AN ANTIGENIC BASIS FOR VIRULENCE IN STRAINS OF SALMONELLA TYPHIMURIUM*

By CHARLES R. JENKIN, Ph.D.

(From the Department of Microbiology, University of Adelaide, South Australia)

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One of the basic questions that host-parasite relationships pose, particularly in relationship to infectious disease, is why should one species of animal be susceptible to certain pathogenic bacteria whilst others and sometimes closely related animals are highly resistant to the same bacteria? Recent studies concerned with the phagocytosis and intracellular fate of bacteria would suggest that in many cases, susceptibility or resistance to a particular pathogen may be correlated with the presence or absence of serum factors (opsonins) which not only enhance phagocytosis but may determine the subsequent fate of the ingested bacteria (1–4).

As a result of such studies we have proposed that the susceptibility of mice to *Salmonella typhimurium* infections is due to an antigenic similarity between host and parasite (5). This antigen, being a "self" component of the mouse, inhibits the formation of specific antibody or opsonins directed against the parasite. Sera obtained from animals naturally resistant to this pathogen protected mice against infection by this strain of bacteria (1). Evidence obtained from the study of the phagocytosis of virulent and avirulent strains of *S. typhimurium* by mouse peritoneal macrophages and the phagocytic elements of the reticuloendothelial system led to the suggestion that the mouse, whilst having opsonins directed against the avirulent strain, lacked or was deficient in opsonins against the virulent strain. However, since virulent strains of *Salmonellae* were phagocytosed to some extent (though poorly, compared with avirulent ones), virulent and avirulent bacteria must both share common antigenic components with which the mouse opsonins reacted. In addition, the virulent strain possessed a further antigen related to a host antigen and against which the host was unable to produce any serum antibodies owing to its resemblance to "self." Animals naturally resistant to *Salmonella typhimurium* infections and whose sera are capable of protecting mice against this infection, possessed opsonins against both antigens. These antigens, the chemical nature of which is at present under investigation, were termed for convenience *V* (virulent) and *A* (avirulent). The relative amounts of V and A in any strain of *Salmonella typhimurium* could conceivably predetermine its virulence for mice (4–6).

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The work presented in this paper was designed to test this hypothesis and presents data supporting both the antigenic relationship and dissimilarity between virulent and avirulent strains of *S. typhimurium*.

Material and Methods

Bacterial Strains.—The virulent and avirulent strains of *Salmonella* used in these studies were *S. typhimurium* C5 (LD_{50}, 2×10^2) and M206 (LD_{50}, 10^6) (1, 4–6). Other species studied were an *Escherichia coli*, *Staphylococcus aureus* (Oxford), and *Klebsiella pneumoniae* NCTC 5054. For RES clearance studies the strains of bacteria were grown either in minimal medium supplemented with Difco cas amino acids (7) or in 10 per cent serum broth. To 50 ml of this medium 1 mc of P^{32} as orthophosphate was added. The inoculated medium was shaken at 37°C for 18 hours. The P^{32}-labelled bacteria were washed three times with 50 ml of saline and finally resuspended in the above medium to give a suspension of 10^9 bacteria/ml. Bacterial suspensions were kept at 4°C and not used for longer than 5 days.

In vivo Clearance Studies of Bacteria by the RES.—The technique used was essentially that described by Biozzi, Benacerraf, and Halpern (8). Blood samples were assayed for radioactivity as previously reported (6). The phagocytic index K, giving a measure of the rate of clearance, was calculated from the equation,

$$K = \frac{\log C1 - \log C2}{T2 - T1}$$

where $C1$ and $C2$ are the concentration of the bacteria at times $T1$ and $T2$ (8). Mice used in the clearance studies were males and females of the LAB grey strain weighing 18 to 20 gm.

Opsonization of Bacteria.—For opsonization 1 ml of the bacterial culture was mixed with 1 ml of serum at 4°C and kept at that temperature for 20 minutes. The mixture was finally centrifuged at 3000 RPM for 15 minutes and resuspended in 1 ml of supplemented minimal medium for injection.

Preparation of Absorbed Sera.—Unless otherwise indicated in the Tables, pig serum was absorbed with 10 mg dry wt of bacteria at 4°C for 18 hours. Following absorption the bacteria were removed by centrifuging at 5000 RPM for 20 minutes.

