STUDIES ON INTRACEREBRAL TYPHOID INFECTION IN MICE. I : CHARACTERISTICS OF THE INFECTION

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THE experimental infection of mice with *Salmonella typhi* has now been used for over two decades, principally in the study of the interaction of typhoid antigens and antibodies, and in the assay of the protective potency of typhoid vaccines. This infection is produced by intraperitoneal inoculation of mice with bacilli (Grinnell, 1932) suspended in either saline or mucin. Even the most virulent strains of *Salm. typhi* fail to infect fatally unless large numbers, of the order of 70 million or more, are employed. Indeed, some workers have routinely used a challenge dose of approximately 250 million bacilli (Felix and Pitt, 1935 ; Felix and Anderson, 1951). However, if the organisms are inoculated as a suspension in gastric mucin, a substance which depresses host defences, relatively few organisms, 1000 or less, suffice to produce a fatal infection (Rake, 1935). Relatively little attention has been given to the nature of the infection initiated by either type of challenge. However, it was observed early that symptoms of acute intoxication arose shortly after the injection of a lethal dose of typhoid bacilli and that nearly all animals succumbed within 24–36 hr. Survivors became asymptomatic rapidly and such animals were found to harbour appreciable numbers of organisms (Ørskov and Kauffmann, 1936). Certain aspects of the infection produced intraperitoneally, such as the overwhelmingly toxic death and either the magnitude of the challenge dose required or the employment of a host-resistance depressant such as mucin, have resulted in criticism of conclusions based on the use of this technique.

Another method, that of infecting mice with *Salm. typhi* by intracerebral injection, was first described by Norton and Dingle (1935). This showed promise in that it was possible to infect mice with a moderate number of bacilli without the use of mucin. The intracerebral route has since proved to be the one of choice for the study of infection and immunity to *Haemophilus pertussis*, and is now the basis for official potency tests of pertussis prophylactic agents (Minimum Requirements : Pertussis Vaccine, 1952). On the other hand, workers concerned with laboratory studies of typhoid infection have generally ignored the intracerebral route of inoculation. We re-investigated this as a possible means of eliminating the features of the intraperitoneal procedure which have been considered most objectionable. It was found that with the use of an appropriate mouse strain and challenge culture, a fatal infection could be established with a small inoculum. This report identifies the factors required for the establishment of this infection and describes certain of its characteristics.

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MATERIALS AND METHODS

Strains of Salm. typhi.—The widely employed vaccine strains, Ty2 and 58, were selected as representing strains containing both Vi and O antigens. Also selected were the international strain O–901 as a pure O strain, and the strain Vi I, as a serologically rough Vi culture containing little or no O antigen. Lastly, a streptomycin-dependent variant was employed which had been isolated from the parent Ty2 strain by our colleague, Dr. L. Baron. This culture was maintained on meat extract agar containing 20 μg. streptomycin per ml.

Preparation and administration of challenge.—Challenge suspensions were prepared by growing cultures on veal infusion agar for 12–15 hr. at 37°. The organisms were harvested and the numbers in the suspensions were standardized spectrophotometrically. Dilutions were made in 1 per cent pancreatic digest of casein (Difco) in saline pH 7·1, so that the desired number of viable bacilli for intracerebral inoculation was contained in 0·03 ml. The number of organisms in the challenge suspensions was checked by plating appropriate dilutions. Challenge suspensions were injected within 1 hr. of preparation. The injections were made intracerebrally through the foramen magnum of mice lightly anaesthetized with ether with short-bevel, ½ inch (0·6 cm.) 25 gauge hypodermic needles and 0·25 ml. tuberculin syringes.

Bacterial counts.—The number of bacilli in the brain and other organs of infected mice were determined as follows: The organ was excised aseptically, ground with sterile sea-sand to a homogeneous paste, and suspended in trypticase-soy broth (Baltimore Biological Laboratories). Serial dilutions of the suspension were made in this broth and in addition appropriate samples were plated on veal infusion agar. The number of organisms in the whole organ was calculated from the plate counts and from the highest dilution of broth exhibiting growth.

