A STUDY OF ANTIGENIC STRUCTURE OF *ESCHERICHIA COLI* $O_{111}B_4H_2$ POSSESSING β ANTIGEN

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The reports of isolation of *Escherichia coli* serotypes $O_{111}B_4$ and $O_{55}B_5$ from sporadic cases and outbreaks of diarrhoea in various localities in Australia (Williams, 1951; Singer & Ludford, 1953; Mushin, 1953) stimulated further search for these organisms. Their significance in infantile gastro-enteritis has been stressed by a number of workers, and recently attention has again been focused on the few serotypes suspected of close association with intestinal infections (Le Minor, Le Minor, Nicolle & Buttiaux, 1954; Ørskov, 1954*a*, *b*; Seeliger, 1954).

In an earlier paper (Mushin, 1949) the presence of a thermolabile surface antigen β was demonstrated in some Gram-negative faecal bacteria, including coliform, paracolon, proteus and shigella strains and the relationship of this factor to both somatic and flagellar types of antigens was described. The present communication is a study of the β antigen in certain cultures of *Esch. coli*. Kauffmann (1951), in his account of the Escherichia types of infantile enteritis, describes two serotypes to which he gives the antigenic formulae $O_{111}B_4$ [H_2] and $O_{111}B_4H_{12}$, the square bracketed H_2 indicating that motile and non-motile forms may occur. Although it was not possible to prove conclusively the presence of the B antigen as a separate entity, Kauffmann considered there was sufficient evidence for its recognition, and his classification has been followed for the description of the strains of *Esch. coli* $O_{111}B_4$ [H_2] with which this paper is concerned.

MATERIALS AND METHODS

A strain of *Esch. coli* $O_{111}B_4H_2$ was kindly supplied by Dr Joan Taylor, a non-motile culture *Esch. coli* $O_{111}B_4$ Stoke W and antiserum by Dr Kauffmann, *Esch. coli* $O_{55}B_5H_2$ and $O_{43}KH_2$ and corresponding antisera by Dr Joyce Wright. Our serotype *Esch. coli* $O_{111}B_4H_2$ was isolated from a small local outbreak of gastro-enteritis in a babies hospital ward, and its characteristics have been described (Mushin, 1953). As shown previously (Mushin, 1953) and in the present experiments, our *Esch. coli* $O_{111}B_4H_2$ and the strain received from Dr Taylor were identical. Also the antisera prepared in our laboratory from the two strains behaved similarly in agglutination and agglutinin-absorption tests, and therefore no distinction need be made when referring to the strains and antisera from the two different sources. The β cultures and β antisera were from our own collection.

The serological methods employed for the preparation of the so-called OB and the O antigens followed the methods recommended by Kauffmann (1951). The antigen for the first named was a living suspension in saline from an 18 hr. agar culture, and the second was a broth suspension which had been steamed for 2 hr.

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Fresh-living suspensions and steamed broth suspensions respectively served as the inocula for the homologous antisera. The β antigen was either a living broth suspension or a saline suspension from an agar culture. The β antiserum was prepared in accordance with the description already published (Mushin, 1949).

β agglutination tests were carried out in Dreyer tubes in a 52° C. water-bath and the results read after 10 min. The other test with living suspensions was carried out in round-bottom tubes incubated at 37° C. for 2 hr. followed by 18 hr. at room temperature before reading. The agglutination tests with the heated broth suspension were read after 20 hr. in a water-bath at 52° C.

The agglutinin-absorption tests were carried out by allowing the various mixtures of bacterial suspensions and antisera to react in a water bath at 37° C. for two 1 hr. periods and then left overnight at +4° C. before centrifugalization and testing the supernatant for unabsorbed agglutinins.

RESULTS

Slide-agglutination tests

It was observed that various β strains were agglutinated by $O_{111}B_4H_2$ antisera and not by $O_{111}B_4$ Stoke W, and Table 1 illustrates the results of these tests.

