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RAPIDLY INDUCED CHANGES IN THE LEVEL OF NON-SPECIFIC IMMUNITY IN LABORATORY ANIMALS

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MANY species of Gram-negative bacteria are rapidly killed when incubated with fresh normal animal or human serum. There is often, however, considerable inter-strain variation in susceptibility to this killing action within any one bacterial species, and it has been suggested (Maaløe, 1948 ; Rowley, 1954) that this variation may account for inter-strain differences in virulence. The necessary presence in the serum of complement and magnesium ions is established but apart from this the chemical mechanisms involved in this bactericidal action are entirely unknown. It was recently suggested that the bactericidal action of serum involves a reaction between serum and some surface substrate of the organism (Rowley, 1955). Support for this suggestion was given by the finding that the injection into mice of a crude preparation of the cell walls of *Bacterium coli* produced a rapid and striking increase in the susceptibility of the animals to a subsequent challenge by *Bact. coli.* This increased susceptibility lasted only a few hours and was followed 24 hours later by a state of immunity in the animals towards challenge by *Bact. coli* which lasted for about a week. Simultaneously with these changes in susceptibility there were corresponding changes in the bactericidal power of the serum. In view of these observations, attempts have been made to obtain in a more purified state the active principle present in the crude cell wall preparations. This has led to the isolation of a lipopolysaccharide, which probably contains the ultimate substrate for the bactericidal reaction in the serum.

MATERIALS AND METHODS

Strains.—The following strains were used :—

Bact. coli 2380, 2206, 75, 145, K12 (Rowley, 1954).

Salmonella paratyphi B—strain Kroger.

Salmonella typhi murium M206.

Proteus vulgaris, Pseudomonas pyocyanea, Klebsiella pneumoniae isolated from routine culture plates.

Corynebacterium diphtheriae strain P.W.8.

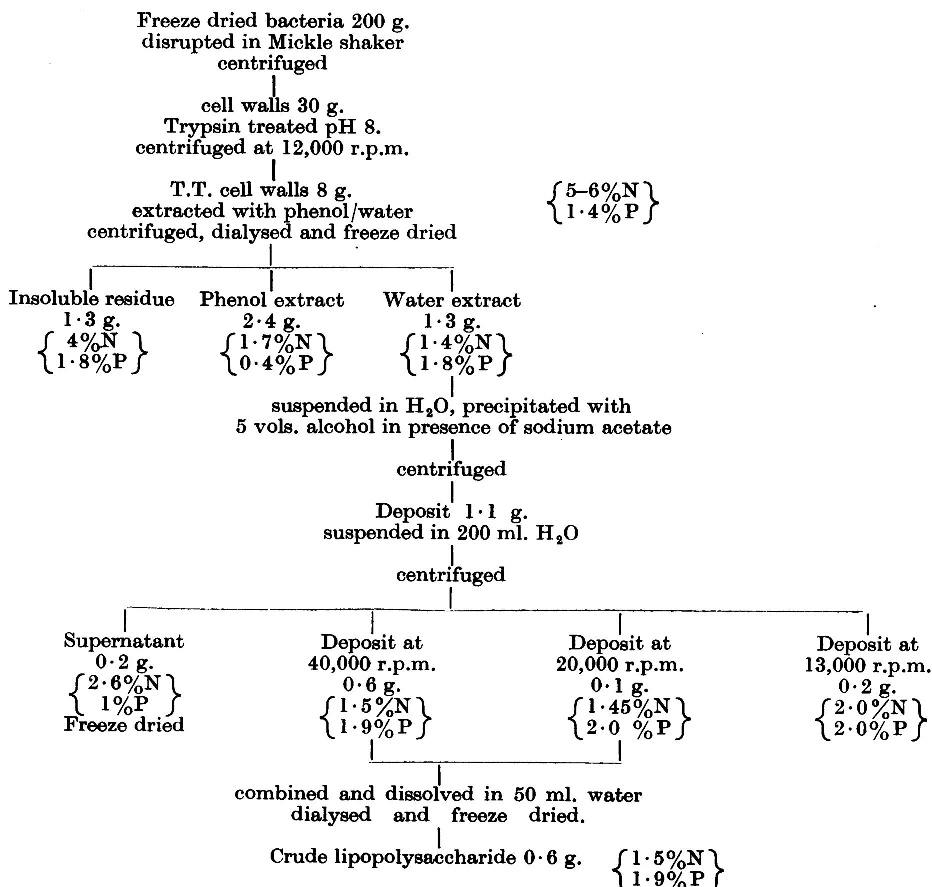
Bacterial extracts.—Bacterial cell wall preparations, made by disintegrating bacteria in a Mickle shaker, were treated with trypsin as previously described (Rowley, 1955). The