Mouse strains.—Animals of both sexes, 5–7 weeks of age and weighing 12–14 g. were used. Eight genetically classified inbred strains of mice were kindly supplied by Dr. George Jay of the National Institutes of Health, Bethesda, Maryland. These are designated in Table I according to the standardized nomenclature for inbred strains of mice (Carter, Dunn, Falconer, Grüneberg, Heston and Snell, 1952). Two additional strains obtained from that organization were randomly-bred Swiss albino mice (GP), and brother-sister matings of the GP mice, referred to as the NIH strain. Six inbred strains were provided by the Department of Animal Husbandry, Walter Reed Army Institute of Research. The history of these strains is recorded at this institution. CFW mice were purchased from Carworth Farms Inc., New York City, New York.

Morbid anatomical examinations.—Those mice destined for histopathological examination were killed with chloroform, autopsied, the brain was removed intact, fixed in neutral formalin, cut coronally into multiple blocks approximately 4 mm. in width, then embedded in paraffin, cut at approximately 7μ and stained with haematoxylin and eosin.

RESULTS

Susceptibility of Different Mouse Strains

The strain of mice generally employed for typhoid studies by one of us (M. L.) has been the well known Bagg strain. Preliminary tests showed that it was possible to produce a fatal infection in these animals by intracerebral inoculation. However, with any of a number of virulent strains of *Salm. typhi*, mortality seldom exceeded 40–50 per cent even when large inocula were used. Consequently, attention was then directed towards the possible importance of genetic differences in mice in determining susceptibility to intracerebral inoculation. A total of 17 strains of mice, most of which were genetically classified, were available for this purpose. In any given test, not less than 20 mice per group were employed. Each mouse strain was tested at least twice and most strains were tested three to five times. It was not possible to carry out comparisons of all strains in a single experiment, but similar test conditions were maintained throughout and

each experiment incorporated the maximum number of strains. The results of this comparison are given in Table I, and show important differences in susceptibility to intracerebral inoculation of approximately 50,000 *Salm. typhi* Ty2, mortality varying from 18 to essentially 100 per cent.

TABLE I.—*Susceptibility of Different Strains of Mice to Intracerebral Typhoid Infection.*

Susceptibility	Strain*	Number of mice tested	Mortality (per cent)
High	BALB/c[1]	227	99·6
	A/He[1]	53	93
	A/L[1]	102	97
	C3H[1]	79	91
	RI[1,2]	75	91
Moderate	DBA[1]	102	82
	CAF1[1]	94	80
	Cinnamon[2]	39	69
	BSVS[2]	39	64
	RN Brown[2]	63	64
	C57BL[1]	70	48
	CFW[3]	57	46
	BALB[2]	115	43
Low	RJ White[2]	33	36
	GP[1]	97	34
	SWR[1]	42	26
	NIH[1]	60	18

All mice challenged with 30,000–50,000 *Salm. typhi* Ty2.
* Obtained from : (1) National Institutes of Health, (2) Walter Reed Army Institute of Research, (3) Carworth Farms.

For convenience in presentation, the mouse strains have been arbitrarily grouped as being of high, moderate or low susceptibility. It is appreciated that were it feasible to carry out complete virulence titrations, *i.e.*, an extended range of challenge doses for each mouse strain, this order and degree of susceptibility probably would shift somewhat. However, it is probable that as the challenge level is reduced, variation in individual mouse susceptibility would become more apparent. Indeed, experiments with BALB/c mice, the strain of highest susceptibility, have shown that while a challenge as small as 20 organisms may induce a fatal infection, the percentage of animals which succumb in any given experiment employing 10–200 cells is highly variable. The variability in distribution of organisms at such low challenge dosages and the heterogeneity of the mouse population probably account for the unpredictability of these results. During the course of this and the accompanying investigation (Landy, Gaines and Sprinz, 1957), 1219 of a total of 1240 normal BALB/c mice inoculated intracerebrally with 5,000–50,000 *Salm. typhi* died, showing that this technique consistently produced a fatal infection. In any case, the results given in Table I serve to emphasize the important part played by genetic constitution in the establishment of a fatal intracerebral infection in the mouse. On the basis of these findings, strain BALB/c was selected for all subsequent work.*

