easily be introduced into scalp veins. Indiscriminate use of plastic catheters, solely for convenience, should be discouraged. However, catheters are generally more secure, and may be indicated in critically ill children for administration of drugs and fluids and for monitoring.

Further investigations are needed in order to clearly understand the mechanisms of bacterial adherence to IV cannulae and to devise ways to prevent it. Our assay for *in vitro* bacterial adherence to cannulae can be used in order to help to produce better designed cannulae, with minimal adherent properties and inflammatory complications.

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The Effect of Postnatal Age on the Adherence of *Shigella flexneri*, *Escherichia coli* 0124, and *E. coli* 0128 to Guinea Pig Intestinal Cells

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ABSTRACT. Since postnatal development of the gastrointestinal tract has an important effect on its microbial flora and may influence the types of intestinal infections, we examined the effect of age on bacterial adherence to intestinal epithelial cells. Radiolabeled bacteria were incubated with guinea pig enterocytes released by treating loops of the intestine with solutions containing EDTA, dithiothreitol, and citrate. Nonbound bacteria were separated from intestinal cells by sedimentation on a Percoll gradient. The colonic cells avidly bound *Shigella flexneri* (64 bacteria per cell), *Escherichia coli* 0124 (59), and *E. coli* 0128 (53).

The adherence process was Ca^{2+} and temperature dependent, was inhibited by fucose, glucose, and mannose, and was shown to be mediated by a carbohydrate-binding protein (lectin) on the colonic cells. Adherence of these bacteria to intestinal cells of newborn animals was only 15–25% of the adherence to adult animal cells and increased gradually, reaching adult values at about 2 weeks of age. The lectin activity, which was determined by agglutination of bacteria, was secreted with the colonic mucus. It was undetectable in the newborn animal, appeared gradually with age, and its titer correlated with the adherent capability of the colonic cells.

E. coli 0128 was the only one of the bacteria tested which significantly adhered to the ileum (19 bacteria per cell) in a process inhibited by mannose. This adherence

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was mediated by a mannose-sensitive lectin in the bacterial pili, and not on the intestinal cells. The postnatal age had no effect on the adherence to the ileum; the newborn animal had the same adherence capability as the adult one. Good correlation was found between the adherence to the suspended intestinal cells and the adherence to intact intestinal surfaces or the *in vivo* adherence to intestinal loops.

These patterns of bacterial adherence can be important in the neonatal microbial colonization of the intestinal tract, and can play a role in the types of intestinal infections during the neonatal period and infancy. (*Pediatr Res* 18:1366-1371, 1984)

Abbreviations

GIT, gastrointestinal tract

MES, 4-morpholineethanesulfonic acid

The adherence of bacteria to epithelial surfaces has been extensively investigated in recent years, and is becoming increasingly recognized as a prerequisite for colonization (19) and virulence (5, 7, 8, 31). The attachment to the intestinal epithelium enable the microorganisms to resist peristaltic clearing mechanisms and enable subsequent colonization, followed sometimes by tissue invasion and enterotoxin production. Enteropathogenic *Escherichia coli* were shown to adhere to the intestinal epithelium, while the nonenteropathogenic strains did not (4, 12). Recently, two reports (33, 37) described cases of protracted diarrhea in infants, caused by bacterial adherence and colonization of the mucosa of the small intestine, without invasion or evidence for enterotoxin production.

In most of the systems that were investigated, the adherence of bacteria to the epithelial surface was mediated by bacterial cell surface appendages, such as pili or fimbriae (15, 17). Some of these protein appendages were shown to possess specific carbohydrate recognition properties, sometimes named adhesins or lectins, which bound to specific receptors, usually carbohydrate moieties, on the mammalian cell surfaces (14). A bacterial adhesin with mannose-binding properties (type I pili) was frequently found (27, 31), although other sugar residues were also recognized as receptors on host cells (22, 36).

In a recent study, we have described a system for studying the adherence of *Shigella flexneri* to guinea-pig intestinal cells (20). We found that the adherence was mediated by a carbohydrate-binding protein (adhesin), found on the colonic mucosal surface, which attached to a receptor structure on the bacterial surface. Characterization and partial purification of this adhesin (28) showed that it was a protein which was secreted together with the intestinal mucus.