Table 1. *Slide-agglutination tests*

Antisera		Agglutination of living suspensions of		
Type	Source	Paracolon 30 β and various β strains	*Esch. coli* $O_{111}B_4H_2$	*Esch. coli* $O_{111}B_4$ (Stoke W)
β	Paracolon 30 β	+ + +	−	−
OB	*Esch. coli* $O_{111}B_4H_2$	+ + +, + +	+ + +	+ + +
OB	*Esch. coli* $O_{111}B_4$ (Stoke W)	−	+ + +	+ + +

+ + + denotes complete agglutination, + + incomplete, − none.

The agglutination with $O_{111}B_4$ sera was a typical β reaction, rapid and floccular, and suggested the presence of β antibody in these sera. It should be noted, however, that *Esch. coli* $O_{111}B_4H_2$ strains did not agglutinate in 30 β antiserum. This could not be due to the dissociation of $O_{111}B_4H_2$ cultures into β minus forms because negative results were constantly obtained.

Tube-agglutination tests

Reactions in Table 2 are typical of many tests with subcultures of *Esch. coli* $O_{111}B_4H_2$ and with various β organisms.

Some results were of special interest. It will be seen that β (living) suspensions of paracolon 30 were agglutinated at high titre as abundant floccular aggregates with OB antisera from *Esch. coli* $O_{111}B_4H_2$ and with β antisera after 10 min. at 52° C., thus demonstrating once again the presence of β antibodies in $O_{111}B_4H_2$ sera. However, the living suspensions of *Esch. coli* $O_{111}B_4H_2$ were not agglutinated by β diagnostic serum which confirmed the results of slide-agglutination tests.

The inagglutinability of the living suspension of *Esch. coli* $O_{111}B_4H_2$ by its homologous O serum is in contrast to the result obtained with the non-motile strain (Stoke W). Kauffmann (1951) indicated that O-agglutinability of these types was variable, but it is of particular interest to note that the serum prepared from the living strain of $O_{111}B_4H_2$ agglutinated paracolon 30 β, whereas the serum from the strain Stoke W failed to react. Whether the variation in agglutinability of the living suspensions of these strains of *Esch. coli* by the homologous O serum is associated with the presence or absence of the β antigen in the particular culture being examined it is not possible to say without extended experience, but the observations appear to be of sufficient interest to record. Living suspensions of paracolon 30 β were not agglutinated by the homologous O antiserum, but some β strains, other than paracolon 30 β, have been found to react to a high titre with their homologous O sera (Mushin, 1949).

Table 2. *Tube-agglutination tests of living and steamed suspensions after* 10 *min. and* 20 *hr.*

Antisera		Agglutination titre against suspension of								
		Paracolon 30 β			*Esch. coli* $O_{111}B_4H_2$			*Esch. coli* $O_{111}B_4$ (Stoke W)		
Type	Source	Living 10 min.	Living 20 hr.	Steamed 20 hr.	Living 10 min.	Living 20 hr.	Steamed 20 hr.	Living 10 min.	Living 20 hr.	Steam 20 h
β	Paracolon 30 β	10,240	10,240	−*	−	−	−	−	−	−
OB	*Esch. coli* $O_{111}B_4H_2$	5,120	5,120	−*	−	320	5,120	−	320	5,1
OB	*Esch. coli* $O_{111}B_4$ (Stoke W)	−	−	−	−	320	5,120	−	320	5,1
O	Paracolon 30 β	−	−	2,560	−	−	−	−	−	−
O	*Esch. coli* $O_{111}B_4H_2$	−	−	−	−	−	5,120	−	80	5,1

Titres are expressed as reciprocals of serum dilutions.
No agglutination at 40 or less is recorded as −.
* In some tests low titre atypical agglutination was observed (see text).

O (steamed) suspensions of paracolon 30 β were not agglutinated by $O_{111}B_4$, and β antisera or in some tests an atypical reaction took place, the agglutinate resembling a small clump of cotton-wool. This was in line with the results of earlier experiments (Mushin, 1949), which indicated the complex character of β antigen containing a thermolabile factor, and occasionally also a more resistant component which gave the atypical 'cotton-wool clump' agglutination.

