INFECTION AND IMMUNITY, Apr. 1972, p. 606-611
Vol. 5, No. 4

Association of *Escherichia coli* with the Small Intestinal Epithelium

II. Variations in Association Index and the Relationship Between Association Index and Enterosorption in Pigs

HANS U. BERTSCHINGER,[1] HARLEY W. MOON, AND SHANNON C. WHIPP

National Animal Disease Laboratory, Veterinary Sciences Research Division, Agricultural Research Service, Ames, Iowa 50010

Received for publication 10 January 1972

The association between small intestinal epithelium and enteropathogenic *Escherichia coli* (EEC) was studied in ligated intestinal loops of pigs and rabbits. The association indexes (degree of association) for each of two porcine EEC strains varied widely among pigs and independently of each other. Significant litter-to-litter variations in association indexes among colostrum-deprived newborn pigs were interpreted to be the result of congenital resistance to association with specific EEC in some pigs. Since enterosorption occurred in loops with low association indexes, it was not necessary for EEC to establish a high association index for them to cause enterosorption in ligated intestinal loops. Two strains of EEC which are enteropathogenic for humans caused enterosorption in ligated loops in pigs 3 weeks old or less but not in 6-week-old pigs.

The accompanying paper reported that enteropathogenic *Escherichia coli* (EEC) have a greater tendency to associate, i.e., occur in a layer contiguous to brush border along villi from tip to base, with porcine small intestinal epithelium than nonenteropathogenic *E. coli* (NEEC; 1). *Vibrio cholerae* has a similar association with intestinal epithelium, and there is evidence that intimate association between vibrio and host cell is necessary if the vibrios are to cause fluid accumulation in ligated intestinal loops, i.e., enterosorption (4). The ability to produce enterosorption was host-specific in certain situations (5, 11). Porcine EEC caused enterosorption in pig intestinal loops, whereas EEC from calves, lambs, and humans did not. Human EEC caused enterosorption in rabbit intestinal loops; EEC from calves, lambs, and pigs did not. This specificity could result from qualitative differences in the enterotoxins produced by EEC of different origins (13). However, rabbit intestine was more sensitive than pig intestine to an enterotoxin produced by a pig EEC (8). Host influence is also evident in pig intestine which develops resistance with age to the enterosorption caused by some porcine EEC (9).

If association between bacterial and host cells is necessary for EEC to cause enterosorption, as for *V. cholerae*, the host specificity of enterosorption that is caused by EEC in intestinal loops may result from species-specific association between EEC and intestinal epithelium. In addition, age resistance of pig intestine might be caused by lack of such association in older pigs. The objective of this study was to see whether such host specificity and age resistance can be explained as a failure of EEC to associate with intestinal epithelium in these situations. During the study, extensive variation in association among pigs exposed to the same EEC was encountered, and the study was expanded to include some sources of variation.

MATERIALS AND METHODS

Bacteria. The porcine EEC strains used were 263 [serotype 08:K87(B) 88a,b(L):H19] and 431 [serotype 0101:KU460(A):NM] which associated with porcine intestinal epithelium and the porcine NEEC strain 123 (serotype 043:K-:H28) which did not (1). The human EEC strains used were B7A (serotype 0148:K?:H28) and B2C (serotype 06:K?:H16). Both of the latter strains were provided by Formal et al. (3) and were known to cause enterosorption in ligated small intestinal loops of rabbits (rabbit loops). Bacterial and enterotoxin inocula were prepared as previously described (1, 8).

Animals. Suckling pigs 1 to 6 weeks old from two herds, N and T, were used. In addition, fasting

[1] Visiting scientist from the University of Zürich, Switzerland, supported by a grant from the Kredit zur Förderung des akademischen Nachwuchses des Kantons Zürich.

hysterectomy-produced, colostrum-deprived (HPCD) pigs were exposed during the first 12 hr after hysterectomy as described (1). Hysterectomies were done when it was judged that the dams would farrow in the succeeding 24 hr; however, the exact gestational ages of HPCD pigs were not known. The rabbits used were 9-week-old New Zealand Whites.

Exposure. When both association and enterosorption were to be studied, ligated jejunal loops in pigs or rabbits were exposed to 3×10^8 *E. coli* cells in 1 ml of broth as described (1), and the animals were necropsied 3 hr later. Pigs in which only enterosorption was studied were necropsied 18 hr after exposure.

