VIRULENCE AND IMMUNOGENIC ACTIVITIES OF *B. TYPHOSUS* IN RELATION TO ITS ANTIGENIC CONSTITUENTS

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Since the work of Arkwright (1921, 1927), Topley and Ayrton (1923–4) and others the close association between "smoothness" and virulence has been well established. This statement, it is true, requires some qualification in so far as many workers have applied the terms "smoothness" and "roughness" to various bacterial species without paying due attention to the exact meaning of the terms. If we limit ourselves to the group of Gram-negative intestinal bacilli, with regard to which the criteria of smooth-rough variation were first defined, the statement is correct that according to the almost universally accepted view smoothness denotes virulence and roughness avirulence. In the case of the flagellated bacilli, as exemplified by the typhoid-paratyphoid group of organisms, the great immunological significance of the smooth O antigen and its corresponding antibody was also well established (Felix, 1924; Felix and Olitzki, 1926; Arkwright, 1927; Topley, 1929; Schütze, 1930).

Recent work, however, showed that the mere presence of smooth O antigen did not connote the highest order of virulence of which smooth strains of *B. typhosus* are capable and that an additional factor, the Vi (virulence) antigen, was required to render the smooth O antigen resistant to the action of the O antibody (Felix and Pitt, 1934*a*, *b*). These workers also showed that the Vi antibody was of primary importance in the defence against attack by virulent strains of *B. typhosus*, though some antibacterial activity of the O antibody, too, could be demonstrated in phagocytic experiments with strains of high virulence (Felix and Bhatnagar, 1935).

In view of the apparent confusion resulting from these recent observations it seemed desirable to investigate the fate of the Vi antigen of virulent strains of *B. typhosus* when the cultures were allowed to undergo the change from the smooth to the rough form.

Vi antigen in rough variants of *B. typhosus*

Rough variants were isolated by plating, at certain intervals, broth cultures kept at room temperature over periods varying from a few weeks to nearly half a year. Nine strains of *B. typhosus*, whose reaction to O agglutinin was known, were included in the present investigation, viz. three strains from each of the three types, inagglutinable, agglutinable and intermediate. It is

https://doi.org/10.1017/S0022172400032459 Published online by Cambridge University Press

known that the first and the third of these types contain Vi antigen, whereas the agglutinable type is devoid of this antigen. We have failed to isolate completely rough variants from one inagglutinable, virulent strain ("Watson") and from one agglutinable, avirulent strain ("Rawlings"). All the other strains yielded completely rough variants along with others which, according to the accepted criteria, were to be classed as partially rough forms. The variant strains were tested for their virulence to mice and for their capacity to induce antibody formation in the rabbit. Table I summarises the main results of these experiments.

Table I. *Vi antigen in rough variants*

Titre of agglutination
Rabbits immunised with live rough variants of strains

	Ty 2 *No. 102*		Giglioli *No. 103*		Mrs S. *No. 100*		Ty 441 *No. 101*		Ty 8 No. 106		Ty 901 No. 107	
Type of antibody	I	II	I	II	I	II	I	II	I	II	I	II
Vi	0	0	0	400	0	0	0	1000	0	0	0	0
O	20	20	20	500	20	20	50	50	50	50	20	20
H	0	500	0	10,000	0	10,000	0	2000	0	5000	0	20,000
Type of parent strain	Inagglutinable Virulence high				Intermediate				Agglutinable Virulence low			
	Virulence for mice											
Dose 400×10^6 intraperitoneally	$\frac{0}{6}$		$\frac{5}{6}$		$\frac{0}{6}$		$\frac{0}{6}$		$\frac{0}{6}$		$\frac{0}{6}$	

Titre 0 = a negative result in a dilution 1 in 10.
The titres of O agglutination were determined with the strain "O 901".
The titres of Vi agglutination were determined with the strain "Watson".
I = titre before immunisation.
II = titre after immunisation.

Six rough variants derived from six different smooth parent strains are described in Table I. The parent strains belonged to the three types of *B. typhosus*, two strains from each type. Saline suspensions of living organisms of the six rough variants were inoculated into rabbits by the intravenous route, following the procedure described in a previous paper (Felix and Pitt, 1934*b*). The only difference was that the rough organisms were injected in much greater doses than those used for the immunisation of rabbits with live smooth bacilli. Four injections were given, the doses being respectively, 200, 800, 1600 and 4000 million organisms of each of the rough variant strains. For each rabbit the titres of H, O and Vi agglutinins are recorded under two headings, viz. (I) the titres observed with the normal serum taken before the commencement of the inoculations; (II) the titres observed with the immune serum taken one week after the last inoculation.

