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Pathogenesis and Immunity in Murine Salmonellosis

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INTRODUCTION

Murine salmonellosis is commonly regarded as an analogous disease to human typhoid fever. By general consensus, *Salmonella* is classified as a facultative intracellular pathogen and acquired immunity to its disease is said to be primarily cell mediated (70, 114). This concept is further popularized by the extensive writings of Collins and his associates in the early 1970s, which culminated in his definitive review on the subject in 1974 (17) and set the direction for most of the research on this topic over the past 15 years. In a conference on host defenses to intracellular pathogens held in Philadelphia in June 1981, experimental data were presented to question the legitimacy of listing *Salmonella typhimurium* as an intracellular pathogen (26). However, recent publications in international journals maintain the classification of *Salmonella* as a facultative intracellular parasite (12, 14, 18, 55, 84, 96). In a current review on microbial pathogenicity (27), the authors cited references on the ability of *Salmonella* to invade nonphagocytic cells, to withstand the antibacterial actions in the phagolysosomes, and to survive within macrophages. Contemporary investigations into the diverse host resistance to salmonellosis among inbred mouse strains attribute its basic difference to the relative capacities of host macrophages to interact with the intracellular pathogen (26, 36, 64, 65, 88).

In spite of the persistent inference of the intracellular survival and multiplication of salmonellae, a thorough search of the literature will reveal that there has never been indisputable experimental evidence of this organism proliferating within host phagocytes. On the contrary, experimental data from independent workers over the years do show the killing of salmonellae within polymorphs (6) and macrophages (11, 126). Also, contrary to the common claim that acquired immunity to the disease is primarily cell mediated, nonviable vaccines, which induce only humoral immunity (19), are shown by many investigators to offer effective protection against subsequent challenges (2, 24, 30, 37, 93). In fact, the reviews by Jenkin and Rowley in 1963 (50) and Roantree in 1967 (101) cited commendable references on the protective value of humoral immunity to salmonellosis in the early literature. As these issues became increasingly polemic, it is not surprising that a unifying understanding of the pathogenesis in murine salmonellosis has yet to emerge out of the deluge of literature on this subject over the past two decades. Unfortunately, rather than reexamining the basic issues in this experimental model, the trend of research has been to perpetuate the assertion of facultative intracellular multiplication of pathogenic salmonellae within macrophages. Without a commonly acceptable tenet on the fundamental mechanism of pathogenesis in murine salmonellosis, continued investigations into the mechanism of acquired immunity, the development of vaccines, and, more recently,

the genetic control of the antibacterial function of macrophages will simply generate more controversies and debates.

We have been involved in the study of host-parasite interactions in murine salmonellosis for over 20 years. Our earlier observations of the killing of salmonellae within macrophages were dismissed as a minority view inconsistent with the prevailing tenet. As our experimental evidence accumulates, it becomes increasingly apparent that the pathogenesis of virulent salmonellae is unlikely to be related to their ability to survive, let alone multiply, within host macrophages. This review will in part present a critical and comprehensive evaluation on both sides of the argument based on experimental data from relevant and representative literature. I believe that the major discrepancies in the literature originate from certain inherent deficiencies in experimental designs and improper interpretations of experimental data, which render the conclusions unreliable. I trust the reader will allow me the latitude of frank critiques as I trace the historical developments of research on this topic, without diminishing the efforts of our colleagues.

This review is primarily intended to be a concise treatise on pathogenesis and immunity in murine salmonellosis as I perceive it from the collective experimental data at hand. It will also serve as a brief article with murine salmonellosis as an experimental model to study the host-parasite interactions in a bacterial infection for the general reader rather than as a complex discourse and exhaustive review of the literature for the selective investigator.

