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Histopathological Study of Protective Immunity Against Murine Salmonellosis Induced by Killed Vaccine

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Swiss-Webster mice were vaccinated with heat-killed salmonellae and then were infected with virulent *Salmonella typhimurium*. Only 1 of the 18 vaccinated mice died from a challenge of 10^4 × the 50% lethal dose, and about 70% of them survived a challenge of 10^5 × the 50% lethal dose. Histopathological examinations of the lesions developed in these vaccinated mice showed that they followed the characteristic features of a primary lesion in murine salmonellosis. There was an early necrosis with infiltration of polymorphonuclear leukocytes and abscess formation within the first 6 to 7 days after infection. However, these abscesses remained small and discrete. By days 7 to 10, the lesions began to transform into granulomas, first with the appearance of peripheral mononuclear cells and then by the replacement of polymorphs. By the third week of the infection, minute and discrete granulomas were seen scattered in the spleen, liver, and lymph nodes. Beyond this stage, healing and tissue regeneration followed. Thus, the characteristics of infectious lesions developed in mice vaccinated with heat-killed salmonellae are distinctly different from those developed in mice protected by the avirulent vaccine.

For many years in the past, some investigators maintained that virulent *Salmonella* was a facultative intracellular parasite (5) and that acquired immunity in salmonellosis was primarily cell mediated (3, 5, 14, 23), i.e., T-lymphocytes played a primary role. This view would imply that virulent salmonellae could survive or multiply within host macrophages, which would in turn play a significant role in the protective immunity to the disease. To the contrary, experimental evidence from this laboratory (10, 12, 13, 20) and elsewhere (4, 22) shows that both *Salmonella typhimurium* and *S. enteritidis* are effectively killed by peritoneal macrophages of guinea pigs and mice. Other investigators reported the killing of salmonellae by polymorphonuclear cells (2, 15, 19).

Previous histopathological findings (17) reveal that the primary lesions of murine salmonellosis are characterized by a rapid, initial accumulation of polymorphonuclear leukocytes, and that the later appearance of mononuclear cells coincides with the emergence of delayed hypersensitivity to bacterial antigens and the terminal stage of a primary infection. This information raises questions as to whether macrophages do in fact play a significant role in the early stage of the disease. In related investigations (16), B-lymphocytes are shown to be more important in protecting mice against salmonellosis than are T-lymphocytes. Mice selectively depleted of B-lymphocytes by cyclophosphamide are unable to control the unlimited multiplication of a viable *S. typhimurium* vaccine and thus die of the vaccination, whereas mice treated with antilymphocytic sera but supplemented with intravenous injections of anti-*Salmonella* sera are capable of suppressing the growth of the vaccine. The immunosuppression of B-lymphocytes by cyclophosphamide significantly increases the susceptibility of mice to infection with *S. typhimurium* (21). Passive transfer of B-lymphocytes derived from immunized mice provides a much greater protection against a normally fatal infection with *S. typhimurium* than the transfer of T-lymphocytes, based on data of the survival rate and of the bacterial multiplication in the liver, spleen, and blood of the infected mice (9).

It is generally agreed that a living attenuated vaccine usually elicits a solid immunity against salmonellosis in the host because of its ability to induce both the cellular and humoral immune responses. Nevertheless, convincing evidence also shows that killed vaccines, which induce only humoral immune response in the host, can provide a significant protection against subsequent challenges (1, 7, 8, 12, 18). The protective mechanism of acquired immunity is ascribed to the antibacterial functions of opsonic and cytophilic antibodies (10, 12, 24).

In contrast to the primary lesions in murine salmonellosis, the secondary lesions produced in mice vaccinated with avirulent salmonellae are marked by an early appearance of mononuclear cells and the formation of granulomas (17). This histopathological feature is clearly a result of the elicitation of delayed hypersensitivity to the pathogen in the vaccinated mice. In the present investigation, the nature of protective immunity in the mice vaccinated with killed salmonellae is studied by histopathological examination of tissues of the infected mice.

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MATERIALS AND METHODS

S. typhimurium. The virulent strain SR-11 was used in this study. Its mean lethal dose was approximately 10 bacteria upon intraperitoneal (i.p.) injection into mice. The stock culture of the bacteria was maintained in tryptic soy agar (TSA, Difco Laboratories, Detroit, Mich.). The bacteria were grown in tryptic soy broth (TSB, Difco) for 6 h and then were washed in saline by the previously described procedure (24). The optically standardized bacterial suspension contained approximately 2.5×10^9 viable organisms per ml.