Table I shows that with the exception of the variant derived from the strain "Giglioli" all the variant strains were completely rough forms, which had entirely lost the smooth O antigen contained in the parent strains. This is evident from the titres of O agglutinins which, in each instance, were no greater after the immunisation than before. The variant of the strain

"Giglioli", on the other hand, was only a partially rough form. The O titre of the rabbit inoculated with this variant rose from 1 in 20 to 1 in 500, indicating the presence in it of some residual smooth O antigen.

While the titres of H agglutination are of no particular significance and are recorded in the Table only for the sake of completeness, those of Vi agglutination are of great interest. It will be seen from Table I that two of the six rough variants stimulated the production of a very considerable amount of Vi antibody, while the other four variants did not. Those two variants were derived from parent strains which contained Vi antigen. It was not surprising to find the variant of the "Giglioli" strain, which was only a partially rough form, still in possession of Vi antigen along with the residuum of the smooth O antigen, but the fact that the completely rough variant of the strain "Ty 441" also contained Vi antigen came as something of a surprise in view of its being entirely devoid of the smooth O antigen.

The results of the virulence tests showed that the five completely rough strains were non-virulent, none being capable of killing mice when injected intraperitoneally in the large dose of 400 million organisms. The rough variant of the strain "Ty 441", which contained Vi antigen, had no greater virulence than the other completely rough strains which were entirely devoid of this antigen. The outstanding exception was the partially rough variant of the "Giglioli" strain. This variant was relatively virulent, a dose of 400 million organisms killing five out of six mice, and it was the one amongst all the variants tested that contained a certain, though small amount of the smooth O antigen.

Absorption tests with a pure Vi antiserum, which are not reproduced here, showed that the avirulent variant of strain "Ty 441" was as rich in Vi antigen as the relatively virulent variant of the "Giglioli" strain. The conclusion appears therefore to be quite obvious that that latter variant owed its virulence to the presence in it of both the Vi and the smooth O antigens.

Active immunisation with rough variants

The rough variants were next tested for their relative values as antigens for the induction of active immunity in the mouse. Previous experiments with vaccines prepared from perfectly smooth strains of *B. typhosus* had shown that only vaccines containing the Vi antigen rendered mice resistant to infection with highly virulent bacilli. It was therefore to be expected that a vaccine made from a rough variant that contained this antigen would also confer protection against the smooth virulent form. Table II shows that this expectation was fully justified.

The vaccines used in the experiment recorded in Table II were prepared exactly in the way described in a previous paper (Felix and Pitt, 1934*b*) and the test was also conducted in the same manner. Two subcutaneous inoculations were given at three days' interval, the doses containing 400 and 800 million

organisms respectively. Twenty-two days later the test dose was inoculated intraperitoneally, consisting of 2 M.L.D. of our most virulent smooth strain "Ty 2". It is seen from Table II that the vaccines prepared from the variant strains of "Giglioli" and "Ty 441", which contained the Vi antigen, conferred protection, whereas those made from the variants of "Mrs S." and "Ty 901", which did not possess this antigen, were devoid of protective action. The smooth strain "Ty 901", representing the type of typhoid bacilli of high agglutinability and low virulence, was included to serve as a control and, in accordance with previous experience, proved to be entirely inefficient in protection against the virulent type of strain.

Table II. *Active immunisation with rough variants*

Vaccines used for the immunisation of mice			Number of mice	
Strain	Variant	Antigens present in the strains	Tested	Survived
Giglioli	Partially rough	H + O + Vi	6	3
Mrs S.	Rough	H	6	0
Ty 441	Rough	H + Vi	6	3
Ty 901	Rough	H	6	0
Ty 901	Smooth	H + O	6	0

Test dose 2 M.L.D. (=160 million organisms of strain Ty 2)

The clear-cut result of this experiment was, that a vaccine made from a completely rough variant, containing Vi antigen but devoid of the smooth O antigen and non-virulent, protected mice against infection with highly virulent smooth bacilli, whereas the vaccine from a smooth strain with a high content of the smooth O antigen was without protective effect. Thus the value of the Vi antigen as an immunising agent and its independence of the smooth O antigen in this respect were again clearly demonstrated.

