# STUDIES ON THE VIRULENCE OF A NATURALLY OCCURRING MUTANT OF SALMONELLA TYPHOSA

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From 1936 to August 1948, cultures of *Salmonella typhosa* isolated from a chronic typhoid carrier in Panama ("the Panama Carrier") were submitted periodically to the Army Medical Service Graduate School for routine examination. In July 1948, an isolate was received which was morphologically, biochemically, and antigenically similar to previous and subsequent samples except that it was essentially avirulent for mice (Batson *et al.*, 1949). This observation was of interest since at that time it was generally considered that the antigenic structure of typhoid strains was the determinant of mouse virulence. Smooth cultures of *S. typhosa* containing equal amounts of Vi antigen—as do the strains obtained from the carrier in Panama—were assumed to be of comparable virulence. Conversely, differences observed in degree of virulence exhibited by various cultures have been associated with quantitative differences in the Vi antigen content of the various strains (Felix and Pitt, 1951).

The Panama Carrier strains are of further interest since one of them (strain 58) is the culture employed in the routine production of typhoid vaccine for use by the U. S. Armed Forces. Vaccines for immunizing the civilian population of the United States also are usually prepared from *S. typhosa*, strain 58.

It is the purpose of this communication to describe further studies which have been made with the "avirulent isolate" from the Panama Carrier. The results substantiate the work of other investigators (Bacon *et al.*, 1950, 1951; Garber *et al.*, 1952) who have shown that nutritional factors as well as antigenic structure may play a role in determining the mouse virulence of certain bacterial cultures.

## MATERIALS AND METHODS

*Cultures.* All cultures used in this work were obtained from the culture collection of the Army Medical Service Graduate School. The virulent strain of *S. typhosa*, strain 58, was labeled 42-A-58 while the avirulent isolate was designated 42-A-58V. Batson *et al.* (1949) have described the general characteristics of these cultures. *S. typhosa*, strain Ty 2, was used also in some experiments since this culture is employed in many laboratories as a representative typhoid strain capable of exhibiting maximum virulence for mice. Strain Ty 2 differs from the Panama Carrier strains mainly in its greater resistance to agglutination by typhoid O antiserum. It is also approximately five times as virulent for mice as the virulent Panama 58 strain when 16 hr cultures are administered as saline challenges.

*Virulence tests.* White mice (Bagg strain) weighing 14 to 16 g were injected intraperitoneally with 0.5 ml quantities of dilutions of 16 hr cultures cultivated on nutrient agar and suspended in either 5 per cent hog gastric mucin or physiological saline. Groups of 10 or 20 mice containing equal numbers of males and females were used to test each dilution. The number of deaths was recorded 72 hr after inoculation, and the $LD_{50}$ and standard error (S. E.) estimated by the method of Miller and Tainter (1944).

When the effect of xanthine in virulence tests was examined, 10 mg (contained in one ml saline) were injected intraperitoneally at the same time as the bacteria. Due to the insolubility of this compound at pH 7, it was administered as a fine suspension. In any experiment in which xanthine was used, mice not receiving the purine were injected with an equal volume of saline.

*Minimal medium.* The minimal medium employed in this work contained: Potassium phosphate (monobasic), 3.0 g; potassium phosphate (dibasic), 7.0 g; ammonium sulfate, 1.0 g; glucose, 2.0 g; agar, 15.0 g; distilled water, 1,000 ml.

*Peritoneal fluid.* Peritoneal fluid was obtained from normal mice by absorbing it directly from

the peritoneal cavity with sterile filter paper discs. These in turn were deposited on the surface of plates containing minimal medium. The plates were seeded with various cultures to determine whether or not the peritoneal fluid would supply the necessary nutritional factors required by the strains.

## RESULTS

The minimal medium did not support the growth of any of the strains used. Further tests revealed that strain Ty 2 required tryptophan, 42-A-58 (virulent) needed tryptophan and cystine, while 42-A-58V (avirulent) required a purine in addition to these two amino acids.

When peritoneal fluid obtained from normal mice was added to the minimal medium, strain Ty 2 grew well, 42-A-58 (virulent) grew only sparsely, the 42-A-58V (avirulent) not at all. This indicated that purines were either absent or present in very low concentration in the peritoneal fluid of the mice used. Thus, a factor necessary for the growth of the avirulent isolate was not available in the very menstruum in which the strain must initially multiply to kill mice.

Experiments were carried out to ascertain the effect of simultaneously injecting xanthine with the avirulent culture. Challenges suspended in both saline and 5 per cent hog gastric mucin were used. The results are summarized in table 1. It may be noted that the virulence of the avirulent isolate was increased significantly by the addition of xanthine when either a saline or a mucin challenge was employed. The addition of xanthine did not increase the virulence of 42-A-58V (avirulent) to the level where it equalled that of 42-A-58 (virulent), possibly because of a relatively rapid diminution in the concentration of the added purine in the peritoneal fluid.