Blockading Experiments.—Mice were injected intravenously with 5×10^9 bacteria and divided into two randomly chosen groups. 30 minutes later one group was challenged intravenously with 2×10^8 isotopically labelled bacteria whilst the other group received a similar dose of bacteria that had been treated with pig serum prepared as indicated in the text. The clearance of the labelled bacteria was followed as above.

Haemagglutination and Haemagglutination Inhibition Studies.—The method used was that described by Crumpton, Davies *et al.* (9, 10).

Test for Incomplete Antibody.—Test for incomplete antibody was carried out by a modification of the indirect Coombs technique (11). 0.5 ml of a 1 per cent *v/v* suspension of sheep red blood cells sensitized with lipopolysaccharides obtained from the strains under investigation (9, 10) were incubated for 60 minutes at 37°C with 0.5 ml of various dilutions of pig serum that had been absorbed with unsensitised sheep red cells to remove all traces of heterophile antibody. After incubation the cells were recovered by centrifuging at 1500 RPM for 10 minutes and washed three times with buffered saline. The washed cells obtained from each dilution of serum were finally resuspended in 0.5 ml of buffered saline pH 7.0 and divided into two equal portions. To one portion was added 0.2 ml of a 1/10 dilution of rabbit anti-pig serum that had been absorbed with sheep red cells to remove heterophile antibodies. To the other portion of cells, 0.2 ml of buffered saline pH 7.0 was added to serve as a control. The

mixtures set out in haemagglutination trays were read after further incubation at 37°C for 60 minutes and after standing at 4°C overnight.

Preparation of O Somatic Antigen and Lipopolysaccharide.—The O somatic antigen and its lipopolysaccharide component were prepared and characterised by methods previously published (12). The *Pasteurella pseudotuberculosis* lipopolysaccharide was kindly supplied by Dr. D. A. L. Davies, Porton, England. Zymosan used in the absorption studies was batch LE-1 obtained from Lederle Laboratories, American Cyanamid Co., Pearl River, New York. Unless otherwise stated all absorption with these various substances took place at 4°C for 18 hours.

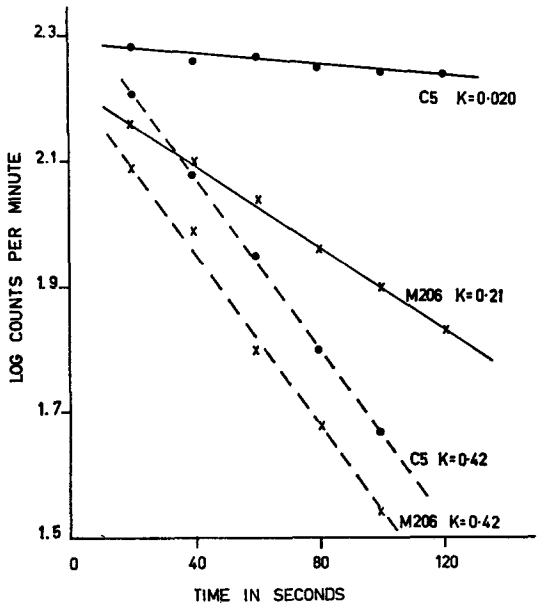

FIG. 1. Rate of clearance of virulent (C5) and avirulent (M206) strains of *S. typhimurium* from the blood stream of normal mice before and after treatment with pig serum. Rate of clearance expressed as the phagocytic index *K*.

RESULTS

Opsonic Studies Using Pig Serum Absorbed with Various Antigenic Fractions From a Virulent (C5) and Avirulent (M206) Strain of S. typhimurium.—Preliminary studies showed that pig serum possessed opsonins against both, the virulent and avirulent strains of *S. typhimurium* (Fig. 1). Titration of the pig serum for specific agglutinating antibody against the two strains showed no agglutination after incubation at 37°C for 2 hours. After standing overnight at 4°C feeble agglutination was recorded for both strains at a dilution of ¼ of the pig serum, though the bacteria were readily suspended on shaking and appeared evenly dispersed. Using a type of indirect Coombs test, no incomplete antibody

to the lipopolysaccharide component of the O somatic antigen could be demonstrated. Absorbing the pig serum with the avirulent strain (M206) removed most of the opsonic activity against itself but left considerable activity against the virulent organism (C5). However, absorption with the virulent strain (C5)

TABLE I

Opsonic Activity of Pig Serum towards an Avirulent (M206) and Virulent (C5) Strain of Salmonella typhimurium after Various Treatments