preparations from *C. diphtheriae*, *S. typhi murium* etc. were used without further purification, but those from *Bact. coli* (2380 and K12) were subjected to further extraction processes. Two methods were used :

(a) *Diethylene glycol extraction* was by the method described by Morgan (1937). This treatment resulted in 90 per cent by dry weight of the cell wall becoming soluble. After removing the solvent by dialysis, the extract was freeze-dried.

(b) *Extraction with* 90 *per cent phenol water at* 65° after the method of Westphal, Lüderitz and Bister (1952) separated the cell walls into three fractions : (*a*) phenol soluble, (*b*) water soluble, (*c*) insoluble residue. The water-soluble fraction was further purified by precipitation with 90 per cent alcohol in the presence of sodium acetate, the precipitate was suspended in water and centrifuged successively at 13,000 r.p.m. and 20,000 r.p.m., in a Serval high speed angle centrifuge, the supernatant was recentrifuged at 40,000 r.p.m. in a Spinco ultracentrifuge. The deposits at 20,000 and 40,000 r.p.m. which proved to possess the greatest biological activitiy, were mixed and re-suspended in water before dialysis and freeze-drying. A chart of the purification process together with the nitrogen and phosphorus analyses of the various fractions is shown in Table I.

TABLE I.—*Preparation of Crude Bacterial Lipopolysaccharide (after Westphal-Lüderitz)*



For nitrogen and phosphorus analyses the methods described by Kabat and Mayer (1948) were used.

In vitro *bactericidal tests*

Dilutions of the serum were made, using minimal medium (Davis and Mingioli, 1950) as diluent in sterile test tubes, the final volume in each tube being 1 ml.; this was incubated for ½ hr. to reach 37° and 0·02 ml. of a dilution of the culture added, such that each tube contained approximately 1,000 organisms. Counts were made on 0·1 ml. samples from each tube spread on nutrient agar plates at times zero, 20 min., 40 min., and 80 min. from the start, during which time the tubes were kept at 37°. Fig. 1 shows a typical result obtained with fresh rat serum and *Bact. coli* K12. Duplicate counts did not differ by more than 20 per cent.

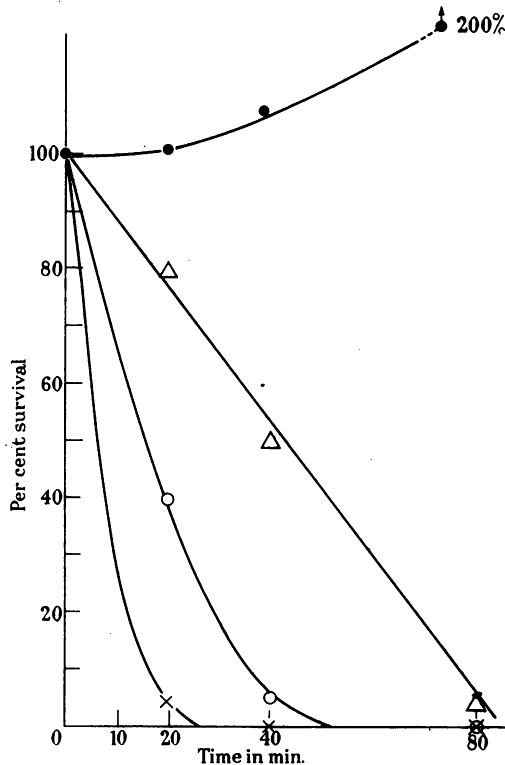


FIG. 1.—Survival of *Bact. coli* K12 in dilutions of rat serum at 37°.
× = neat rat serum and 1/5.
○ = dilution 1/10.
△ = dilution 1/40.
● = diluent (minimal medium).

