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The relative potencies of heat-killed and acetone-killed vaccines against *Salmonella typhimurium* in mice

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SUMMARY

Mice were immunized against *Salmonella typhimurium* with graded doses of heat-killed (HK) and acetone-killed (AK) vaccines and then challenged by the oral or intraperitoneal routes with two doses of *S. typhimurium*. HK and AK vaccines gave good protection against an intraperitoneal challenge, but failed to protect against an oral challenge which is presumably the natural mode of infection. HK vaccine was as potent as AK vaccine in reducing the mortality rate among mice challenged by the intraperitoneal route but, unlike HK vaccine, AK vaccine was also able to reduce the infectivity rate. With a small intraperitoneal challenge dose it was observed that a gradual increase in vaccine dose is associated with a corresponding fall in mortality rate, but with a larger challenge dose an increase in vaccine dose was associated with a corresponding increase in mortality rates. It was concluded that the protective potency of this type of vaccine may partly depend upon the total amount of antigen in the animal, i.e. including both the vaccine and the challenge organisms, at a critical time after challenge.

INTRODUCTION

'Mouse typhoid' or *Salmonella typhimurium* infection of mice has many interesting parallels in common with enteric fever in man; for example, the best form of protection against the experimental disease in mice appears to be that conferred by recovery from a virulent attack or prior infection with an avirulent strain of the causative organism (Topley, Wilson & Lewis, 1925; Hobson, 1957*a*, *b*). It is extremely difficult to immunize mice effectively against the disease, although many killed vaccines have been reported that have been moderately successful in protecting against an intraperitoneal challenge (Topley, Greenwood & Wilson, 1931; Hobson, 1957*a*). Raistrick & Topley (1934) reported that an acetone-killed vaccine was able to confer better protection against an intraperitoneal challenge than a heat-killed vaccine. MacLeod (1954) demonstrated that a heat-killed vaccine capable of conferring good protection against an intraperitoneal challenge was unable to protect mice against a challenge by the oral or natural route of infection. The present investigation compares the protective potencies of graded doses of heat-killed (HK) and acetone-killed (AK) organisms against challenge by the oral or the intraperitoneal routes.

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MATERIALS AND METHODS

Animals

Male Swiss white mice weighing 19–22 g. were used.

Bacterial cultures

Salmonella typhimurium strain 1566, phage type 1*a*/u57, derived from freeze-dried stock was used for preparing vaccines and for challenge.

Preparation of vaccines

Heat-killed phenol-preserved vaccine and acetone-killed freeze-dried vaccine were prepared as described in an earlier paper (Cronly-Dillon, 1972). These vaccines were administered in a volume of 0·1 ml. as a single subcutaneous injection at the root of the tail.

The challenge organisms were suspended in 0·1 M phosphate buffer at pH 8 and the actual dose administered in each case was determined by a viable count done by the method of Miles & Misra (1938). The challenge inoculum was administered in a volume of 0·1 ml. by the intraperitoneal route or by the oral route.

Design of experiments

Mice were prepared for oral challenge by starving overnight to ensure a speedy passage of the inoculum to the intestines. The infecting dose was administered by a narrow polythene cannula (0·5 mm. bore) passed into the stomach of the animal.

The protective potencies of HK and AK vaccines were assessed by challenging groups of immunized mice in parallel by the oral route or the intraperitoneal route. A batch of 280 mice was used for the oral challenge experiments and a similar number for the interaperitoneal challenge work.

In each batch of animals a group of 120 mice were immunized with HK vaccine and a similar number with AK vaccine. The 40 non-immunized mice remained as controls. Among the two vaccinated groups, sets of 40 mice each were given approximately 10^3, 10^5 and 10^7 vaccine organisms as a single subcutaneous injection. All the animals were challenged on the 15th day after vaccination. Twenty mice in each set of 40 animals were challenged with a small dose of *S. typhimurium* strain 1566 and the remaining mice were challenged with a large dose of organisms. For the intraperitoneal challenge, the small dose consisted of 20 organisms and the large dose was 2×10^4 organisms. The doses used for oral challenge were 3×10^4 and 3×10^7 organisms per mouse.

After challenge mice were housed in threes in cages that were cleaned out frequently, and the animals were transferred to freshly sterilized cages during the course of the experiment to reduce the chances of cross and auto-infection.

The animals were examined daily, deaths were recorded and an immediate necropsy was performed. The liver and spleen of each dead animal was cultured to test for the presence of *S. typhimurium*. On the 28th day after challenge, all surviving animals were killed and examined *post mortem* for bacteriological evidence of infection. The percentage mortality, infectivity and mean time to death of those mice that died were then calculated for each group.