Pig serum absorbed with	Reduction in the phagocytic index K	
	Avirulent	Virulent
	per cent	*per cent*
S. typhimurium C5, *8 mg*	90	100
S. typhimurium M206, *7 mg*	70	19
Lipopolysac. from C5, *250 μg*	91	27
Lipopolysac. from M206, *250 μg*	62	36
O somatic antigen C5, *300 μg*	65	30
O somatic antigen M206, *300 μg*	65	20
Lipopolysac. *P. pseudotuberculosis, 250 μg*	100	25
Zymosan, *1 mg*	80	0

TABLE II

Opsonic Activity of Pig Serum towards an Avirulent Strain of S. typhimurium M206 after Absorption with Various Amounts of the Virulent Strain (C5) and Avirulent Strain (M206)

Pig serum absorbed with	Reduction in the phagocytic index K
	per cent
S. typhimurium C5, *8 mg*	100
S. typhimurium M206, *7 mg*	60
S. typhimurium C5, *4 mg*	80
S. typhimurium M206, *4 mg*	40
S. typhimurium C5, *2 mg*	50
S. typhimurium M206, *2 mg*	20

removed opsonins directed against both (Table I). On a dry weight basis it was found that the virulent organism was much more active in removing opsonins against the avirulent strain (M206) than was M206 itself (Table II). Since both strains possess identical O somatic antigens as revealed by haemagglutination and haemagglutination inhibition studies (Table III, *a* and *b*), it seemed possible that this component of the cell wall might well be one of the common antigenic components against which some of the pig serum opsonins

were directed. Absorption studies, using the lipopolysaccharide component of the O somatic antigen from both strains and the whole antigen itself, showed that the opsonins to the avirulent strain could be almost completely removed but those against the virulent organism were only partially reduced (Table I). However, absorbing with lipopolysaccharide was relatively "non-specific" since absorbing the pig serum with lipopolysaccharide derived from *Pasteurella pseudotuberculosis* antigenically unrelated to either strain, or by zymosan,

TABLE III

(*a*) *Antigenic Relationships between Lipopolysaccharides from S. typhimurium Strains C5, M206, and Pasteurella pseudotuberculosis*

Sheep red cells sensitised with lipopolysaccharides from	Maximum dilution to produce haemagglutination		
	C5 O antiserum	M206 O antiserum	*P. pseudotuberculosis* O antiserum
C5	1/2000	1/2000	<1/10
M206	1/2000	1/2000	<1/10
P. pseudotuberculosis	1/10	1/10	1/400

(*b*) *Inhibition of Haemagglutination between C5 and M206 0 Antiserum and Sheep Red Cells Sensitised Homologous Lipopolysaccharides, by Lipopolysaccharides from C5, M206, and Pasteurella pseudotuberculosis*

O antiserum to	Amount of lipopolysaccharide inhibiting 4 haemagglutinating doses of antiserum		
	C5	M206	*P. pseudotuberculosis*
	μg	μg	μg
C5	0.5	0.5	>5
M206	0.5	0.5	>5

greatly reduced the titre of serum opsonins against the avirulent organism, though the titre of opsonins against the virulent strain was only partially reduced (Table I).

Nature of the Opsonins Against the Virulent Strain (C5).—Experimental studies reported elsewhere (5) have suggested an antigenic relationship between the virulent strain of *S. typhimurium* and the tissues of the susceptible mouse host. Pig serum that had been absorbed with the avirulent organism (M206) and which had lost half of the opsonic activity against this strain was absorbed with mouse red blood cells. This twice absorbed serum was tested for its opsonic activity against C5 (Table IV). That the reduction in opsonic titre against the virulent organism which occurred as a result of the absorption with mouse red blood cells was a specific effect is shown by the fact that no further reduction

in the titre of opsonins against the avirulent strain (M206) took place as a consequence of this. Absorption with sheep red blood cells at the same concentration did not remove any of the opsonic activity against C5 (virulent).

Nature of the Opsonins Present in the Serum of Mice.—The experimental data presented so far is in keeping with the hypothesis advanced earlier. In order to

TABLE IV

Effect of Absorbing Pig Serum with M206 (Avirulent) followed by Absorption with Mouse Red Blood cells on its Opsonizing Capacity toward C5 (Virulent) and M206 (Avirulent) Strains of S. typhimurium

Pig serum absorbed with	Reduction in the phagocytic index *K*	
	Avirulent M206	Virulent C5
	per cent	*per cent*
Avirulent M206, *7 mg*	53	37
Mouse red blood cells, *0.5 ml w/v*	55	63

All absorptions at 4°C.