Necropsy. Enterosorption was evaluated as described (7). The association index was determined by microscopy examination of duplicate frozen sections stained with either fluorescent antibody against *E. coli* or with Toluidine Blue as described (1). An association index of 25 means there were numerous *E. coli* contiguous to brush border at the bases of villi; whereas, an index of 1 means there were no *E. coli* at the bases of villi and that *E. coli* were not contiguous to the brush border. Total viable *E. coli* counts were performed, and paraffin sections of Formalin-fixed tissues were prepared and examined as described (1).

RESULTS

Three hours postexposure, the number of viable *E. coli* which belonged to the strain inoculated varied from 0.2×10^9 to 7.1×10^9 per loop in both pigs and rabbits, regardless of the strain of *E. coli* inoculated, and there were no consistent strain or host differences within this range. Bacteria other than the strain inoculated were frequently recovered from loops in suckling pigs but never from HPCD pigs.

The NEEC strain 123 did not cause enterosorption in any pig loops and, as described (1), this strain tended to be randomly distributed in the intestinal lumen, resulting in low (<10) or intermediate (>10 <20) association indexes in all pigs (Tables 1 and 2). The association indexes for this strain were higher in HPCD than in suckling pigs.

TABLE 1. *Association indexes for three porcine Escherichia coli strains in ligated intestinal loops of suckling pigs obtained from two herds*

E. coli strain	Source of pigs	No. of		Association index	
		Pigs	Loops	Mean	Range
263	Herd N	10	20	17.1	8–25
	Herd T	6	12	2.9	1–6
431	Herd N	10	20	6.5	1–20
	Herd T	6	12	3.7	1–8
123	Herd N	10	20	5.1	1–12
	Herd T	6	12	2.3	1–4

TABLE 2. *Association indexes for three porcine and one human Escherichia coli strains in ligated intestinal loops of newborn fasting HPCD[a] pigs*

E. coli strain	Litter	No. of		Association index	
		Pigs	Loops	Mean	Range
263	A	1	2	25.0	25–25
	B	2	4	12.2	9–16
	C	6	12	6.7	2–16
	D	4	8	5.8	2–16
	E	4	8	18.9	12–25
431	A	1	2	20.5	16–25
	B	2	4	22.8	16–25
	D	3	6	23.3	20–25
	E	3	6	17.5	12–25
123	A	1	2	9.5	3–16
	B	2	4	14.0	8–16
	D	1	2	8.5	6–9
	E	1	1	6.0	
B7A	C	6	12	7.8	1–12
	D	1	2	9.0	9–9
	E	1	2	12.0	12–12

[a] Hysterectomy-produced, colostrum-deprived.

Variations in association indexes. Indexes for porcine EEC in suckling pigs from herd N were higher than in those from herd T. In pigs from herd N, indexes were higher for EEC strain 263 than for EEC strain 431 and NEEC strain 123. Association indexes of EEC in herd T varied in the same range as those for the NEEC strain 123 (Table 1). Indexes for each strain varied widely among pigs the same age from the same herd and between loops in the same pig. The distribution of EEC in sections from suckling pigs with intermediate association indexes (>10 <20) was frequently irregular; i.e., some villi had a layer of EEC continguous to brush border, whereas others in the same section did not.

The association indexes for either porcine EEC strain also varied in HPCD pigs (Table 2). Those for strain 263 in some pigs varied in the same range as for the NEEC strain 123. Indexes from the 34 loops exposed to strain 263 (Table 2) were analyzed statistically (hierarchal or nested analysis) with the following results: 62.9% of the total variation occurred among litters, 30.7% among pigs within litters, and 6.3% among loops within pigs. Differences among litters and among

pigs within litters were statistically significant ($P < 0.01$).

Association indexes for the two porcine EEC strains varied independently within individual HPCD pigs. For example, in litters B and D (Table 2), indexes for strain 431 were high (>20) or intermediate, whereas for strain 263 in different loops from the same pigs they were low or intermediate. The four pigs in litter E and one pig in litter D had an unusual pattern of partial association. In these, strain 263 occurred in layers contiguous to brush border at the bases of the villi, as the association index emphasizes bacterial at the villous base (1), it was high to intermediate in these pigs. However, there were very few bacteria associated with the upper portions of the villi (Fig. 1). Some crypts in these sections were filled with strain 263. In the same pigs, strain 431 was distributed along the villi as usual.