Since the postnatal age has a significant influence on the development of the GIT and the appearance of mucosal surface proteins (2, 23), on the growth of the normal microbial flora (26), and the types of intestinal infections (1, 3, 13), we studied the effect of age on bacterial adherence to the intestinal tract and on the appearance and secretion of this adhesin.

MATERIALS AND METHODS

Bacterial adherence to guinea pig intestinal cells and intestinal epithelium was quantitatively determined by the use of radiolabeled bacteria.

Bacteria. Clinical isolates of *E. coli* 0124 and *S. flexneri* 1b were obtained from Dr. G. Altmann, Dept. of Microbiology, Tel-Hashomer Hospital, Israel. *E. coli* 0128:H4 was obtained from Dr. N. Gerber, Bar-Ilan University, Israel. *E. coli* strains were grown in 1% peptone (Difco Laboratories, Detroit, MI), 0.5% yeast extract, and 0.5% NaCl. *S. flexneri* was grown in Luria broth, supplemented with 5 mM CaCl₂ and 0.2% glucose.

The invasiveness of *S. flexneri* and *E. coli* 0124 was repeatedly tested by the guinea pig eye inoculation technique (35).

Radiolabeled bacteria were prepared by adding D-[¹⁴C]glucose (239 mCi/mmol, 0.1 Ci/ml; The Radiochemical Centre, Amersham, England) as described (20). The radioactivity of the labeled bacteria was counted in a Tricarb liquid scintillation spectrometer (model 3255, Packard Instrument Co.) with 10 ml Triton X-100-toluene scintillation fluid. The usual specific radioactivity obtained was 1 cpm/1–2 × 10³ bacteria. Precipitation in 5% trichloroacetic acid showed that more than 90% of the radiolabeled precursor was incorporated into macromolecular components of the bacteria.

Preparation of intestinal epithelial cells. Dunkin-Hartley non-inbred guinea pigs of different ages were sacrificed by cervical dislocation. The intestinal epithelial cells were released essentially as described by Weiser (38). The colon, or other region of the intestine, was removed and washed with prewarmed (37° C) solution of 0.154 M NaCl and 1 mM dithiothreitol. Its end was tied and it was filled with 50 mM phosphate buffer containing 27 mM sodium citrate and incubated for 15 min at 37° C. The solution was discarded and the intestine was refilled with a prewarmed solution containing phosphate-buffered saline, 1.5 mM EDTA, and 0.5 M dithiothreitol and incubated for 45 min. The solution containing suspended mucosal cells was carefully removed; the cells were combined, sedimented by centrifugation (500 × g, 10 min), washed and resuspended in 20 mM MES buffer containing 140 mM NaCl and 1 mM CaCl₂, in a final concentration of 5 × 10⁶ cells/ml. The viability of the epithelial cells was monitored by trypan blue exclusion. The animal age was precisely determined from birth.

Assay for bacterial adherence to epithelial cells. A suspension of epithelial cells (200 µl; 5 × 10⁶ cells/ml) was incubated with radiolabeled bacteria (100 µl; 2 × 10⁹ bacteria/ml; 200 bacteria/epithelial cell) at 37° C in a rotating rack (16 rpm). After 45 min, the interaction was terminated by adding saline (2.5 ml) and then centrifuging (500 × g, 5 min) for sedimentation of epithelial cells, and the supernatant, containing mainly the nonadhered bacteria, was discarded. In order to separate the epithelial cells from the remaining nonadhered bacteria, we used discontinuous density gradient centrifugation with Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). The gradient was built on top of the sediment, consisting of epithelial cells and bacteria, by layering 0.5 ml of 100% Percoll, followed by 1 ml of 60% (diluted with saline), 2 ml of 40%, 1 ml of 30%, and 0.5 ml of 10% Percoll. After centrifugation (500 × g, 20 min) the bacteria formed a layer between 100 and 60% Percoll and were not observed in the epithelial cell layer that banded at 30%. The epithelial cell layer was collected, with the bacteria adhered to it, and counted for radioactivity.