* It is noteworthy that the strain of highest susceptibility (BALB/c) is an inbred variant of the Bagg strain (BALB) used in these laboratories and initially found to be of relatively low susceptibility to intracerebral challenge (43 per cent mortality).

Virulence of Different Strains of Salm. typhi

The availability of a mouse strain of uniformly high susceptibility permitted a direct comparison of the virulence of different strains of *Salm. typhi*. Table II shows the results of one of several experiments in which 4 strains, which have been studied extensively in many laboratories during the past 20 years, and which represent combinations of the major antigenic components of this organism, were compared. It is apparent that strains such as 58 and Ty2 which possess both Vi and O antigens, were highly lethal, since the LD_{50} of both was less than 200 cells. On the other hand, strains containing only one of these antigens showed limited virulence. Thus, the LD_{50} for the serologically rough Vi strain is in excess of 10 million, while that for the O strain is approximately 5 million cells. It is of interest and probably significant that the relative mouse virulence of these strains by the intracerebral route is, for the most part, similar to that by the intraperitoneal route.

TABLE II.—*Virulence of* S. typhi *for BALB/c Mice Inoculated Intracerebrally: Comparison of Strains.*

Strain	Antigenic components	Challenge dose				LD_{50}
		10,000,000	1,000,000	100,000	10,000	
O-901	O	13/20*	8/20	8/20	5/20	5,000,000
Vi I	Vi	2/20	0/20	2/20	1/20	>10,000,000
		200,000	20,000	2,000	200	
Ty2	Vi+O	19/19	20/20	20/20	15/19	<200
58	Vi+O	20/20	20/20	19/22	13/20	<200

* Fractions = Number of deaths/total number mice.

Relationship between Bacterial Multiplication, Tissue Changes and Mortality in Mice

It was felt that a study of the sequence of events following intracerebral inoculation, such as determination of the rate of multiplication of the inoculum, the dissemination of bacilli in organs and the progressive development of characteristic lesions in the brain, might provide a better understanding of the nature of the infective process. Accordingly, a number of experiments with these objectives in mind were performed, of which the following is typical. Three hundred mice were inoculated intracerebrally with 40,000 *Salm. typhi* Ty2, the animals randomly distributed 5 to a jar, and the jars assembled into groups of 100 mice. One group was employed for the purpose of following bacterial numbers and distribution, another for study of the development of brain lesions, and the third for the mortality rate. At 6 and 12-hour intervals, jars of 5 mice from each of the first 2 groups were selected in accordance with a predetermined schedule and the animals sacrificed for bacteriological and histopathological study, while mortality in the third group was recorded.

Table III shows the multiplication of challenge, the dissemination of bacilli in several organs, as determined by viable count, and the number of mice which succumbed at indicated intervals following challenge. It will be noted that at zero time (actually 10 min. post-challenge), essentially all the inoculum was

accounted for. In the brain significant, but not extensive, multiplication of organisms occurred as early as 12 hr. after inoculation. Numbers increased rather rapidly after 18–24 hr., attaining a maximum of several hundred million bacilli, which was maintained until all animals succumbed. In other experiments, maxima of 500–1000 million organisms were encountered. By 12 hr., bacilli were found in the heart's blood, spleen and liver. In contrast to the findings in the brain, much smaller numbers of bacilli were demonstrable in these organs. The viable counts in this and other experiments ranged from 100 to 10,000 bacilli per ml. of blood or entire spleen or liver. Within these limits the numbers varied widely among mice at any given time and from one time period to another, and thus provided no evidence of progressive increase in total count in these organs. It is likely that the presence of bacilli in spleen and liver reflect their presence in the blood stream rather than separate foci of multiplication.

