THE SENSITIVITY OF *SALMONELLA TYPHI* TO THE BACTERICIDAL ACTION OF ANTIBODY

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THE first demonstration of the ability of antibody to sensitise *Salmonella typhi* to the lethal action of complement was made by Felix and Olitski (1926) when investigating O antibody. Later it was found that rough (R) strains were susceptible to R antibody (Adler, 1953*b*) and recently it has been shown that Vi antibody may also cause specific sensitisation (Muschel and Treffers, 1956). There is no evidence of similar activity on the part of H antibody. In the present work the activity of the bactericidal antibodies is considered in relation to the antigenic structure of the bacterial cell.

MATERIALS AND METHODS

The classical strains used were supplied by the National Collection of Type Cultures. They included *Salm. typhi* 0901 (NCTC 5759), Ty2 (NCTC 4446), "Watson" (NCTC 5761), Ty6S (NCTC 8383), ViI (NCTC 8222) and a completely rough variant of strain "Mrs S" (NCTC 8396). Several recently isolated strains, including T13 (T 5277), were from the Enteric Reference Laboratory, Colindale. *Salm. ballerup* (NCTC 6021) was received from Dr. F. Kauffmann with the strain number K 107, 7851, and is assumed to be the original type strain. A rough strain of *Salmonella paratyphi* C (R30) was from the London School of Hygiene.

Except where otherwise stated unabsorbed immune rabbit antiserum was employed in the experiments to be described. The method of preparation of this and also absorbed guinea-pig serum, which was used as a source of complement, has already been described (Nagington, 1956).

Bactericidal titration was performed by the technique described in the preceding paper. In this method, inocula of about 10 viable organisms were sensitised by the addition of antiserum and then complement was added to produce killing. To facilitate the detection of survivors use was made of the ability of growing cells to reduce tetrazolium salt to a red formazan. The bactericidal titre of a serum is expressed as the greatest dilution of serum capable of producing sufficient sensitisation of the cells in the inoculum for them to be killed by the addition of adequate complement.

Agglutination tests and the maintenance of strains were according to the methods of Felix and Pitt (1951).

To compare the agglutinating and bactericidal titres of immune rabbit O and Vi antisera on strains of organisms containing both O and Vi antigens (O + Vi strains) the following method was based on that used by Felix and Pitt for the preparation of Table 2 in their 1951 paper. Cultures of *Salm. typhi* Ty2, "Watson" and a recently isolated wild strain, T 13, were subcultivated daily until judged to be in a state of maximum Vi development. A culture of 0901 was maintained fully smooth and subjected to identical treatment. Fresh 18 hr. cultures of all 4 strains were harvested in sterile saline and divided into 3 parts. One part was diluted in half-strength tryptic digest broth for use as the inoculum in bactericidal titrations. The second part was killed with 0·05 per cent formalin and the third killed

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by heating at 100° for 2 hr. Then the agglutinability of both killed suspensions by O and Vi antisera was measured and compared with the bactericidal activity of the same sera on the living organisms.

RESULTS

The Sensitivity of O + Vi Typhoid Strains

The O + Vi strains of *Salm. typhi* are the smooth virulent strains such as Ty2, " Watson " and the majority of naturally occurring strains. A set of titrations to illustrate their sensitivity to bactericidal action compared with their agglutinability is reproduced in the form of a histogram in the Figure, in which the bactericidal and agglutination titres of the O and Vi antisera against each suspension are expressed as a percentage of the possible maximum titre attainable.

The strains are arranged in the form of a series with increasing Vi development, from 0901 (smooth and completely devoid of Vi antigen) to Ty2 with the greatest amount of Vi. The development of O-inagglutinability with increasing amount of Vi antigen is well demonstrated. The effect described by Felix and Pitt (1934; 1951) of less than maximum Vi-agglutinability in forms with the greatest Vi development is also illustrated.