Preparation of heat-killed vaccine. The heat-killed *Salmonella* vaccine was prepared from the SR-11 strain. Tubes containing about 7 ml of saline suspension with approximately 2.5×10^9 viable bacteria per ml were heated in a boiling water bath for 30 min. After the suspensions were checked for sterility, they were centrifuged at $1,400 \times g$ for 20 min. The supernatant fluid was discarded. The sediments were washed once with the same volume of saline and collected again by centrifugation. The final sediments were resuspended in the same volume with saline and used as the heat-killed vaccine. This vaccine preparation therefore contained approximately 2×10^9 heat-killed salmonellae per ml.

Mice. Male mice of the Swiss-Webster strain (RFW), weighing 25 to 30 g each, were used in this study. They were purchased from Rockland Farm (Gilbertsville, Pa.) and housed in the Central Animal Care Facility of the Medical College of Virginia.

Vaccination and infection of mice. The heat-killed *Salmonella* vaccine was administered to mice in two successive doses of 0.1 ml each by i.p. injections 3 weeks apart. About 10 days after the booster vaccination, the mice were challenged i.p. with 0.2 ml of saline suspension containing 10^5 or 10^6 virulent *S. typhimurium*. At the same time, normal control mice were infected i.p. with the same preparation containing 10^5 virulent salmonellae.

Determination of delayed hypersensitivity to *Salmonella* antigens in vaccinated mice. The ammonium sulfate-precipitated antigens described in a previous publication (13) were used as the test *Salmonella* antigens. This preparation was shown to be capable of eliciting the in vitro migration inhibition of peritoneal cells obtained from *Salmonella*-infected guinea pigs. Its active components were considered to be protein in nature because of its lability to proteolytic enzymes. A volume of 0.05 ml of the antigenic preparation was injected into one of the hind footpads of the mouse, and a control preparation derived from TSB was injected into the other hind footpad. The delayed hypersensitivity was manifested as a firm swelling of the footpad injected with the antigenic preparation (6).

Determination of bacteremia in infected mice. When the infected mice were sacrificed for autopsy, approximately 0.1 ml of blood was withdrawn directly from the exposed heart with a tuberculin syringe and needle. Half of this blood sample was inoculated into a TSB tube, and the other half was inoculated onto a TSA plate for streaking. Bacterial growth was determined after 24 h of incubation at 37°C. Confirmation of *Salmonella* colonies was done whenever necessary by specific slide agglutination test, using antiserum bled from guinea pigs infected with *S. typhimurium*.

Preparation of tissue specimens from infected mice for histopathological examination. At intervals, tissue sections were removed from various organs of the infected mice, fixed in 10% buffered Formalin, and imbedded in paraffin. Sections of the tissues (2 to 3 μm thick) were cut and stained with hematoxylin and eosin (H & E). Others were stained with Giemsa or Gram stain.

RESULTS

Protective effect of heat-killed vaccine. Mice vaccinated with heat-killed salmonellae were challenged i.p. with either 10^5 or 10^6 virulent *S. typhimurium*. At the same time, normal control mice were infected i.p. with 10^5 bacteria. Infected mice from each of these groups were randomly removed and sacrificed for autopsy at intervals. The remaining mice were kept so that their survival time after infection could be recorded. Three experiments were done with this protocol. The survival time of each of these three groups of mice over a 3-week period after infection, excluding those removed for autopsy within the first 2 weeks, was pooled from these experiments and is shown in Fig. 1.