TABLE V

The Clearance (as Measured by the Phagocytic Index K) of Virulent (C5) and Avirulent (M206) Strains of Salmonella typhimurium from the blood Stream of Normal and Blockaded Mice, before and after Treatment with Pig Serum Absorbed with Avirulent M206

Strain of *S. typhimurium*	The phagocytic index *K*			
	Normal mice		Blockaded mice	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Virulent C5	0.021	0.017	0.010	0.008
Virulent C5 (opsonized)	0.29	0.24	0.080	0.09
Avirulent M206	0.19	0.20	0.030	0.070
Avirulent M206 (opsonized)	0.22	0.22	0.015	0.070

For opsonization, bacteria treated with pig serum absorbed with M206 for 20 minutes at 4°C.

show that the mouse possessed opsonins directed against shared antigenic components between virulent and avirulent bacteria but in general lacked or was deficient in opsonins against the virulent strain, the following experiment was performed. Mice were injected with a blockading dose of M206 (avirulent), and the clearance from the blood stream of a further injection of isotopically labelled bacteria followed before and after treatment (opsonization) with pig serum that had been absorbed with the avirulent organism such as to remove all opsonins against this strain. It may be seen from the results presented in

Table V that treatment with the absorbed pig serum enhanced only the clearance of the virulent strain C5. The significance of these results will be discussed further.

Heat Stability of the Opsonins in Pig Serum.—Unabsorbed pig serum and pig serum that had been absorbed with the avirulent organism were heated at 56°C. The results illustrated in Fig. 2 show that opsonins corresponding to the antigenic components shared between the two strains were relatively heat-stable

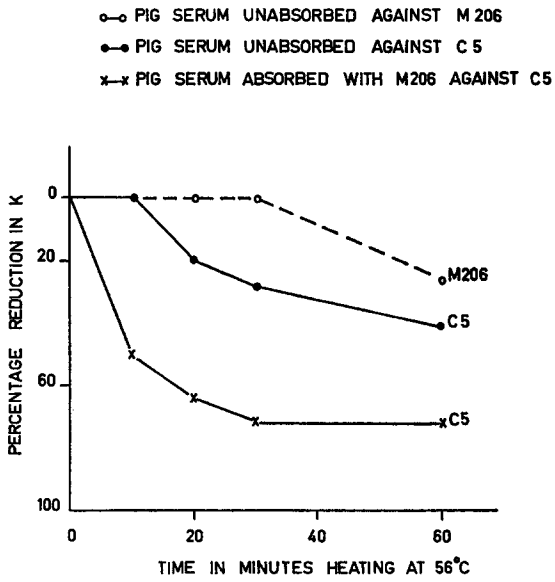

FIG. 2. Effect of heingat at 56°C, on the opsonic properties of pig serum, and pig serum absorbed with M206. Opsonic activity titrated against virulent (C5) and avirulent (M206) trains of *S. typhimurium.*

whilst some of those directed specifically against the virulent strain were comparatively heat-labile.

Nature of the Opsonins against the Virulent Strain C5.—Previous experiments reported above have shown that treating pig serum with either the avirulent strain M206, mouse red blood cells, or heating at 56°C reduced the opsonic activity of the pig serum against C5. This suggested that the opsonins against C5 were of three types: (*a*) those directed against the antigen shared between C5 and M206, (*b*) those directed against the antigen shared between C5 and mouse red blood cells, and finally (*c*) heat-labile opsonin(s). Absorption of pig serum with M206, followed by heating at 56°C for 60 minutes, and finally absorption of this serum with mouse red blood cells removed completely all of the opsonic activity of the pig serum against the virulent strain (Table VI). Combinations of any of the above two procedures failed to remove completely

the serum opsonins against C5, and only when the above three procedures were followed, irrespective of any order, was this achieved. The heat-labile opsonins appear unrelated to complement since absorption of pig serum at 4°C for 18 hours with the virulent strain, whilst removing all of the opsonic activity, still left the full titre of haemolytic complement. Likewise treating pig serum with sheep red blood cells sensitised with rabbit haemolysin whilst removing comple-

TABLE VI

Effect of Various Cumulative Treatments on the Opsonic Activity of Pig Serum towards the Virulent Strain of Salmonella typhimurium C5

Stepwise treatment of pig serum	Reduction in the phagocytic index *K*
	per cent
Absorbed M206 overnight at 4°C, *10 mg*, +	33
Heating 56°C for 60 minutes, +	72
Absorption 1 ml packed mouse red blood cells	100

TABLE VII

Opsonic Activity of Pig Serum Absorbed with Various Strains of Bacteria against these Same Strains

Strain tested	Pig serum absorbed with				
	C5	M206	5054	E. coli	*S. aureus*
C5	100*	19	32	10	10
M206	100	100	72	100	67
5054	80	30	80	50	50
E. coli	0	30	0	92	8

* Figures within the chequer-board indicate the percent reduction in the phagocytic index *K*.

ment did not affect the opsonic properties of the pig serum towards the virulent strain.

