JOURNAL OF BACTERIOLOGY, Sept., 1966
Vol. 92, No .3

Heat-Labile Antigens of *Salmonella enteritidis*

II. Mouse-protection Studies

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Received for publication 13 May 1966

ABSTRACT

COLLINS, F. M. (University of Adelaide, Adelaide, South Australia), AND MARGARET MILNE. Heat-labile antigens of *Salmonella enteritidis*. II. Mouse-protection studies. J. Bacteriol. **92**:549–557. 1966.—A number of extracts prepared from a virulent and an avirulent strain of *Salmonella enteritidis* were used to immunize mice. Living and alcohol-killed whole-cell vaccines were also used to compare the relative protective value of the various preparations. All mice were challenged intravenously with 100 to 1,000 LD_{50} of *S. enteritidis*. Daily counts of the liver, spleen, and blood populations of vaccinated and control mice revealed that the challenge organism was rapidly eliminated only in those mice which had been immunized with a living vaccine. Immunization with extracts resulted in rapid clearance of *S. enteritidis* from the blood, but, after a delay of 24 to 48 hr, the bacterial populations increased until a maximal liver and spleen population of approximately 5×10^8 was reached. Between 55 and 100% of the immunized animals died, compared with 95 to 100% of the controls. With all four extracts, it was only the first antigenic fraction eluted from diethylaminoethyl cellulose which had any detectable effect on host resistance. The ineffectiveness of vaccines prepared with the various extracts or with whole killed bacteria relative to the protection observed after immunization with living organisms is discussed.

Living vaccines are generally recognized to produce excellent protection against reinfection by the homologous organism (8, 9; Mackaness, Blanden, and Collins, J. Exptl. Med., *in press*). The evidence for the effectiveness of killed vaccines is much more controversial. Most workers agree that dead vaccines of gram-negative bacteria usually produce only marginal protection against virulent challenge (1, 5, 17, 20; Collins, Mackaness, and Blanden, J. Exptl. Med., *in press*). Auzins and Rowley (2) reported that a heat-labile antigen of *Salmonella typhimurium* provoked an immune response which resulted in an increased rate of phagocytosis by mouse peritoneal macrophages. In a more recent paper, Jenkin and Rowley (11) claimed that alcohol- or acetone-killed vaccines of *S. typhimurium* afforded greater protection to mice than that obtained with heat-inactivated cells. They went on to isolate a fraction from cells treated with sodium dodecyl sulfate, and showed that this material was also protective. In the present study, the protective abilities of purified extracts obtained from a virulent and an avirulent strain of *S. enteritidis* with sodium dodecyl sulfate or sodium deoxycholate (16) are compared with those produced by living or dead whole-cell vaccines

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MATERIALS AND METHODS

Organism. S. enteritidis (NCTC 5694) was obtained from Collindale, England. This organism had an intravenous LD_{50} of 10 organisms.

Vaccines. Whole cells, cell walls, and cell wall extracts of *S. enteritidis* (strain Se795) were prepared as described in an earlier paper (15). Lipopolysaccharide was prepared by the method of Westphal et al. (19). All vaccines were tested for sterility as described elsewhere (Collins et al., J. Exptl. Med., *in press*). Cell wall vaccines were suspended in 70% ethyl alcohol at 4 C overnight. The walls were centrifuged at $20,000 \times g$ for 30 min, resuspended in sterile saline, and diluted to contain 1 mg (dry weight) per ml.

Sodium deoxycholate and sodium dodecyl sulfate primary extracts and the separated fractions were dissolved in sterile saline at a concentration of 1.0 mg (dry weight) per ml. The resulting opalescent solutions were sterilized by passage through a membrane filter (pore size, 0.45 μ, Millipore). Sterility tests were

carried out on five 1.0-ml samples. The vaccines were dispensed in 2.0-ml samples. These were stored at −20 C until required, used once, and then discarded.