BACKGROUND

Basic Terms and Concepts

Facultative intracellular parasite. The classification of pathogenic bacteria as facultative intracellular parasites was initially introduced by Suter (114) to describe those organisms capable of survival or proliferation within phagocytes and in extracellular space during certain stages of the host-parasite relationship. According to past reviews (17, 18, 114, 115), this group of pathogens includes *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Brucella* spp., and *Salmonella* spp. The basic mechanism of pathogenesis in these organisms is attributed to their ability to propagate within host phagocytes, in particular, macrophages. Acquired immunity to their diseases is primarily cell mediated. With recent experimental evidence showing virulent salmonellae multiplying within parenchymal cells (27, 61), some investigators may disagree with the restrictive definition of this term.

Cellular immunity. The concept of, though not the term, antibacterial cellular immunity originated from Metchnikoff (74). The term has traditionally taken the meaning of the innate ability of host phagocytes (polymorphs and macrophages) to ingest and digest invading bacteria. Alternatively, in acquired immunity, cellular immunity may be manifested as an increased ability of phagocytes to ingest bacteria with the aid of opsonic antibodies or as an enhanced capacity of macrophages from an immune host to destroy or suppress the growth of intracellular bacteria (13, 35, 38, 50, 115). The "immune macrophages" exhibiting the latter phenomenon are also referred to as "activated" or "angry" macrophages (67, 68). This usage of the term cellular immunity in bacterial infections was clear in the literature until the late 1960s. As the role of T lymphocytes in cell-mediated immune responses became rapidly understood, the term cellular immunity was widely used interchangeably with the term cell-mediated immunity to describe the immunological mechanisms of antitissue immunity and delayed hypersensitivity, including the expression of migration inhibition factor in vitro (69).

Acquired immunity and DTH in bacterial infections. Tuberculosis is probably the most thoroughly studied and best-understood model of infectious disease caused by facultative intracellular bacteria. There is little question that virulent *M. tuberculosis* replicates freely within host macrophages both in vitro and in vivo (43, 44, 66). It is generally accepted that acquired cellular immunity to the disease is primarily manifested by an enhanced bacteriostatic action of macrophages from an immune host. Delayed-type hypersensitivity (DTH) to bacterial antigens emerges simultaneously with acquired immunity to the disease. While it has long been recognized that these two immunological responses to infections are not synonymous (35, 54, 55, 66, 132), the modern concept in immunobiology supports their manifestations to be mediated through the immunologically committed T lymphocytes, hence, the term cell-mediated immunity. The discerning investigator is more apt to draw a distinction between cellular hypersensitivity and cellular immunity when describing the cellular role in DTH and antibacterial immunity, respectively (83, 111). Unfortunately, the frequent indiscriminant use of the term cellular immunity in reference to these two distinctly separable immunological manifestations has created confusion in the literature, which in turn contributes to the current controversies in the common understanding of many infectious diseases.

Technical Considerations in Experimental Designs

Cell culture methods. Advances in cell culture techniques in the 1950s and 1960s provided a unique opportunity to study the direct interactions between bacteria and isolated host cells in a controlled in vitro environment. It also offered the advantage of assessing the effect of antiserum on the host cell-parasite relationship. However, a number of inherent pitfalls in the experimental designs need to be considered when a cell culture procedure is used.

One major objective of using cell culture experiments is to answer the question of what happens to the bacteria after they are ingested by the phagocytes (polymorphs and macrophages), i.e., to determine the fate of the ingested bacteria. Hence, the following basic criteria in the experimental design must be maintained: (i) the phagocytes harvested from the host animals must be allowed to ingest the bacteria in vitro within a reasonably short period of time; (ii) the infected phagocytes are then cultured in a medium closely resembling the in vivo conditions; (iii) the fate of intracellular bacteria must be assessed by the changing numerical ratio derived from the quantitation of the populations of viable intracellular bacteria and host cells at various intervals; and (iv) the viable extracellular bacterial population must be inhibited at a negligible level to prevent the continuous phagocytosis of bacteria by the host cells during the period of observation. The critical issue here is that one is dealing with a dynamic state in which the populations of the surviving host cells and the viable intracellular bacteria fluctuate continuously. Their interactions may be further complicated by the extracellular bacterial population.