TABLE III.—*Multiplication and Dissemination of Typhoid Bacilli in Mice Following Intracerebral Inoculation.*

Hours post-challenge	Percentage mortality (cumulative)	Bacteriological findings			
		Brain* (whole organ)	Blood† (per ml.)	Spleen† (whole organ)	Liver† (whole organ).
0	—	40,000	0	0	0
6	0	20,000			
12	0	500,000	600	500	6,000
18	2	12,500,000			
24	6	25,000,000	4,000	3,700	400
36	8	35,000,000			
48	10	35,000,000	3,300	4,000	2,800
60	13	80,000,000			
72	20	79,000,000	5,500	500	7,000
84	42	200,000,000			
96	59	400,000,000	2,000	500	200
108	73	300,000,000			
120	85	350,000,000	500	1,900	3,300
132	95	95,000,000			

All mice challenged with 40,000 *Salm. typhi* Ty2.
* Mean values for groups of 5 mice.
† Mean values for groups of 3 mice.

The only extensive multiplication of the inoculum occurred in the area of deposition, the brain. The relationship between the rate of multiplication in this organ and the concomitant mortality is depicted graphically in the Figure.

There was a lag of approximately 3 days between the development of large numbers of bacilli in the brain and progressive rise in mortality. Few deaths occurred during the first 2 to 3 days although most animals appeared ill as evidenced by a ruffled coat, a gummy exudate sealing the eyelids, a characteristic hunched posture and markedly reduced activity. Beginning on the 3rd or 4th day the animals generally exhibited paralysis and thereafter rapidly succumbed, mortality usually reaching 100 per cent by the 6th to the 10th day depending on the dose.

A smaller inoculum generally produced a similar picture with regard to the course of disease and multiplication of organisms, except for a slower rate of development; the bacterial populations ultimately attained in the brain were comparable to those resulting from inoculation with 50,000 cells.

Examination of brains harvested according to the schedule as outlined previously reveals a characteristic sequence of events in the evolution of the inflammatory process. The size of the inoculum apparently only influences the speed with which the changes occur, but not the sequence as such, nor the pattern and distribution of the lesion or the type of cellular exudate. In an experiment in which 5000 organisms were injected, the first changes noted within a few hours were a fresh haemorrhage along the needle track and an early glial-mesenchymal response. At the same time an early meningitis was present, exudate containing polymorphs and mononuclear cells. From the beginning the meningitis was diffuse and became progressively more severe. Already at 24 hr. the spread of