Table 1. *Assessment of the protective potencies of heat-killed and acetone-killed vaccines against an intraperitoneal challenge with* S. typhimurium *strain* 1566 *over a period of* 28 *days after challenge*

Subcutaneous immunization						
Vaccine treatment	Dose	Challenge dose	No. of mice	Mortality rate (%)	Infectivity rate (%)	Mean survival time of mice that died (days)
Heat killed	10^3	20	20	20	90	14
		2×10^4	20	53*	100	13
	10^5	20	20	5*	90	15
		2×10^4	20	70*	100	13
	10^7	20	20	0*	100	...
		2×10^4	20	100	100	10
Control		20	20	42	100	15
		2×10^4	20	100	100	9
Acetone killed	10^3	20	20	25	65*	24
		2×10^4	20	50*	100	12
	10^5	20	20	15	90	16
		2×10^4	20	80	100	13
	10^7	20	20	0*	50*	...
		2×10^4	20	40*	100	13

* Indicates that the result was statistically significant when compared with observations on non-immunized controls (χ^2 test of probability).

Statistical analysis

The results were analysed by the χ^2 test of probability incorporating a formula that makes allowance for small numbers.

$$\chi^2 = \frac{\{ad - bc - \frac{1}{2}(a+b+c+d)\}^2 (a+b+c+d)}{(a+b)\,(c+d)\,(a+c)\,(b+d)}.$$

When the value for P was 0·05 or less, the result was considered as unlikely to be due to chance variation.

RESULTS

Assessment of protection against intraperitoneal challenge

The results of this study are given in Table 1. When unimmunized control mice were challenged with the test strain of *S. typhimurium* given intraperitoneally in two different doses (20 and 2×10^4 organisms per mouse) the mortality rates were 42 % and 100 % respectively, the mean time to death of the fatal cases was 15 and 9 days, and the infectivity rate was 100 % in each group.

Heat-killed vaccine

Mice that had been immunized with certain doses of HK vaccine showed a significantly reduced mortality rate, but the infectivity rates were not greatly reduced. The mean survival time did not appear to be related to the mortality rate. With a low challenge of 20 organisms per mouse statistically significant

Table 2. *The relationship between heat-killed vaccine dose, intraperitoneal challenge dose, and observed mortality in groups of Swiss white mice observed for* 28 *days after challenge*

Intraperitoneal challenge dose (no. of *S. typhimurium* organisms)	Vaccine dose (no. of HK organisms per mouse)	Mortality rate (%)
20	10^3	20
	10^5	5
	10^7	0
2×10^4	10^3	53
	10^5	70
	10^7	100

protection (as measured by a reduction in mortality) was observed in those mice vaccinated with 10^5 and 10^7 HK vaccine organisms. When a larger intraperitoneal challenge of 2×10^4 *S. typhimurium* per mouse was given, statistically significant protection was observed in animals vaccinated with 10^3 and 10^5 HK vaccine organisms.

It is of interest that, with a low challenge dose of 20 *S. typhimurium* per mouse, a graded increase of the vaccine dose from 10^3 to 10^5 to 10^7 organisms was associated with a corresponding fall in the values for percentage mortality (see Table 2) whereas, with a large intraperitoneal challenge dose of 10^4 *S. typhimurium* per mouse, a gradual increase in vaccine dose is related to a corresponding increase in mortality.

Acetone-killed vaccine

Vaccination with certain doses of AK vaccine conferred significant protection as measured in terms of reduced mortality and infectivity. Although mean survival times were prolonged in all the vaccinated animals they did not provide a good index of the degree of protection. With a small intraperitoneal challenge of 20 *S. typhimurium* it was seen that mice vaccinated with 10^7 AK vaccine organisms showed a statistically significant reduction in mortality and infectivity rates. Vaccination with 10^3 AK vaccine organisms significantly reduced the infectivity rate, and prolonged the mean survival time of those mice that died, but the mortality rate was not reduced to a significant degree. When the intraperitoneal challenge dose was 2×10^4 *S. typhimurium*, those mice that had been vaccinated with 10^3 or 10^7 AK vaccine organisms showed a statistically significant reduction in mortality rates.

Here again, it was seen that in those mice challenged intraperitoneally with 20 *S. typhimurium*, a graded increase in vaccine dose from 10^3 to 10^5 to 10^7 was associated with a graded fall in mortality rates. When the mice were challenged with an intraperitoneal dose of 10^4 organisms an increase in vaccine dose from 10^3 to 10^5 was associated with an increase in mortality from 50 to 80%, but a vaccine dose of 10^7 organisms was associated with a subsequent mortality of only 40% (see Table 3).

Table 3. *The relationship between acetone-killed vaccine dose, intraperitoneal challenge dose, and observed mortality in groups of Swiss white mice observed for a period of* 28 *days after challenge*

Intraperitoneal challenge dose (no. of *S. typhimurium* organisms)	Vaccine dose (no. of AK organisms per mouse)	Mortality rate (%)
20	10^3	25
	10^5	15
	10^7	0
2×10^4	10^3	50
	10^5	80
	10^7	40

Table 4. *Assessment of the protective potencies of heat-killed and acetone-killed vaccines against an oral challenge with* S. typhimurium *strain* 1566 *over a period of* 28 *days after challenge*