Assay for bacterial adherence to intact intestinal epithelial surface. Guinea pig intestinal sections were obtained after washing the intestine with saline. Radiolabeled bacteria were added to areas of exposed intact mucosa and incubated as described (20). The reaction was terminated by washing the tissue three times with saline. The area of the intestinal mucosa that had been exposed to the labeled bacteria was cut and heated (100° C, 15 min) in a tube containing sodium dodecyl sulfate (1 ml; 2%). The adhered radiolabeled bacteria were solubilized under these conditions (6) and their radioactivity was determined as described above.

Assay for *in vivo* bacterial adherence to intestinal loops. The guinea pig was anesthetized by intramuscular injection of 0.4–1.0 ml of the following mixture: 2% Rompon [2-(2,6-xylylidino)-5,6-dihydro-4H-1,3-thiazine HCl] (Bayer), 1% Combelen [N-(3-dimethylaminopropyl)-3-propionyl phenothiazine] (Bayer), 10% Vetalar (ketamine HCl) (Parke-Davis), in a ratio of 2:2:1. Bacterial adherence was determined essentially as described (18). The peritoneal cavity was opened and loops of 2-cm length were created in the intestine by surgical sutures. Radiolabeled bacteria (200 µl, 10⁹ bacteria/ml) were carefully introduced into the loops

with a 26-gauge needle, and then the loops were replaced in the peritoneal cavity and the abdomen covered for 45 min. Following the incubation period, each loop was cut from the intestine, washed with saline and then the intestine was spread and scraped gently with a glass slide. The removed tissue was suspended in 5 ml saline, sedimented ($500 \times g$, 5 min) and the nonadhered bacteria were separated from the intestinal mucosa by the Percoll gradient as described above, and the radioactivity of the radiolabeled bacteria that adhered to the mucosa was determined as described before. Ten per cent of the suspended mucosal scraping was taken for protein determination according to Lowry *et al.* (25) and the results were expressed as adherence per g protein.

Assay for secreted agglutinin activity of the colonic cells. The suspended colonic epithelial cells (10 ml , 5×10^6 cells/ml) released clear mucus when incubated at 37°C for 45 min. After incubation, the colonic cells were sedimented ($500 \times g$, 5 min) and the supernatant was centrifuged again ($100,000 \times g$, 60 min). The clear supernatant solution obtained was extracted with light petroleum ether (volume ratio of 1:1). The aqueous phase, which was recovered, contained the agglutinating activity. Five μl of bacterial suspension (10^9 bacteria/ml) were incubated (5 min) on a glass slide with 5 μl of the clear colonic mucus solution or with MES-Ca buffer (control) and observed under a microscope. Agglutination activity was determined when clumps of 10 bacteria or more were seen, and the titer was determined by serial 2-fold dilutions of the solution. The results of the agglutinating titer were reproducible in numerous animals.

All the experiments were done in triplicate, and results are expressed as mean $\pm$ SEM.

Scanning electron microscopy. After the assay for bacterial adherence, the intestinal cells or intestinal surface were washed in saline, fixed with 2% glutaraldehyde (1 h), and washed with 0.1 M sodium cacodylate buffer (pH 7.2). It was then incubated with 1% osmium tetroxide (30 min) and dried to a critical point. The tissue was fixed to planchets, coated with gold, and examined with a JSM-35C scanning electron microscope (JEOL, Japan).

RESULTS

E. coli 0128:H4 cells agglutinated mannan-containing yeast cells (27) and guinea pig (but not human) erythrocytes. Electron micrographs of negatively stained cells revealed that they contained type 1 pili (17). *E. coli* 0124 and *S. flexneri* 1b did not have any cell surface appendages, and did not agglutinate yeast cells or erythrocytes.

The adherence of these bacteria to epithelial cells released from various regions of the intestine showed that attachment was highest to the colonic cells especially from transverse and descending regions (Table 1). *E. coli* 0128 was the only bacteria which also adhered to the small intestine (17–20 bacteria/cell).

