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K99 Surface Antigen of *Escherichia coli*: Antigenic Characterization

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K99 prepared by acid precipitation hemagglutinated guinea pig erythrocytes, whereas K99 prepared by chromatography on diethylaminoethyl-Sephadex did not. K99 purified by either procedure hemagglutinated horse erythrocytes. K99 prepared by acid precipitation contained a second antigen not present in the K99 prepared by chromatography on diethylaminoethyl-Sephadex. This antigen could be detected by immunoprecipitation with some, but not all, sera prepared against K99-positive *Escherichia coli* strains. It was assumed that this second antigen is not K99 and is responsible for the guinea pig erythrocyte hemagglutination reaction. Furthermore, the second antigen has an isoelectric point of 4.2, which has been reported by Morris and co-workers to be the isoelectric point of K99.

K99 is a pilus found on most, if not all, enterotoxigenic *Escherichia coli* that cause diarrheal disease in neonatal calves (4, 8, 10, 11, 15; R. E. Isaacson, H. W. Moon, and R. A. Schneider, Am. J. Vet. Res., in press). It is likely that in vivo K99 confers an adhesive ability to *E. coli* cells which produce it and thus promotes their colonization of the small intestine (16). In vitro, K99 is directly involved in adhesion of K99-positive *E. coli* to isolated porcine intestinal epithelial cells (6) and bovine small intestinal brush borders (1). K99 is also produced by enterotoxigenic *E. coli* strains which cause diarrhea in neonatal lambs (16) and by some enterotoxigenic *E. coli* which cause diarrhea in neonatal pigs (9).

Purified K99 is composed primarily of protein subunits of 22,500 and 29,500 daltons and has an isoelectric point of 10 (5). The purified pili do not hemagglutinate guinea pig erythrocytes. Recently, Morris et al. reported that K99 isolated in their laboratory hemagglutinated guinea pig erythrocytes (12) and had an isoelectric point of 4.2 (13). However, the *E. coli* strain used by Morris et al. was not the same one used in this laboratory, although both strains possess the same K99 plasmid. Burrows et al. (1) reported that cell-free K99 hemagglutinated sheep erythrocytes. Ørskov et al. (14) reported that some but not all K99-positive *E. coli* strains caused guinea pig erythrocytes to hemagglutinate, whereas Tixier and Gonet (17) reported that K99-positive enterotoxigenic *E. coli* hemagglutinated horse erythrocytes.

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The purpose of this report is to describe the results of experiments which investigated the apparent discrepancy in isoelectric points and hemagglutination properties of K99 prepared in different laboratories and from different strains.

MATERIALS AND METHODS

K99 preparations. K99 was purified from *E. coli* K12 strain 1474 and from enterotoxigenic *E. coli* strain B41 by the diethylaminoethyl (DEAE)-Sephadex procedure previously described (5) (referred to as DEAE-K99-1474 and DEAE-K99-B41, respectively) and by a series of five acid precipitations as described by Morris et al. (12) (referred to as acid-K99-1474 and acid-K99-B41, respectively). K99 isolated from *E. coli* strain B41 by acid precipitation was also obtained from J. Morris.

Sera. Hyperimmune rabbit sera against *E. coli* strains 1474 (O^-:K99), 431 (O101:K30,99), 637 (O64:K^+, 99), and 1351 (O101:K30,99) were prepared by the procedure of Edwards and Ewing (2). Standard absorbed anti-K99 serum was prepared by absorption of rabbit serum against *E. coli* strain 1474 with *E. coli* strain 1475, the $K99^-$ isogenic parent of strain 1474 (5, 10). Other serum absorptions were done as described by Edwards and Ewing (2). K99 was assayed by the double diffusion procedure (5), using these sera.

Hemagglutination. The microhemagglutination test described by Jones and Rutter (7) was employed, using washed erythrocytes obtained from guinea pigs, sheep, and horses. All reactions were run in the presence of 0.5% D-mannose. Serum inhibition of K99-mediated hemagglutinations were performed by mixing a constant amount of serum with an equal volume of diluted K99 (twofold dilutions) and incubating for 30 min at room temperature. Washed erythrocytes were added and incubated as for the microhemagglutination procedures.

Electrophoretic procedures. Sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Weber and Osborn (18). Gels from SDS-PAGE were stained with Coomassie brilliant blue (3). Isoelectric focusing in pH 3 to 10 gradients was as previously described (5).