The Specificity of the Opsonins in Pig Serum.—Pig serum was absorbed with various strains of bacteria, and the opsonic activity of each absorbed serum was tested against the bacteria used in the absorptions (Table VII). It is apparent from these results that the opsonins in pig serum are a complex group of substances, the results of these cross-absorptions showing that whilst some species of bacteria removed wholly or partially the opsonins against another strain, the opsonins against a third strain were unaffected. The significance of these results will be discussed further.

DISCUSSION

The results reported in this paper whilst not defining in chemical terms the important antigenic differences between the virulent and avirulent strains of *Salmonella typhimurium* in relation to virulence, do show that whilst both strains share common antigenic components the virulent strain in addition possesses an antigen which bears some relationship to an antigen of the mouse, the susceptible host.

In view of the possible importance of such a relationship between host and parasite in determining host susceptibility (5) it would be well to examine in some detail the experimental data contained in this paper in relation to that which has already been published. The role of serum opsonins in promoting phagocytosis of bacteria by the fixed cells of the reticuloendothelial system and those wandering free in the tissues and body cavities have pointed to the importance of such factors in determining an animal's susceptibility or resistance to infection by these pathogens (1, 4, 5).

We have been concerned mainly with a study of the factors involved in the pathogenesis of *S. typhimurium* infections in different animal hosts, and it has become increasingly clear that phagocytic cells from animals differing widely in their susceptibility to this infection, have similar bactericidal potentials providing that certain serum factors are added to the cellular system (5, 13). Thus, peritoneal macrophages taken from rats highly resistant to infection by *S. typhimurium*, are basically no more efficient in dealing with this pathogen than are those of the mouse, (the susceptible host) if the bacteria have first been treated with mouse serum. Conversely, mouse peritoneal macrophages are as efficient as the corresponding rat cells in killing this pathogen in the presence of rat serum. These *in vitro* studies are supported by previous observations that *S. typhimurium* C5 is less virulent for the mouse after treatment with rat serum (1). It should perhaps be emphasized that treatment or opsonization of the bacteria with the serum in no way affects their viability nor does it cause any visible agglutination. These results together with the findings of other research workers on the role of serum factors in phagocytosis of a great variety of bacteria (14–19) have led us to postulate that the susceptibility of certain strains of mice to infection by *S. typhimurium* is determined by their inability to produce opsonins against this organism. One reason for this defect may be due to antigenic resemblances between host and parasite. There is good evidence in the literature that such resemblances do in fact exist but as far as we know, no one has suggested that this may be an important factor in determining host susceptibility to parasitic bacteria (20–24).

Pig serum has also been shown to protect mice against this infection and to enhance the clearance of the virulent strain from the blood stream of normal mice by RES. (1, 5, 6). Since this serum could be obtained in considerable quantity it seemed suitable material for more extensive biological and chemical studies. It is obvious since the virulent *S. typhimurium* C5 is cleared from the blood stream of normal mice (but at a much slower rate than the avirulent M206) that the mouse must possess some opsonins against this organism, since our previous studies have shown that no phagocytosis takes place in the absence of serum factors. (14, 15). Pig serum, whilst dis-

