IMMUNITY TO SALMONELLA INFECTION IN MICE*

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INTRODUCTION

Certain aspects of immunity, such as resistance to infection by the natural route, can be examined only with an organism which is a natural pathogen for the experimental animal. It seemed to me, therefore, of some interest, as it has to a number of workers in the past, to study immunity in mice to organisms that cause naturally occurring infections in these animals.

The purpose of these studies was to investigate the degree and duration of resistance, produced by inoculation with vaccines of certain members of the *Salmonella* group, to infection by artificial and by natural routes. Resistance to an artificial route of infection was tested by intraperitoneal challenge, resistance to the natural route was tested by oral challenge. Though the method of testing resistance by exposure to infected animals, after the manner of Greenwood, Topley & Wilson (1931) was not attempted directly, it did in effect come into operation, in certain experiments, in the form of secondary animal-to-animal spread following the primary infective dose.

The duration of immunity to intraperitoneal injection was followed because of the scarcity of reported observations on the maintenance of specific resistance following inoculation of vaccine, despite numerous observations on the persistence of immunological reactions such as agglutination. Parallel observations on immunity to oral challenge were not found possible because of the small degree of protection against infection by this route.

MATERIALS AND METHODS

Bacterial strains. A strain of *Salmonella dublin* was used in most of the experiments. It was one of high virulence for mice, its LD_{50} by intraperitoneal inoculation being approximately four organisms. A strain of *S. typhi-murium* of comparatively low virulence for mice was also used. Its LD_{50} was approximately 1,000,000 organisms.

Animals. White mice bred and used in these laboratories for a number of years were employed. No spontaneous infections with salmonellae were encountered during the period these experiments were carried out.

Vaccines. Vaccines of each strain were prepared by washing cultures from agar slopes, heating for $1\frac{1}{2}$ hr. at 56° C. and adding 0·25 % phenol. The density was adjusted to approximately 10^9 organisms per ml. using McFarland standards. Each

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dose was 0·25 ml. The interval between doses was 2 weeks. Intramuscular inoculation was employed in certain experiments, intraperitoneal inoculation in others.

Immunity testing by intraperitoneal challenge

Mice were allotted at random to groups of which certain were inoculated with vaccine of the test organisms and others kept as controls. The animals were kept in large cages taking up to fifty mice, except where stated otherwise. Vaccinated and control mice were challenged at various intervals after immunization of the former, the survival rate being the measure of immunity. In experiments with *S. dublin* the test dose was 0·5 ml. of an 18 hr. broth culture diluted 1 in 10^7 in normal saline. No appreciable change in the viable count occurred during the period required for any experiment. In two experiments, E1 and E2, beef-infusion broth was the culture medium used. In all other experiments the medium was meat-extract broth. In an attempt to keep variation in the number of viable bacteria at a minimum, the same batch of medium was used throughout any one experiment except for the 12-month test in experiment E4. Plate counts were made of the dilution used for the challenge dose. These indicated that the dose was sufficiently constant within each experiment to allow comparison of the results. In all experiments the dose was such that it killed over 90% of controls.

In experiments with *S. typhi-murium* the challenge dose was 0·5 ml. of a 1 in 100 dilution of broth culture. Here an estimate only of the number of viable organisms, based on plate counts, could be made.

Immunity testing by oral challenge

An 18 hr. broth culture was fed by dropping into the mouth from a syringe fitted with a no. 26 gauge needle giving 60 drops per ml. The amount which mice would take varied from 3 to 5 drops but the average volume was equal in both the vaccinated and control groups.