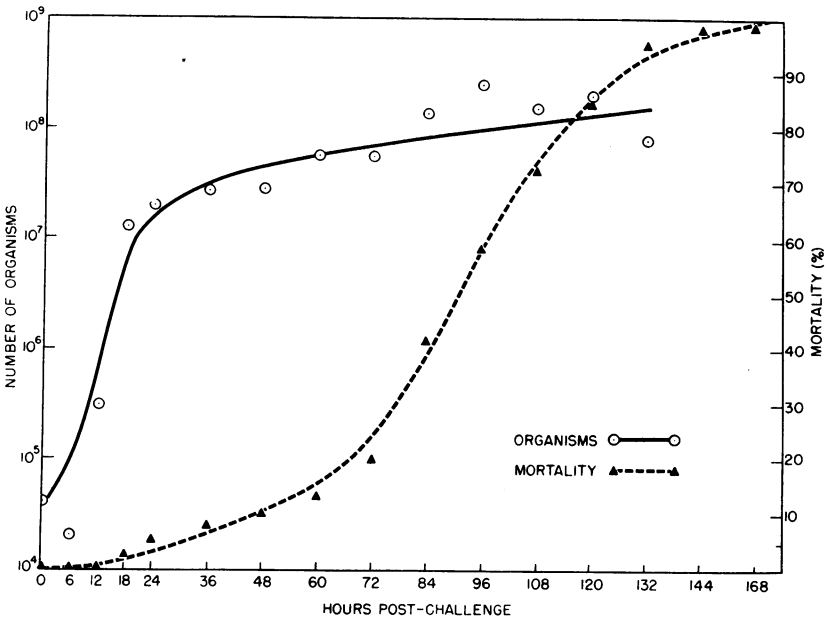

FIGURE.—Bacterial multiplication (in brain) and mortality following intracerebral inoculation of mice with 40,000 *Salm. typhi* Ty2.

the inflammation along the Virchow-Robin spaces was noticeable. At the inoculation site a small abscess had formed. There was a moderate degree of hyperaemia which progressively increased. At 36 hr. this had affected the subependymal vessels and there was a minimal diffuse inflammatory infiltration of the connective tissue stroma of the choroid plexus. The ventricles were still free of exudate. At 48 hr. a significant symmetrical, bilateral extension of the inflammatory process within the ventricular system had taken place, producing an early purulent ependymitis, particularly noticeable in the lateral ventricles. At about 96 hr. the ventricular system was completely filled with pus. There was now a quite extensive liquefaction necrosis of the subependymal brain tissue resulting from an extension of the inflammatory process beyond the confines of the lateral ventricles with separation of the neocortex from the hippocampus. At 120 hr. the intensity of the inflammatory process had further increased. There is evidence of definite obstruction of the ventricular system and distension of the ventricles

with pus. The meningitis had likewise continued to increase in severity. There was some further progression of the inflammatory reaction at the 168-hr. interval, the last studied. The animals at this time were succumbing to a diffuse purulent meningo-encephalitis and particularly to a purulent ependymitis with severe dilatation of the ventricular system due to obstruction. The histological findings correlate well with the clinical course and the mortality curve, as well as with the propagation rate of the organisms.

In as much as endotoxin is recognized as the principal noxious component elaborated by the typhoid bacillus it was of interest to determine the extent to which it contributed to the outcome of the infective process. Observations up to this point had suggested that if endotoxin *per se* played a rôle in the evolution of the process it was only in so far as it was connected with the virulence of the challenge strain which permitted the organism to multiply progressively.

In order to test this assumption experiments were done to ascertain the rôle of endotoxin in the pathogenesis of the intracerebral infection. Purified *Salm. typhi* endotoxin (Landy and Johnson, 1955), in a dosage range of 1 to 30 μg. (estimated to be equal to 0·07 to 2 billion organisms) proved highly toxic but not lethal for mice when injected intracerebrally. The animals showed symptoms indistinguishable from those of infected mice at 2 to 4 days but recovered within a few days. Histological examination gave variable results in two experiments using 15 μg. of endotoxin. In one group of animals a number of deaths resulted from extensive haemorrhage and necrosis of the midbrain, the site into which the injection was made, while in a subsequent experiment using the same dose and the same strain of mice, these findings could not be duplicated. Instead the inflammatory reaction never exceeded the 24-hr. phase of the infection of the brain described above. The peak of the endotoxin response was reached within 24 hr. and subsequently rapid healing took place corresponding to the clinical course. These experiments leave little doubt about the immediate and direct effect of purified endotoxin on brain tissue. However, this effect is rather rapidly dissipated and is not entirely consonant with the slight histological lesion observed. In contrast, in the animals challenged with bacteria, lesions of similar intensity are associated with only a moderate indisposition of the animal.