Vaccination programs. All vaccines were diluted to the required strength and injected within 60 min. Swiss-Webster white mice (18 to 24 g) were used throughout. Doses of the whole bacterial vaccines started with three injections of 10^6 bacteria per mouse per week, rose to 10^7, and rose again to 10^8 in subsequent weeks. The mice were then rested for 7 days. Prior to challenge, a number of mice from each group were randomly selected; their livers and spleens were removed and homogenized in broth. The homogenates were incubated and examined for *S. enteritidis* to establish the sterility of the vaccines used for immunization. Doses of cell walls and the various purified fractions were graded so that the amount injected was equivalent on a weight-for-weight basis with the calculated amount of antigenic material present in the original whole cells. All the vaccinated mice, together with groups of normal mice of the same age, were challenged intravenously (iv) with 1,000 LD_{50} of *S. enteritidis*. The numbers of organisms in the livers, spleens, and blood of five randomly selected mice were estimated after 1 hr and at daily intervals after challenge by the method described by Mackaness et al. (J. Exptl. Med., *in press*). A group of mice were always put aside so that the progressive number of deaths in immunized and control mice could be compared.

RESULTS

Mouse-protection tests were carried out to compare the "protective" value of the dodecyl sulfate and the deoxycholate extracts obtained from the virulent and avirulent strains of *S. enteritidis* (15). Protection was assessed on the basis of the ability of the immunized mice to inhibit the in vivo growth of the challenge organism, as well as by the actual survival of a higher proportion of treated mice.

Protection of mice immunized with the primary dodecyl sulfate extracts. Intravenous challenge of mice immunized with extracts prepared from virulent or avirulent *S. enteritidis* was characterized by a rapid clearance of the organisms from the blood with an average recovery of only 5 to 10% of the inoculum as viable organisms in the liver and spleen after 1 hr (Fig. 1). Growth of the challenge organism in the spleen and liver of mice immunized with extracts prepared from either virulent or avirulent strains was essentially identical.

On the other hand, normal control mice often failed to clear the blood by the end of 60 min. The blood-borne population was sufficiently high to account for up to 10% of the challenge organisms injected 1 hr previously. At the same time, the liver and spleen population represented 50% or more of the initial challenge dose (Fig. 6). Basically, this was the only significant difference between the behavior of the organism in the normal and in the vaccinated mice. In the control mice, the liver and spleen populations rose rapidly to an average of 10^8 by the 3rd day, at which time

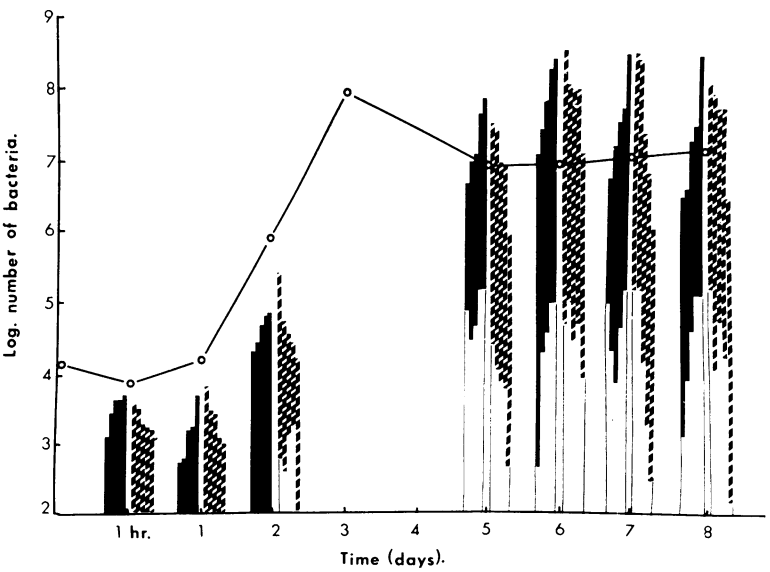

FIG. 1. *Number of Salmonella enteritidis in the blood and in livers and spleens of randomly selected animals after iv challenge of mice immunized with dodecyl sulfate (SDS) primary extracts of S. enteritidis. Solid bar, SDS primary extract prepared from the virulent strain of S. enteritidis Se795; hatched bar, SDS primary extract prepared from the avirulent strain;* ○, *average liver and spleen population in five randomly selected normal mice; open bar, numbers of S. enteritidis per milliliter of blood.*

deaths began to occur. By day 14, all unvaccinated mice were dead (Table 1). The mortality of the vaccinated mice was 55 to 85% of that observed in controls. However, from the liver and spleen populations present from day 2 onwards, it was obvious that none of the extracts had been able to generate sufficient response in the host to *prevent* the growth of the challenge organism, or even to reduce substantially the severity of the infection.

