of the bowel throughout the experiment. The mean

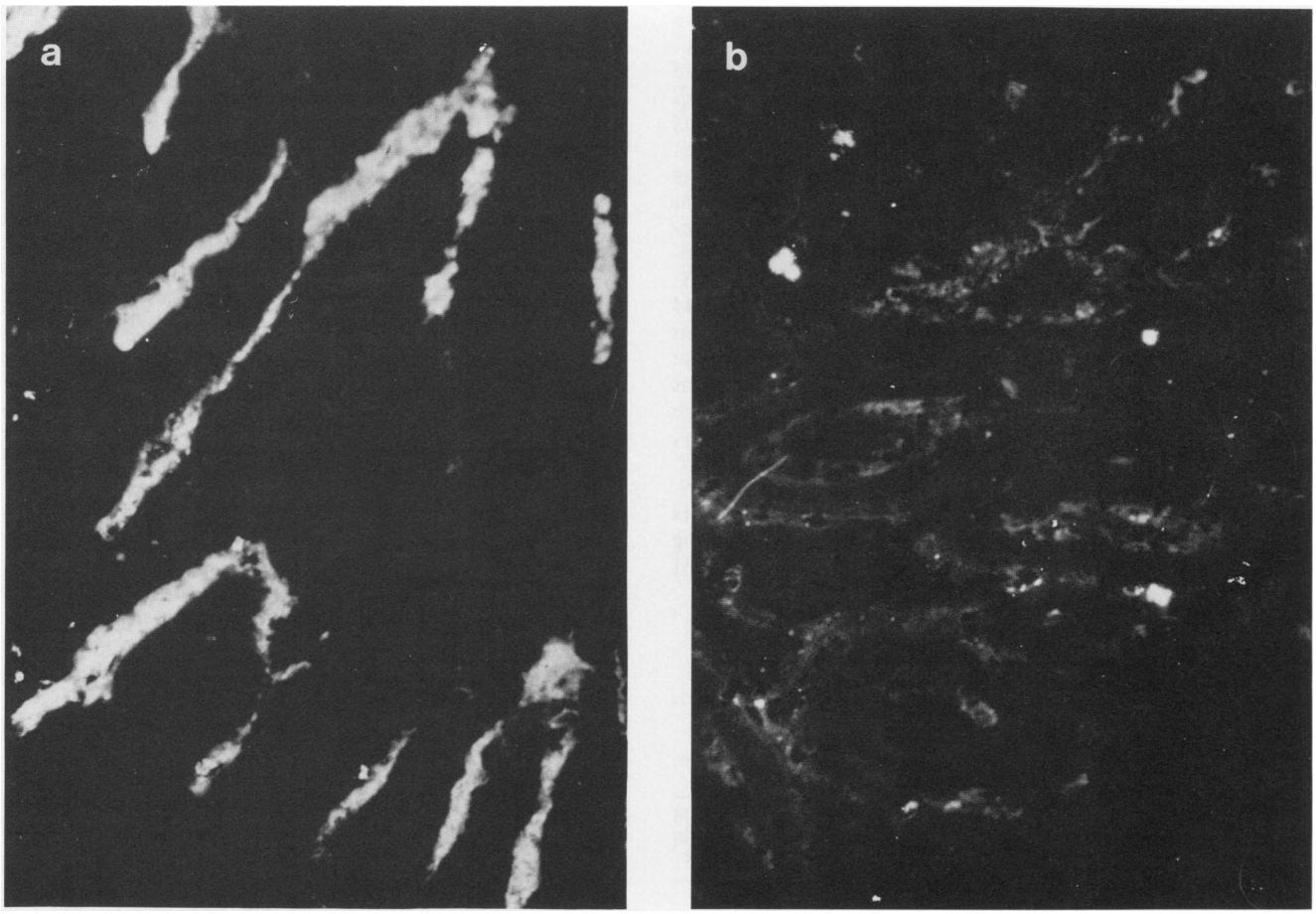

FIG. 1. Immunofluorescence staining of bacteria. (a) In vivo production of K88 antigen and adherence of bacteria to the surface of villi in the ileum of a susceptible piglet orally infected with *E. coli* WBL1212(pMK005). (b) Nonadherence of bacteria to the intestinal villi in the ileum of a susceptible piglet orally infected with WBL1212.

numbers of viable WBL1212(pMK005) detected in the intestinal contents of the two piglets necropsied at 162 h postinfection were 3.56 $\log_{10}$ CFU/ml in upper small bowel, 5.32 $\log_{10}$ CFU/ml in middle small bowel, and 8.56 $\log_{10}$ CFU/ml in lower small bowel. These counts are also representative of those seen at earlier times. Interestingly, none of the piglets in this experiment exhibited obvious symptoms of diarrhea.