playing considerable opsonic activity against the virulent C5, also possesses a high titre of heterophile antibody against the mouse red blood cell, and in addition gives rise to anaphylaxis in the mouse if injected intravenously (5). Chemical studies to be reported elsewhere on the nature and purification of the opsonins in pig serum have shown that the mouse haemagglutinating activity closely parallels the opsonic activity of a given fraction. In addition such fractions displayed opsonic activity against the virulent only and not against the avirulent strain M206. These observations strongly support the contention that the virulent strain possesses an antigen(s) not present (or masked) in the avirulent strain, and that this antigen is related to an antigen of the mouse. Evidence for this relationship is further supported by the biological experiments presented in this paper. It has been found that absorbing pig serum with the avirulent strain M206 only partially reduces the opsonic activity against the virulent strain C5 whilst removing completely the homologous opsonic activity. However, absorption of the serum with C5 not only removes all the opsonins against itself but also those against M206. These results show that C5 and M206 share common antigenic components but the virulent strain C5 possesses additional antigens which may not be present in M206, and against which pig serum possesses opsonins. If pig serum absorbed with M206 so as to remove all opsonic activity against this strain, is heated at 56°C for 60 minutes and this is then followed by absorption with mouse red blood cells, all the opsonic activity against the virulent strain is removed. Heating unabsorbed pig serum or absorbing it with mouse red blood cells is not effective in removing any of the opsonins against M206, (avirulent). The heat-labile opsonins against the virulent strain C5 appear unrelated to complement, since under suitable experimental conditions it is possible to remove all opsonic activity against C5 whilst leaving the full titre of haemolytic complement. Opsonins common to both strains may be removed by absorption with Gram-negative lipopolysaccharides. This apparent non-specific effect may in reality be specific, in the sense that these opsonins may be directed against common antigenic arrangements in the polysaccharide molecule even though these appeared antigenically unrelated when titrated against specific antisera. The antigenic specificity of these macromolecules depends on the arrangement of their terminal sugars (25, 26). The opsonins of the mouse appear to be those directed against both strains as shown by the fact that mice blockaded with M206 cleared a second injection of isotopically labelled C5 or M206 at a much slower rate than did normal unblockaded control mice. However, when both strains were opsonised with pig serum absorbed with M206 thus removing common opsonins to both strains in this serum, only the clearance of the virulent strain from the blood stream of the blockaded mice was enhanced. The experiments with pig serum absorbed with various strains of bacteria and then titrated for its opsonic activity against these same strains, though few in number, reveal the presence of shared antigenic components between these bacteria and also point to the heterogeneity of the opsonin pool.

The natural selection theory of antibody production as postulated by Jerne and extended by Burnet (27, 28) would appear to offer a satisfactory explanation for the presence of opsonins or natural antibodies in the serum of animals toward a whole range of different bacteria. Some of these opsonins would display greater specificity than others depending on the extent to which certain antigens were shared within this group of parasites. Recent observations by several workers on the bactericidal activity

of normal serum against various strains of Gram-negative bacteria before and after absorption with these strains support the above statement (29–31). The titre of these factors may well be determined by previous contact with the antigen (7). Thus it seems to us that given a potential pathogen that has the capacity to multiply within the host environment, the extent to which it will do so will depend in most cases on the ability of the host to produce the necessary opsonins which ultimately lead to the destruction of the bacteria. On this basis, host susceptibility to infection by a particular pathogen could fall into two categories. Firstly, whilst the animal may have the capacity to respond by producing opsonins against the parasite, the titre of these may be very low, either owing to the rarity of the particular antigenic structure of the parasite, or owing to the isolation of the animal population. In both these cases once infection has been established, even though this may initially be severe and lead to death of a high percentage of the infected animals, the host may emerge as a resistant animal, not because of any genetic selection within the population but merely because a percentage of the animals have had sufficient time to develop a high titre of specific opsonins against the parasite. The second category and of more immediate interest is one in which despite non-specific stimulation, which raises the titre of opsonins, and attempts at specific immunisation, the host is still susceptible to infection by the parasite. This is particularly so in the case of *S. typhimurium* infections of mice where the degree of protection afforded by non-specific stimuli such as lipopolysaccharides from Gram-negative bacteria, or even specific immunisation is of a very low order, and in most cases may be measured in terms of survival time rather than differences in over-all mortality (32, 33). It would seem that in this latter instance there is a failure on the part of the host to respond in an immune fashion to some of the antigens of the parasite. This seems to be the case if one considers the parasite *Salmonella typhimurium* and the susceptible mouse host. In this connection it is interesting to record that using a strain of rats highly resistant to this infection, made partially tolerant to the tissues of the mouse (susceptible host), we have been able to show that such rats are now susceptible to infection by this pathogen. Whilst these results are of a preliminary nature they strongly support the general hypothesis of host susceptibility outlined in this and a previous publication. (5).

SUMMARY

A study has been made of the antigenic relationships between a virulent and an avirulent strain of *Salmonella typhimurium*. Evidence is presented which supports the hypothesis that the susceptibility of the mouse to infection by *S. typhimurium* is due to an antigenic relationship between host and parasite. The antigen shared between host and parasite, appears to be absent or is masked in the avirulent strain.

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