THE PROTECTIVE POWER OF THE Vi ANTIBODY ELABORATED IN RESPONSE TO IMMUNISATION WITH ROUGH NON-VIRULENT VARIANTS OF *B. TYPHOSUS*

Some of the rabbit sera described in Table I were also tested for their protective values in passive immunisation of mice. It has been shown in a preceding paper of this series (Felix and Bhatnagar, 1935) that the Vi antibody resulting from immunisation with formolised Vi antigen is not in all respects identical with that elaborated in response to immunisation with the "natural" Vi antigen contained in the living virulent bacilli. The protective power and the phagocytosis-promoting activity of the former are much inferior to those of the latter, though there is no difference in the agglutinating properties of the two varieties of antibody. In view of this startling fact great care was taken in the exact quantitative estimation of the effects of the sera in Vi agglutination and in protective action. The technique of the protection tests was that employed in previous experiments (Felix and Pitt, 1934*a*, *b*).

Table III shows that the protective values of the two sera Nos. 62 and 101 and their Vi titres, as estimated by the agglutination reaction, run parallel.

In this respect, therefore, there is no difference between the sera from rabbits immunised with live vaccines of smooth or of rough bacilli, provided they contain the Vi antigen. Three Vi antisera, produced by immunisation with formolised Vi antigen from smooth strains, were included in this experiment as controls. It is seen from the Table that the inferior protective effects of these sera were quite out of proportion to their high titres of Vi agglutinin.

Table III. *Protective values of Vi antisera from rabbits immunised with Vi antigen from smooth and rough strains of* B. typhosus

Immune sera			Titre of antibodies estimated by agglutination		Dose	Number of mice	
Rabbit No.	Immunised with strains	Type of vaccine	O	Vi	of serum in c.c.	Tested	Survived
62	Ty 2 smooth	Live	5,000	400	0·2	4	4
					0·1	4	1
					0·04	4	0
101	Ty 441 rough	Live	50	1000	0·2	4	4
					0·1	4	4
					0·04	4	1
113	Watson smooth	Formolised	10,000	1000	0·2	4	4
					0·1	4	2
					0·04	4	0
79	Formolised extracts from smooth		10,000	600	0·4	4	0
82	strains Ty 2, Watson and Giglioli		5,000	600	0·4	4	0

Test dose 3 M.L.D. (240 million organisms of the smooth strain Ty 2)

Phagocytosis experiments with virulent smooth strains of *B. typhosus*, conducted in the manner described by Felix and Bhatnagar (1935), also clearly indicated that the Vi antibody derived from immunisation with the avirulent rough variant of the strain "Ty 441" was as potent in its sensitising activity as was the Vi antibody in the sera of rabbits, which had been inoculated with virulent smooth bacilli.

The rough variant of the strain "Ty 441", since it was first isolated from its smooth parent more than a year ago, retained its cultural properties, its antigenic composition and its low degree of virulence entirely unaltered. It could therefore safely be used as the source of the "natural" Vi antigen required for the preparation of a therapeutic anti-typhoid serum and the difficulties and risks experienced with the immunisation of horses with highly virulent bacilli in the living state were thus overcome.

Discussion

The experiments recorded in this paper clearly indicate that the Vi antigen, contained in virulent strains of *B. typhosus*, is not necessarily lost when the cultures undergo the change from the smooth to the rough form. The Vi antigen may survive unimpaired, though the smooth O antigen, or its specific polysaccharide component, has disappeared in the process. It has also been shown that both the Vi and the smooth O antigen may be lost simultaneously. From the small number of our observations it cannot be stated which of the two

alternatives occurs the more often. The important fact, however, is that the smooth → rough variation, while accomplishing the loss of the smooth O antigen, may leave the Vi antigen entirely unaffected.[1]

Various reasons combined to make this observation at first appear rather startling. It was not the mere fact that an extremely labile component, such as the Vi antigen, was kept intact, whereas the so-called stable O antigen was lost, that caused the surprise. An analogous condition is known to exist in the case of the labile H antigen. It is well established that the H → O variation is independent of the smooth → rough variation. It seemed, however, from previous work that there was good reason for the belief that the association between the smooth O and the Vi antigen was much more intimate in nature than that between the smooth O and the H antigen.