When mice were immunized with the separate fractions, the growth curves shown in Fig. 2 were

TABLE 1. *Progressive mortality in mice immunized with dodecyl sulfate (SDS) extracts prior to intravenous challenge with 1,000 LD_{50} of Salmonella enteritidis*

Vaccine	Time (days)											
	4	5	6	7	8	9	10	11	12	13	14	28
Living Se795 S^{Ra}	0	0	0	0	0	0	0	0	0	0	0	0/20[b]
Ethyl alcohol-killed cells	1	7	7	11	11	13	15	15	15	16	16	16/20
Washed cell walls	—	3	9	9	10	10	13	15	16	16	17	17/20
SDS virulent primary extract	—	1	4	4	7	7	10	12	12	13	13	13/20
SDS avirulent primary extract	—	—	3	5	10	10	11	11	11	12	12	12/20
SDS virulent ethyl alcohol precipitate	—	1	1	4	4	4	7	7	7	9	10	11/20
Control	7	12	13	18	19	19	19	19	19	19	20	20/20
SDS virulent peak 1	1	4	6	11	11	11	12	12	13	13	13	13/20
SDS avirulent peak 1	3	3	3	5	7	11	11	13	13	13	13	14/20
SDS virulent peak 2	3	5	10	15	17	17	17	17	17	17	17	17/20
SDS avirulent peak 2	2	6	14	20	20	20	20	20	20	20	20	20/20
Control	3	7	12	16	19	19	19	19	19	19	19	19/20

[a] *S. enteritidis* Se795 resistant to 50 μg/ml of streptomycin.
[b] Dead/total.

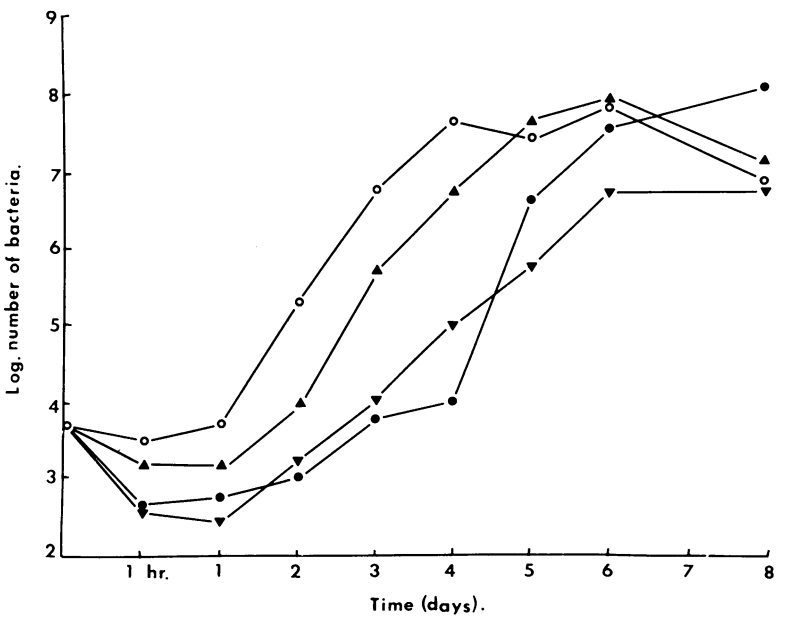

FIG. 2. *Average liver and spleen populations of Salmonella enteritidis after iv challenge of mice immunized with separate fractions prepared from the primary dodecyl extracts. Symbols: ○ = normal control; ▼ = SDS virulent strain peak 1; ● = SDS avirulent strain peak 1; ▲ = SDS virulent strain peak 2 and 3. The avirulent strain peak 2 and 3 curves were essentially identical in shape to that shown for the control animals.*

obtained. Clearly, only the first fraction obtained by elution of both the virulent and avirulent cell extracts from the diethylaminoethyl (DEAE) cellulose had any influence on the in vivo growth of the challenge organism, an effect reflected in the survival data recorded in Table 1. Tests of the later fractions showed that, although these were also capable of producing antibodies which resulted in rapid blood clearance, they were unable to influence the subsequent course of the infection. With the failure to show any real protection from the dodecyl sulfate extracts, attention was turned to the deoxycholate extracts to see whether they were any more effective as immunizing agents.