In other experiments groups of 20 mice each were given 10, 100, or 1000 million heat-killed *Salm. typhi* Ty2 intracerebrally. Relatively little evidence of toxicity was manifested by these animals, and only 2 of the mice receiving the largest dose succumbed. Brains of mice killed 192 hr. following the challenge showed only a slight to moderate hyperaemia. There was a slight haemorrhage at the inoculation site together with a mild glial-mesenchymal response. There was no sign of meningitis or ependymitis. Only those animals receiving the highest dose showed in addition a slight exudate of mononuclear cells in the pia-arachnoid. These findings compare well with the clinical course and show that heat-killed organisms do not elicit a significant inflammatory reaction; they produce less effect than an equivalent amount of purified endotoxin.

Another experiment was designed to determine whether viable cells *per se* are more toxic than those killed by heat. Groups of 40 mice each were inoculated intracerebrally with a streptomycin-dependent variant of the Ty2 strain, *i.e.*, a viable, but non-multiplying challenge. In the groups inoculated with 100 million or less of these organisms, few if any mice died (3 deaths in 40 mice injected with

100 million bacilli). It was only when 1000 million bacilli were administered that a considerable number of deaths (75 per cent) occurred. This experiment was repeated with essentially similar findings.

Histologically the brains of mice inoculated with 100 million of these organisms and killed approximately 7 days later showed fairly massive abscess formation with necrosis of the surrounding brain tissue at the inoculation site in the midbrain. There was a slight to moderate degree of meningitis and ependymitis but no spreading encephalomyelitis. With an inoculum of 1 million bacteria there was, at the same time interval, a distinct tendency towards healing of the needle track with only a slight leucocytic infiltration, while compound granular cells were very much in evidence. There was a sprouting of capillaries and a slight glial-mesenchymal response. This pattern of response differed from the previously described reaction to infection with virulent bacilli in that the inflammation was practically limited to the inoculation site where it was severe with the higher dosage. Massive abscess formation at the inoculation site was only observed in these mice infected with streptomycin-dependent organisms; it was not seen following inoculation with purified endotoxin. Endotoxin appeared to act primarily on the capillaries and in certain instances caused massive haemorrhage and necrosis. The degree of meningitis and ependymitis seen following inoculation with streptomycin-dependent organisms was distinctly less than that present at the same time interval following inoculation of 5000 *S. typhi* Ty2 as previously described. Endotoxin did not induce ependymitis and only a slight degree of meningitis while the animal inoculated with virulent bacilli succumbed to a spreading, very severe acute purulent meningo-encephalitis with particular involvement of the ventricles. The death of the animals inoculated with viable but non-multiplying organisms was attributable to the massive degeneration of the midbrain, the site of deposition of the inoculum.

DISCUSSION

The evidence presented here shows that under appropriate conditions, mice may be fatally infected with small numbers of *Salm. typhi* inoculated intracerebrally. In addition to a susceptible host, the antigenic composition of the organism is important in producing this fatal infection. The combination of Vi and O antigens in *Salm. typhi* appears to be essential in establishing a fatal infection in the presence of those anti-bacterial factors which are effective against strains containing only Vi or O antigen alone. These observations fully confirm the main facts established for mice infected intraperitoneally, as first reported by Felix and Pitt (1935) more than 20 years ago and subsequently by other workers. It is significant that despite the obvious differences in the nature of the infective processes initiated by these two routes, a fatal infection in both instances is produced only by strains of *Salm. typhi* possessing the two major antigenic components. It is apparent that, regardless of the challenge route, the body defence mechanisms, probably humoral, are capable of controlling infections initiated by Vi or by O strains of *Salm. typhi*. Differences in the virulence of challenge strains may be greatly magnified by the intracerebral route. For example, the LD_{50} of strains Ty2 and O–901, administered intraperitoneally as saline suspensions, differ by approximately 20-fold (Felix and Pitt, 1951), while by the intracerebral route these differences are enlarged to more than 1000-fold (see Table II).