The inhibitory effect of various bacterial fractions on the bactericidal reaction was shown by incorporating varying amounts of the fraction into a duplicate set of tubes, together with the serum dilutions and organisms as above. Fig. 2 shows how the addition of 0·1 mg. trypsin-treated cell walls from *Bact. coli* (2380) prevented the killing of *Bact. coli* (2206) by fresh guinea-pig serum. This test was done with all the bacterial extracts preliminary to *in vivo* tests.

In vivo *tests*

All materials injected into mice were in a volume of 0·2 ml. and with intravenous injections were given into one of the tail veins ; the challenge organisms were always given by the intraperitoneal route suspended in 1·7 per cent hog mucin and 0·4 per cent charcoal (Rowley,

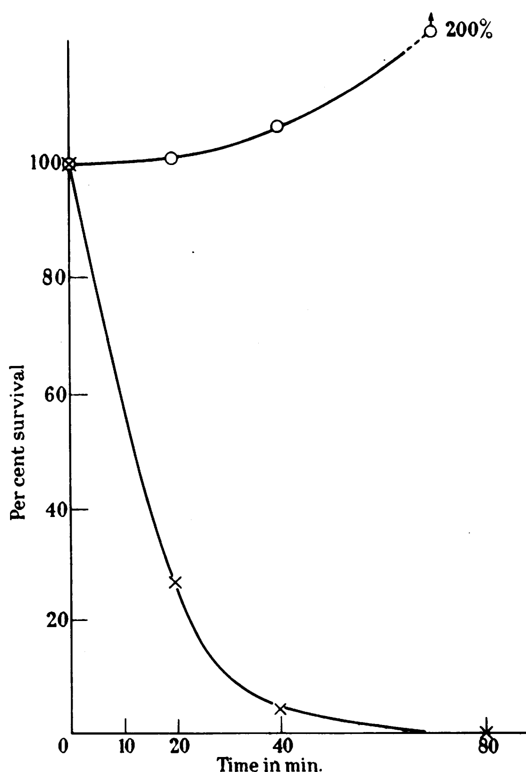


FIG. 2.—Survival rate of *Bact. coli* 2206 in guinea-pig serum with and without the simultaneous addition of 0·1 mg./ml. *Bact. coli* 2380 cell walls.

× = guinea-pig serum alone.
○ = same serum plus 0·1 mg./ml. *Bact. coli* 2380 cell walls.

1954). For viable counts the drop method of Miles and Misra (1938) was used in triplicate on the injected dilutions.

The cumulative results from about 500 mice with each bacterial strain have been used to assess the LD_{50} values by the method of Reed and Muench (1938). These are :

Bact. coli	75 and 145	10^3 organisms	
,,	2380	10^4 ,,	
,,	2206	10^7 ,,	(Rowley, 1954).

RESULTS

Effects of Bacterial Cell Walls on the Susceptibility of Animals to Bact. coli.

Trypsin-treated cell walls of *Bact. coli* 2380 were injected intraperitoneally (i.p.) into mice (0·1 mg./mouse) and the mice challenged within 1 hr. with various strains of *Bact. coli.* Table II shows the results, and it is evident that the mice given cell walls were, at this time, much more susceptible to the experimental infection than were the normal control mice.

On the other hand, when mice were challenged at 24 or 48 hr. after giving an injection of cell wall preparation, a quite different state of affairs existed. Table

TABLE II.—*Effect of Injecting Bacterial Cell Walls on the Immediate Susceptibility of Mice to* Bact. coli *Challenge*

Injected with cell walls.	Time before challenge.	No. of organisms in challenge dose.		Deaths. Treated.	Deaths. Controls.
Bact. coli 2380 0·05–0·1 mg. i.p.	30 min.	*Bact. coli* 2206	2×10^5	19/21*	4/15*
			2×10^4	16/21	2/15
			4×10^3	18/21	0/15
Bact. coli K12R 0·2 mg. i.p.	30 min.	*Bact. coli* 2206	2×10^4	4/6	0/6
			4×10^3	3/6	0/6

* Numerators = numbers of deaths. Denominators = total numbers challenged.