RESULTS

Hemagglutination. Hemagglutination of guinea pig, sheep, and horse erythrocytes was attempted by using DEAE-K99-1474 and acid-K99-B41 (prepared by J. Morris). DEAE-K99-1474 did not hemagglutinate guinea pig erythrocytes, whereas the acid-K99-B41 did (Table 1). *E. coli* strains 1474, 1475 (the K99$^-$ parent of 1474), and B41 all caused guinea pig erythrocytes to hemagglutinate. When sheep erythrocytes were used, both K99s acted as hemagglutinins (Table 1). The acid-K99-B41-mediated reaction was not temperature dependent, whereas the DEAE-K99-1474-mediated reaction occurred only in the cold. Both K99s also hemagglutinated horse erythrocytes (Table 1). Pretreatment of K99 with standard absorbed anti-K99 serum reduced the horse erythrocyte hemagglutination by seven wells (twofold dilutions in microtiter plates) for both types of K99 (Table 2). However, this serum did not reduce the hemagglutination of guinea pig erythrocytes by acid-K99-B41.

TABLE 1. *Hemagglutination of erythrocytes by K99*

K99 prepn[a]	Hemagglutination with erythrocytes from:		
	Guinea pig	Sheep	Horse
Acid-K99-B41	1,024[b]	2,048	2,048
DEAE-K99-1474	0	256	4,096

[a] Acid-K99 (23 mg/ml of protein) was obtained from J. A. Morris. DEAE-K99 (2 mg/ml) was as described in the text.

[b] Reciprocal of the highest twofold dilution of K99 to yield a positive hemagglutination reaction.

TABLE 2. *Serum inhibition of K99-mediated hemagglutinations*

K99 prepn[a]	Serum (1:60 dilution)	Hemagglutination with erythrocytes from:	
		Guinea pig	Horse
Acid-K99-B41	Normal rabbit	2,048[c]	2,048
	Standard absorbed anti-K99	2,048	16
DEAE-K99 1474	Normal rabbit	0	4,096
	Standard absorbed anti-99	0	32

[a] Acid-K99 (23 mg/ml of protein) was obtained from J. A. Morris. DEAE-K99 (2 mg/ml) was as described in the text.

[b] Serum and K99 (twofold dilution) were preincubated and then mixed with erythrocytes. Number is the reciprocal of highest twofold dilution of K99 to yield a positive hemagglutination reaction.

Electrophoretic properties of various K99 preparations. The K99 from *E. coli* strains 1474 and B41, purified by both procedures, and the K99 (B41) from J. Morris, acid precipitated five times, were compared by SDS-PAGE. DEAE-K99 from both strains yielded identical patterns. Two Coomassie brilliant blue-stained bands were present, corresponding to molecular weights of 22,500 and 29,500. The three acid-K99 preparations yielded somewhere between 5 and 10 Coomassie brilliant blue-stained bands. The bands tended to run together, preventing an accurate determination of the number of components.

All five samples were also analyzed by isoelectric focusing in pH 3 to 10 gradients. The two DEAE-K99 samples focused as a single band at pH 10 (Fig. 1A). The only K99 activity detected in the entire gradient was also at pH 10. On the other hand, the three acid-K99 samples focused into four discrete bands (Fig. 1B), and K99 activity was only found at pH 10 when standard absorbed anti-K99 serum was used.

Antigenicity. Because the K99 prepared by Morris et al. had an isoelectric point of 10 in our laboratory and was reported to have an isoelectric point of 4.2 (a pH 4.2 band was observed [13]), it was possible that the discrepancy was

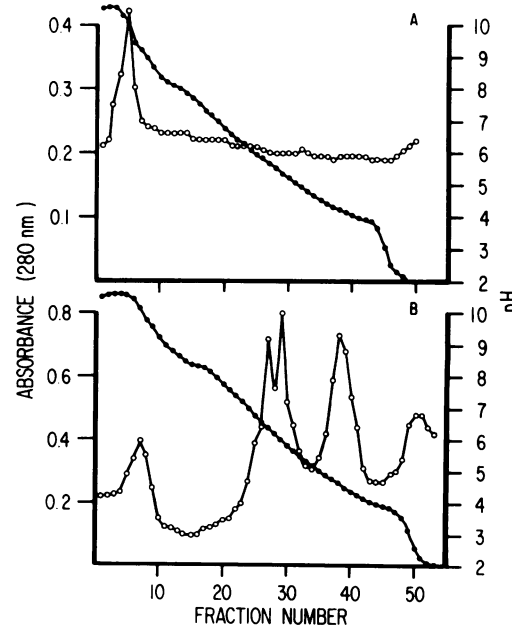

FIG. 1. *Isoelectric focusing of DEAE-K99-B41 (A) and acid-K99-B41 (B). Conditions are described in the text.*