Protection of mice immunized with the deoxycholate extracts. The growth of *S. enteritidis* after iv challenge of mice immunized with deoxycholate extracts of the virulent and avirulent strains is recorded in Fig. 3. The curves are comparable in every respect with those obtained with the dodecyl sulfate extracts. The survival data in Table 2 shows that up to 45% of the mice survived the challenge, but the in vivo growth data indicates again that immunization contributed little or nothing to the host's capacity to influence the ultimate size of the infecting population. The purified deoxycholate fractions behaved in a similar manner to the dodecyl sulfate extracts. Only the first DEAE cellulose fraction, from either virulent or avirulent organisms, influenced the growth rate of *S. enteritidis* in vivo (Fig. 4, Table 2).

The poor protection obtained in the present study was conceivably due to the destruction of the "protective" antigens during the extraction process. To determine whether this had in fact occurred, and also to place the above results in a better perspective, mice were immunized with a living vaccine of an avirulent strain and also with an alcohol-killed vaccine consisting of whole cells of the virulent strain of *S. enteritidis.*

Protection of mice immunized with whole-cell vaccines. Mice were immunized by the iv injection of 10^5 living *S. enteritidis* (streptomycin-resistant). Twenty-eight days later, the survivors were found to be completely resistant to 1,000 LD_{50} of the virulent organism (Table 1). Enumeration of both the challenge and vaccinating organisms in the livers and spleens of these mice revealed a rapid and complete elimination of the reinfecting organisms so that, by 4 days, only the residual vaccinating strain could be detected (Fig. 5).

After repeated injections of alcohol-killed whole cells, an iv challenge with 100 LD_{50} of *S. enteritidis* killed 80% of vaccinated mice compared with 100% of the controls (Table 1). Enumeration of the liver, spleen, and blood populations in these mice showed that immunization with dead organisms had enhanced blood clearance and temporarily delayed the rise in liver and spleen populations (Fig. 6). This effect was entirely comparable with that already observed after immunization with the most effective of the bacterial extracts. The failure of bacterial extracts

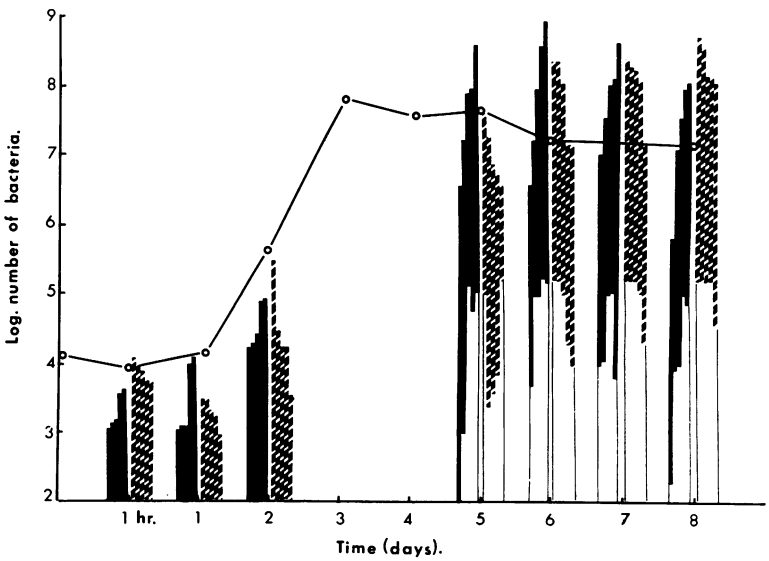

FIG. 3. *Numbers of Salmonella enteritidis in the blood and in the livers and spleens of mice immunized with the deoxycholate primary extracts of S. enteritidis and then challenged iv with 1,000 LD_{50} of S. enteritidis. Solid bar, SDC primary extract of the virulent strain; hatched bar, SDC primary extract of the avirulent strain;* O, *normal controls (average of five mice); open bar, numbers per milliliter of blood.*