The most important consideration associated with this experimental design is the quantitation of the viable intracellular bacteria at each interval. Aside from enumerating the population of viable host cells in the culture, these cells must be selectively disintegrated so that the intracellular

bacteria can be released unharmed for viable count. We have conducted an extensive study on this issue, as described later. Water is used for this purpose by some investigators (28, 79); however, it can be observed microscopically that this method can only devitalize the cells by hypotonicity but cannot disrupt them to release their intracellular bacteria as dispersed single organisms for individual colony count on bacteriological media. Chemical detergents are usually highly effective in dissolving mammalian cells, but unfortunately many bacteria are also sensitive to such a treatment. Sodium deoxycholate (SDC) is highly effective in disintegrating leukocytes for the recovery of *M. tuberculosis* (43) but inadequate for the recovery of *S. typhimurium* (45). Among the physical methods of lysing host cells, mechanical grinding (40), Ballotini beads (53), and sonic vibration (5) have been used for the release of intracellular organisms. We found that sonication could recover consistently more salmonellae than SDC or water from identical suspensions of infected macrophages (45, 49).

If these logical considerations are thoughtfully included in an experimental design, it becomes obvious that cell culture procedures are necessarily tedious and time-consuming. In the early days, the common method was to culture the infected macrophages on glass slides (29, 78). At designated intervals, the slides were removed, fixed, and stained. The number of intracellular bacteria were counted microscopically and tabulated to show a changing pattern of the total bacterial population within the cultured cells. The obvious flaw in this method is that the stained bacteria may or may not be viable. The phagocytes may continue to ingest live and dead bacteria from the medium, and the subsequent accumulation of the intracellular bacteria would be misinterpreted as bacterial growth.

The incorporation of antibiotics into the culture medium prevents microbial contaminations of the culture as well as controls the extracellular proliferation of bacteria. The latter is particularly necessary to prevent the continuous phagocytosis of viable bacteria by the cultured cells, which would otherwise complicate determination of the fate of the intracellular pathogen. The favorite criticism here is that the antibiotics might penetrate the host cells and thereby interfere with the determination of host-parasite interactions (94). If the intracellular bacteria are shown to be replicating by this method, it would be easier to argue against such a likelihood. On the other hand, if the intracellular bacteria happen to be decreasing in number, then it is going to be difficult to rule out such a possibility. Furthermore, if the growth rate of an intracellular pathogen (e.g., *M. tuberculosis*) is shown to be retarded within macrophages of an immune host as compared with that in the normal host, there is still the criticism that the immune cells are metabolically more active and therefore allow the antibiotics to permeate the cells faster (94). There is probably no foolproof method to dispute these challenges, unless one can trace the biologically active molecules of antibiotics from the culture medium to their contact with the intracellular bacteria.

Many of the early experiments intending to show salmonellae as facultative intracellular bacteria and the role of macrophages in acquired immunity in salmonellosis were done with cell cultures. Improper designs in techniques and interpretations of data in many of these experiments undoubtedly contributed to the current discrepancies in these issues. In any case, cell culture experiments remain an artificial environment. The host cell population is never replenished, as it is in vivo by inflammation.

Determination of bacterial growth in liver and spleen. A common way of showing bacterial growth in vivo is to grind up the liver or spleen of infected mice at periodic intervals and assay the viable bacterial population by colony counts on bacteriological media (17). This method gives a crude estimate of the rate of bacterial multiplication, especially suitable for comparative purposes, but it is unlikely to effect a complete disruption of all infected cells or to achieve a thorough dispersion of their intracellular bacteria for quantitation. Since the liver and spleen are organs of the reticuloendothelial system (RES), there is the tendency to suppose that the bacteria are trapped and therefore multiply within resident macrophages, e.g., Kupffer cells. Such an assumption is clearly erroneous. Organisms can proliferate just as well in the sinusoids and intercellular spaces of these organs. More realistically, once they are established in the tissues, there will inevitably be an inflammatory response through which a massive infiltration of leukocytes will dominate the site of infection. Such an explanation is based on the most fundamental understanding of tissue pathology and well documented by our experimental observations in murine salmonellosis (61, 81, 82, 129). Hence, an increase of the bacterial population in the liver and spleen does not in any sense represent bacterial multiplication within macrophages, nor does the suppression of it by an immune host necessarily reflect the manifestation of acquired cellular immunity.