III summates many such experiments, the results of which were completely opposite to those in Table II since the treated mice were now much more resistant than the untreated control animals.

TABLE III.—*Effect of Injecting Trypsin-treated Cell Walls of* Bact. coli 2380 *on the Susceptibility of Mice to* Bact. coli *Challenge* 24–48 *hr. Later*

Dose of cell walls.	Route of injection.	Numbers of organisms in challenge dose. 10^7.	10^6.	10^5.	Percentage deaths.
			Strain 145		
0·1 mg.	i.p.	4/21*	2/21	2/21	12
0·05 mg.	i.v.	6/27	3/27	2/27	13
Controls, Strain 145	—	32/36	30/36	26/36	81
			Strain 75		
0·1 mg.	i.p.	4/11	0/12	0/12	11
Controls, Strain 75	—	12/12	12/12	12/12	100
			Strain 2380		
0·1 mg.	i.p.	3/12	0/12	0/12	8
0·05 mg.	i.v.	0/5	0/5	0/3	0
Controls, Strain 2380	—	9/9	9/9	7/9	93

* Numerator = numbers of deaths. Denominator = total numbers challenged.

This rapidly developing immunity had disappeared by about seven days (see also Field, Howard and Whitby, 1955), its duration depending to some extent on the dose of cell walls initially injected. The antigenic structure of the challenge organism was of no consequence ; from Table III it is seen that cell walls of *Bact. coli* 2380 gave good protection against either the homologous organisms or against two other strains, *Bact. coli* 75 and 145. The latter strains were not typable with any of the usual *Bact. coli* antisera whereas strains 2206 and 2380 belong to type 0111 B4.H12. Moreover, mice immunised with living cells of *Bact. coli* 2206 and challenged 12 days later with strains 2380, 145 and 75 were found to be immune to the antigenically homologous strain 2380 but normally susceptible to strains 145 and 75 (Table IV).

Exactly similar changes in susceptibility to *Bact. coli* challenge could be brought about by the previous injection of the cell walls of *S. typhi murium*, *Ps. pyocyanea*, *P. vulgaris* or *K. pneumoniae*.

TABLE IV.—*Cross Protection Tests with Mice Immunised I.P. with* 10^7 *Organisms of Living* Bact. coli 2206 *and Challenged with Different Strains after* 12 *Days*

Challenge strain.	Number of organisms in challenge dose + 1·75 per cent hog mucin.		
	10^6.	10^5.	10^4.
Bact. coli 145 . . .	3/3	2/3	3/3
Bact. coli 75 . . .	3/3	2/3	3/3
Bact. coli 2380* . .	0/3	0/3	0/3

* Strains 2206 and 2380 are antigenically similar.

This early immunity is not associated with any detectable change in the level of agglutinating antibody to the challenge organisms, nor with the appearance of measurable amounts of whole haemolytic complement in the serum of protected mice.

Although most of the present experiments have involved mice, the same biological changes occurred in other animals. It is not possible to demonstrate protection against *Bact. coli* in rats, since normal animals can withstand 10^7 organisms in mucin ; challenge within a short time of injecting *Bact. coli* cell walls, however, resulted in an increase of susceptibility (Table V). Guinea-pigs being moderately susceptible to challenge with *Bact. coli* + mucin behaved very much like mice in their changes in immunity following administration of trypsin-treated cell walls.

TABLE V.—*Changes in Susceptibility to* Bact. coli 145 *of Guinea-pigs and Rats following Injection of* Bact. coli 2380 *Cell Walls.*

Dose of cell walls.	Time before challenge.	Numbers of organisms in challenge dose.			
		10^7.	10^6.	10^5.	10^4.
Guinea-pigs					
1 mg. i.p. . . .	30 min. .	—	6/6	4/6	5/6
None (controls) . .	— .	—	3/4	1/4	0/4
1 mg. i.p. . . .	48 hr. .	0/3	0/3	0/3	—
1 mg. i.p. (controls)	. No challenge 0/12				
Rats					
0·2 mg. i.v. . . .	30 min. .	—	8/8	5/8	5/8
None (controls) . .	— .	—	0/4	0/4	0/4
0·2 mg. i.v. (control)	. No challenge 3/12				

It should be noted that the cell wall material produced early immunity whether given i.p. or i.v. ; on the other hand, substances such as starch or broth which have been shown to produce a transient immunity (Pfeiffer and Issaeff, 1894), or hog mucin, must be given i.p. in order to be effective. When these substances were given intravenously there was no effect on the subsequent i.p. challenge (see Table VI).