due to differences in the sera used to detect K99. Using unabsorbed sera against four K99-positive *E. coli* strains (431, 637, 1351, and 1474), double diffusion assays were run with DEAE-K99-1474 and acid-K99-B41 of Morris (Fig. 2). Sera against strains 637 and 1474 yielded a single precipitin line with both K99s. Sera against strains 431 and 1351 produced the same precipitin lines, but also had an additional nonidentical precipitin line with the acid-K99-B41. The 431 and 1351 sera were then used to reassay the fractions from the isoelectric focusing experiments. The DEAE-K99 fractions still had activity only at pH 10. However, the acid-K99 fractions had activity at pH 4.2 and 10.

If the pH 4.2 material (presumably the second precipitin line in double diffusion when anti-431 or -1351 sera were used) is K99, then absorption of sera with this activity by K99-positive *E. coli* strains should remove it. Anti-431 serum was absorbed with *E. coli* strains 431 and B41 until agglutinating activity was removed. Each strain was grown at 18°C (a temperature which represses K99 expression) and at 37°C. When cells grown at 18°C were used to absorb the serum, no changes in the precipitin lines in double diffusion were observed (Fig. 3). However, when the cells were grown at 37°C, the precipitin band identical to the one that was formed when standard absorbed anti-K99 serum was used disappeared. The second precipitin line was not absorbed out. When DEAE-K99 was used, precipitin bands were formed only with the serum absorbed with the cells grown at 18°C.

DISCUSSION

K99s purified by the two published procedures (5, 12) are hemagglutinins. The spectra of hemagglutination of erythrocytes from various animals do differ. DEAE-K99 hemagglutinates sheep erythrocytes in the cold and horse erythrocytes, whereas acid-K99 hemagglutinates guinea pig, sheep, and horse erythrocytes with no temperature effect. Inclusion of standard absorbed anti-K99 serum in the hemagglutination mixtures inhibits the horse erythrocyte reaction for DEAE-K99 and acid-K99, but has no effect on the guinea pig reaction with acid-K99. This result is consistent with the conclusion that the acid-K99 preparations contain a second antigen which is responsible for the guinea pig erythrocyte hemagglutination. Also consistent with this conclusion is the observation by Morris et al. that the isoelectric point of acid-K99 is 4.2 (13). SDS-PAGE of acid-K99 results in the separation of 5 to 10 Coomassie brilliant blue-stained components. One or more of these components, other than the previously described K99 components, could easily be the guinea pig erythrocyte hemagglutinin. The lack of K99 activity at pH 4.2 in isoelectric focusing experiments when acid-K99 is used also suggests that the standard absorbed anti-K99 serum does not have antibodies specific for the second antigen.

The use of several sera prepared against different K99-positive *E. coli* strains in double diffusion experiments revealed that some sera contained precipitating activity against a second component of the acid-K99 (different from the antigen precipitated by standard absorbed anti-K99 serum). This precipitating activity, however, is not universal. Sera against other K99-positive *E. coli* strains do not possess precipitating activity for the second antigen. When sera with the two precipitating activities were used, a second antigen was identified at pH 4.2 in isoelectric focusing experiments of acid-K99.