Association index and enterosorption. The association indexes in two loops exposed to human EEC strain B7A and two more exposed to strain 263, in each of the same three rabbits, ranged from 1 to 3 and 1 to 2, respectively. Neither strain caused enterosorption in rabbits during the 3-hr exposure time used to determine association indexes. However, strain B7A did cause enterosorption in 26 of 30 rabbit loops exposed for 18 hr, whereas strain 263 did so in only 10 of 33 such loops.

Live bacterial suspensions of human EEC strains B7A and B2C caused enterosorption in pigs 3 weeks old or less, but not in 6-week-old pigs. Enterotoxins from these strains also caused enterosorption in pigs, and part of this toxicity remained after heating at 100 C for 15 min (Table 3). Unheated enterotoxic preparations (Table 3) contained the following numbers of rabbit jejunal loop units (6) per ml: strain 263, three; strain B7A, four; and strain B2C, one. However, in contrast to pig loops, rabbit loops

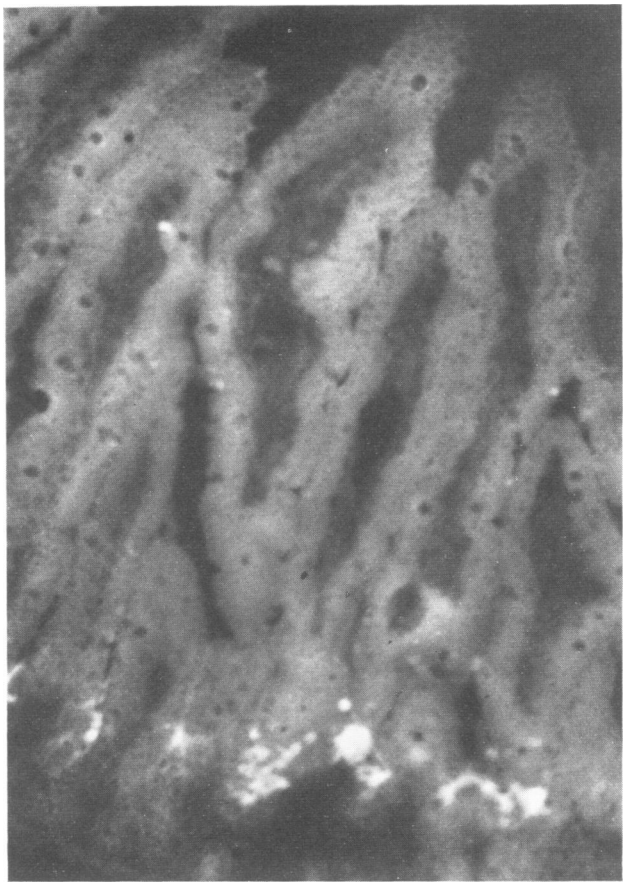

FIG. 1. *Unusual distribution of pig EEC strain 263 at the bases of villi, in contrast to the usual association of this strain with epithelium all along villi. From fluorescent antibody-stained frozen section of ligated jejunal loop in newborn colostrum-deprived pig prepared 3 hr postexposure to E. coli.*

TABLE 3. *Enterosorption in pig intestinal loops caused by living Escherichia coli and cell-free E. coli enterotoxins*

Pigs		Inoculum										
		Living *E. coli* strain				Enterotoxin[a] from *E. coli* strain						
						263		B7A		B2C		Control Saline
Age	No. tested	263	B7A	B2C	123	Unheated	Heated[b]	Unheated	Heated	Unheated	Heated	
HPCD[c]	17	17/34[d]	15/16	ND[e]	0/19	ND	ND	ND	ND	ND	ND	ND
5 days	8	8/8	7/8	6/8	1/8	ND	ND	ND	ND	ND	ND	ND
3 wks	7	11/11	4/6	5/7	ND	4/4 (36.2)[f]	0/4 (0)	7/7 (47.1)	3/4 (11.2)	6/6 (30.0)	4/4 (13.8)	0/4 (0)
6 wks	4	8/8	0/8	0/8	0/8	ND	ND	ND	ND	ND	ND	ND

[a] Each loop was injected with 7 ml of enterotoxin or saline.
[b] Heated at 100 C for 15 min.
[c] Hysterectomy-produced, colostrum-deprived fasting pigs, exposed for 3 hr during the first 12 hr after hysterectomy. All other pigs were sucklings, exposed for 18 hr.
[d] Number of loops with enterosorption over number tested.
[e] Not done.
[f] Mean fluid volume (milliliters) per loop is in parentheses.