Effect of Purified Extracts on Susceptibility to Bact. coli *in Mice.*

All the Gram-negative bacterial cell walls which were examined contain material which produced the early changes in immunity described above. The active material could be extracted into diethylene glycol or into the water phase

TABLE VI.—*Effect of Injecting Various Materials on the Susceptibility of Mice to* Bact. coli 145 *Challenge* 48 *hr. Later.*

Material.	Dose (mg.).	Route.	Challenge dose of *Bact. coli* 145 10^6.	10^5.	10^4.	Total deaths.
Starch	0·1	i.p.	0/3	0/3	2/3	2/9
,,	0·1	i.v.	3/3	3/3	3/3	9/9
Hog mucin	3	i.p.	1/3	0/3	0/3	1/9
,,	3	i.v.	3/3	3/3	3/3	9/9
Bact. coli K12R T.T. cell walls	0·05–0·1	i.v.	4/9	0/9	0/9	4/27
Controls	—	—	15/15	15/15	12/15	42/45
C. diphtheriae T.T. cell walls	0·2–0·5	i.v.	7/15	4/15	3/15	14/45
Ditto	0·1	i.v.	3/3	0/3	1/3	4/9
,,	0·04	i.v.	3/3	3/3	3/3	9/9
Controls	—	—	12/12	10/12	9/12	31/36

of a phenol/water mixture as described earlier. This extracted material contained 2·0 per cent phosphorus and 1·5 per cent nitrogen ; it was very stable at 100°, the biological activity being little impaired after 7 hr. boiling in water. These properties were similar to those of the lipopolysaccharides of Gram-negative bacteria obtained by similar extraction procedures by Westphal and Lüderitz (1954).

Table VII shows that the minimum amounts of the present lipopolysaccharide material required to protect mice against subsequent challenge was of the order of 1 μg. per 20 g. mouse, compared with a value of approximately 10 μg. using the most active purified lipopolysaccharides of Westphal. This difference may have been due to the bacterial strain from which the lipopolysaccharides were derived, since the lipopolysaccharides kindly supplied by Professor O. Westphal from twelve different Gram-negative bacteria varied considerably in protective ability.

TABLE VII.—*The Susceptibility of Mice to Challenge by* Bact. coli 145, 48 *hr. after the I.V. Injection of Purified Lipopolysaccharide from* Bact. coli 2380

Dose of lipopolysaccharide injected.	Challenge dose of *Bact. coli* 145 10^6.	10^5.	10^4.	Percentage deaths.
0·05 mg.	4/21	1/21	1/21	9
0·01 mg.	15/21	6/21	2/21	36
0·001 mg.	10/15	5/15	4/15	42
Controls	15/15	11/15	13/15	86

The question of the exact identity of the bacterial substrate is still open. There are several reasons for suspecting that the whole lipopolysaccharide molecule may not be the ultimate substrate ; for example, a sample of "degraded" polysaccharide from *Shigella shigae* (Davis, Morgan and Record, 1955) kindly supplied by Dr. W. T. J. Morgan produced immunity 48 hr. following injection of 0·1 mg./mouse, while the Friedlander polysaccharide of Ginsberg, Goebel and Horsfall (1948) was similarly active at a dose of 0·1 mg./mouse. Fairly active materials have also been extracted from Gram-positive organisms (see Table VI).

Toxicity of fractions

Since the various fractions increased the susceptibility to *Bact. coli* infection, it was important to determine their toxicity. From Table II it can be seen that of the cell walls of *Bact. coli* 2380 approximately 1/20 of an LD_{50} dose produced an increase in susceptibility to immediate challenge. It does not seem likely that this immediate depression in immunity can be due to the summation of two sub-lethal effects. The early stimulation of immunity has been produced by less than 1/100 of an LD_{50} dose (Table VIII).