TABLE 2. *Progressive mortality in mice immunized with sodium deoxycholate (SDC) extracts prior to intravenous challenge with Salmonella enteritidis*

Vaccine	Time (days)											
	4	5	6	7	8	9	10	11	12	13	14	28
SDC virulent primary extract	—	1	2	2	4	7	8	10	10	10	12	12/20
SDC avirulent primary extract	—	—	1	2	2	4	6	6	7	9	9	11/20
SDC virulent peak 1	1	1	4	6	7	11	11	13	13	13	13	13/20
SDC avirulent peak 1	2	3	5	7	10	10	10	10	11	11	11	11/20
SDC virulent peak 2	5	5	7	12	17	18	18	20	20	20	20	20/20
SDC virulent peak 3	5	7	13	18	20	20	20	20	20	20	20	20/20
SDC avirulent peak 2	3	4	5	10	14	16	16	17	17	17	17	17/20
SDC high-speed deposit	1	2	9	12	12	12	15	19	19	20	20	20/20
Control	3	7	12	16	18	19	19	19	19	19	19	19/20

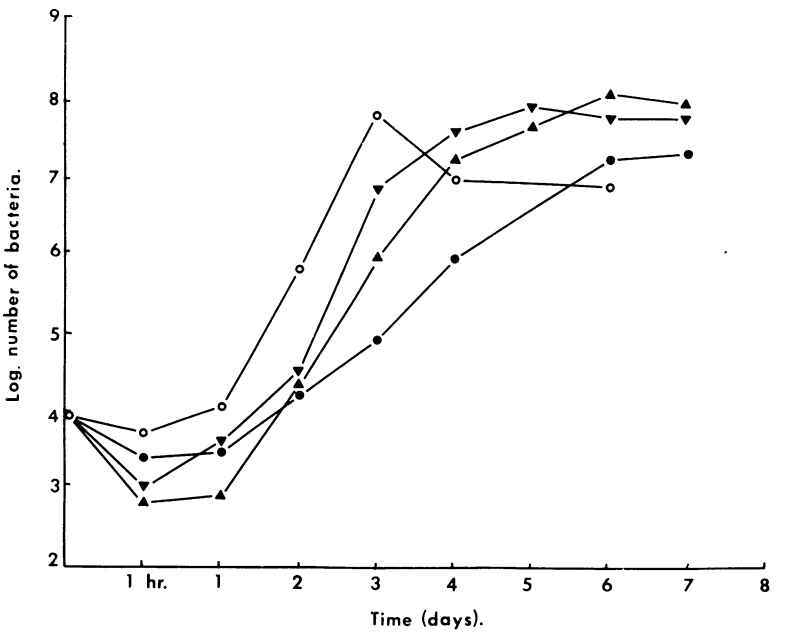

FIG. 4. *Average liver and spleen populations of Salmonella enteritidis after iv challenge of mice immunized with separate fractions prepared from the primary deoxycholate extracts. Symbols: ○ = normal control; ● = SDC virulent strain peak 1 and SDC avirulent strain peak 1; ▲ = SDC virulent strain peaks 2 and 3; ▼ = SDC avirulent strain peak 2. The high-speed deposit vaccinated mice had a growth curve similar to the avirulent peak 2 mice.*

to protect mice could not, therefore, be explained by the destruction of "protective" antigens during the extraction procedure. The survivors from the above protection experiment were challenged again, this time with 1,000 LD_{50} of a virulent streptomycin-resistant strain of *S. enteritidis*. Liver and spleen enumeration showed that the reinfecting population was rapidly and completely inactivated (Fig. 7). This clearly demonstrates the ineffectiveness of any immunization program employing dead cells or cell extracts, and reemphasizes the superiority of the resistance mechanism generated by an active infection.