The data shown in Fig. 1 were statistically compared by using the Mantel-Haenszel test. It may be concluded that the survival patterns of these three groups of infected mice were significantly different ($P < 0.0001$). The control mice challenged with 10^5 bacteria began to die rapidly beginning from day 4 after infection, and none of them survived beyond day 12. In contrast, the 18 vaccinated mice infected with the same dose of bacteria as the control mice survived the challenge with only one death. However, when the vaccinated mice were challenged with a 10-fold increase of the pathogen, the survival rate was obviously decreased. About 30% of these infected mice began to die from day 8 after infection. Nevertheless, 70% of these mice remained alive at the end of 3 weeks (Fig. 1). There was no

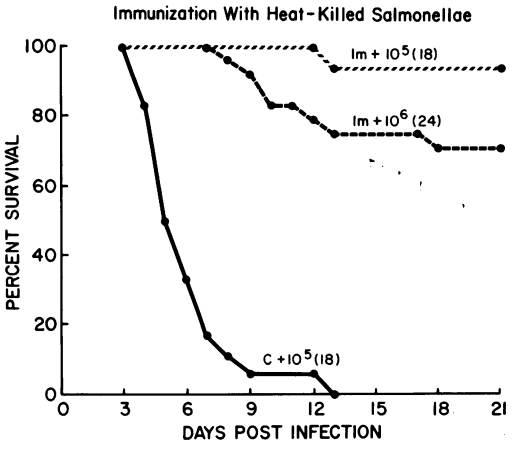

FIG. 1. Survival rate of mice injected with virulent *S. typhimurium* cells. C, Control mice; Im, mice immunized with heat-killed vaccine. These mice were challenged with either 10^5 or 10^6 bacteria as indicated. Numbers in parentheses indicate the total number of mice from which the data were determined in each group.

change in the survival pattern beyond 21 days.

Hematogenous dissemination of pathogen in infected mice. When infected mice were sacrificed for autopsy, blood samples were obtained by cardiac puncture and cultured in TSB tubes and on TSA plates. In all studies of this aspect, including but not limited to the experiments shown in Fig. 1, the control mice infected with 10^5 salmonellae consistently showed positive blood culture as early as 2 days after infection. Other studies in this laboratory also showed that salmonellae were recovered from the blood circulation within 2 h after an i.p. inoculation of 10^3 virulent *S. typhimurium*. In contrast, the blood samples taken from vaccinated mice infected with 10^5 or 10^6 bacteria were usually sterile. For example, of a total of 13 vaccinated mice challenged with either 10^5 or 10^6 salmonellae and sampled within 10 days after infection, only 1 infected with 10^5 salmonellae showed a positive blood culture on day 4 after infection. Although the blood samples were sterile, there was undoubtedly transient bacteremia occurring in these vaccinated mice, since lesions were seen in the liver and spleen by day 4 after an i.p. infection.

Delayed hypersensitivity to *Salmonella* antigens in vaccinated mice. Randomly selected mice vaccinated with killed salmonellae were tested on the hind footpads with the *Salmonella* protein antigens 2 or 3 days before the challenge with virulent *S. typhimurium*. They consistently showed an absence of firm swelling at the site of injection, indicating that they did not develop a delayed hypersensitivity to salmonellae. In contrast, mice vaccinated with avirulent salmonellae consistently developed a firm swelling in the footpad after the injection with the same antigenic preparation. Therefore, it may be concluded that the killed vaccine does not induce in the host a delayed hypersensitivity to salmonellae, as the attenuated vaccine does.

Histopathological evidence of protective immunity induced by killed vaccine. Among control mice infected with 10^5 salmonellae, a small number of acute, microscopic lesions appeared in the liver and spleen as early as 2 days after infection. These lesions rapidly increased in size and in number by day 3, and they spread across as much as 70% of the tissues in these organs by day 4 in some of the mice. The lesions seen on day 3 consisted of numerous confluent abscesses, vascular thrombosis, and extensive areas of necrosis (Fig. 2A). Beginning from day 4, infiltration of mononuclear cells became apparent in some of the lesions. This group of infected mice began to die at this time (Fig. 1). Among the survivors autopsied between days 5 and 9, the infectious process spread to the lungs, heart, and lymph nodes, causing severe interstitial pneumonitis, myocarditis, and lymphadenitis. The inflammatory reaction consisted of a mixture of acute, frequently confluent, abscesses (Fig. 2B), increasing number of granulomas (Fig. 2C), and numerous microinfarcts, which led to a rapidly invasive destruction of the involved organs. None of the infected mice in this group survived beyond 12 days.