TABLE VIII.—*Toxicity for Mice of Various* Bact. coli *Fractions*

Material.	Toxic dose LD_{50}/20 g. mouse. (mg.).	Minimum dose per mouse giving 48 hr. protection. (μg.).
Bact. coli 2380 trypsin-treated cell walls	1	10
Bact. coli 2380 crude lipopolysaccharide	0·3	1
Bact. coli 2380 residue from phenol/H_2O extraction	4	10
Bact. coli K12 trypsin-treated cell walls	10	50
Bact. coli K12 crude lipopolysaccharide	3	10
Bact. coli K12 residue from phenol/H_2O extraction	10	50

Changes in the bactericidal property of serum from animals injected with cell wall fractions

Experiments were made to investigate the bactericidal properties of serum from animals treated with bacterial extracts. Since mouse serum is non-bactericidal when drawn it was not possible to determine with this serum whether changes occurred. Changes were however seen in guinea-pig and rabbit serum following injection of lipopolysaccharides. In one experiment three guinea-pigs were bled and their sera pooled ; each was then injected i.p. with 5 mg. of cell walls of *Bact. coli* (2380) suspended in saline, and each was bled after 1 hr. and 24 hr. The pooled serum from the untreated animals was able to destroy 1,000 cells/ml. of *Bact. coli* (2206) when diluted 1/2 with saline ; 1 hr. after the injection of cell walls the pooled serum was not bactericidal even when undiluted, whereas after 24 hr. the pooled serum was active when diluted 1/8 in saline.

Fig. 3 presents data from a similar experiment in which the guinea-pigs were injected i.p. with 5 mg. of the cell walls of *P. vulgaris.*

Relationship between cell wall activity and virulence

It has been suggested (Rowley, 1954) that the variations in virulence for mice from one strain of *Bact. coli* to another may depend on the susceptibility of the strains to lysis by complement-containing sera. Such variations must depend either on different quantities or accessibility of the bacterial substrate between the strains. When the cell walls were prepared from two such strains, *viz.*, *Bact. coli* 2380 (virulent) and *Bact. coli* 2206 (avirulent) it was found that those of the virulent strains were more active in protection experiments, suggesting that the virulent strain had a higher content of the active lipopolysaccharide. This was further supported by determining the minimum amounts of these bacteria and their cell walls which would inactivate the C′3 content of 1 ml. of human serum. I am grateful to Professor L. Pillemer and Miss Leona Wurtz for these determina-

tions. Table IX shows that the virulent strain was consistently more active in this respect.

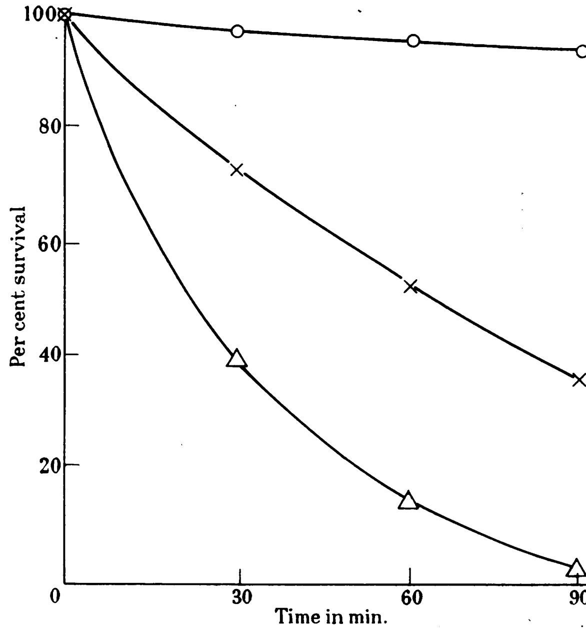


FIG. 3.—Bactericidal power toward *Bact. coli* 2206 of pooled guinea-pig serum drawn before, and at intervals after the injection of *P. vulgaris* cell walls. Each of 4 guinea-pigs injected i.p. with 5 mg. *P. vulgaris* cell walls. Serum diluted 1 vol. in 2 vols. minimal medium.
× = before injection with cell walls.
○ = 80 min. after injection.
△ = 24 hr. ,, ,,