The adherence of *E. coli* 0128 to the ileum and *E. coli* 0124 to the colon is shown in Figure 1. The attachment to the ileum was to the cell microvilli, while in the colon the bacteria attached also to the crypt areas. We did not observe phagocytosis of the

bacteria or bacteria inside the cells in our experimental conditions.

Bacterial adherence to the colonic cells. The attachment of the three bacterial strains to guinea pig colonic cells was found to be quite similar. The adherence was optimal at 37°C , required Ca^{2+} ions, and the optimal pH was 6.2.

Preincubation of the colonic cells with unlabeled *S. flexneri* or *E. coli* 0128 competitively inhibited the adherence of radiolabeled *E. coli* 0124 by 45 and 37%, respectively (Fig. 2), indicating that the attachment of these bacteria to colonic cells is probably by the same mechanism.

Significant inhibition of the adherence (about 80%) was achieved (Table 2) by fucose (50 mM) and glucose (100 mM), and about 50% inhibition by mannose (100 mM), while other carbohydrates had no inhibitory effect as previously studied (20). Lipopolysaccharides of *S. flexneri* and *E. coli* 0124, prepared by the phenol-water extraction procedure (39) and containing the carbohydrates (32), were also potent inhibitors of the adherence of the respective bacteria (Table 2).

Pretreatment of the epithelial cells by fixation with glutaraldehyde, proteolytic digestion, or heat inactivation (20) completely abolished their capability to adhere to bacteria. Preincubation of the colonic cells with fucose (10 mg/ml, 37°C , 45 min), followed by washing of the cells, was also sufficient to inhibit the attachment. On the other hand, similar pretreatments of the bacteria did not affect their subsequent adherence to the colonic cells.

The adherence of *E. coli* 0128 to ileal epithelial cells. *E. coli* 0128 was the only one of the bacteria tested which significantly adhered to the ileal cells. In contrast to the adherence of *E. coli* 0128 to the colonic cells, the adherence to the ileal cells was significantly inhibited (>50%) by mannose (10 mg/ml) while other carbohydrates, including fucose and glucose, had no inhibitory effect (Table 3). In contradiction to the finding with the colonic cells, the various pretreatments of bacteria described above (using mannose as an inhibiting carbohydrate) significantly inhibited (>90%) the adherence, while pretreatments of the ileal cells did not affect the process. This indicated that the adherence to the ileal cells was mediated by a bacterial adhesin.

Bacterial adherence to intestinal surfaces and to intestinal loops *in vivo*. These experiments were performed in order to correlate bacterial adherence to a suspension of epithelial cells with adherence to the intestinal surface or the binding to the intestine *in vivo*. The attachment to the intestinal surface is shown in Figure 1, indicating that *E. coli* 0124 (and the other bacteria examined) avidly adhered to colonic surfaces, and *E. coli* 0128 also adhered to the ileal surface. Quantitative determinations indicated that the adherence of *E. coli* 0124 to the colonic surface was $1.63 \times 10^7 \pm 2 \times 10^6$ bacteria/cm². Since the number of colonic cells released from the colon was $2.52 \times 10^5 \pm 4 \times 10^4$ cells/cm², the calculated adherence was 63 bacteria/cell. This value is similar to the number of bacteria that adhered to a suspension of colonic cells. The adherence both to the intestinal surface and to intestinal loops *in vivo* was Ca^{2+} dependent, and was inhibited by fucose or glucose (adherence to the colon) and by mannose (adherence to the ileum), in accordance with the *in vitro* findings.

The effect of postnatal age on bacterial adherence. The adherence of *S. flexneri* and *E. coli* 0124 to the colonic cells of a newborn guinea pig (less than 24 h after birth) was only 15–25% of that of an adult animal (Fig. 3). Adherence increased gradually with age and at the age of about 2 wk the number of bacteria adhering was similar to the adult level, and this remained constant throughout life (examined till the age of 2 years). Although adherence to the colonic cells of the young animal was reduced, it had the same characteristics as the adherence in the adult animal with regard to dependence on time, temperature, pH, and Ca^{2+} ions. Moreover, it was inhibited by fucose and glucose to the same extent as in the adult.