Observation of animals after challenging

Deaths were recorded daily so that survivors at various periods after challenge could be noted. Missing mice were counted as dead. The effect of such losses was negligible as almost none occurred during the first 28 days of observation, the period used for comparisons of survival rates. In certain experiments where observation was continued for more than 28 days some losses occurred after this time but were not greater than 5% in any cage. Though the total numbers of survivors were used to measure the results of challenging, cultures of heart's blood and spleen were made from mice dying on test unless the animals had been destroyed by other mice or were otherwise unsuitable for culturing. Almost all control mice gave positive cultures, while only about two-thirds of the vaccinated mice were positive. No clear reason for this difference can be given, but it would appear to be associated with the longer period between challenge and death in vaccinated mice, that is, a less acute infection. These proportions remained fairly constant so that comparisons were not unduly affected. Two exceptions are noted.

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Cultures were evidence of the specific cause of death in most animals and gave indication of the presence of certain other intercurrent infections.

The selection of an observation period upon which to base survival rates was made from the following considerations. While the object was to measure the effect of a definite challenge dose, infection once initiated in a cage tended to spread and eventually cause deaths in animals which had survived the primary infecting dose. It was found in preliminary experiments that almost all deaths from the challenge dose took place within the first four weeks, while few deaths from the spread of infection resulted in this period. Two experiments with *S. dublin* are shown in Table 1, in which half of a group of vaccinated mice were kept in individual cages

Table 1. S. dublin. *Comparison of survivors of vaccinated mice kept in large cages and in individual cages, after intraperitoneal challenge*

Expt. no.	Type of cage	Challenge dose no. of organisms	No. of mice challenged	No. of survivors at 2 wk.	4 wk.	6 wk.	8 wk.
E6	Large	23	40	38	36	35	33
	Individual		40	39	36	36	36
E8*	Large	95†	21	14	12	12	8
	Individual		21	15	14	14	14

* Blood cultures taken on all mice dying on test except one at 8 weeks. All cultures positive. Spleen cultures taken on all survivors. All cultures negative.

† Dilution of broth culture 1 in 2 million.

to prevent transfer of infection, and half in the usual type of large cage. Deaths in both individual and large cages were approximately equal up to 4 weeks, after which time there were no deaths in individual cages though they continued to occur in the large cages. A similar distribution of deaths in immunized mice kept in individual cages can be seen in an experiment with *S. typhi-murium* (*S. aertrycke*) reported by Ibrahim & Schütze (1928).

That deaths from secondarily acquired infection were relatively few during the first 4 weeks after challenge was shown more clearly by four experiments in each of which thirty normal mice were kept in the same cage with twenty mice infected with *S. dublin*, deaths in the normals then being due to this cause only. When normal mice were exposed to intraperitoneally injected mice, three deaths occurred within 4 weeks in an experiment with males. No deaths resulted in a similar experiment with females. When infection was initiated by feeding, there were two deaths in the normal males and one in females. The survival rate at 4 weeks, even when mice were kept in large groups, appeared then to be a reasonable measure of immunity to the challenge dose and satisfactory at least for comparative purposes.

RESULTS

(1) *Immunity to intraperitoneal challenge.* In Table 2 are shown the number of survivors following intraperitoneal challenge at various periods after immunization with *S. dublin.* In the various experiments almost all of the controls died within

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the first 4 weeks after challenging, at which time many of the vaccinated animals survived. The time from challenge to death tended to be longer in the vaccinated animals than in the controls, even within the first 4 weeks. When mice were observed for longer periods after challenging, as in Expts. E1 and E2, deaths continued to occur, most of the mice eventually dying. It would appear, therefore, that inoculation with vaccine gave considerable protection against intraperitoneal challenge, but did not prevent deaths resulting from natural animal-to-animal spread of infection.