TABLE IX.—*Comparison between* Bact. coli 2380 (*Virulent*) *and* 2206 (*Avirulent*) *for Their Capacity to Bind the Third Component of Complement in Human Serum*

Substance.	Amount in mg. required to inactivate C′3 in 1 ml. human serum. Av.	V.
Living bacteria	2·0	$<$1·0
Killed bacteria (60° for ½ hr.)	$>$5·0	5·0
Cell walls	$>$2·0	0·2

If the content of bacterial lipopolysaccharide is a determinant of sensitivity to the bactericidal power of serum, as the above suggests, then it is to be expected that changes in strains from S→R which are known to cause a decrease in the amount of O antigen and hence of the associated lipopolysaccharide, will result in changes in the sensitivity of the strain to serum. The action of serum on six bacterial strains and on the rough mutants derived from them by growth in O antisera, was examined. In every case the rough strain was more rapidly killed ; examples are shown in Fig. 4.

Another way of showing this increase in susceptibility is to determine the maximum dilution of the serum which will cause a standard amount of killing in a given time. In one such experiment the smooth strain of *Bact. coli* (2206)

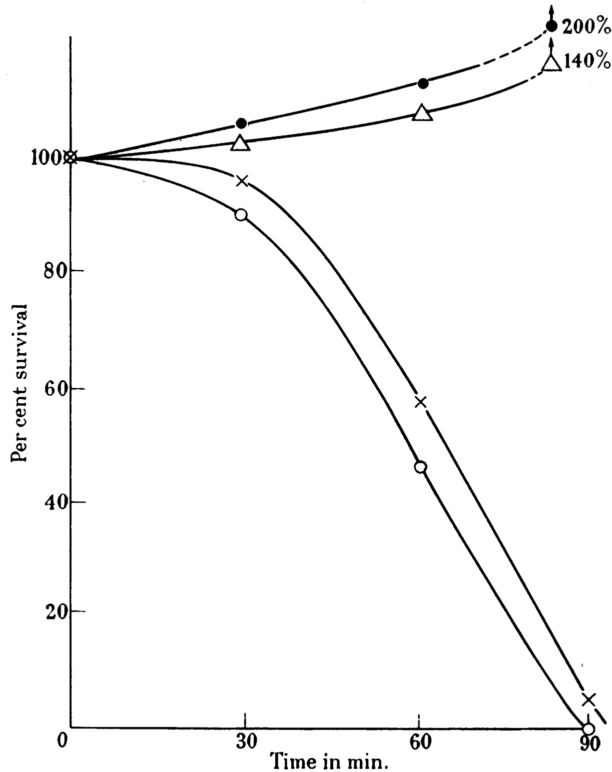


FIG. 4.—Survival rates of rough and smooth mutants during incubation in guinea-pig serum diluted 1 vol. in 4 vols. minimal medium.
● = *S. paratyphi* B (Kröger) smooth.
○ = *S. paratyphi* B (Kröger) rough.
△ = *Bact. coli* 2206 smooth.
× = *Bact. coli* 2206 rough.

showed 50 per cent survival after 90 min. at a dilution of guinea-pig serum of 1/2 in minimal medium, whereas a rough mutant of the same strain showed the same survival at a dilution of 1/16.

DISCUSSION

It has been shown that the early changes in the resistance of animals to infection with coliform organisms are accompanied by simultaneous variations in the bactericidal powers of the serum and the possibility exists that these bactericidal variations are the cause of the immunity effects. If this is so, the results obtained here are consistent with the hypothesis that the bacterial lipopolysaccharide contains the reactant for the bactericidal reaction. In this event the injection of this bacterial substrate rapidly inactivates the presumed enzyme system in serum, which in a few hours is replaced and the loss is over-compensated

in the manner of an adaptive enzyme. In support of this view is the finding that the early immunity can be transferred to some extent by the serum of protected animals to normal mice.