In contrast, the lesions in the vaccinated mice infected with the same number (10^5) of salmonellae began to appear later, i.e., on day 4, and consisted of a few isolated microabscesses in the liver (Fig. 3A) and a mild increase of polymorphs in the subcapsular sinuses of the spleen. With the exception of a slight splenomegaly, all other organs appeared normal on gross examinations at this time. By day 7, there were limited numbers of microscopic foci of necrosis in the liver and spleen, and occasionally in the lymph nodes. About this time, polymorphs in the subcapsular sinuses of the spleen and the lymph nodes were replaced by macrophages. Between days 7 and 10, the minute acute lesions began to transform into granulomas with the appearance of mononuclear cells around their periphery (Fig. 3B and C). However, they did not enlarge in size and remained few in number. From week 2 of infection onward, only very few residual microscopic lesions were found in the liver and spleen, most of which were minute granulomas with occasional lesions composed of a mixture of polymorphs and macrophages (Fig. 3D). After week 3, most of the tissues appeared regenerated and normal, with rarely an occasional microscopic granuloma (Fig. 3E). In general, gross

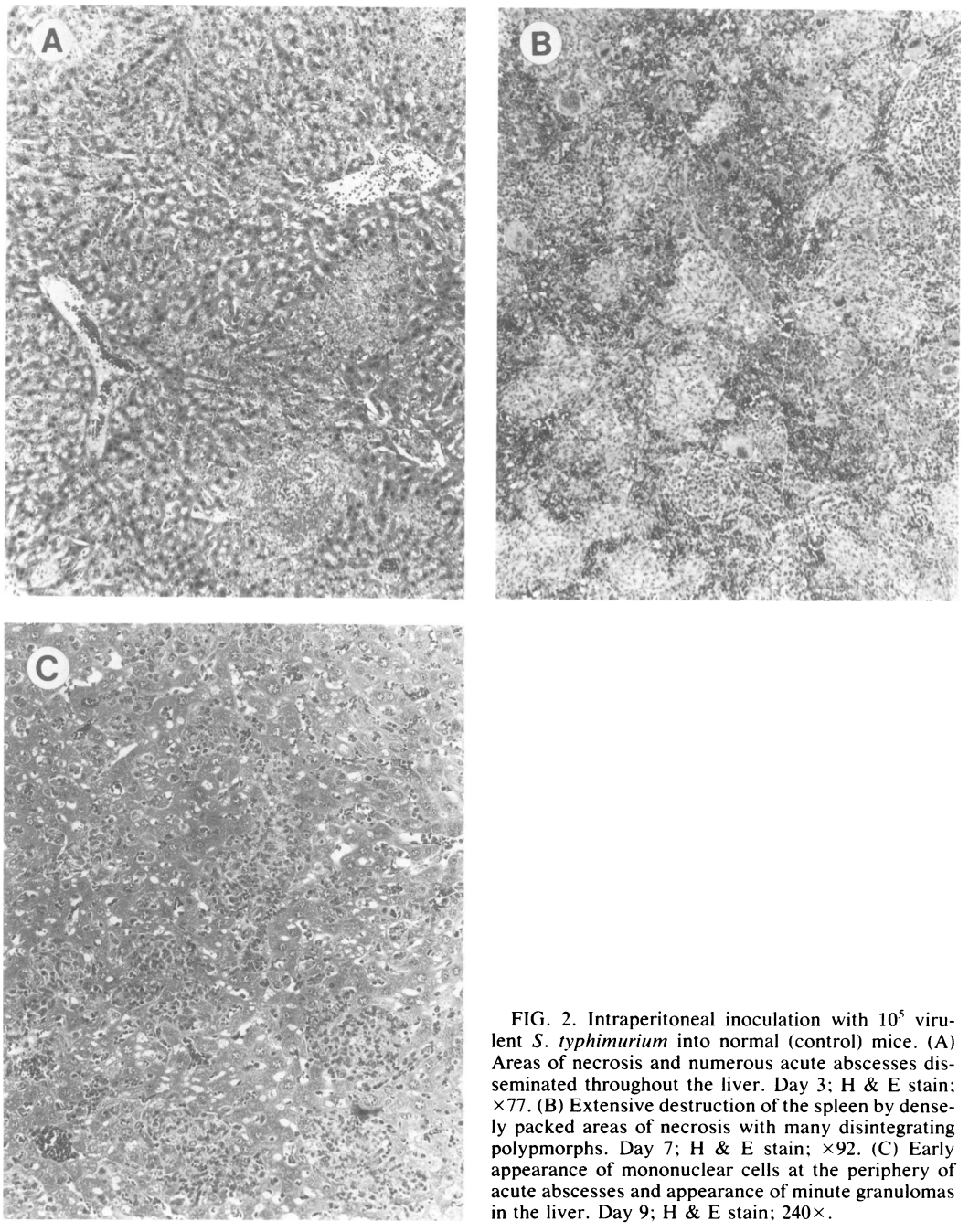

FIG. 2. Intraperitoneal inoculation with 10^5 virulent *S. typhimurium* into normal (control) mice. (A) Areas of necrosis and numerous acute abscesses disseminated throughout the liver. Day 3; H & E stain; ×77. (B) Extensive destruction of the spleen by densely packed areas of necrosis with many disintegrating polypmorphs. Day 7; H & E stain; ×92. (C) Early appearance of mononuclear cells at the periphery of acute abscesses and appearance of minute granulomas in the liver. Day 9; H & E stain; 240×.

observations at autopsy of this group of infected mice revealed the liver to be normal and the spleen to be congested and somewhat enlarged. A few pinpoint-sized, grayish nodular lesions in the liver or the spleen or both were noted in some mice during the weeks 2 and 3 of the infection.

On the other hand, the vaccinated mice challenged with 10^6 salmonellae presented a more accelerated development of the infectious process than those infected with 10-fold-fewer bacteria. A few microabscesses were already found in the liver on day 3 after infection. Moderate numbers of polymorphs appeared in sinusoids of