On the basis of the above experiments one must postulate that the agglutination of β suspensions with sera from *Esch. coli* $O_{111}B_4H_2$ was due to the presence of β antibodies in these sera and not to O, B or H antibodies. This was supported by the following evidence: (i) No agglutination was obtained with living and steamed suspensions of paracolon 30 β against antiserum from *Esch. coli* $O_{111}B_4$ Stoke W (Table 2), thus showing no reaction with O and B antibodies. An additional test was performed with O antiserum from *Salmonella adelaide* in which antigen XXXV was proved to be almost identical with somatic antigen $O_{111}B_4$ (Kauffmann, 1952;

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Olarte & Varela, 1952) and again no agglutination was recorded. (ii) The absence of H_2 antibodies in $O_{111}B_4$ sera was shown by negative reactions with actively motile *Esch. coli* strains $O_{55}B_5H_2$ and $O_{43}KH_2$. Beside, β suspensions of paracolon 30 used as diagnostic antigen were non-motile after ten successive passages through semi-solid agar in Craigie tubes at 22° C. and did not agglutinate with $O_{55}B_5H_2$ and $O_{43}KH_2$ antisera.

Agglutinin-absorption tests

The results of absorption tests with OB antisera from *Esch. coli* $O_{111}B_4H_2$ are presented in Table 3.

Table 3. *Agglutination tests with unabsorbed and absorbed OB antiserum from* Esch. coli $O_{111}B_4H_2$

		Agglutination titre against suspension of			
		Esch. coli $O_{111}B_4H_2$		Paracolon 30	
OB antiserum from *Esch. coli* $O_{111}B_4H_2$		OB	O	β	O
Unabsorbed		320	2,560	10,240*	–
Absorbed with suspension					
Type	Source				
OB	*Esch. coli* $O_{111}B_4H_2$	–	–	–	–
O	*Esch. coli* $O_{111}B_4H_2$	–	–	640*	–
β	Paracolon 30 β	320	2,560	–	–
O	Paracolon 30 β	320	2,560	640*	–

* Denotes reading after 10 min. at 52° C.

Absorption of OB antiserum with the homologous OB suspensions was complete and removed agglutinins for OB and O suspensions of *Esch. coli* and for β suspensions of paracolon 30. Absorption with O suspensions of *Esch. coli* left β antibodies in the serum, thus giving a positive test with paracolon 30 β but to a lower titre and negative reactions with *Esch. coli* O and OB suspensions. This conforms with results of tests presented in Table 2 and shows that β antigen in paracolon 30 β was active and in *Esch. coli* $O_{111}B_4H_2$ inactive in agglutination reactions.

Absorption of OB antiserum with β suspensions of paracolon 30 removed β but failed to remove B and O antibodies as shown by positive tests with *Esch. coli* OB and O antigens. Absorption with O suspensions of paracolon 30 β left B and O antibodies and also β antibodies, but at a lower titre.

Results of agglutinin-absorption tests with β antiserum from paracolon 30 presented in Table 4 show that β antibody was removed completely by absorption with paracolon 30 β suspensions and markedly reduced with living suspensions of the *Esch. coli* but not by heated suspensions of these organisms.

The above tests confirmed the presence of β antibodies in sera prepared from these particular cultures of *Esch. coli* $O_{111}B_4H_2$. The presence of β antigen in these organisms could not be demonstrated by direct agglutination tests, but was shown in an indirect manner by its ability to produce and to absorb β antibodies.

EXPERIMENTS WITH FEEDING AND INOCULATING RABBITS USING β AND β MINUS ORGANISMS

In a personal communication Dr Taylor suggested that the presence of β antibody in the OB antisera from *Esch. coli* $O_{111}B_4H_2$ could have been due to 'some antigenic stimulus from organisms present in the gut' of rabbits. It seemed worth while to follow this line of thought, and an experiment was planned to throw light on the subject.