Subcutaneous immunization						
Vaccine treatment	Dose	Challenge dose	No. of mice	Mortality rate (%)	Infectivity rate (%)	Mean survival time of mice that died (days)
Heat killed	10^3	3×10^4	20	20	35	20
		3×10^7	20	35	95	18
	10^5	3×10^4	20	40	80	18
		3×10^7	20	40	85	16
	10^7	3×10^4	20	16	63	15
		3×10^7	20	50	100	16
Control		3×10^4	20	21	32	13
		3×10^7	20	45	100	15
Acetone killed	10^3	3×10^4	20	17	50	15
		3×10^7	20	42	95	19
	10^5	3×10^4	20	28	44	19
		3×10^7	20	28	83	15
	10^7	3×10^4	20	7	20	23
		3×10^7	20	24	71	14

Assessment of protection against oral challenge

The results of these studies are summarized in Table 4. In unvaccinated control mice, oral challenge with two doses of *S. typhimurium* (3×10^4 and 3×10^7 organisms per mouse) produced mortality rates of 21 and 45 % respectively, infectivity rates of 32 and 100 %, and the mean times to death for those mice that died were 13 and 15 days respectively.

The results of oral challenges (with doses of 3×10^4 and 3×10^7 organisms per mouse) in groups of mice immunized with different doses of HK or AK vaccine show that no significant protection (measured in terms of reduced mortality or infectivity) was conferred. It was again evident that survival times did not correspond with lowered mortality rates.

DISCUSSION

The results of the present study confirm the earlier observation (MacLeod, 1954) that a vaccine which is potent in reducing the mortality of mice against an intraperitoneal challenge may fail to confer any protection against an oral challenge. Mice vaccinated with whole heat-killed or acetone-killed vaccines and challenged intraperitoneally showed significant protection as measured in terms of reduced mortality, as well as prolonged survival time of those mice that died. However, when the challenge was given by the oral route no protection was observed. Resistance to infection by an artificial route is thus not a reliable measure of resistance to infection by the natural route. This observation has already been reported in an earlier paper (Cronly-Dillon, 1972), which also described the preparation of a potent vaccine against oral challenge, viz. Mickle disintegrated HK organisms.

It is interesting that graded doses of whole HK and AK vaccines followed by a small intraperitoneal challenge (20 *S. typhimurium*) produced a gradual fall in mortality rate – i.e. the protection afforded improved gradually with increasing vaccine doses. It appears, in this case, that up to a certain extent an increase in the vaccine dose improved the degree of protection conferred. However, when a larger intraperitoneal challenge dose was used (10^4 *S. typhimurium*) the mortality rates rose in a step-like manner as the vaccine dose was increased gradually from 10^3 to 10^5 to 10^7 organisms per mouse – i.e. the protection afforded decreased with an increasing vaccine dose. With both challenge doses there was a very obvious gradient in the response observed. In the case of AK vaccine, however, mice that received a large dose of vaccine (10^7 AK organisms) and a large intraperitoneal challenge dose (10^4 *S. typhimurium*) showed a significantly lowered mortality rate. This result did not correspond with that obtained with the HK vaccine. The salient feature was the paradoxical result that a small vaccine dose of 10^3 HK or AK organisms per mouse failed to protect the group of mice challenged intraperitoneally with a small dose of *S. typhimurium* (20 organisms per mouse) but offered potent protection against a larger intraperitoneal dose (10^4 organisms per mouse). It is possible that the important factor in protection here is the total amount of antigenic material in the animal, i.e. including both the vaccine and the challenge organisms; it may be that the protective mechanisms are triggered when the critical level of antigen is reached. It is reasonable to assume that beyond a certain critical level there is depression of the response, since 10^7 HK vaccine organisms failed to protect against an intraperitoneal challenge with 10^4 organisms. It would be interesting to study the effects of some higher doses of vaccine organisms since it is possible that there may be a further phase of stimulation following the depression. The effect of 10^7 AK vaccine organisms against 10^4 intraperitoneal challenge organisms may very well point towards this explanation.

It is well known that endotoxin acts on the reticuloendothelial system causing first a depression in activity followed by a phase of increased activity associated with enhanced non-specific resistance to infection. However, in the mouse this phase of immunity is only observed when the challenge organism is one to which

the host is partially resistant or when the organism is not a facultative intracellular parasite of the host phagocytes (Rowley, 1963). Hence, it would appear that endotoxin-induced immunity is unlikely to play any major part in the response of vaccinated mice challenged with *S. typhimurium* which is a facultative intracellular parasite for this animal.

Since both whole HK and AK vaccines produced fairly similar significant reductions in mortality rate against intraperitoneal challenge, it is not possible to say which was superior in this respect. However, AK vaccine reduced the infectivity rates significantly when the animals were challenged intraperitoneally, and the mean time to death of those mice that died was also prolonged in this group. This may explain to some extent the earlier report of Raistrick & Topley (1934) that AK vaccine was superior to the heat-killed form against challenge with virulent *S. typhimurium*.

No protection was conferred against oral challenge by vaccinating mice with whole HK or AK vaccines. Although survival times were prolonged in some groups this was not considered to be a reliable index of protection unless associated with a reduction in mortality or infectivity rates, or both. It should be mentioned that the author has been able to prepare a vaccine, by Mickle disintegration of HK organisms, that was able to confer statistically significant protection by reducing the mortality rate against an oral challenge.

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