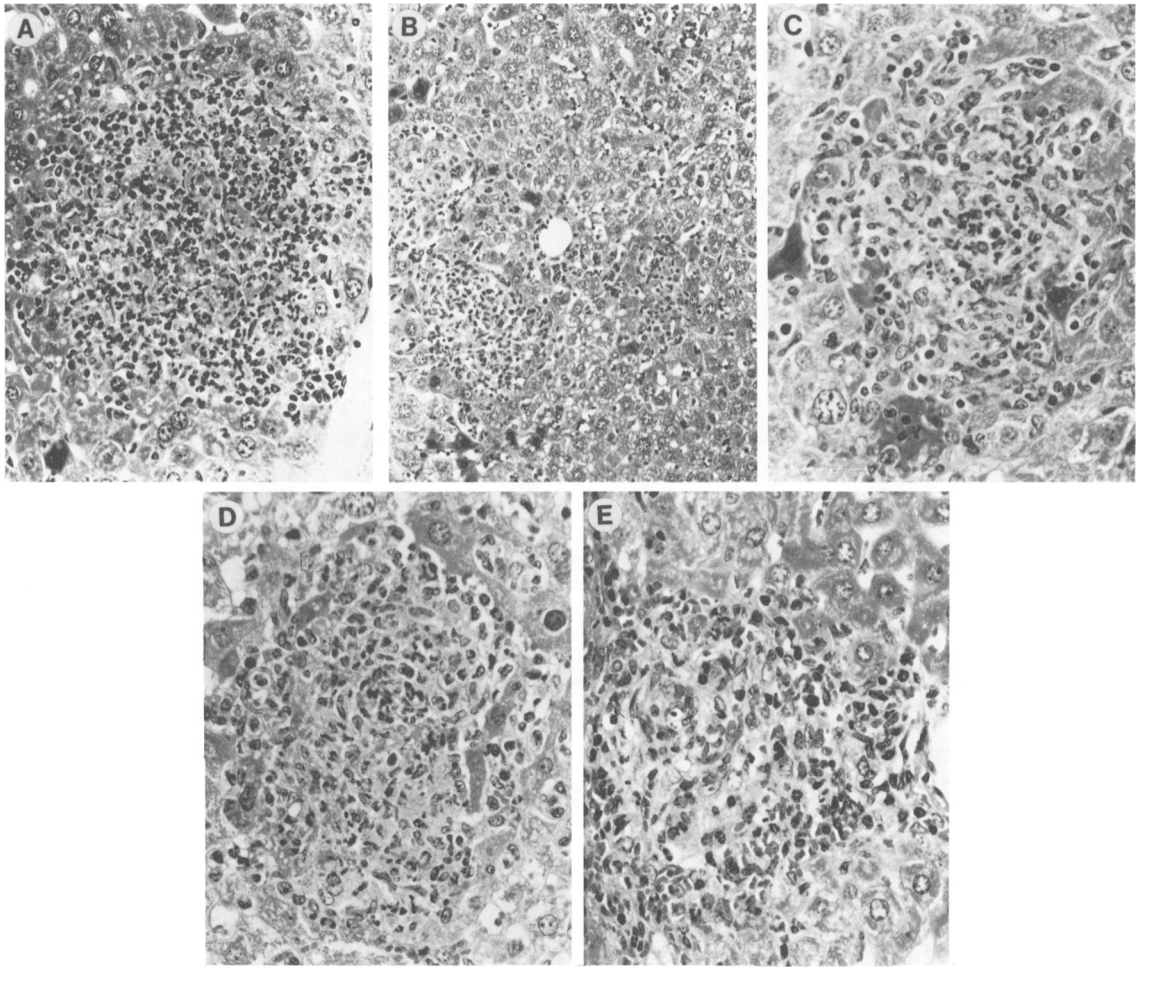

FIG. 3. Intraperitoneal inoculation with 10^5 virulent *S. typhimurium* into vaccinated mice. (A) An isolated microabscess in the liver. Day 4; H & E stain; ×170. (B) Few scattered microabscesses in the liver showing early transformation into granulomas. Day 10; H & E stain; ×135. (C) Higher magnification of an acute microabscess in the liver showing an early transformation into granuloma. Note the mononuclear cells infiltrating the periphery of the lesion. Day 10; H & E stain; ×225. (D) Late transformation of an acute microabscess into granuloma in the liver with only a few foci of disintegrating polypmorphs left. Day 15; H & E stain; ×225. (E) A well-formed granuloma in the liver. Day 21; H & E stain; ×270.

the liver, in subcapsular sinuses of the spleen, and in the medulla of lymph nodes. The areas of necrosis expanded, and the number of microabscesses increased on days 4 and 5. By day 6, the acute lesions began to transform into granulomas, as evidenced by the appearance of mononuclear cells in the vascular channels of the involved organs, their aggregation around and infiltration into the acute lesions, and their progressive replacement of polymorphs within the lesions. This was accompanied by the concomitant nodular proliferation of the reticuloendothelial cells. In the majority of these infected mice, the acute lesions were transformed into small discrete granulomas between days 7 and 10 after infection. Gross examinations of this group as a whole at autopsy revealed a slightly greater number of minute lesions on the surface of the liver and spleen than in the preceding group of vaccinated mice. Microscopic examinations also revealed that these lesions were self-limiting and rarely became confluent. Autopsies performed on the surviving mice between the days 15 and 48 disclosed the presence of a few minute, healing granulomas in the liver, spleen, or lymph nodes.

DISCUSSION