A basic understanding of tissue pathology in bacterial infections. A common point of debate in experimental salmonellosis relates to the wisdom of using the intraperitoneal (i.p.) or intravenous (i.v.) route of challenge as opposed to the natural oral route of infection. Other than the high dosage necessary to effect an infection by the oral route, the more cogent fact is that, once a pathogen can successfully invade the local tissue, bacteremia will likely ensue, leading to a systemic disease. The i.p. and i.v. routes are ways to initiate a bacteremia. Hence, the outcome of a systemic infection is identical regardless of the route of inoculation. Even if the organisms are localized at the site of entry, inflammation is almost inevitable. Some investigators question the relevance of studying peritoneal macrophages in experimental salmonellosis, since the natural site of infection is in the gut. Others postulate that the pathogen may invade epithelial cells of the intestines and resident macrophages or some subsets of phagocytes in the lymphoid tissues of the gut. The animal host is not a test tube, but rather a dynamic and fluctuating system governed by multiple factors. In reality, it is unlikely that a pathogen could selectively invade isolated cells in the host tissues without eliciting any immediate tissue response. Although it may be conceivable that the organism could stay dormant within a few cells, once some minor tissue injury is initiated, the host will promptly mount an inflammatory response. Thus, regardless of the site of initial infection, be it the gut, the peritoneal cavity, or the liver, the invading organisms are inevitably surrounded by inflammatory cells of hematogenous origin. On this basis, it clearly makes no difference where or how the animal is challenged, so long as the experiment is focused on the interactions of the pathogen and the inflammatory cells. Undoubtedly, there are differences in the functional activities of various phagocytes, but it is irrelevant to speak simply of the resident macrophages of the RES or the peritoneal cavity when dealing with an in vivo infection.

Along this line, the major deficiency in past studies of murine salmonellosis is that the groundwork on the pathology of the disease has never been systematically established before the role of macrophages in the infectious process is

pursued. In contrast, for example, the investigations of tuberculosis evolved through a much more logical sequence of research over the years. Here, the pathology of the disease was thoroughly studied and carefully documented, identifying the role of macrophages in the disease before direct experiments on them were conducted (66).

I have detailed the above background information in the hope that the reader will keep it in mind as we review the historical development in experimental salmonellosis. It will become obvious that many of the current discrepancies and controversies in the literature on this subject can be traced to these very basic issues.

REVIEW OF RELEVANT LITERATURE

Basis for Intracellular Growth of Salmonellae and for Acquired Cellular Immunity

Pathological findings of typhoid fever usually describe the characteristic lesions as typhoid nodules, i.e., focal aggregates of predominantly mononuclear cells, including lymphocytes and macrophages. One of the early descriptions of intracellular salmonellae was reported by Goodpasture (32) and later confirmed by Adams (1). They observed gram-negative bacilli in the cytoplasm of young plasma cells and macrophages in intestinal lymphoid tissues and in lesions at autopsy of cases of typhoid fever. It was assumed that *S. typhi* was capable of growing within these cells. Perhaps the close resemblance of typhoid nodules and tubercles might have also contributed to the initial presumption that *Salmonella*, like *M. tuberculosis*, is a facultative intracellular pathogen.