Bactericidal sensitivity can be seen to be parallel, but not coincident, with agglutinability. This might be expected as a result of the far greater sensitivity of bacteria to the bactericidal than to the agglutinating action of antibody. When Vi antigen has covered or in some other way affected the surface of the organism to reduce O-agglutinability, there will presumably still be ample O receptors left uncovered to enable O antibody to kill. According to Adler (1953*b*) the combination of antibody with one in a hundred receptors on the surface of 0901 is sufficient to enable complement to kill.

Strains possessing intermediate amounts of Vi antigen showed greatest sensitivity to Vi antibody killing, but Ty2 which, according to Felix and Pitt (1951) can be shown by quantitative agglutinin absorption tests and by its antigenicity to contain more Vi antigen than " Watson," was less sensitive both to agglutination (as shown by Felix and Pitt, 1951) and to killing. Felix and Pitt explain their observations by supposing that Ty2 contains an excess of Vi antigen, making it inagglutinable by the higher dilutions of Vi antibody. A similar explanation, amplified in the Discussion below, may serve to explain the low sensitivity of Ty2 to Vi antibody killing.

It is conceivable that an organism may develop such an amount of Vi antigen that Vi antibody may no longer be able to exert any bactericidal action at all. This may be the case in strains such as *Salm. ballerup* which was found resistant not only to typhoid Vi antibody but also to rabbit antiserum prepared against the homologous living organisms. This insensitivity of *Salm. ballerup* to the bactericidal action of Vi antiserum was noted also by Muschel and Treffers (1956).

The Sensitivity of R + Vi Strains

There are two classical strains of *Salm. typhi* in which the predominating antigens are R and Vi. Strain Ty6S (Felix and Petrie, 1938) is sometimes called a " pure " Vi variant (Felix and Pitt, 1951) or " Vi half-smooth form " (Scholtens 1937) because it is antigenically " rough ", that is, completely devoid of O antigen. Morphologically it does not appear to be rough and it is stable in 5 per cent saline.

Strain ViI (Bhatnagar, Speechly and Singh, 1938) is similar but contains a small amount of O antigen. This is insufficient to cause agglutination by O antiserum but gives rise to the production of O antibody when ViI is used as a vaccine (Felix, 1938). In many ways ViI behaves as intermediate between Ty6S and the O + Vi strains. It is convenient to consider the two strains ViI and Ty6S together since their major antigens are the same, apart from the presence of the trace of O in ViI.

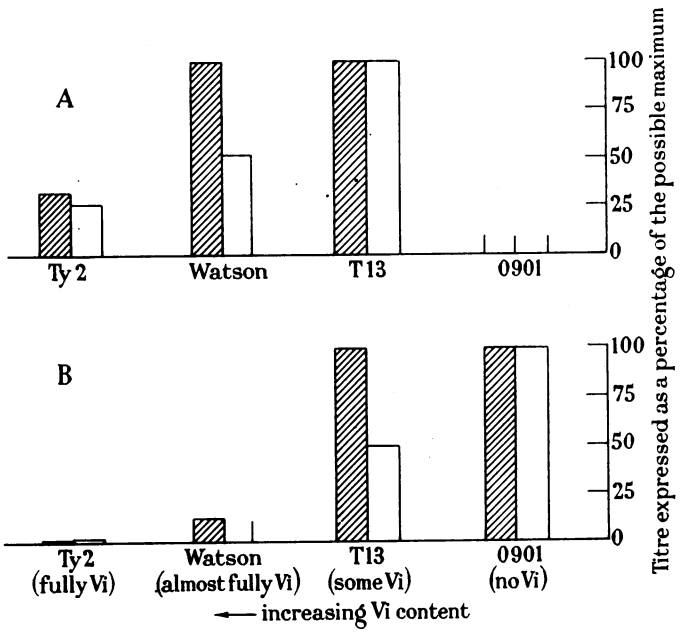

FIGURE.—Histogram of the agglutinability and bactericidal sensitivity of 0 + Vi strains of *Salm. typhi.* A. Vi antiserum (54/2). B. O antiserum (54/1). Titre expressed as a percentage of the possible maximum. Bactericidal action shown by hatched columns. Agglutination shown by white columns.