The present study shows that the killed vaccine preparation used here offered protection to practically all of the mice challenged with $10^4 \times$ the 50% lethal dose and to 70% of those challenged with $10^5 \times$ the 50% lethal dose, and significantly prolonged the survival time of the remainder. Although in comparison it is by no means as effective as the avirulent vaccine, *S. typhimurium* RIA, which was found in this laboratory to protect all RFW mice against challenges as high as 10^7 virulent salmonellae, the present killed vaccine must still be considered highly immunogenic.

It is known that avirulent vaccines induce both the cellular and humoral immune response to the pathogen (6, 10, 12, 13, 24). A direct way to discern this is by histopathological examinations of lesions in infected animals. Previous investigations (17) have shown that the initial stage of primary lesions in murine salmonellosis is characterized by multiple foci of necrosis and acute microabscesses with predominantly polymorphonuclear cells. These primary lesions enlarge and gradually transform into granulomas by the progressive infiltration of mononuclear cells and replacement of polymorphs. This transformation coincides with the development of cellular (delayed hypersensitivity) and humoral immune responses. In contrast, the lesions induced in mice protected by an avirulent vaccine (17) are marked by the early appearance of well-formed granulomas, which usually are few in number, remain small, discrete, and without central necrosis, and progress to healing with the regeneration of normal tissues. The early appearance of granulomas in these vaccinated mice clearly indicates the elicitation of delayed hypersensitivity to the bacterial challenge. The prompt and effective termination of bacterial invasion seen in these vaccinated mice is undoubtedly the result of the synergistic protective effect provided by the cellular as well as the humoral immune responses.