The advent of cell culture techniques offered the opportunity to determine whether virulent salmonellae could grow within isolated host cells, especially the macrophages, and whether antibacterial cellular immunity played a role in the infectious process. Among the earliest workers to take advantage of this experimental approach, Gelzer and Suter (29) cultured rabbit peritoneal exudative macrophages infected with *S. typhimurium* on cover slips and reported the intracellular bacterial multiplication based on the microscopic counting of bacteria within host cells on the stained cover slips. Likewise, Mitsuhashi et al. (78) and Sato et al. (106) infected mouse macrophages from peritoneal exudates or from the liver and cutaneous tissues with *S. enteritidis* and cultured them on cover slips. They concluded that the virulent strain multiplies intracellularly while the avirulent strain does not. By comparison, macrophages derived from mice immunized with live bacteria are capable of inhibiting the intracellular growth of virulent pathogens independent of the presence of antiserum.

Since microscopic counting of stained bacteria within a given number of infected macrophages in culture cannot distinguish live from dead organisms, the increase in the intracellular microbial population may simply reflect an accumulation of bacteria. This deficiency is compounded by a continuous phagocytosis of extracellular bacteria, especially when the extracellular bacterial multiplication is not properly controlled. Organisms released from dying host cells will also contribute to this population of extracellular bacteria. A more reliable method is to assay the viable intracellular bacteria. Furness (28) and Morello and Baker (79) attempted to recover viable intracellular bacteria by treating infected macrophages in individual culture tubes with distilled water. In both of these studies, the authors showed initial killing of both avirulent and virulent *S. typhimurium* within resident and stimulated peritoneal macrophages of normal mice. Furness found that many of the surviving virulent organisms go on to multiply. Since distilled water does not disintegrate host cells, the changing bacterial count can only represent the number of infected cells, each of which will generate one bacterial colony regardless of the number of bacteria within it, but not the total viable bacterial population within the infected cell population. This quantitation will also be dependent on the initial percentage of infected cells as well as the initial ratio of bacteria within the individual cells. Another critical variable not taken into consideration in their method is the continuously declining population of infected cells in different culture tubes and at different intervals of sampling.

The publication by Blanden et al. (8) is a frequently cited experimental documentation of cellular immunity in murine salmonellosis. *S. typhimurium* was injected i.p. into normal or specifically immunized mice. Shortly thereafter, peritoneal washings were removed and cultured in individual tubes. At intervals, an entire tube of the infected cell culture and a tube of culture supernatant with the cells removed were plated out separately for bacterial colony counts. The difference of the two variables represented the changes in the cell-associated bacterial population over the period of observation. Their results showed that there is a substantially greater bactericidal activity of immune peritoneal macrophages than normal macrophages, and about 50% of the cell-associated bacteria survive within the normal cells at the end of 60 min. A similarly enhanced killing of salmonellae is also seen in peritoneal macrophages of *Listeria*-infected mice. Hence, it was concluded that the expression of cellular immunity is nonspecific. In this experimental design, the initial ratio of bacteria and macrophages in culture between the two groups of mice might not be comparable and the variable populations of macrophages in each culture tube at different intervals of sampling were not taken into account. Since no effort was made to disrupt the host cells and to release the intracellular bacteria, the clumping of immune cells (due to their cytophilic antibodies) would result in many fewer colonies as compared with the dispersed suspension of normal cells. Thus, their data cannot provide reliable support of cellular immunity.

To some workers, the expression of acquired cellular immunity is viewed not as an altered ability of immune macrophages to inactivate salmonellae, but rather as a cellular function aided by antibodies. Rowley and his associates concluded that antiserum is necessary for the phagocytosis of virulent *S. typhimurium*, but, once ingested, both normal and immune macrophages are equally capable of killing the pathogen (104, 131). They contended that, in salmonellosis, the enhanced resistance to superinfection is due to the production of antibodies during the carrier state (51, 119), and the successful passive transfer of immunity with macrophages can be attributed to the presence of cytophilic macroglobulins (103). Their cell culture technique involved the maintenance of infected macrophages on cover slips in Porter flasks. At sampling intervals, the cover slips were removed and rubbed vigorously on agar plates to recover the cell-associated salmonellae. It is doubtful that this method could adequately disintegrate the macrophages and distribute the intracellular organisms for proper colony counts, not to mention the variation in the macrophage populations on the cover slips at different sampling times.