The question remains as to whether the two antigens in acid-K99 that were identified by double diffusion and in isoelectric focusing experiments are both K99. The results of the ab-

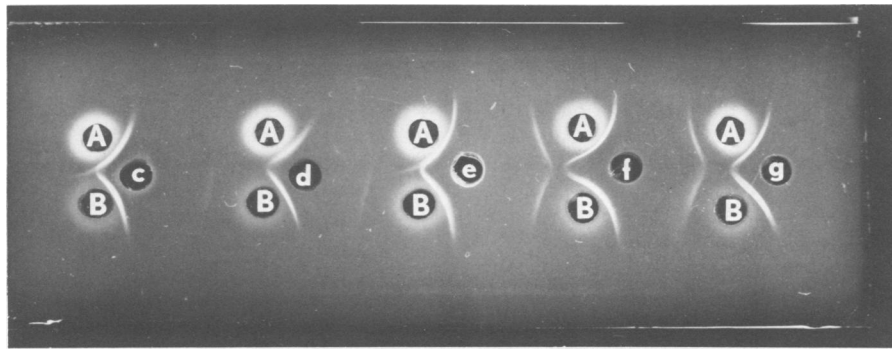

FIG. 2. *Assay of acid-K99-B41 prepared by J. Morris (A) and DEAE-K99-1474 (B) with sera prepared against E. coli strains 431(c), 637(d), 1351(e) and 1474(f) and standard absorbed anti-K99 serum (g).*

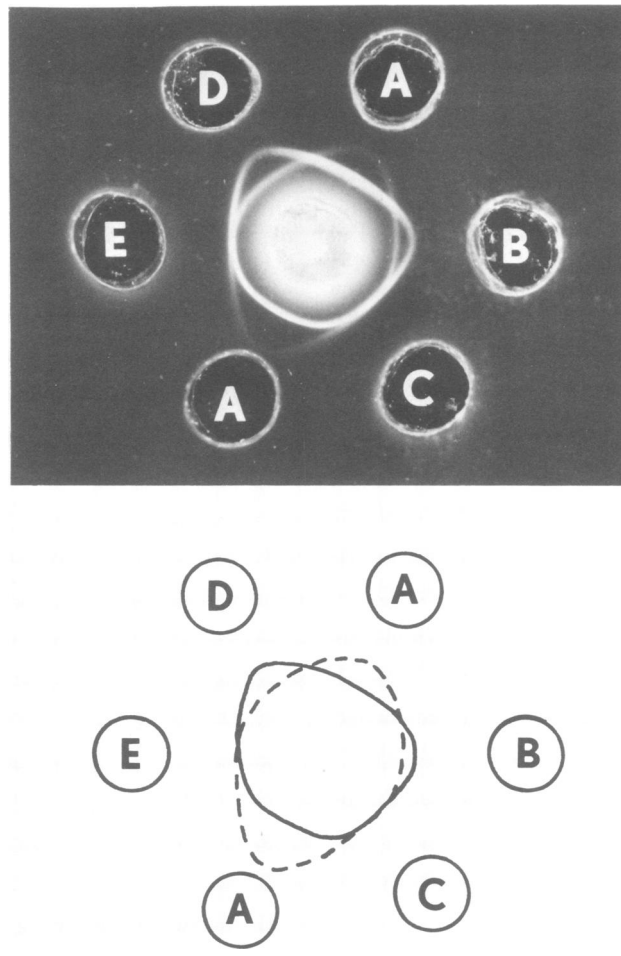

FIG. 3. *Assay of acid-K99-B41 with standard absorbed anti-K99 serum (A), or anti-431 serum absorbed with B41 cells grown at 37°C (B) or 18°C (C) or absorbed with 431 cells grown at 37°C (D) or 18°C (E). The diagram of the precipitin pattern is included as an aid in identifying the precipitin lines.*

sorption experiments described when serum prepared against the K99-positive *E. coli* strain 431 was used provide evidence that the second antigen (the anionic component, with an isoelectric point of 4.2) in the acid-K99 is not K99. Cells grown at a temperature where K99 is expressed (37°C) did not remove the precipitating activity against the second antigen from the serum. This result was obtained even when the absorbing *E. coli* strain was B41, the strain from which the two antigens were extracted during acid purification of K99. One explanation of the results is that the second antigen (anionic component) is a non-K99 antigen which is extracted from *E. coli* strain B41 at 60°C and is concentrated by acid precipitation. That this component is not an exposed surface antigen is demonstrated by the inability of whole cells to absorb antibodies against it from serum. Furthermore, not all K99-positive *E. coli* strains express this antigen. Other explanations include the possibility that the *E. coli* strains used in the absorption were not grown properly to express the antigen. Also, it is possible that the anionic component is buried in the cell envelope and is responsible for attachment of the pilus structure to the cell surface. Thus, it would not be available for serum absorption, could be antigenically different from the exposed antigen, and could still be part of the total K99 structure.

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