Table 2. S. dublin. *Results of intraperitoneal challenge at various periods after inoculation of vaccine*

Expt. no.	Vaccination of mice	Challenge: Time after last dose of vaccine (months)	Challenge: No. of organisms	No. of mice challenged	No. of survivors at 2 wk.	4 wk.	6 wk.	8 wk.	10 wk
E1	Intramuscular, 2 doses (males)	1	75	Vacc. 50	31	20	11	2	0
				Con. 10	0	—	—	—	—
		3	65	Vacc. 37*	23	15	8	4	0
				Con. 10	1	1	0	—	—
		5	70	Vacc. 41*	27	15	7	—	—
				Con. 10	0	—	—	—	—
E2	Intraperitoneal, 2 doses (males)	1	72	Vacc. 46	40	39	31	28	23
				Con. 7	2	0	—	—	—
		3	58	Vacc. 37*	32	28	22	17	7
				Con. 10	1	0	—	—	—
		5	70	Vacc. 47	40	33	—	—	—
				Con. 10	1	1	—	—	—
E4	Intramuscular, 3 doses (females)	1	35	Vacc. 50	48	43	—	—	—
				Con. 49	9	2	—	—	—
		7	36	Vacc. 25†	24	22	—	—	—
				Con. 6	2	0	—	—	—
		12	38	Vacc. 38	20	18	—	—	—
				Con. 10	0	—	—	—	—

* Loss in numbers before challenge apparently due to fighting.
† Loss in numbers before challenge due to pneumonia of unknown aetiology.

Table 3 shows the results of two experiments with the strain of *S. typhi-murium*. About the time of the 6-month testing, cross-infection with *S. dublin* occurred, deaths from this cause in the vaccinated mice equalling those from the intraperitoneal challenge dose of *S. typhi-murium*. The specific immunity was almost certainly higher than the survivals indicate.

In Table 4 the percentage surviving at 4 weeks after challenge is shown for each experiment. From experiments with *S. dublin*, it is seen that the level of immunity reached was well maintained for at least 5–7 months and to a moderate degree for 12 months. In the 12-month test two positive cultures only were obtained. These

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mice were some 15 months old. The effects of age and possibly unknown infections consequent upon long confinement in a large group may well have affected survival. Specific immunity was probably higher than indicated by the survival rate. The results of experiments with *S. typhi-murium*, though obscured by cross-infection,

Table 3. S. typhi-murium. *Results of intraperitoneal challenge at various periods after inoculation of vaccine*

(Challenge dose 4 million organisms.)

Expt. no.	Vaccination of mice	Time after last dose of vaccine (months)	No. of mice challenged	No. of survivors at 2 wk.	No. of survivors at 4 wk.
T1	Intramuscular, 2 doses (females)	2	Vacc. 49	43	42
			Con. 10	0	0
		6	Vacc. 37	28	23*
			Con. 20	3	3
T2	Intraperitoneal, 2 doses (females)	2	Vacc. 36	35	29
			Con. 10	0	0
		5	Vacc. 55	38	30*
			Con. 20	3	3

* 50 % of deaths due to cross-infection with *S. dublin*.

Table 4. *Survival rate 4 weeks after intraperitoneal challenge at various periods after immunization*

Expt. no.	Organism	Route of vaccine inoculation	Time after last dose of vaccine (months)	Survival rate (%)
E1	*S. dublin*	Intramuscular	1	40
			3	40
			5	37
E2	*S. dublin*	Intraperitoneal	1	85
			3	76
			5	70
E4	*S. dublin*	Intramuscular	1	86
			7	88
			12	47*
T1	*S. typhi-murium*	Intramuscular	2	86
			6	62†
T2	*S. typhi-murium*	Intraperitoneal	2	81
			6	55†

* Many non-specific deaths.
† 50 % of deaths due to cross-infection.

indicated maintenance of considerable immunity for at least 6 months. Since immunity was maintained after either intramuscular or intraperitoneal vaccination no question of local immunity arises.

(2) *Immunity to oral challenge.* The results of tests with *S. dublin* are shown in Tables 5 and 6. Though in only two experiments was the difference between

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vaccinated and control mice statistically significant (Expt. 3*a*, $P = 0{\cdot}04$; Expt. E 16, large cage, $P = 0{\cdot}001$), when differences appeared in other groups the mortality was less in the vaccinated animals. This suggests that there was a real though small degree of protection. It might be considered that in such experiments exposure to other infected animals increases the effective dose above the actual amount fed, and that resistance, though not raised sufficiently to withstand repeated infections, might be relatively efficient against a single infection by mouth. That spread of infection was not sufficient to cause more than an occasional death during the 4-week period of observation was shown, however, by two experiments described above. Moreover, it is to be noted that when mice were kept in individual cages no greater difference between vaccinated and controls appeared than when the mice were kept in large cages.