Among the facultative intracellular bacteria, the expression of acquired cellular immunity against *M. tuberculosis* and *L. monocytogenes* can be nonspecifically mediated by

lymphokines elicited from antigen-stimulated committed lymphocytes, as demonstrated in cell culture experiments. Lymphokines induced from bovine gamma-globulin-stimulated lymphocytes of guinea pigs confer on peritoneal macrophages, after 3 days of culturing, an enhanced phagocytic action against *M. tuberculosis* (83). The addition of splenic lymphocytes from H37Ra-immunized mice to normal macrophage cultures enables the host cells to inhibit the intracellular growth of virulent *M. tuberculosis* H37Rv (95). Similarly, the addition of lymphocytes, derived from guinea pigs immunized with either *M. bovis* BCG (111) or *Toxoplasma gondii* (57) and stimulated with the specific antigens, to macrophage monolayers also augments their antibacterial activity against *L. monocytogenes*. The data from these studies were based on either the microscopic counting of the number of bacteria per 100 cells on stained cover slips (57, 95) or viable bacterial count after lysis of host cells with water (111).

Contrary to the assertion of intracellular multiplication of salmonellae, some in vitro studies showed the innate ability of murine macrophages (11, 126) and polymorphs (6) to destroy the pathogen. Consistent with this view, the virulence of salmonellae is directly influenced by their resistance to phagocytosis (120) and is related to the nature of their O-antigenic structure (101, 122). Furthermore, the O-antigenic polysaccharide affects the rate of complement activation via the alternative pathway and, in turn, the susceptibility of the organisms to phagocytosis (59, 109). Others proposed that the innate bactericidal capacity of polymorphs is dependent on their synergistic action with antiserum (5). In most of the cell culture experiments, macrophages are infected with salmonellae, since the definition of facultative intracellular bacteria was originally confined to phagocytic cells. Some workers believed that the pathogen is likely to invade nonphagocytic cells of the intestinal mucosa and cultured cell lines, as recently reviewed by Finlay and Falkow (27). For example, there are reports of the growth of virulent *S. typhimurium* in cultured HeLa cells (31) and monkey kidney cells (28) and of *S. typhosa* in a leukemic monocytic cell line (56) and in a mouse fibroblast cell line (110). It appears that the fate of salmonellae is different within phagocytic and nonphagocytic host cells.

One of the frequently debated issues in cell culture experiments involves the incorporation of antibiotics in the culture medium to control the extracellular bacterial population. Both penicillin (22) and streptomycin (9) were shown to bind to cultured mammalian cells, although their biological activities against the intracellular bacteria were not established. Prolonged exposure of cultured cells to antibiotics would promptly inhibit the multiplication of intracellular *S. typhosa* and later eradicate them (40, 110). The suppressed multiplication of *M. tuberculosis* within cultured immune macrophages was attributed to an accelerated penetration of streptomycin into these cells as compared with the nonimmune cells (94). Since the latter observation was based on the microscopic counting of intracellular bacteria on stained preparations and no data of the extracellular bacterial population were given, it is difficult to exclude the likelihood of continuous phagocytosis of live and dead bacteria by host cells. The significance of this issue was demonstrated in cell cultures infected with *Staphylococcus aureus*, in which the intracellular bacterial population will not increase as long as the extracellular organisms are inhibited by either streptomycin or repeated washings (53).