The effect of age on the adherence of *E. coli* 0128 (Fig. 4) showed two distinct patterns. The attachment to the colonic cells was reduced in the newborn animal (16%) and increased gradu-

Table 1. Adherence of bacteria to guinea pig intestinal epithelial cells released from different regions of the intestine*

Region in the intestine	Adherence (bacteria/cell)		
	<i>S. flexneri</i> 1b	<i>E. coli</i> 0124	<i>E. coli</i> 0128
Duodenum	3 ± 1	2 ± 1	20 ± 4
Jejunum	3 ± 1	$1 \pm <1$	17 ± 2
Ileum	6 ± 2	$1 \pm <1$	19 ± 2
Ascending colon	17 ± 3	18 ± 6	14 ± 3
Transverse colon	52 ± 5	79 ± 9	32 ± 6
Descending colon	78 ± 7	68 ± 10	52 ± 6

* Adherence of bacteria (2×10^8) to epithelial cells (10^6), as described under "Materials and Methods."

Fig. 1. Scanning electron micrographs of bacterial adherence to intact intestinal surfaces of guinea pig. *A*, adherence of *E. coli* 0128 to the ileum. The bacteria are seen on the epithelial surface ($\times 3,000$). *B*, high magnification ($\times 22,000$) showing bacteria attached to the brush border of an ileal cell. *C*, adherence of *E. coli* 0124 to the colonic surface ($\times 5,400$). *D*, high magnification ($\times 15,000$) showing bacteria attached to a colonic cell. For detailed experimental conditions and the preparations for the electron microscopy, see "Materials and Methods."

Fig. 2. Inhibition of the adherence of radiolabeled *E. coli* 0124 (2×10^8 bacteria) to colonic cells (10^6 cells) by the addition of unlabeled *E. coli* 0128 or *S. flexneri* (4×10^8 bacteria/ml), during the incubation period (37°C , 45 min).

ally, giving the same picture as the other bacteria. On the other hand, the adherence of *E. coli* 0128 to ileal cells showed no age dependency: similar values were obtained in the newborn animal as in the adult one, and they remained constant throughout life. Determination of the *in vivo* adherence to intestinal loops in a living animal showed the same results: decreased adherence to the colon in the young animal.

The effect of age on the secreted agglutinin activity of the colonic cells. The soluble agglutinin released by the colonic cells agglutinated the three types of bacteria tested, as well as other

Table 2. Inhibition of adherence of bacteria to guinea pig colonic cells*

Compound added (10 mg/ml)	Adherence (% of control)		
	<i>S. flexneri</i> 1b	<i>E. coli</i> 0124	<i>E. coli</i> 0128
Fucose	24 ± 3	21 ± 3	48 ± 8
Glucose	26 ± 4	27 ± 6	50 ± 3
Mannose	52 ± 5	54 ± 3	43 ± 3
Rhamnose	86 ± 5	97 ± 10	90 ± 2
Lactose	97 ± 4	98 ± 11	95 ± 9
Lipopolysaccharide†	7 ± 2	21 ± 1	

* The results represent means $\pm$ SEM of 10 experiments for fucose, glucose, and mannose or three experiments for the others.

† Lipopolysaccharides prepared from *S. flexneri* and *E. coli* 0124 were used for inhibition of the respective bacteria (at 1 mg/ml concentration). The preparation procedure was as described (39).

Shigella and *E. coli* strains, as reported before (28). No such agglutinating activity was seen in the mucus released by the colonic cells of a newborn animal (Table 4). The agglutinating activity was first observed at the age of 2 days, and its titer increased gradually to the adult level.

DISCUSSION

Our study indicates that the adherence of *S. flexneri* and *E. coli* 0124 and 0128 to the colon is dependent upon a mucosal

Table 3. Inhibition of adherence of *E. coli* 0128 to guinea pig ileal cells*

Carbohydrate added (10 mg/ml)	Adherence (% of control)
Mannose	56 ± 3
Fucose	101 ± 16
Glucose	98 ± 10
Rhamnose	102 ± 2
Lactose	102 ± 13

* The results represent means ± SEM of 10 determinations for mannose and of three determinations for the other carbohydrates.