Table 4. *Agglutination tests with unabsorbed and absorbed β antiserum from paracolon* 30 *β*

		Agglutination titre against suspension of			
		Paracolon 30 β		*Esch. coli* $O_{111}B_4H_2$	
Antiserum from paracolon 30 β		β	O	OB	O
Unabsorbed		10,240*	–	–	–
Absorbed with suspension					
Type	Source				
β	Paracolon 30 β	–	–	–	–
O	Paracolon 30 β	5,120*	–	–	–
OB	*Esch. coli* $O_{111}B_4H_2$	160*	–	–	–
O	*Esch. coli* $O_{111}B_4H_2$	5,120*	–	–	–

* Denotes reading after 10 min. at 52° C.

Rabbits were selected for the experiment after test bleedings had been examined to ensure the absence of natural antibodies to β and any of the antigens of *Esch. coli* O_{111}.. In addition, faecal specimens of the rabbits were plated out on sheep-blood agar, plain deoxycholate agar and SS ('Difco') medium and representative colonies were tested. Four rabbits, whose faecal flora were free of Gram-negative bacilli reacting with $O_{111}B_4$ and β sera, were selected. The following procedure was used:

Rabbit 1—fed once per week for 6 weeks with 1 ml. of living *Esch. coli* $O_{111}B_4H_2$ suspensions containing 1000×10^6 organisms mixed with a small amount of meal.

Rabbit 2—fed as above and given once a week intravenous injections with increasing doses of O (steamed) suspensions of *Esch. coli* $O_{111}B_4H_2$.

Rabbit 3—injected intravenously once a week with increasing doses of OB (living) suspensions of *Esch. coli* $O_{111}B_4H_2$.

Rabbit 4—injected as rabbit 3 with O (steamed) suspensions of *Esch. coli* $O_{111}B_4H_2$.

A day before each injection or feeding and on two consecutive days after, faecal specimens from rabbits were cultured on sheep-blood agar, plain deoxycholate agar and SS medium and their rectal temperatures taken. Agglutination tests with sera so obtained were carried out as indicated previously.

The rectal temperatures fluctuated between 98 and 102° F. After the feeding tests *Esch. coli* $O_{111}B_4H_2$ organisms appeared in faeces for a day or two and occasionally longer and then disappeared, hence there was no evidence of persistent infection.

The results in Table 5 show that β antibodies appeared only in serum of rabbit 3, indicating that β antibodies found in OB antisera from *Esch. coli* $O_{111}B_4H_2$ strains have formed in response to β antigen present in the suspensions.

Table 5. *Agglutination titres of sera of rabbits fed or immunized with β and β minus bacilli*

		Agglutination titres against suspensions of		
		Paracolon 30β	*Esch. coli* $O_{111}B_4H_2$	
Rabbit	Treatment	β	OB	O_{111}
1	Fed with *Esch. coli* $O_{111}B_4H_2$	–	–	–
2	{Fed with *Esch. coli* $O_{111}B_4H_2$ / Injected with *Esch. coli* O_{111}}	–	–	5,120
3	Injected with *Esch. coli* $O_{111}B_4H_2$	10,240	1,280	5,120
4	Injected with *Esch. coli* O_{111}	–	–	10,240

DISCUSSION

The presence of β antibodies in antisera prepared by immunization of rabbits with two strains of *Esch. coli* $O_{111}B_4H_2$ is of interest in the study of the antigenic pattern of this organism. These antibodies have been demonstrated (Mushin, 1949) in the normal sera of some rabbits and this must obviously be considered in the interpretation of experimental results. In the present work this aspect has been excluded because the sera of rabbits were tested with β suspensions as a routine procedure prior to inoculation. The suggestion that β antibodies were due to the presence of β forms in the intestine of the experimental rabbits was also eliminated (Table 5). Therefore it became evident that the β antibodies in our antisera appeared in response to β antigen present in two strains of *Esch. coli* $O_{111}B_4H_2$.