Vi antibody prepared from smooth vaccine strains

Two rabbit antisera (53/7 and 54/2) prepared against smooth fully Vi living strains of *Salm. ballerup* (NCTC 6021) were without effect on both Ty6S and ViI. Yet both sera contained abundant Vi antibody and this was fully bactericidal against O + Vi typhoid strains (Table I).

Immune antiserum prepared against a formolised suspension of Ty2 was equally ineffective against Ty6S and ViI. It has been shown that Vi antibody prepared from formolised Ty2 is " functionally inadequate " in passive-protection tests in mice (Felix and Bhatnagar, 1935; Felix, 1938; 1952) and also in complement fixation tests (Henderson, 1939), but there is no evidence that it is other than functionally adequate in its bactericidal action against the O + Vi strains Ty2 and " Watson " when the present method is used (Table I).

From these results it is thought probable that Vi antibody which is derived from smooth vaccine strains is without action on R + Vi strains of *Salm. typhi.*

TABLE I.—*Insensitivity of Strains Ty6S and ViI to Vi Antibody from Smooth Vaccine Strains*

Rabbit serum.	Vaccine.	Maximum bactericidal titre recorded against the following test strains. Ty2 (O + Vi).	0901 (O).	"Mrs S" (R).	Ty6S.	ViI.
54/2	*Salm. ballerup* (living)	100,000	100	100	30	<10
53/7	„ „	50,000	300	<10	<10	<10
53/4	Ty2 (formolised)	(10,000)*	300,000	<30	100	<30
53/4 (after absorption with 0901)	„ „	3,000	<30	<30	<30	<30

* Part of this titre is due to killing by the O antibody.
Note: titres of 100 or less are probably due to normal antibodies.

Vi antibody prepared from rough vaccine strains

Rabbit antiserum was prepared against two vaccines of Ty6S—alcoholised and heat-killed (100° for 2½ hr.), a vaccine of ViI which had been treated with N-HCl for 20 hr. at 37° (Felix, 1952) and an alcoholised vaccine of the rough strain of *Salm. paratyphi* C (R 30).

All of these strains produced Vi antibody which was measurable by agglutination. This Vi antibody differed from that produced by antigenically smooth strains in that it was fully bactericidal to R + Vi strains of *Salm. typhi*. It was fully bactericidal also to O + Vi strains of *Salm. typhi* (Table II) and so could readily be distinguished from R antibody.

TABLE II.—*Sensitivity of Strains Ty6S and ViI to Vi Antibody from a Rough Vaccine Strain and also to R Antibody*

	Maximum bactericidal titre recorded against the following test strains. "Mrs S" (R).	Ty2 (Vi).	Ty6S.	ViI.
Immune rabbit serum 54/13 prepared against an alcoholised vaccine of *Salm. paratyphi* C rough strain (R30)—				
Unabsorbed	3,000	30,000	30,000	30,000
Absorbed twice with an alcohol-killed *Salm. ballerup* suspension to remove Vi antibody	3,000	<10	1,000	1,000
Absorbed twice with *Salm. ballerup* and then twice with heat-killed "Mrs S." to remove Vi and R antibodies	<10	<10	<10	<10
Absorbed twice with a heat-killed suspension of "Mrs S" to remove R antibody	<10	10,000	10,000	30,000
Immune rabbit serum 54/8 prepared against a heat-killed vaccine of *Salm. typhi* "Mrs S"	3,000	<10	1,000	1,000

Serum 54/13 (unabsorbed) had a standard Vi agglutination titre of 160 measured against the Standards Laboratory agglutinating suspension. A bactericidal titre of 100 against *Salm. typhi* 0901 and an agglutination titre of less that 40 is taken to indicate only a trace of normal O antibody.

When antigenically smooth Vi strains of *Salm. typhi* and *Salm. ballerup* were used to absorb these sera it was found that all Vi agglutinins were removed and also all the Vi antibody bactericidal to O + Vi strains. The results of absorptions with *Salm. ballerup* are illustrated in Table II. The bactericidal activity against

ViI and Ty6S was almost completely removed. On some occasions the activity of the absorbed serum towards Ty6S did not show the same drop in activity as against ViI. Assuming that there are only the two antigens Vi and R present in Ty6S the remaining factor was therefore either unabsorbed Vi or R antibody. Since absorption with a suspension of the pure R strain " Mrs. S " completely removed this residual activity the factor was almost certainly R antibody.