Table 5. S. dublin. *Results of oral challenge tests in vaccinated* and control mice*

Expt. no.	Challenge dose dilution of broth culture	Number of mice challenged	Survivors at 4 weeks	
			No.	%
E 3*a*†	1/10	Vacc. 47	44	94
		Con. 46	35	76
E 3*b*‡	1/5	Vacc. 52	23	44
		Con. 42	14	33

* Vaccine given intramuscularly.
† Challenged 2 months after last dose of vaccine.
‡ Challenged 5 months after last dose of vaccine.

Table 6. S. dublin. *Results of oral challenge* tests. A portion of vaccinated† and control mice kept in individual cages*

Expt. no.	Type of cage	No. of mice challenged	Survivors at 4 weeks	
			No.	%
E 10	Large	Vacc. 47	34	72
		Con. 47	26	55
	Individual	Vacc. 24	17	75
		Con. 23	13	56
E 16	Large	Vacc. 49	39	80
		Con. 50	18	36
	Individual	Vacc. 25	17	68
		Con. 25	17	68

* Broth culture diluted 1/3 fed 1 month after last dose of vaccine.
† Vaccine given intramuscularly.

In the experiment with *S. typhi-murium*, shown in Table 7, no evidence of protection was obtained. This strain proved to be of too low virulence to be satisfactory for oral tests, even though in this experiment the animals were observed for 6 weeks with the probable inclusion of deaths from spread of infection within the cages.

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Table 7. S. typhi-murium. *Results of oral challenge* tests. Vaccinated and control mice kept in same cages*

Expt. no.	Route of vaccine inoculation	No. of mice challenged	Survivors No. 4 wk.	Survivors No. 6 wk.	Survivors % 4 wk.	Survivors % 6 wk.
T5	Intraperitoneal	Vacc. 48	46	38	96	79
		Con. 40	36	30	90	75
	Intramuscular	Vacc. 49	47	43	96	88
		Con. 40	37	32	93	80

* Broth culture fed 1 month after last dose of vaccine.

DISCUSSION

From these results it is seen that, with two strains of salmonellae, a degree of immunity against intraperitoneal challenge, such that the majority of vaccinated animals could withstand an infecting dose which killed almost all controls, was produced by inoculation with vaccine, and that this was well maintained for at least 6 months. In the experiments with *S. typhi-murium* the decrease in survival rate at 6 months was partly, if not chiefly, due to deaths from cross-infection. In the *S. dublin* experimental group kept for 12 months the recovery of positive cultures was low, and many of the deaths were due to non-specific causes associated perhaps with age. Appreciable immunity was, however, still present. A possible explanation for the maintenance of the level of protection for at least 6 months with little or no apparent decrease is suggested by some observations reported in another paper (Reid & MacLeod, 1954). In passive protection tests it was found that the survival rate after challenge decreased very little with each tenfold decrease in the amount of serum given. A considerable fall in antibody level could occur with little change in survival rate.

When tested by introducing culture into the mouth, vaccinated mice showed no such increased resistance over unvaccinated animals. Nor did vaccination prevent animal-to-animal spread of infection in mice that appeared to have resisted intraperitoneal challenge. Webster (1922) challenged vaccinated and control mice with a mouse typhoid strain by intraperitoneal inoculation and by inoculation through a tube into the stomach, concluding that protection was shown against both routes. The differences between vaccinated and control mice after *per os* inoculation were, however, small and, in terms of the numbers used, inconclusive. Pritchett (1924) reported some protection against *per os* inoculation with the strain used by Webster but not with another strain. The differences between vaccinated and control groups were again small and not conclusive. Carnochan & Cumming (1952) reported reduced death-rates in mice given Typhoid Combined Vaccine containing *S. typhi* (*Eberthella typhosa*), *S. paratyphi* and *S. Schottmuelleri* as compared with those in unvaccinated mice, during an outbreak of *S. enteritidis* infection in a breeding colony. However, the composition of the various groups and the methods of comparison used do not appear to warrant

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a definite conclusion. Moreover, they stated that the vaccine, in the doses used, did not protect against intraperitoneal inoculation of organisms.