In the foregoing experiments, it was thought significant that immunization with nonliving vaccines failed to influence the ultimate size of the bacterial populations reached in the livers and spleens after a challenge infection. In unvaccinated animals, deaths were observed once the average viable population in the liver and spleen had reached a level in excess of 10^8. In vaccinated mice, on the other hand, a proportion of the

animals survived, although the average liver and spleen population reached even higher levels than in control mice. This suggested that the increased survival rate in the vaccinated animals was due to the antitoxic effects of the immunity produced by nonviable vaccines rather than to an antibacterial immunity. To test this possibility, the susceptibility of immunized mice to the lethal effect of large doses of dead organisms was examined.

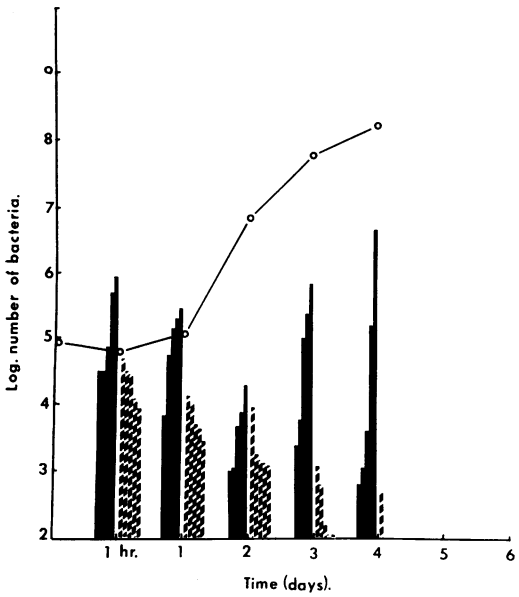

FIG. 5. *Numbers of residual Salmonella enteritidis S^R and the virulent challenge strain (NCTC 5694) in the livers and spleens of mice immunized 28 days previously with 1.0 × 10^5 S. enteritidis S^R. Solid bar, S. enteritidis S^R; hatched bar, S. enteritidis NTCC 5694; O, normal controls (average of five mice).*

Lethality of dead organisms in normal and vaccinated mice. Mice were immunized with alcohol-killed suspensions of *S. enteritidis* as in the previous experiments. They were then challenged with increasing doses of ethyl alcohol-killed *S. enteritidis* or with purified lipopolysaccharide prepared from this organism. The results in Table 3 show a significant increase in resistance to the toxic effects of dead bacteria or their products in the immunized animals. Although the degree of protection was small, it seems sufficient in itself to explain the equally small reduction in mortality observed in the immunized mice.

DISCUSSION

Jenkin and Rowley (11) reported that a "protective" antigen could be isolated from *S. typhimurium* which, they claimed, was highly effective in protecting mice against fully virulent organisms. They found that this antigen was preserved in an active form in alcohol-inactivated bacteria, and went on to show that sodium dodecyl sulfate extracts of whole cells were also protective. The present study was undertaken to determine whether similar antigens are present in *S. enteritidis*. Although this organism belongs to a different Kauffmann-White group, it causes a clinically identical infection. It is true that *S.*

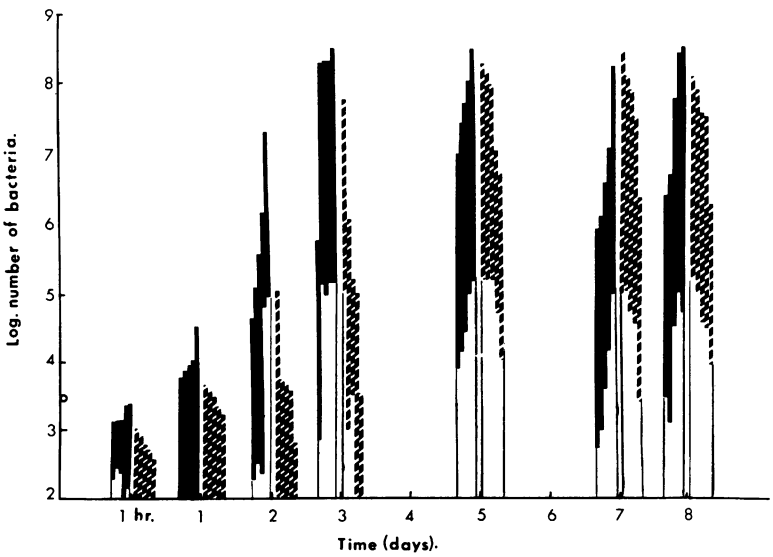

FIG. 6. *Numbers of Salmonella enteritidis in the liver and spleens of randomly selected mice challenged iv after immunization with alcohol-killed S. enteritidis vaccine. Solid bar, normal controls; hatched bar, vaccinated mice; open bar, S. enteritidis per milliliter of blood.*