We have also found β antibodies at a low 1:160 titre in a diagnostic $O_{111}B_4$ serum kindly provided a few years ago by Lieut.-Col. Bensted. Dr Joyce Wright (private communication) tested some of her *Esch. coli* antisera with our β strains and obtained agglutination reactions. Also Dr Hall (private communication) found antibodies against β antigen in $O_{111}B_4$ sera used in his laboratory.

There are difficulties in demonstrating β factor. β antigen-antibody reaction is apparently ineffective in agglutinating *Esch. coli* $O_{111}B_4H_2$ strains (Tables 1 and 2), but the reaction is demonstrated indirectly by absorption of β antibodies with these organisms (Tables 3 and 4).

Another difficulty is the unpredictable dissociation of β into β minus forms. Thus only OB antisera from some *Esch. coli* $O_{111}B_4H_2$ strains will contain β antibodies or they may be found in a titre much lower than in our antisera. Comparison with some other surface antigens is of interest. The loss of Vi factor in strains of *Salmonella typhi* is well known, also the disappearance of α antigen, a common agglutinogen found in coliform and paracolon bacilli (Stamp & Stone, 1944). Although OB strains of *Esch. coli* do not dissociate spontaneously (Le Minor *et al.* 1954) it was stated by Hilton & Taylor (1951) that increase in motility of *Esch. coli*

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$O_{111}B_4H_2$ strains by passage through semi-solid agar at 22° C. was associated with decrease in B_4 antigen.

The complex character of β antigen was noticed in heat-inactivation tests (Mushin, 1949) as some components were apparently more labile than others. In a large number of tests performed with various β strains it became evident that although the agglutinin-binding capacity was impaired by heat, occasionally conflicting results were obtained. It is possible that several antigens contribute to the 'β antigen' complex. Felix (1952), in his studies on *Salm. typhi* antigen, suggested that 'the physico-chemical behaviour of an antigenic substance, in this case the TVi antigen, may vary as the result of the presence in, or absence from, the bacterial cell of some other substance, which itself may be either antigenic or non-antigenic in nature'. β antigen may be similar. Some β forms are highly agglutinable but the β antigen in *Esch. coli* $O_{111}B_4H_2$ cell is inactive while still showing undoubted antigenicity as evidenced by β antibody response (Tables 1 and 2).

Agglutination tests with living suspensions and homologous O antisera may also show variation in results (Table 2), and warrants further investigation. This is, however, outside the scope of the present paper.

With the growing knowledge of the serology of coliform bacilli it may be expected that new antigenic factors will be encountered. So far, about 120 somatic O, over 60 surface K and over 30 flagellar H antigens have been identified (Kauffmann, 1954). New relationships between various serogroups have been reported by Seeliger (1954).

It is evident from the experiments described that certain strains of *Esch. coli* O_{111} carry the surface antigen β. It has been presumed that the so-called B_4 antigen, so constantly associated with this type, is a separate entity and is also a surface antigen, but proof of this is at present lacking. Until complete evidence is produced the complex antigenic structure of this and probably other similar *Esch. coli* can only be assumed. Whatever may be finally accepted as the general formula for *Esch. coli* O_{111} carrying H_2 antigen it is suggested that it be followed by an additional factor: [β] to indicate that some of these strains may also carry the β antigen.

Because previous work (Mushin, 1949) has shown that the β antigen can be carried by a number of otherwise serologically unrelated organisms its identification in other antigen complexes is a relatively simple laboratory procedure. But the possibility of complete determination of the antigenic structure of many of the coliform bacilli will not be possible until the separate components can be identified individually.

SUMMARY

1. Coliform and paracolon β strains agglutinated to a high titre with OB antisera from two strains of *Escherichia coli* $O_{111}B_4H_2$.

2. The presence of β antigen in some strains of *Esch. coli* $O_{111}B_4H_2$ has been shown and therefore such strains should be classified as plus β forms. This factor could not be demonstrated by agglutination tests but was evident by its ability to produce and absorb β antibodies.

3. The dissociation and complex nature of β forms was discussed.

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