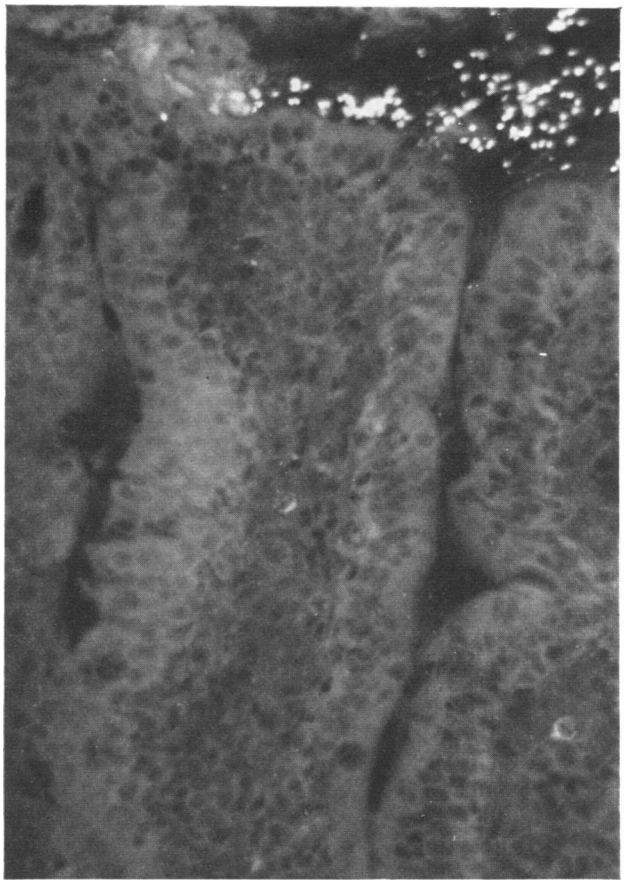

FIG. 2. *Pig EEC strain 431 with association index of 2 in ligated jejunal loop of a suckling pig resistant to association with this strain. Enterosorption occurred frequently in loops with such low association indexes. From fluorescent antibody-stained frozen section prepared 3 hr postexposure to E. coli.*

did not have enterosorption after exposure to heated enterotoxins from strains B7A or B2C. Strain B7A tended to be randomly distributed in the central intestinal lumen. In contrast to strain B7A, the porcine EEC strains frequently had intermediate to high association indexes (Table 2). However, when all pig loops exposed to porcine EEC strains 263 and 431 for 3 hr (Tables 1 and 2) were considered, there was a total of 59 loops in which enterosorption occurred. In 22 of these 59 loops with enterosorption (12 exposed to strain 263 and 10 exposed to strain 431), the association index was low (<10) (Fig. 2). There were no consistent differences in the association indexes for strain 431 in suckling pigs of different ages (Table 1). Association indexes for the human EEC in suckling pigs were not determined.

DISCUSSION

Previously, we observed high association indexes between porcine EEC and pig small intestinal villous epithelium and low indexes for NEEC (1). As more pigs were included in similar experiments reported here, considerable variation in association between EEC and epithelium were observed. Qualitative variations were observed. In some pigs, EEC strain 263 was associated with epithelium at the bases of villi and in crypts (Fig. 1), in contrast to the usual distribution of EEC along villous epithelium. Quantitative variations were demonstrated as differences in association indexes for the same EEC in different pigs. For example, in some pigs (Tables 1 and 2), association indexes for EEC strains 263 and 431 were no greater than those for NEEC strain 123. Variations in association indexes among HPCD litters and pigs were statistically significant and consistently greater than variation among loops within these pigs. Thus, it is unlikely that the variations were caused by the technique, and we conclude that the variations recorded reflect in vivo variations.