The crucial point to keep in mind is that, in every cell culture experiment, the data on the fate of intracellular bacteria must be interpreted by a correlation between the viable intracellular bacteria and the viable host cell population without interference of the extracellular bacterial population. Even if there is a decline in the intracellular bacterial population in the presence of antibiotics in the culture medium, it may simply reflect the reduction of a continuous phagocytosis of extracellular bacteria along with the intracellular killing of bacteria by host cells, rather than the penetration of antibiotics into the host cells. For example, in experiments with antibiotics, both Mitsuhashi et al. (78) and Sato et al. (106) showed intracellular bacterial growth, while Furness (28) reported an initial killing followed by the multiplication of intracellular salmonellae. None of these investigators provided any data on the extracellular bacterial population.

In essence, the early investigations on the fate of intracellular salmonellae, using cell culture methods, have produced two diverse views. One view favors the multiplication of virulent salmonellae within murine macrophages and cellular immunity as the primary expression of acquired immunity to the disease. The other stresses the intracellular killing of the pathogen facilitated by opsonic antibodies. In light of the technical complexity involved with the cell culture methodology, it is not difficult to see why we have come to this state of uncertainty in the simple question of whether *Salmonella* can adequately be regarded as a facultative intracellular parasite.

Basis for Acquired Cellular Immunity from In Vivo Experiments

The most thorough discussion on the cellular basis of immunity in experimental salmonellosis has been presented in the authoritative review by Collins (17), in which the principal data were based on in vivo experiments showing the bacterial growth rate in the liver and spleen of infected mice. The major assumptions are that (i) unstimulated mouse macrophages are inherently capable of inactivating a considerable portion of the infecting salmonella population, but with the highly virulent organisms the survivors begin to multiply intracellularly and may eventually overwhelm the host (8); and (ii) in a systemic disease, the pathogens reach the fixed macrophages of the liver and spleen, where they proliferate extensively to lethal proportions. Thus, the basis for antibacterial cellular immunity to the disease is determined by the suppressed bacterial growth in the liver and spleen of the immune mice. Macrophages are activated by mediators released from immunocompetent T lymphocytes interacting with antigens of actively multiplying bacteria in the tissues. The induction and elicitation of cellular immune response occur in the T cells, while the activation of macrophages is a nonspecific antibacterial function. Both the immunocompetent T lymphocytes and the nonsensitized macrophages are required for a successful adoptive transfer of both DTH and cellular immunity to heavily irradiated recipients. Since salmonellae are regarded as facultative intracellular bacteria in this context, there appears to be a close similarity between the immune response to tuberculosis and that to salmonellosis, in that both DTH to bacterial antigens and acquired immunity to the disease emerge at about the same time. This basic concept is supported by the failure to protect mice passively with hyperimmune serum, which can substantially increase the initial inactivation of the challenging inoculum but cannot control the subsequent multiplication of the surviving pathogens, and which can only extend the survival time of the protected mice (16).

Contrary to this popular view of a cellular basis of acquired immunity in murine salmonellosis, other investigators found that mice selectively depleted of B lymphocytes with cyclophosphamide are unable to control the multiplication of a live *galE* mutant *S. typhimurium* vaccine and eventually die from the vaccination (80). Mice deprived of both T and B cells with antilymphocyte sera can still inhibit the growth of the vaccine if they are supplemented with an i.v. injection of antiserum. In contrast, immunosuppression with cyclophosphamide significantly increases the susceptibility of mice to infection with *S. typhimurium* (121). In passive transfer experiments, purified bone-marrow-derived (B) lymphocytes from immune mice yield a better survival rate in recipients against challenges with *S. typhimurium* than thymus-derived (T) lymphocytes (39). The bacterial populations recovered from the liver, spleen, and blood after challenges are significantly lower in mice supplemented with B cells than in those supplemented with T cells. Collectively, these observations would indicate that B lymphocytes are more important in protecting mice against salmonellosis than T lymphocytes.