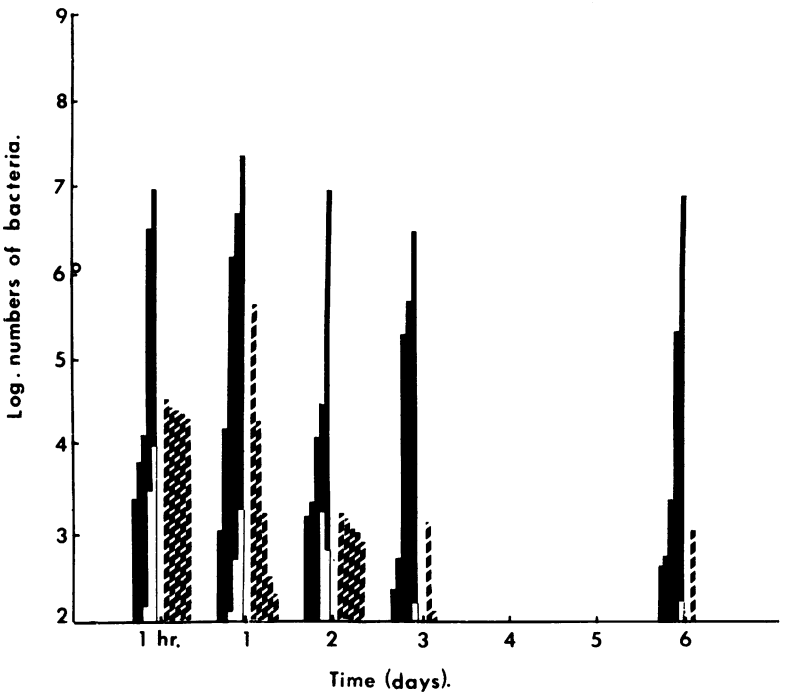

FIG. 7. *Intravenous rechallenge of surviving mice (see Fig. 6) with 1,000* LD_{50} *Salmonella enteritidis* S^R. *Solid bar = S. enteritidis in livers and spleens (residual vaccine); hatched bar, S. enteritidis* S^R *in livers and spleens; open bar, S. enteritidis per milliliter of blood.*

TABLE 3. *Toxicity of whole cells and the purified lipopolysaccharide of Salmonella enteritidis for mice immunized with ethyl alcohol-killed S. enteritidis*

Vaccine	Challenge	Dose	Deaths/total[a]	LD_{50}
		mg		*μg*
S. enteritidis (ethyl alcohol-killed)	*S. enteritidis* (whole cells, ethyl alcohol-killed)	10.0	10/10	5,000
		5.0	5/9	
		2.5	1/10	
		1.0	0/10	
Saline	*S. enteritidis* (ethyl alcohol-killed)	10.0	9/10	3,000
		5.0	9/10	
		2.5	1/10	
		1.0	1/10	
S. enteritidis (ethyl alcohol-killed)	*S. enteritidis* (lipopolysaccharide)	5.0	10/10	1,250
		1.0	2/10	
		0.5	0/10	
		0.25	0/10	
Saline	*S. enteritidis* (lipopolysaccharide)	5.0	10/10	800
		1.0	7/10	
		0.5	2/10	
		0.25	0/10	

[a] Read 48 hr after injection.

enteritidis does not contain antigen 0–5. However, it seems likely that other labile cell wall antigens could serve an analogous role.