Fig. 3. Adherence of *S. flexneri* and *E. coli* 0124 to colonic epithelial cells as a function of the age of the guinea pig. The experiments were done under standard conditions (37° C, 45 min). Ten animals of each age group were examined.

Fig. 4. Adherence of *E. coli* 0128 to ileal and colonic epithelial cells as a function of guinea pig age. All the experiments were done under standard conditions (37° C, 45 min. incubation) as described in text. Ten animals of each age group were examined.

carbohydrate-binding protein (adhesin or lectin) that is secreted together with the colonic mucus and coats the colonic cells. This is in contrast to systems investigated before, where the adhesins were always found on the bacterial surface, and these mediated the binding to a receptor site on the mammalian cells (14, 30). The adhesin mediates the binding of certain bacteria to mucosal cell surfaces by recognizing distinct receptors on the bacterial surface polysaccharide. The mucosal adhesin was recently characterized and purified using affinity chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (28).

The physiological role of this adhesin is still unclear. It appears, however, that the constant secretion of this adhesin, with the colonic mucus, may have a sort of defensive function which is to agglutinate bacteria and remove them from the intestinal tract. Indeed, experimental development of shigellosis in guinea

Table 4. The effect of the guinea pig age on the secreted agglutinin activity in intestinal mucus*

Age	Titer of agglutination	
	<i>S. flexneri</i> 1b	<i>E. coli</i> 0124
1 day	0	0
2-6 days	1:1	1:1
1 wk	1:4	1:8
1 month	1:8	1:8
6 month	1:16	1:16
2 year	1:16	1:8

* The agglutination was performed by incubating (5 min) bacterial suspension of either *E. coli* 0124 or *S. flexneri* 1b with soluble colonic mucus. Agglutinating activity was determined when clumps of 10 bacteria or more were seen under the microscope. The titer was determined by serial 2-fold dilutions of the mucus. Agglutinations were inhibited by fucose (0.1%) or glucose (0.5%) solutions.

pigs (16), which, together with humans and rhesus monkeys (29), are among the few mammals that can be infected by *Shigella*, required starvation and opiate administration, which reduced mucus secretion and intestinal motility. Thus, our finding may lend further support to the previous observations on the important defensive role of human intestinal mucus (10, 21).

One of the most noteworthy findings of this investigation was the fact that the mucosal adhesin was not present in the colon of the newborn animal, and began to appear gradually after a few days. Consequently, bacterial adherence to the colon was significantly reduced in the young animal, as was the secreted agglutinating activity of the colonic cells. This finding is in accordance with the developmental changes occurring in the GIT after birth (2, 23), such as the gradual appearance and secretion of considerable digestive enzymes (23).

The adherence of *E. coli* 0128 to the ileum, which was shown in this study to depend upon a mannose-binding bacterial adhesin, was not affected by the animal age. A preliminary report suggested (9) that in the rabbit the postnatal age affected the appearance of the receptors for *E. coli* on the intestinal cells. But in the guinea pig we found that the receptors for *E. coli* 0128 were present immediately after birth, and in the same amount as in the adult animal.

The applicability of these findings to humans should be made with caution. The adhesin has not as yet been found in the human intestinal tract, and the effect of age on bacterial adherence was not documented because of the technical difficulties of such studies. However, the adherence of *E. coli* to biopsies of human colon (19), taken during colonoscopic examination, is very similar to our scanning electron micrographs of guinea pig colonic cells, where the adhesin was found. Recently, a way for obtaining human intestinal cells from long-standing ileostomies was described (12). Using this approach, the validity of these findings to human GIT is currently under investigation in our laboratory.

The gradual increase in the adherence of bacteria to the colon during the early stages of postnatal life, and the correlation with the appearance of the colonic mucus lectin, suggest that the adherence may play a role in the gradual neonatal colonization of the GIT (24, 26) and in the well documented effect of age on the types of intestinal infections (11, 34). Moreover, it is possible that quantitative or qualitative interference with the secretion of the described intestinal adhesin can alter bacterial adherence to the gut and increase susceptibility to infections. Considerable work is still needed to clarify these as yet unproven important possibilities, and elucidate the observed developmental changes of the GIT.

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