A reversion of the avirulent culture to the xanthine independent state was obtained by inoculating $10^{10}$ cells on plates of minimal medium enriched with cystine and tryptophan. This culture was then similar in its nutritional requirements to the original strain 42-A-58. Virulence tests were performed with the reverted culture employing challenges suspended in both mucin and saline. The data are summarized in table 2. They show that when reversion to xanthine independence occurs, strain 42-A-58V (avirulent) becomes as virulent as strain 42-A-58 (virulent).

TABLE 1

*Effect of the inoculation of xanthine on the mouse virulence of Salmonella typhosa, strain 42-A-58V (avirulent)*

| CHALLENGE SUSPENSION ADMINISTERED IN 5 PER CENT HOG GASTRIC MUCIN | NUMBER OF ORGANISMS INJECTED | | | | | $LD_{50}$ | S.E. |
|---|---|---|---|---|---|---|---|
| | $7.5 \times 10^7$ | $7.5 \times 10^6$ | $7.5 \times 10^5$ | $7.5 \times 10^4$ | $7.5 \times 10^3$ | | |
| 42-A-58V (avirulent) | 18/20* | 9/20 | 2/20 | 0/20 | 0/20 | $900 \times 10^4$ | $450 \times 10^4$ |
| 42-A-58V (avirulent) plus 10 mg xanthine† | 19/20 | 18/20 | 17/20 | 13/20 | 8/20 | $2 \times 10^4$ | $6.6 \times 10^4$ |
| 42-A-58 (virulent) | | | | 20/20 | 20/20 | | |
| CHALLENGE SUSPENSION ADMINISTERED IN PHYSIOLOGICAL SALINE | NUMBER OF ORGANISMS INJECTED | | | | | | |
| | $4.5 \times 10^9$ | $8.1 \times 10^8$ | $1.6 \times 10^8$ | $3.6 \times 10^7$ | $7.3 \times 10^6$ | | |
| 42-A-58V (avirulent) | 17/20* | 4/20 | 0/20 | 0/20 | 0/20 | $170 \times 10^7$ | $30 \times 10^7$ |
| 42-A-58V (avirulent) plus 10 mg xanthine† | 19/20 | 19/20 | 18/20 | 3/20 | 1/20 | $7.5 \times 10^7$ | $1.2 \times 10^7$ |
| 42-A-58 (virulent) | 20/20 | 20/20 | 18/20 | 9/20 | 2/20 | $4.2 \times 10^7$ | $0.95 \times 10^7$ |

* Numerator denotes the number of deaths in 72 hours; denominator, total mice injected.

† Control mice receiving 10 mg xanthine exhibited no adverse effects.

TABLE 2

*Effect of the reversion to the xanthine independent state on the mouse virulence of Salmonella typhosa, strain 42-A-58V (avirulent)*

| CHALLENGE SUSPENSION ADMINISTERED IN 5 PER CENT HOG GASTRIC MUCIN | NUMBER OF ORGANISMS INJECTED | | | | | | | $LD_{50}$ | S.E. |
|---|---|---|---|---|---|---|---|---|---|
| | $5 \times 10^6$ | $5 \times 10^5$ | $5 \times 10^4$ | $5 \times 10^3$ | $5 \times 10^2$ | $5 \times 10$ | 5 | | |
| 42-A-58V (avirulent) | 7/10* | 1/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | $2.2 \times 10^6$ | $1.3 \times 10^6$ |
| 42-A-58V (avirulent) reverted to xanthine independence | 10/10 | 10/10 | 10/10 | 8/10 | 8/10 | 5/10 | 1/10 | 68 | 76 |
| 42-A-58 (virulent) | | | | 10/10 | 10/10 | 7/10 | 2/10 | 20 | 16 |

| CHALLENGE SUSPENSION ADMINISTERED IN PHYSIOLOGICAL SALINE | NUMBER OF ORGANISMS INJECTED | | | | | $LD_{50}$ | S.E. |
|---|---|---|---|---|---|---|---|
| | $7.5 \times 10^8$ | $1.5 \times 10^8$ | $3 \times 10^7$ | $6 \times 10^6$ | $1.2 \times 10^6$ | | |
| 42-A-58V (avirulent) | 5/20 | 0/20 | 0/20 | | | | |
| 42-A-58V (avirulent) reverted to xanthine independence | 18/20 | 13/20 | 8/20 | 1/20 | 0/20 | $5.8 \times 10^7$ | $2.8 \times 10^7$ |
| 42-A-58 (virulent) | | 16/20 | 8/20 | 2/20 | 0/20 | $4.4 \times 10^7$ | $1.6 \times 10^7$ |

* Numerator denotes number of deaths in 72 hours; denominator, total mice injected.