R antibody

The pure R antibody serum produced by a vaccine of *Salm. typhi* " Mrs. S." is bactericidal to both Ty6S and ViI (Table II).

O antibody

There is no evidence that O antibody can kill either ViI or Ty6S, although it is conceivable that under the appropriate conditions some action on ViI might occur.

It is concluded that both ViI and Ty6S are sensitive to the action of Vi antibody produced in rabbits against antigenically rough Vi strains, *i.e.*, strains which are capable also of stimulating the production of R antibody. Ty6S is very sensitive to the R antibody produced both by these strains and purely R strains. ViI tends to be less sensitive to R antibody but is still highly sensitive to it. This is what might be expected in a strain in which there is still a small amount of O antigen.

According to Kennedy and Stuart (1950) R-agglutinability in R + Vi strains can be masked by the Vi antigen. This effect may be reflected by variation in the sensitivity of the strains to the bactericidal action of R antibody. If Vi antigen development is sufficiently marked it is reasonable to assume that some hindrance will be produced to the accessibility of the R antigen to its antibody, which will reduce the bactericidal efficacy of the R antibody in the same way that Vi antigen reduces the agglutinability and bactericidal power of O antibody in O + Vi strains.

R antibody

The discovery of the sensitivity of the R + Vi strains to the bactericidal action of R antibody as well as to the Vi antibody produced by rough vaccine strains prompted further investigation of the antigenicity of the R substance. There is considerable confusion in the literature and much of this appears to be due to the similarity of the properties of the R and Vi antibodies (Kennedy and Stuart, 1950).

Table III shows the results of the bactericidal titration of immune rabbit sera produced against various vaccines. The bactericidal method is more sensitive than agglutination methods of measuring antibody and it is of especial value in the case of R antibody where agglutination methods are of limited value. Strains 0901 and "Mrs. S." are sensitive only to O and R antibody respectively, but Ty2 is usually sensitive to O as well as Vi antibody. Unfortunately, no typhoid strain has yet been found which is sensitive to the bactericidal action of Vi antibody alone. Consequently the titres against Ty2 in the Table are of unabsorbed sera for those in which there is no appreciable O antibody and after absorption with 0901 for those sera with appreciable O antibody.

TABLE III.—*Antigenicity of R in Relation to the Amount of O and Vi Antigen Present in Various Vaccine Strains.*

Immune antibodies present in the antiserum.			Immune antiserum. Rabbit.	Nature of vaccine used.	Maximum bactericidal titres recorded against the following test strains. Ty2 (Vi).	0901 (O).	" Mrs S " (R).
Vi	—	—	54/2	*Salm. ballerup* (live)	100,000	100	100
Vi	—	—	53/7	„ „ „	50,000	300	<10
—	O	—	54/1	0901 (alcoholised)	(3,000)	300,000	10
Vi	O	—	53/4	Ty2 (formolised)	3,000*	300,000	<30
Vi	O	R	53/5	ViI (alcoholised)	10,000*	10,000	1,000
(Vi)	O	R	53/3	„ (heated)	(300)	1,000	1,000
Vi	—	R	53/1	„ (HCl-treated)	3,000*	300	3,000
Vi	—	R	54/11	Ty6S (alcoholised)	100,000	300	1,000
Vi	—	R	54/12	„ (heated)	10,000	100	1,000
Vi	—	R	54/13	*Salm. paratyphi* C (R30—alcoholised)	30,000	100	3,000
—	—	R	54/6	*Salm. paratyphi* C (R30—heated)	(300)	300	1,000
—	—	R	54/8	Mrs S (heated)	<10	100	3,000

Titres marked with an asterisk are following absorption with 0901 to remove all the O antibody. The Ty2 titres shown in brackets are partly or entirely due to O antibody. All titres are the reciprocal of the initial dilution of serum, *i.e.*, before admixture with any of the other reagents. Normal antibody may give titres up to 300.