In the past, few antigenic differences have been detected between highly virulent and avirulent strains of salmonellae (10, 13, 17, 18). The present attempts to detect chemical or haptenic differences between virulent and avirulent strains of *S.*

enteritidis were also unsuccessful (15). Furthermore, it was not possible to demonstrate any significant differences in the immunogenicity of extracts prepared from the two strains. It is possible, however, that the tests employed were capable of detecting only gross differences in immunogenicity. The only heat-labile antigen shown to give any increase in resistance was found in the first fraction eluted from the DEAE cellulose column. This was true regardless of which organism or extractant was employed. However, the interpretation of these results is critically dependent on the criteria used to assess protection. If only the progressive mortality figures and the mean time to death are taken into account, immunization with any of the primary extracts, or with the material in the first DEAE cellulose fractions, gave significant protection. On the basis of percentage mortality, these extracts were comparable in their "protective" ability to to the dodecyl sulfate extracts obtained from *S. typhimurium* (11). However, when the growth of the challenge organism was followed each day in the liver, spleen, and blood of randomly selected animals, a different picture emerged. The best that could be achieved in mice immunized either with whole dead cells, or the most active extracts, was a rapid and complete clearing of the blood and a 24- to 48-hr delay in the growth of organisms in the liver and spleen. Thereafter, rapid growth occurred until a maximum of 10^8 to 10^9 organisms was reached. At this time, death usually intervened. In the groups of animals showing "significant" protection, there was a 48-hr delay before any significant increase in bacterial numbers was observed in spleen and liver. Thereafter, the rate of bacterial growth in normal and vaccinated animals was similar. However, this initial delay meant that the maximal bacterial populations were not reached until 6 to 8 days, as compared with the 3 to 4 days required in unvaccinated mice. The initial reduction in bacterial numbers presumably reflects the increased efficiency of phagocytosis in the immunized mice. Since a high proportion of ingested organisms are killed by the cells of normal mice (Blanden, Mackaness, and Collins, J. Exptl. Med., *in press*), the increased rate of phagocytosis would be expected to produce a more extensive killing of the initial population. Such an effect was, in fact, observed in immunized animals in the present study. It is clear, however, that this immune mechanism failed to influence the subsequent behavior of those organisms which survived inactivation during the initial stages of infection.

The behavior of virulent organisms in animals immunized with living vaccines revealed, by contrast, the inadequacy of the immunity produced with killed vaccines or cell wall extracts. Thus, the present results indicate that the superior immunity produced with the living organisms (1, 10, 16, 20) is the result of an antibacterial mechanism not found in animals immunized with whole dead organisms or bacterial extracts. Recently, it was shown that infection immunity produces the only effective mechanism against *Salmonella* infections, and that humoral factors have little influence on the course of experimental infections initiated by intravenous inoculation (Blanden et al., J. Exptl. Med., *in press*; Collins et al., J. Exptl. Med., *in press*; Mackaness et al., J. Exptl. Med., *in press*). The present results are entirely consistent with this view. However, the increased resistance of mice immunized with killed *S. enteritidis* vaccines to the toxic effects of killed whole cells, or purified lipopolysaccharide, points to an antitoxic role for the antibodies produced in response to the injection of dead vaccines. The exact mechanism of endotoxin tolerance has been the subject of some controversy (3, 4, 6, 7, 12), and evidence for the participation of both humoral and cellular factors has been adduced. Recently, however, there has been an increasing body of evidence to suggest that resistance to the biological effects of endotoxin depends largely upon specific neutralization of the lipopolysaccharide moiety by humoral factors (14). It is possible, therefore, that, in the present studies, the specific neutralization of the endotoxin by humoral antibody could explain the survival of a proportion of vaccinated animals despite the presence in the liver and spleen of bacteria in sufficient numbers to insure the death of unvaccinated mice.

If antibody plays such a relatively minor role in the course of the infection, it seems hardly surprising that the present attempts to isolate a "protective" antigen from *S. enteritidis* failed so consistently. Thus, although the heat-labile antigens of the salmonellae remain of considerable academic and taxonomic interest, they do not appear to determine the virulence of *S. enteritidis* so far as the mouse is concerned. There seems, therefore, little point to further attempts at isolating and purifying these antigens as a means of studying the immune response to this organism. Investigation of the cellular changes associated with the use of living vaccines would appear to be a more attractive line of approach to this problem.

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