On the other hand, it is generally agreed that killed-*Salmonella* vaccines induce only the humoral immune response to the pathogen. This contention can be supported by the present study through the development of lesions seen in mice vaccinated with heat-killed salmonellae. In contrast to the rapidly progressive and fatal infection seen in control mice, and unlike the infected mice immunized with an avirulent vaccine (17), these vaccinated mice developed lesions, which were similar in character to primary lesions, with an acute inflammatory response of predominantly polymorphs and were indicative of the absence of delayed hypersensitivity to bacterial antigens at this stage of the infectious process. The crucial difference between these lesions and those seen in control mice was that the former remained essentially minute and isolated, and transformed into granulomas later.

It is therefore apparent from this study that the control mice die from salmonellosis because they fail to contain the rapid proliferation of the pathogen, despite a massive accumulation of polymorphs at the site of infection, whereas the vaccinated mice are able to eliminate the invading pathogen effectively by the influx of polymorphs, whose antibacterial action at the site of infection is undoubtedly facilitated by opsonic antibodies. This contention is consistent with in vitro observations of the antiphagocytic property of virulent *S. typhimurium*, the digestive capacity of phagocytes, and the opsonic action of antibodies (4, 10, 12, 13, 22, 24). Furthermore, the killed vaccine endows the host with an effective capability to localize the pathogens at the site of inoculation and to retard their systemic dissemination, as evidenced by the delayed appearance of lesions in the organs after the initial i.p. infection and by the usual absence of septicemia in the vaccinated mice. It differs from the avirulent vaccine in that it induces only the humoral immunity but not the delayed hypersensitivity to bacterial antigens in the recipients, as evidenced by the absence of granulomatous lesions at the initial phase of the disease and of footpad swelling in the vaccinated mice tested with *Salmonella* protein antigens. The delayed transformation into granulomas seen in these

vaccinated mice in comparison with the controls reflects a decreased antigenic induction of delayed hypersensitivity as a result of an early removal and killing of bacteria by the acute inflammation in the presence of opsonic antibodies.

This study lends further support to a previous assertion (17) that the pathogenicity of *S. typhimurium* is somewhat analogous with that of *Streptococcus pneumoniae*; namely, it is dependent on the antiphagocytic property and the extracellular multiplication of the pathogen. Unfortunately, efforts to discern the location of bacterial proliferation in tissue lesions by using common histological staining procedures such as the Gram stain and the Giemsa stain have so far been unsuccessful, possibly because of the inability of the stains to differentiate clearly the minute salmonellae from tissue debris in the inflammatory lesions.

To recapitulate, when mice vaccinated with killed salmonellae are challenged i.p. with a relatively large dose (up to 10^6 bacteria) of the pathogen, the initial bacterial dissemination from the site of inoculation appears to be retarded. Even when the organisms reach the organs of the reticuloendothelial system, they are effectively localized and inactivated by an acute inflammation aided by humoral immunity. However, in the absence of delayed hypersensitivity to the pathogen, these vaccinated mice are unlikely to overcome an overwhelming dose ($>10^6$ bacteria) of challenge. In mice vaccinated with killed salmonellae, the bacterial infection itself serves as a stimulus for the anamnestic humoral response and for the induction of delayed hypersensitivity. In contrast, mice vaccinated with avirulent salmonellae are already armed with both humoral immunity and delayed hypersensitivity against the pathogen at the time they are challenged. Even when they are faced with an overwhelming dose (10^7 bacteria) of challenge, the inflammatory response in the form of granulomas is far more effective in annihilating the pathogen and terminating the disease. In addition, it has been shown that delayed hypersensitivity enhances the influx of cellular and humoral elements to the site of reaction in favor of the host defense (11).

In the final analysis, as stated in previous publications (12, 13, 17), the innate capacity of phagocytic leukocytes to destroy ingested salmonellae, the enhanced cellular activities of macrophages mediated by cytophilic antibodies, the opsonic and agglutinating functions of the immune serum, and the accelerated inflammation as a result of delayed hypersensitivity (i.e., the influx of antibodies and mononuclear cells) constitute the integral components of acquired immunity of the host working in synergism against salmonellosis. Although the killed vaccine is incapable of inducing delayed hypersensitivity to *Salmonella* antigens, it generates an adequate humoral immunity in the host for a significantly effective, though not necessarily complete, protection against subsequent challenges.

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