It is known that the presence or absence of the Vi antigen has a far-reaching effect on the behaviour of the smooth O antigen, whereas there is no analogous relationship between the O and H antigen. In fact, the discovery of the new antigenic constituent of *B. typhosus* was mainly due to the striking differences exhibited by the smooth O antigen as a result of the presence or absence of the Vi antigen. In teleological language the statement would appear to be quite appropriate that the Vi antigen owed its existence to its being required for the protection of the smooth O antigen against the action of the O antibody, natural or immune. The resistance of the smooth O antigen to the agglutinating, bactericidal and opsonising effects of the O antibody entirely depend on the presence of the Vi antigen. As a result of this protective effect of the Vi on the smooth O antigen, smooth strains of *B. typhosus*, which contain the Vi antigen, are virulent, whereas strains of equally perfect smoothness, which do not possess the Vi antigen, are avirulent.

The presence of an abundant amount of Vi antigen in a rough variant, which was entirely devoid of the smooth O antigen, did not cause any appreciable increase in its virulence over that of other rough strains, from which the Vi antigen was absent. On the other hand, the combination of the Vi antigen even with a small amount of the smooth O antigen, as instanced in a partially rough variant, was reflected by a considerable degree of virulence of a strain which showed most of the accepted characters of roughness.

In view of these facts doubts will possibly be voiced as to whether the name "Virulence (Vi) antigen" was appropriately chosen for the new antigenic constituent of *B. typhosus*. We still think it was. It is generally agreed that the rough form of *B. typhosus* is an artificial product, due to deterioration through the laboratory methods of artificial culture. Typhoid fever is invariably caused by the smooth bacterium, which, consequently, is often referred to as the "normal" form. Now, the only difference between the virulent and avirulent types of the normal smooth typhoid bacillus, which so

[1] The occurrence of rough strains of *B. typhosus* containing the Vi antigen is mentioned in a recent paper by Kauffmann (1935), but there is no evidence to show that the author was dealing with a culture of *B. typhosus* entirely devoid of the smooth O antigen.

far has been detected, is the possession by the former of that antigenic constituent that has been designated as the "Virulence (Vi) antigen". It seems to us that there was, and still is, no better alternative. The fact that this antigen is still present in the deteriorated, avirulent, rough variant of a smooth Vi strain is merely another instance of the more general rule, according to which the various characters or functions of a bacterium may vary independently of each other (for references see Arkwright, 1930). Our observation simply illustrates the fact that the Vi, like the H antigen, is unaffected by the changes which the process of smooth → rough variation imposes on the polysaccharide component of the O antigen. There can be little doubt that the three kinds of antigen, viz. the O, H and Vi, differ profoundly in their chemical composition. Their different behaviour in a process involving chemical change is, therefore, not at all surprising.

The mechanism by means of which the Vi antigen protects the smooth O antigen against the action of the O antibody still remains obscure. It has been stated in a previous paper (Felix and Pitt, 1934*b*) that "the supporters of the surface theory of antigen-antibody action undoubtedly will welcome the phenomenon as additional evidence of the correctness and general validity of the theory". If the modern conception of the spatial arrangement of the antigenic constituents of a micro-organism be applied to the case of the virulent smooth typhoid bacillus, the dominant position on the surface of the bacterial cell would be assigned to the Vi antigen, which determines the immunological behaviour of this type of *B. typhosus*. In so doing a simple explanation could be offered of the protective action of the Vi on the smooth O antigen, the latter implicitly being placed beneath the surface. The fact, however, that animals inoculated with live virulent strains of *B. typhosus* invariably produce antibodies to both the Vi and O antigens already appears to be in conflict with the surface theory in its extreme form (see particularly discussion by White (1933) and Topley (1933)). Again, the suppression of the development of the Vi antigen by growing the cultures on phenol agar or at unsuitable temperatures, and the consequent unhindered reaction between the unmasked O antigen and its corresponding antibody, could also be explained by the surface theory in a simple and logical manner. On the other hand, the smooth → rough variation of the virulent Vi-containing strains, described in this paper, would not fit in at all. In this case one would have to assume that the surface of the cell occupied by the Vi antigen remained unchanged, while the smooth O antigen, that originally lay underneath, disappeared. Though we are unable to suggest what exactly the mechanism is by means of which the Vi antigen inhibits the reaction between the O antigen and the O antibody, it seems to us that some of the facts established in connection with the phenomenon do not conform with the theory, already referred to, of the spatial arrangement of antigens contained in a bacterial cell.