Tissue sections were prepared from the intestinal mucosa of infected piglets. Sections of frozen tissue were incubated with rabbit anti-K88 antibodies followed by anti-rabbit antibody conjugated to fluorescein isothiocyanate (Sellwood, Ph.D. thesis). No K88 antigen was detectable by immunofluorescence in the tissues of piglets orally infected with WBL1212. In contrast, all of the tissues prepared from sections of the intestine containing high levels of WBL1212 (pMK005) fluoresced strongly, indicating that the K88 antigen was expressed in vivo and that the organisms were adhering to the epithelial surfaces of the gut (Fig. 1). Taken together, the above results suggest that pMK005 encodes sufficient genetic information to promote attachment of WBL1212 to the small bowel of young piglets and that the K88 determinant is expressed in vivo. Thus, the cloned K88 determinant may be of value for constructing candidate live vaccine strains.

Expression of K88 antigen in *Salmonella typhimurium*. Young piglets can be passively protected against diarrheal disease caused by K88-producing enterotoxigenic *E. coli* if they are allowed to suckle colostrum from sows orally or parenterally immunized with K88 preparations (12). Live attenuated bacterial strains may be useful vectors for delivering antigens to the immune system to stimulate effective active immunity (9). The fact that nonpathogenic *E. coli* strains harboring the K88 antigen induce diarrheal symptoms in young piglets could preclude their use as live oral vaccines (17). The cloned K88 determinant, however, may be used as a model system to test whether live attenuated *Salmonella* strains can be used as carriers to deliver potentially protective antigens to the immune system. The mouse is a convenient host in which to test the immunogenic properties of *S. typhimurium* strains expressing the K88 antigen. SL1344 *his*$^-$ is a mouse-virulent *S. typhimurium* strain. SL3261 is an avirulent isogenic derivative of SL1344, attenuated because it contains a deletion in the *aroA* gene, making it dependent on aromatic metabolites for growth (6). Both strains were kindly provided by B. A. D. Stocker of Stanford University, Stanford, Calif. Plasmid pMK005 was introduced into *S. typhimurium* SL3261, and all of the ampicillin-resistant *S. typhimurium* SL3261 cells tested harbored pMK005 in its normal extrachromosomal form, as assayed by running plasmid DNA preparations on agarose gels. Unlike SL3261 cells, SL3261(pMK005) cells agglutinated strongly in anti-K88 sera, bound to isolated porcine intestinal brush border cells in the presence of 0.1 M D-mannose, and expressed K88 fimbriae on their surfaces, as detected by immuno-gold electron microscopy (results not shown).

TABLE 2. SL1344 challenge of BALB/c mice previously immunized with SL3261(pMK005)

Route of immunization[a]	$\log_{10}$ LD_{50} for the following route of challenge[b]	
	Oral	i.v.
Oral	>10.36	5.48
i.v.	>10.36	>6.36
None	<6.66	<1.5

[a] All mice survived the immunizing infections both oral and i.v.

[b] Values are expressed as the log of the 50% lethal dose ($\log_{10}$ LD_{50}). All immunized mice survived a fully virulent challenge except for those mice immunized orally and challenged with 7.2×10^5 CFU i.v., of which four of five died. All nonimmunized mice died.