Also consistent with this interpretation is the finding that rough strains are much more sensitive to the bactericidal system than are the smooth strains from which they were derived. The rough strains contain less O antigen and therefore less of the particular lipopolysaccharide material which is a component of it. This smaller amount of substrate in rough strains is analogous to the smaller amount in one smooth avirulent strain as compared to an antigenically similar virulent strain. It seems likely that the speed with which the small quantities of reactant present in rough or avirulent organisms can be attacked is a major determinant of their lack of virulence.

The question arises whether the virulence-enhancing powers of mucin can be due to an interference with the humoral defences of the host such as that described here. Hog mucin is known to be anti-complementary in relatively large doses (Lambert and Richley, 1952); nevertheless there is one piece of evidence against a humoral action. If hog mucin is given intravenously and the organisms given ntraperitoneally there is no enhancement of virulence (personal observations); moreover intravenous injection of hog mucin does not produce any early non-specific immunity.

The effect of the lipopolysaccharide is further distinguishable from that of hog mucin by the finding that injection of active bacterial substrate fractions either i.p. or i.v. together with *Bact. coli* but without mucin does not have any marked virulence-enhancing powers. It seems that hog mucin inhibits the action of the cellular defences and resolves the struggle between bacteria and host into one with the humoral defences (Olitzki, 1948), and it is for this reason that the changes in the bactericidal powers of the serum are so markedly paralleled by the degree of resistance to this artificial infection.

Our understanding of the nature of the bactericidal reaction in normal serum has been considerably advanced recently by Pillemer, Blum, Lepow, Ross, Todd and Wardlaw (1954); they have shown that besides complement and magnesium ions, a protein factor named properdin is necessary for bactericidal activity. This factor, properdin, is removed from serum *in vitro* by incubation in the presence of a yeast cell wall preparation called zymosan. It has been found that i.v. injection of zymosan into mice, rats or guinea-pigs produces very similar changes in early immunity to those produced by cell walls of Gram-negative bacteria (Rowley 1955). Pillemer and Ross (1955) have also shown that following the injection of zymosan or *Bact. coli* cell walls into rats or mice there is a rapid fall in the serum properdin which is rapidly replaced and within 24–48 hr. reaches a level often considerably higher than normal; this increased level falls down to normal around the eighth day.

Owing to the scarcity of purified properdin it has not so far been possible to prove conclusively that these early changes in immunity are a result of the simultaneous changes in properdin levels. However, three mice each injected with 20 units of properdin survived immediate challenge with *Bact. coli* (145) whereas the three control animals died; this slight evidence may be coupled with the finding that rat serum which is rich in properdin will effectively protect mice against *Bact. coli* challenge.

The evidence is in favour of the early immunity effects being due to changes

in available active properdin, these changes being induced by contact with the bacterial substrate which itself appears to be a part of the very high molecular lipopolysaccharide fraction in bacteria.

SUMMARY

The injection of Gram-negative bacterial cell walls into animals causes rapid changes in the susceptibility of the animals to subsequent infection with *Bact. coli* + mucin. During the first hour the animals are much more susceptible, but after 24 hr. a state of resistance develops.

The active material in the bacterial cell walls has been shown to occur in the lipopolysaccharide fraction. After injection of this active material into animals there is a rapid decrease followed within 24 hr. by an increase in the bactericidal powers of the animals' serum. It is suggested that the changes in bactericidal power of the serum are the cause of the changes in the immune state of the animal.

One smooth virulent strain of *Bact. coli* has been shown to have a higher content of active material than one smooth avirulent strain of the same antigenic type. Rough strains (like smooth avirulent strains) are more sensitive to the killing action of serum than are the smooth parent strains from which they were derived.

The possible relationship between these immunity effects and the concurrent changes which occur in serum properdin levels is discussed.

I am indebted to Professor L. Pillemer of Western Reserve University, Cleveland, for materials and valuable discussions, to Dr. H. Ginsberg for the Friedlander polysaccharide, to Professor O. Westphal of the A. Wander Institute, Säckingen, Germany for samples of purified lipopolysaccharides and to Dr. E. Kröger, Hygiene Institut, Göttingen, Germany, for various rough and smooth pairs of bacteria. I wish to thank the Medical Research Council for a grant toward the cost of animals. The excellent technical assistance of C. R. Jenkin is gratefully acknowledged.

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