The results of the present investigation corroborate the earlier conclusion that the virulence of *B. typhosus* depends on the presence in the bacilli of

both the smooth O and the Vi antigen. At the same time they indicate clearly, again in full accord with the results of previous experiments, that the Vi antibody is the one of outstanding importance in protection against infection with highly virulent strains of *B. typhosus*. Thus, a rough variant, which was entirely devoid of the smooth O but possessed the Vi antigen, could be used successfully for active immunisation and for the preparation of a serum of high potency in the passive immunisation of mice. The discrepancy between our results and the earlier observations of Arkwright (1927) is readily understood on account of the fact that no strain of the highly virulent type was included in his experiments and that none of his rough variants contained the Vi antigen. Three strains of *B. typhosus* were used by Arkwright, viz. the strains "Rawlings" and "Ty 901", both now known to be devoid of the Vi antigen, and the strain "Mrs S." which belongs to the intermediate type. The latter strain in our hands, too, yielded only rough variants which did not contain the Vi antigen (see Table I) and consequently had no protective value (see Table II). Some of the results recently published by Maltaner (1934), who, working with the rabbit as experimental animal, established protective action of vaccines prepared from rough strains of *B. typhosus*, were probably due to the presence in his strains of the Vi antigen, of which he was not aware.

It is not suggested that vaccines of rough variants of *B. typhosus*, which contain the Vi antigen, should be adopted in the practice of antityphoid inoculation, though theoretically such a procedure would appear to be feasible. It has been shown in phagocytic experiments (Felix and Bhatnagar, 1935) that the O antibody has a certain sensitising effect on strains of the highly virulent type, though the powerful activity of the Vi antibody tends to overshadow the feeble action of the O antibody. From this it must be inferred that in protection against attack by strains of this type the O antibody also contributes towards the sum total of protective action, although this contribution may appear to be almost negligible. Furthermore, cases of the human disease occur, probably in a very small minority, where the infecting bacilli are of the type of low virulence, entirely or almost entirely devoid of the Vi antigen (Felix, Krikorian and Reitler, 1935). Protection against this type of *B. typhosus* can only be afforded by the use of vaccines containing the smooth O antigen.

On the other hand the rough variant of the strain "Ty 441", containing the Vi antigen, proved to be of immediate practical value in the preparation of a therapeutic antityphoid serum. Large doses of a live vaccine of this avirulent culture could be inoculated into horses without those harmful effects that follow the injection of the smooth virulent bacilli in the living state. The Vi antibody in the serum of rabbits and horses immunised with this rough variant was found to be as potent in protective action and in its phagocytosis-promoting activity as was the Vi antibody derived from immunisation with the smooth and highly virulent bacilli. It is known that the efficacy of a therapeutic antityphoid serum also depends on the presence in it of an adequate amount of the O antibody, which is responsible for effecting

the neutralisation of the endotoxin of *B. typhosus*. This antibody can be produced by injecting a dead vaccine containing the smooth O antigen, whereas the live rough variant serves as the source of the "natural" Vi antigen required for the elaboration of the Vi antibody.

B. typhosus provides an illuminating example of the diversity of functions assigned to the various antigenic constituents of the bacterial cell. While it is rather depressing to confess that the biological function of the H antigen is as obscure today as it was fifteen years ago, some light has been thrown on the O and Vi antigens, which, according to present knowledge, seem to share between them the decisive rôles in the pathogenicity and immunogenic activities of the typhoid bacillus. It seems to be almost a matter of course to assume that similar complex interactions between various antigenic constituents are at play in other pathogenic bacteria. The fact that virulence may be determined by the combined activity of two (or more) antigenic components, whereas one only may be required to induce a state of efficient immunity, opens a new avenue of approach to the solution of numerous practical problems of immunisation. Deteriorated "rough" variants, which possibly have some precursors in the "attenuated" cultures of earlier days, may yet rise to prominence in this field.

SUMMARY

1. Rough variants, derived from strains of *B. typhosus* which possess the Vi antigen, may still contain this antigen though the smooth O antigen has been lost.

2. Such variants, which are non-virulent, are yet capable of inducing active and passive immunity.

3. The virulence of *B. typhosus* depends on the combined activity of the smooth O and the Vi antigen. Nevertheless the Vi antibody alone is sufficient to protect against infection with strains of the highly virulent type.

4. The use of avirulent, rough, but Vi-containing variants as vaccines and in the preparation of therapeutic sera is discussed.

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(*MS. received for publication* 20. VII. 1935.—Ed.)