Since variations occurred, resulting in low to intermediate association indexes in some unfed HPCD pigs less than 12 hr old, we assume that these pigs were congenitally resistant to association with certain porcine EEC. Variations for a single EEC in HPCD pigs were greater among litters than among pigs within litters and greater among pigs within litters than among loops within pigs. Thus, we suggest that variations in association indexes may have been the result of genetically controlled differences in the ability of intestinal epithelium to associate with EEC. Evidence for genetic differences in resistance to enteric *E. coli* infections in pigs has been reported by others (12, 14). The exact gestational ages of the HPCD pigs were unknown, and slight variations in gestational age may have contributed to variations in association indexes in HPCD pigs.

Another possible source of variations in association indexes is antibody. Freter has shown that specific antibody inhibits association between *V. cholerae* and intestinal epithelium in rabbits (4). If one assumes the HPCD pigs were devoid of antibody (2), then antibody did not cause the variations in these pigs. However, some antibody can occur in newborn colostrum-deprived pigs (10) and thus cannot be unequivocally dismissed as a possible source of congenital resistance to association in HPCD pigs.

Association indexes for all three procine strains and particularly for strain 431 tended to be lower in suckling than in HPCD pigs (Tables 1 and 2). Although these differences may have been the result of chance alone, antibody from milk may have contributed to low association indexes in some suckling pigs although they were weaned the day before tests were done. For example, passive protection against experimental cholera was detectable in mice 3 days after they were weaned from cholera-immune mothers (15). In addition, suckling pigs may have acquired active immunity.

It would be useful if resistance to association could be applied in attempts to inhibit colonization of small intestine by EEC. However, resistance to association with EEC strains 263 and 431 varied independently within the same pig or litter (Tables 1 and 2). Thus, whatever the cause(s) of the spontaneous resistance, it was strain-specific. There are numerous serotypes of *E. coli* which cause enteric colibacillosis in pigs. Thus, it would be difficult to control the disease by enhacing or selecting for such resistance in an attempt to inhibit colonization by all pig EEC.

Porcine EEC produce heat-labile (LT) and heat stable (ST) enterotoxins (6). Rabbit loops are more sensitive than pig loops to LT, and pig loops are more sensitive than rabbit loops to ST (6, 7). The enterotoxins from human EEC strains B7A and B2C were partially heat-labile when tested in pig loops (Table 3). In contrast to pig loops, rabbit loops did not have enterosorption after exposure to heated enterotoxins from these strains. From this, we conclude that the human EEC strains B7A and B2C produced both LT and ST.

Living bacteria of strains B7A and B2C caused enterosorption in pigs 3 weeks old or less but not in 6-week-old pigs. Such resistance with age to enterosorption that was caused by certain living strains isolated from pigs designated as class 2 EEC was characteristic of pig intestine (8). None of the human EEC strains which Gyles and Barnum (5), and Smith and Halls (11) tested in pigs 3- and 7- to-12-weeks-old, respectively,

caused enterosorption. During the first 3 weeks of life, pigs developed progressive resistance to the effects of enterotoxins prepared from porcine EEC; i.e., the dose of toxin per unit of body weight required to produce diarrhea increased with age (J. B. Stevens, M. S. thesis, University of Guelph, Ontario, 1971). We assume this resistance to toxin with age at least partially explains the failure of living human EEC strains B7A and B2C as well as the class 2 pig EEC mentioned above to cause enterosorption in 6-week-old pigs.

Enterosorption occurred frequently in pig loops in which the association indexes for EEC were as low as those for NEEC in the same pigs. The possibility that enterosorption might reduce the association index was considered. However, this seems unlikely because in previous work (Fig. 6 of reference 1) the association index did not decrease during fluid accumulation from 1.5 to 6 hr postexposure. In addition, association indexes for strain B7A in rabbit loops, where it causes enterosorption in 18-hr tests, were low. For these reasons, we conclude that the association between host epithelium and EEC which resulted in intermediate to high association indexes and which characterized the differences in histological distribution between EEC and NEEC in this and our previous study (1) is not a prerequisite for enterosorption induced by living EEC. Thus, host specificity and age resistance of EEC-induced enterosorption in pig intestine cannot be explained as a failure of EEC to establish high association indexes.