It is obvious that the reaction of the mouse to a given strain of *Salm. typhi* is very different in the two sites. A small inoculum in the peritoneum is rapidly eliminated since the organisms are dispersed throughout the peritoneum and are brought into immediate contact with phagocytic cells. A similar inoculum in the brain of BALB/c mice initially is restricted more or less to the area of deposition. The cellular defence mechanism, the microglia, as well as the blood-borne macrophages and leucccytes are promptly mobilized to cope with the challenge. Nevertheless, the infective process becomes established and progresses to a characteristic meningo-encephalitis. These facts support the view that in the procedure here described the brain does not function merely as a suitable culture medium, but rather that the interaction between host and organism in brain tissue gives rise to a characteristic sequence of events. The primary determinants of the ensuing inflammatory process are the effective multiplication of the micro-organisms and the rate of mobilization of defence mechanisms, cellular and humoral, their interaction and their effect on brain tissue. It is noteworthy that mice inoculated intracerebrally exhibit an early bacteraemia which persists until death. The lesions in the brain are of such magnitude that the bacilli have ready access to the general circulation and it can be safely assumed that the brain serves as a focus from which bacilli continue to enter the blood stream and organs such as spleen and liver. The circulation normally provides an effective clearing mechanism. There is little doubt that this dissemination of organisms is of secondary importance compared to multiplication in the brain, and probably contributes little, if anything, to the fatal result.

The marked variation in susceptibility of genetically different mouse strains is of unusual interest. Genetic differences are known to play an important part in the susceptibility of mice to certain bacterial (Gowen, 1948) and virus (Sabin, 1952) infections. The variation in susceptibility of mice of different genetic constitution as here reported may be a reflection of inherently different levels of natural anti-bacterial factors, differences in the concentration of essential metabolites or variation of susceptibility of brain tissue. At present these remain to be determined.

Results of experiments with isolated endotoxin and viable, non-multiplying, or killed organisms, undertaken to define the rôle of endotoxin in the death of infected animals, indicate that this substance probably is not the major determinant of death. It must nevertheless be remembered that the doses tested correspond only to the numbers of bacilli found at a given time and not necessarily to the total amount of endotoxin formed during fatal infection. Consequently, these experiments do not entirely rule out the possibility of endotoxin being a primary cause of death, since accumulation of endotoxin during the infection undoubtedly results in considerably greater total quantities than were administered. It is not possible to distinguish histologically the leucocytic response produced by endotoxin from that provoked by the organisms themselves.

The important inference to be drawn from the experiments with killed typhoid bacilli, isolated endotoxin, and a streptomycin-dependent variant of this organism is that the lethality of *Salm. typhi* may be attributed mainly to the growth of the organisms in brain tissue. The relatively small initial dose of viable bacilli, which increases in number concomitantly with the progressive fatal infection, in itself attests importance of the changes resulting from this multiplication of organisms in the brain.

SUMMARY

A study was made of the intracerebral infection of mice with *Salm. typhi* in which it was shown that a susceptible strain of mice and a challenge culture possessing both Vi and O antigens were required for the production of an infection which terminated fatally. A great variation in susceptibility of genetically classified mouse strains was found. Under optimal conditions, *i.e.*, use of BALB/c mice and *Salm. typhi* Ty2, few organisms sufficed to initiate a fatal infection.

After intracerebral inoculation with virulent bacilli a period of approximately three days elapsed during which there occurred extensive bacterial multiplication in the brain. This was followed by a progressive rise in mortality, which reached 100 per cent in 6–10 days. The histopathological changes in the brain proceeded at a rate similar to that of bacterial multiplication. The animals finally succumbed to an acute purulent meningo-encephalitis and a secondary cerebral oedema and pyocephalus.

Intracerebral typhoid infection in contrast with intraperitoneal, can be established with small numbers of organisms, requires no adjuvant and produces a progressively developing fatal infection with a well defined incubation period, and characteristic dissemination of organisms and tissue changes.

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