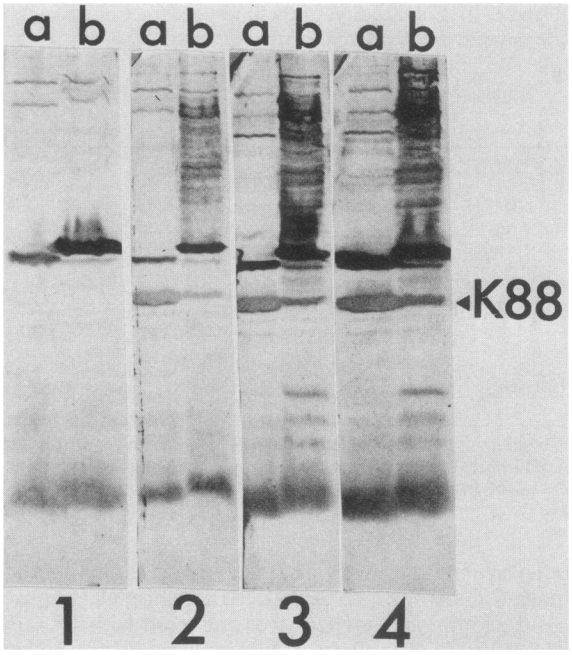

FIG. 2. Western blotting analysis of serum obtained from mice immunized with SL3261 or SL3261(pMK005). Cells of *E. coli* K-12 C600(pMK005) or SL3261(pMK005) were scraped from the surface of L agar plates (8) and suspended in sodium dodecyl sulfate-polyacrylamide gel final sample buffer (8, 19), and the samples were boiled for 2 min. Portions (20 μl) of these preparations were then electrophoresed on a 12.5% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel, and the separated polypeptides were transferred to nitrocellulose filters by the method of Towbin et al. (18). Western blotting was performed by using serum samples prepared from immunized mice at a final dilution of 1:500 as described previously (19). Alkaline phosphatase-labeled goat anti-mouse immunoglobulins (Sigma, Scunthorpe, United Kingdom) were used to detect bound mouse immunoglobulins exactly as described by Blake et al. (1). Lanes represent *E. coli* K-12 C600(pMK005) lysates (lanes a) and SL3261(pMK005) samples (lanes b). K88 indicates the position of the K88 fimbrial subunit polypeptide. This can be located precisely on gels and filters, as it can be visualized as a major polypeptide after staining with nonselective protein stains such as Coomassie blue. It is also absent from lysates of cells which do not harbor pMK005. Mouse serum used was from mice that were i.v. immunized with SL3261 (track 1), i.v. immunized with SL3261(pMK005) (track 2), i.v. immunized with SL3261(pMK005) and boosted i.v. with SL3261(pMK005) (track 3), or immunized orally with SL3261(pMK005) and boosted i.v. with SL3261(pMK005) (track 4).

Strains SL3261 and SL3261(pMK005) were avirulent in BALB/c male mice with 50% lethal doses in excess of 10^{10} organisms after oral infection or 10^7 after intravenous (i.v.) infection. Thus, acquisition of pMK005 did not affect the avirulence of SL3261 for mice. To test the immunogenic properties of SL3261(pMK005), 40 BALB/c male mice 6 to 8 weeks old were infected orally with 3.7×10^9 CFU of SL3261(pMK005) by using a gavage tube, and 40 mice were infected i.v. with 3.7×10^5 CFU of the same strain. There was no mortality after these infections. Immunized mice and age- and sex-matched unimmunized controls were chal-

lenged orally or i.v. with the virulent *S. typhimurium* SL1344 16 days after immunization. Each 50% lethal dose covered a range of four dilutions, with five mice challenged per dilution. The oral dilution range was 7.2×10^6 to 7.2×10^9 CFU (log 6.86 to 9.86), and the i.v. dilution ranges were 7.2×10^2 to 7.2×10^5 CFU (log 2.86 to 5.86) for immunized mice and 7.2×10^1 to 7.2×10^4 CFU (log 1.86 to 4.86) for unimmunized control mice. The 50% lethal doses were calculated after 3 weeks by the method of Reed and Muench (13).

Mice immunized with SL3261(pMK005) were strongly protected against challenge with SL1344, compared with unimmunized controls (Table 2). Sera prepared from blood samples taken from the tail vein of mice 14 days after immunization with either SL3261 or SL3261(pMK005), however, failed to agglutinate SL3261(pMK005) or *E. coli* C600(pMK005) cells (7). This serum was then used in Western blotting experiments against whole-cell lysates of C600(pMK005) and SL3261(pMK005) (Fig. 2, tracks 1 and 2). Serum from mice infected with SL3261 or SL3261 (pMK005) contained antibodies which bound to a number of *Salmonella* and *E. coli* antigens, particularly polypeptides which appeared to correspond to major outer membrane proteins of these organisms. However, only serum from mice immunized with SL3261(pMK005) contained anti-K88 antibodies. Mice immunized with SL3261(pMK005) were boosted by the i.v. route with 10^5 CFU of SL3261(pMK005), and serum samples were again obtained 10 days later. Serum from these reimmunized mice was able to agglutinate C600(pMK005) cells at a dilution of between 1 in 16 and 1 in 64. The presence of anti-K88 antibodies in this serum was confirmed in Western blotting experiments (Fig. 2, tracks 3 and 4). These results therefore demonstrate that attenuated *Salmonella* strains may be of value for presenting *E. coli* antigens to the immune system.

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