The observation that living EEC can cause enterosorption in loops without a high association index is apparently in contrast to those made on experimental cholera. Freter presented evidence that "adsorption" of vibrios to mucosa (association) is necessary if vibrios are to cause enterosorption in rabbit loops (4). However, direct comparisons between our results and those of Freter cannot be made, as the techniques used to quantitate association were different. In comparing his photographs (Fig. 1 of reference 4) and morphological descriptions of vibrios in rabbits with ours for EEC in pigs (Fig. 2 and 3 of reference 1 and Fig. 2 of this report), we would have calculated low or intermediate association indexes for the vibrios. However, his observations were made from washed and ours from unwashed loops. Thus, "adsorption" of the nature and degree reported by Freter for *V. cholerae* may have occurred in loops exposed to EEC that had low association indexes in the study reported here. This question could be resolved if a technique for consistently removing nonadsorbed EEC from pig loops were developed and if the techniques used by Freter for rabbit intestine could be successfully applied to rabbit intestine exposed to human EEC.

High association indexes are not necessary for EEC to produce enterosorption in loops where the washout effects of intestinal motility are eliminated. However, the ability of EEC to develop high association indexes is probably an attribute of virulence which enables them to colonize the enterotoxin-sensitive small intestine of intact pigs more readily than NEEC. Variations in resistance of pigs to such association may be a reason for differences in susceptibility of pigs to enteric colibacillosis.

ACKNOWLEDGMENT

We thank G. D. Booth for the statistical analyses.

LITERATURE CITED

1. Bertschinger, H. U., H. W. Moon, and S. C. Whipp. 1971. Association of *Escherichia coli* with the small intestinal epithelium. I. Comparison of enteropathogenic and non-enteropathogenic porcine strains in pigs. Infect. Immun. 5:595–605.
2. Brambell, F. W. R. 1970. The transmission of passive immunity from mother to young. North-Holland Research Monographs Frontiers of Biology, vol. 18. North-Holland Publishing Co., Amsterdam.
3. Formal, S. B., H. L. Dupont, R. Hornick, N. J. Snyder, J. Libonati, and E. H. LaBrec. 1971. Experimental models in the investigation of the virulence of dysentery bacilli and *Escherichia coli*. Ann. N. Y. Acad. Sci. 176:190–196.
4. Freter, R. 1969. Studies of the mechanisms of action of intestinal antibody in experimental cholera. Texas Rep. Biol. Med. Suppl. 1 27:299–316.
5. Gyles, C. L., and D. A. Barnum. 1967. *Escherichia coli* in ligated segments of pig intestine. J. Pathol. Bacteriol. 94: 189–194.
6. Gyles, C. L. 1971. Heat-labile and heat stable forms of the enterotoxin from *E. coli* strains enteropathogenic for pigs. Ann. N. Y. Acad. Sci. 176:314–322.
7. Moon, H. W., and S. C. Whipp. 1971. Systems for testing the enteropathogenicity of *Escherichia coli*. Ann. N.Y. Acad. Sci. 176: 197–211.
8. Moon, H. W., S. C. Whipp, G. W. Engstrom, and A. L. Baetz. 1970. Response of the rabbit ileal loop to cell-free products from *Escherichia coli* enteropathogenic for swine. J. Infect. Dis. 121:182–187.
9. Moon, H. W., and S. C. Whipp. 1970. Development of resistance with age by swine intestine to effects of enteropathogenic *Escherichia coli*. J. Infect. Dis. 122:220–223.
10. Myers, W. L., and D. Segre. 1963. The immunologic behavior of baby pigs. III. Transplacental transfer of antibody globulin in swine. J. Immunol. 91:697–700.
11. Smith, H. W., and S. Halls. 1967. Observations by the ligated intestinal segment and oral inoculation methods on *E. coli* infections in pigs, calves, lambs, and rabbits. J. Pathol. Bacteriol. 93:499–529.
12. Smith, H. W., and S. Halls. 1968. The production of edema disease and diarrhea in weaned pigs by the oral administration of *Escherichia coli*: factors that influence the course of the experimental disease. J. Med. Microbiol. 1:45–59.
13. Smith, H. W. 1970. *E. coli*: a wolf in sheep's clothing. N. Scient. 47:26–27.
14. Sweeney, E. J. 1968. *Escherichia coli* in enteric disease of swine: observations on herd resistance. Irish Vet. J. 22: 42–46.
15. Ujiiye, A., and K. Kobari. 1970. Protective effect on infections with *Vibrio cholerae* in suckling mice caused by the passive immunization with milk of immune mothers. J. Infect. Dis. (Suppl.) 121:50–55.