It will be seen that after inoculation with fully O strains no R antibody is produced (the course of injections was of not less than 5 injections on alternate days, which made a total dose of approximately 15×10^9 cells). This would perhaps seem to be a restatement of an obvious and well known fact: But the point to which it is wished to draw attention is that a progressive loss of O antigen leads to an equally progressive increase in the antigenicity of the R substance; R antibody is produced and this process is quite independent of the presence of the Vi antigen. Hence the use of " pure Vi " strains for the production of sera is attended by the production of considerable R antibody and this antibody can react with such " pure Vi " strains in a closely similar manner to Vi antibody.

This may explain the agglutination by normal sera of a ViI suspension used by Brower (1944) who noted that the effect was due to an antibody other than Vi. Wheeler and Stuart (1946) found a " Vi-like " antigen in several coliform and paracolon strains, and Kennedy and Stuart (1950) christened it the ' Er ' antigen, *i.e.*, *Enterobacteriaceae* rough. It was found in many organisms known to possess Vi antigen and they found that sera thought to contain only Vi antibody may contain also ' Er ' antibody.

If, for " Vi-like " and Er, we substitute R antibody the picture becomes clearer. A recent example of the occurrence of this antibody in " pure Vi " antiserum may be provided by the additional specificity of typhoid Vi antibody (produced against ViI extract) when compared with *Salm. ballerup* Vi antibody which was described by Chi-Yen Chu and Hoyt (1954). When Ty6S was substituted for ViI in the preparation of sera by the technique they described, a high-titre R serum was produced with so little Vi antibody that the method might well

be used for the preparation of R antiserum. It may be that other antigens which have been described, such as the X antigen of Topley and Ayrton (1924) would, on re-examination, be found to be R antigen.

The Bactericidal Sensitivity of the Classical Typhoid Strains

The classical strains of *Salm. typhi* are well defined stable types which are particularly useful in serological work. Their sensitivity to bactericidal antibody is shown in Table IV. Those which are sensitive to only a single antibody are

TABLE IV.—*Bactericidal Sensitivity of the Classical Strains of* Salm. typhi.

Strain.	Antigenic structure.				Antibodies which can exert a bactericidal action.				Remarks.
Ty2	H	Vi	O	—	—	Vi	O	—	Sensitive to all Vi antibodies. Insensitive to O antibody when fully Vi.
"Watson"	H	Vi	O	—	—	Vi	O	—	
H901	H	—	O	—	—	—	O	—	
0901	—	—	O	—	—	—	O	—	
ViI	H	Vi	O	R	—	Vi	—	R	Sensitive only to the Vi antibody produced against R vaccines.
	tr.		tr.						
Ty6S	H	Vi	—	R	—	Vi	—	R	
	tr.								
"Mrs S" (rough variant)	H	—	—	R	—	—	—	R	

Table based on that of Felix and Pitt (1951), from which the production of R antibody was omitted.

straightforward (H901, 0901, "Mrs. S."). Others, which are sensitive to two antibodies (Ty2, "Watson") need greater care in their use as test strains, and control titrations must always be employed to determine their senitivity to each antibody on every occasion. The R + Vi strains (Ty6S, ViI) are the least suitable of the group on account of their peculiar sensitivities and, compared with the other strains, they also show a much greater degree of variation in susceptibility.

DISCUSSION

A culture of *Salm. typhi* on first isolation from a case of enteric fever may be considered to represent the nearest approach to full antigenic development *in vitro*. The surface of such organisms is almost always composed of Vi antigen. On continued subculture the Vi antigen is gradually lost unless certain conditions are observed. The O antigen then enters into the composition of the surface layer and with complete loss of Vi it forms the entire surface. Further "loss variation" (Kauffmann, 1951) with reduction in the quantity of O antigen is accompanied by the development of antigenicity by what is apparently an underlying substance, the R substance. This process produces a mixture of O and R antigens on the cell surface in the same way as during the loss of Vi it was composed of Vi and O. At the completion of the process the surface is probably again uniform, the organism completely rough and the surface composed of R antigen.