TABLE 3

*Effect of the reversion to the cystine independent state on the mouse virulence of Salmonella typhosa, strain 42-A-58 (virulent)*

| CHALLENGE SUSPENSION ADMINISTERED IN PHYSIOLOGICAL SALINE | NUMBER OF ORGANISMS INJECTED | | | | | $LD_{50}$ | S.E. |
|---|---|---|---|---|---|---|---|
| | $2.3 \times 10^8$ | $4.6 \times 10^7$ | $9.2 \times 10^6$ | $1.8 \times 10^6$ | $3.7 \times 10^5$ | | |
| 42-A-58 (virulent) | 17/20* | 12/20 | 4/20 | 1/20 | 0/20 | $45 \times 10^6$ | $21 \times 10^6$ |
| 42-A-58 (virulent) reverted to cystine independence | 17/20 | 10/20 | 4/20 | 0/20 | 0/20 | $38 \times 10^6$ | $16 \times 10^6$ |
| Ty 2 | 19/20 | 17/20 | 11/20 | 4/20 | 0/20 | $7.6 \times 10^6$ | $4 \times 10^6$ |

* Numerator denotes number of deaths in 72 hours; denominator, total mice injected.

In a manner similar to that described above, a reversion of strain 42-A-58 (virulent) was obtained which did not require cystine. Thus, with respect to its nutritional requirements, it resembled strain Ty 2. Virulence tests were carried out using as challenges, suspensions of strain 42-A-58 (virulent), the cystine independent reversion, and strain Ty 2. There was no indication that the reverted culture was any more virulent than the parent strain, and neither challenge was as virulent as that of Ty 2 (table 3).

## DISCUSSION

It is now generally appreciated that the phenomenon of bacterial virulence is complex and that the ability of a bacterial species to invade and cause the death of an animal host may be the summation of a number of different and independent attributes. Certain of these (such as the Vi antigen in the case of *S. typhosa*) originally were considered to be *the* virulence factor but are no longer so regarded. In a situation where a number of conditions must be met in order to infect and overwhelm the host, it is obvious that any one may become the limiting factor and hence appear to be the determinant of virulence.

Bacon and his co-workers, using the technique of Davis (1948) and Lederberg and Zinder (1948)

to isolate auxotrophic mutants, showed that purine requiring or *p*-aminobenzoic acid requiring mutants of *S. typhosa* were less virulent for mice than their prototroph parents. Employing the same techniques, Garber showed that purine requiring mutants of *Klebsiella pneumoniae* were less virulent for mice than the parent strain. When purines were injected with a purine requiring challenge suspensions, a significant decrease in the number of cells necessary to kill mice was noted. The data obtained in the present investigation confirm the findings of these two groups of investigators. However, their greatest importance lies in the demonstration that loss of synthetic ability (and mouse virulence) can and does occur under natural conditions. Whether or not the mutant was present in large numbers in the feces of the carrier is not known. It may have been just a chance isolation, and in any case was at most a "transient" (Sears *et al.*, 1949) since typhoid isolates obtained from the carrier one month previously and a month later exhibited the usual virulence for mice.

Although the ability to grow without xanthine appears to be the dominant factor in determining the virulence of the Panama 58 strains, reversion of these cultures to the tryptophanless state does not render them as virulent as strain Ty 2 when administered to mice as a saline challenge. Here another factor, namely the Vi antigen content of the respective strains, may very well play the deciding role. Felix and Pitt (1951) have shown that typhoid strains with the largest amounts of Vi antigen are the most virulent for mice. Since it is not agglutinated to a significant titer by typhoid O antiserum, strain Ty 2 is assumed by most workers to possess more Vi than the Panama 58 strains which agglutinate to the titer of the serum. Studies in this laboratory, using both biological assay and chemical isolation procedures, have demonstrated that strain Ty 2 contains approximately five times the amount of Vi antigen as the Panama strains (Landy, 1953*a*,*b*). The precise role that the Vi antigen plays in virulence has not been established. However, Bhatnager (1935) has suggested that its presence on the bacterial surface renders the cells resistant to phagocytosis.

Consequently, it now appears that at least two factors are of major importance in determining the mouse virulence of smooth typhoid strains: The Vi antigen content of the cells and the nutritional requirements of the culture. At the present time, only the Vi antigen has been implicated as a human virulence factor. Findlay (1951) and Felix and Anderson (1951) have shown that strains of *S. typhosa* isolated from mild cases of typhoid fever were deficient in Vi antigen and were of low virulence for mice. Cultures obtained from severe outbreaks contained greater amounts of Vi antigen and were more virulent for mice. Whether or not a similar correlation can be made in the case of nutritional variants remains to be established.

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SUMMARY

A mouse virulent and a mouse avirulent strain of *Salmonella typhosa*, both isolated from the Panama Carrier, were shown to have different nutritional requirements. The strain virulent for mice requires cystine and tryptophan for growth, while its naturally occurring avirulent mutant requires a purine in addition to these two amino acids. It has been shown that the peritoneal fluid of mice, which is the menstruum in which the challenge suspension must multiply initially, does not supply the nutritional requirements of purine deficient strains. The injection of xanthine into the peritoneum of mice challenged with the avirulent, purine requiring mutant resulted in a significant increase in virulence of this strain. A reversion of the avirulent culture to purine independence was accompanied by a marked increase in virulence. These results obtained with a naturally occurring mutant substantiate the work of other investigators who have shown that nutritional factors as well as antigenic structure may play a dominant role in determining the mouse virulence of certain bacterial cultures.

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