Greenwood *et al.* (1931), in extensive experiments with *S. typhi-murium* (*Bacterium aertrycke*), observed average survival times of 30–35 days, excluding animals surviving more than 60 days, in vaccinated mice as compared with some 26 days in controls, where the observed mice were exposed to infected animals. This difference they showed to be of statistical significance and, though comparatively small, they considered it to be of possible epidemiological importance. They also gave the results of experiments in which intraperitoneal challenge was carried out. Almost 400 mice were used consisting of four groups given *S. typhi-murium* vaccine, one group given *Pasteurella pseudotuberculosis* vaccine and three groups unvaccinated. In the groups given the specific vaccine the average survival times, in experiments limited to 28 days, were between 20 and 23 days with survival rates of about 40 %, as compared with average survival times of between 5 and 8 days with survival rates of 0–1 % in the other groups. Thus, out of a total of 194 vaccinated mice, 83 survived for 28 days as against 1 out of 200 mice unvaccinated or given non-specific vaccine. They recorded these results as evidence that the vaccine contained 'antigenic factors that should produce an efficient immunizing response'. They attributed the relatively poor protection afforded by vaccine against naturally spreading infection to the conditions of such experiments which were considered to involve severe and prolonged exposure. If, however, the difference lies in the route of infection rather than in the degree of exposure then the results reported in the present study could be considered as not inconsistent with and, indeed, essentially similar to those of Greenwood *et al.* (1931). The results of their experiments and those reported here indicate that vaccination can produce a moderate to high survival rate after intraperitoneal injection of a dose that kills most controls but, by contrast, does not give more than a small degree of protection against ingested organisms. Webster's observations (1922) would not be inconsistent with such a conclusion.

Certain questions arise out of this contrast. Whether a higher level of immunity, if such could be produced, would give satisfactory protection against oral infection is not known, but the similarities in the results of different studies employing different doses of vaccine and different methods of challenging make this unlikely. While the reason for the difference between protection against intraperitoneal and natural routes of infection is quite obscure, a possible mechanism might be considered. If increased resistance to intraperitoneal inoculation with *S. dublin* in vaccinated mice is due mainly, if not entirely, to humoral antibody (Reid & MacLeod, 1954), the availability of antibody in the tissues, a subject which is discussed in some detail by Topley, Wilson & Miles (1946), may be of particular importance. If bacteria entering the tissues from the intestinal tract initially reach sites, perhaps intracellular, not accessible to antibody, multiplication might then proceed until a sufficient number of organisms is produced to set up fatal infection in spite of a high level of serum antibody.

Two only of the many types of salmonellae have been examined in this study. However, the similarity in the effects of immunization with these strains which

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represent almost the two extremes in virulence, as well as with those used by Greenwood *et al.*, suggest that the same effects are likely to be found with various members of the group. The relative inefficiency of immunization against natural infection by mouth with salmonella organisms may be a characteristic of the mouse only, but it is at least a possibility that it is a characteristic of other species also.

SUMMARY

Experiments on immunity produced by vaccine were carried out in mice with two strains of salmonella.

Against intraperitoneal challenge protection was such that 40–80 % of vaccinated mice survived a dose that killed almost all controls. This level of immunity appeared to be maintained with little change for at least 5–7 months. An explanation for this stability is suggested.

Against infection by the natural route, either by direct feeding or by exposure to infected animals, protection from vaccine was of a comparatively low order.

Resistance to infection by an artificial route was, therefore, not a measure of resistance to infection by the natural route.

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