It is to be expected that most strains of *Salm. typhi* in the laboratory will have a surface layer composed of a mixture of two antigens. They can therefore be sensitive to the bactericidal action of two antibodies. If however only one bacteri-

cidal antibody is presented to such a cell then susceptibility to this will depend on the amount of that particular antigen, that is " susceptible " antigen, which is accessible to the antibody. In consequence there are at least three possible ways in which reduction or loss of sensitivity to bactericidal action may be expected to occur :

(*a*) Decrease in the actual quantity of susceptible antigen in the cell.

(*b*) Increase in the quantity of insusceptible antigen as in the case smooth O + Vi strains and probably also in R + Vi strains.

(*c*) Increase in the quantity of *susceptible* antigen.

This proposed third mechanism is analogous to the reduction in Vi-agglutinability when Vi antigen increases in quantity past a certain point, which is shown well with Ty2 and " Watson ". The mechanism is the same as in (*b*) but, instead of the coating of insusceptible antigen making inaccessible the susceptible antigen, in this case the increasing thickness of susceptible antigen itself acts as a barrier. The reduced sensitivity of Ty2 to the action of Vi antibody is considered to be an example of this effect. It may also provide an explanation for the insusceptibility of *Salm. ballerup* and some *Bacterium coli* strains to the bactericidal action of Vi antibody.

It is probable that the effect is only seen in Vi and similar antigens which can form a relatively thick envelope around the bacterial cell. It has been shown that in O-inagglutinable Ty2 there may be as much as ten times the quantity of Vi antigen that is present in O-agglutinable strains such as the American " 58 " strain (Webster, Sagin, Anderson, Breese, Freeman and Landy, 1954). The mechanism protecting the bacterial cell from the Vi antibody is probably not a simple one but may well consist of several parts. Antibody may be rendered harmless by combination with soluble Vi antigen and hapten at the periphery of the Vi envelope. It may be held away from the vital part of the cell purely by the thickness of the Vi layer and perhaps even be actively repelled by *Quelling* (Landy, 1954) when it attaches itself to the antigen. In each case the subsequent combination of complement with the antigen-antibody complex then takes place so far removed from the vital part of the cell wall that it is rendered impotent and the cell is undamaged. It is suggested that this is a possible way in which the invading typhoid bacillus is protected from the antibody defences of the host.

Another possible method by which the typhoid organism can be protected from the bactericidal action of antiserum was proposed by Adler (1953*a*, *b*) who suggested that R antibody could, by attachment to the cell, impede the action of O antibody on 0901. The demonstration of this effect depends on the simultaneous introduction of all three factors, antigen, antibody and complement. Competition for complement may therefore be a major factor. Unfortunately, even after alteration of the method and sequence of addition of the reagents, it has not been possible to elicit the effect by means of the present method of bactericidal titration.

A special problem is presented by the antigenically rough strains Ty6S and ViI. They are susceptible to killing by R antibody and also by Vi antibody, but apparently only homologous Vi antibody or that produced against rough vaccine strains. This would seem to provide evidence of the necessity for a particular fit of Vi antibody, determined by its formation against Vi antigen in close proximity to R antigen. This might provide a clue to the curious ability of a Vi-phage to lyse organisms which contain no Vi antigen which was reported

by Cherry, Davis and Edwards (1954). The Vi-phage may have been able to utilise R antigen receptors.

SUMMARY

The bactericidal action of O antibody on *Salm. typhi* can be blocked by Vi antigen covering the surface of the cell in the same way that Vi antigen can produce O-inagglutinability. This effect is thought also to occur with R antibody and R + Vi strains (Ty6S and ViI) where Vi antigen covers the R antigen. Such R + Vi strains are apparently sensitive only to homologous Vi antibody and not to that formed against smooth vaccine strains.

The sensitivity of the classical strains of *Salm. typhi* to bactericidal antibodies is presented.

A mechanism by means of which the bacterial cell may be protected against Vi antibody through the increased development of Vi antigen is discussed, as is the R antigen and the confusion its presence in Vi strains may cause.

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