Perhaps one of the difficulties in interpreting data from in vivo experimentation is the lack of a systematic description of the development of histopathology in murine salmonellosis. The common assumption that salmonella replication is primarily confined to the fixed macrophages of the liver and spleen is clearly untenable. A dense cellular infiltrate consisting mainly of granulocytes is seen in the cecum of germfree mice 24 h after an oral infection with *S. panama* (105). The liver of mice infected orally with *S. typhimurium* (4) contains necrotic lesions, with infiltration of macrophages and hypertrophy of Kupffer cells from day 4 or 5 on. Thrombosis of the branches of the portal vein is found with many macrophages after day 8. However, the sequential development in the histopathology of a lesion was not elucidated.

Efficacies of Vaccine Preparations

There are clearly dichotomous views on the protective values of various vaccine preparations in murine salmonellosis. Proponents of cellular immunity as the effective immune response against a subsequent challenge stated that only live, attenuated vaccines are fully protective (16, 17, 20). While it is generally agreed that viable vaccines induce both the cellular and humoral arms of immune response and afford the host with a solid acquired immunity against the disease, the contention of antibody-mediated immunity is strengthened by reports of significant protection offered by killed bacteria. Varying degrees of success have been achieved with nonviable, intact salmonellae, which are usually heat or acetone killed (2, 3, 24, 30, 37, 93). The nonviable vaccines are less effective than the viable ones because they provide only a humoral immunity to the pathogen. This subject has been critically reviewed by Eisenstein and Sultzer (26).

Aside from using inactivated intact bacteria as vaccines to induce protective immunity in murine salmonellosis, there are two major categories of nonviable vaccines prepared from components of salmonellae. One consists of crude ribonucleic acid preparations derived from the organism (112, 127, 128). Some investigators in this area believe that acquired immunity in the vaccinated animals is primarily cell mediated, and there is evidence to show the induction of DTH to bacterial antigens (113). The mechanism of immunogenicity for the ribonucleic acid vaccines is unknown. However, it is thought that the immunogenicity of the ribosomal vaccines is in part due to their contamination with O antigens (23, 62, 75), while other data show that the ribosomal proteins (76) or contaminant bacterial surface proteins (77) contribute at least part of their protective immunogens. The other category of vaccines is essentially composed of cell wall lipopolysaccharide (24), based on the belief that the acquired resistance to salmonellosis (21, 63), or, conversely, the virulence of salmonellae (71, 101, 121, 122), is directly related to the somatic antigens of the pathogen. The inherent endotoxic effect of these vaccines has not been taken into consideration in the experimental studies. Mouse antisera (107, 108) or monoclonal antibodies (15) of immunoglobulins G and M isotypes with specificities for O-antigenic polysaccharide of *S. typhimurium* are effective in passive protection. Associated with the somatic antigens, porins (proteins from the outer cell membrane) and octasaccharide derived from O antigens of *S. typhimurium* are identified as protective immunogens (52, 58, 116, 117). Vaccination with the octasaccharide-porin conjugate provides a greater protection against *Salmonella* infection than vaccination with either of these components separately. However, neither of these two antigens in separate components or in conjugates is a potent protective immunogen.

The interpretation of the relative efficacies of various vaccine preparations frequently depends on the view of the individual investigator concerning the nature of acquired immunity to the disease. It also relates to how protection is defined. Some experiments are based on the increased survival time or survival rate, while others are based on the decreased bacterial population in the vaccinated animals. Eisenstein and her associates (25, 26) proposed that the intricate protective immunity is an interplay of three variables; namely, the genetic constitutions of the mouse strains, the route of challenge, and the nature of the vaccines. There are the factors that govern the relative contributions of cellular or humoral immunity to the infection. Thus, viable vaccines, inducing cellular immunity, will be protective in the highly susceptible mice with an i.v. challenge, whereas nonviable vaccines, eliciting only humoral immunity, will be effective in the highly resistant mice with an i.p. challenge.

Genetic Control of Host Resistance

It has been known for some time that host resistance to murine salmonellosis is under genetic control (97, 102). A better understanding of the subject has since emerged from several laboratories. With the prevalent view of macrophages being the effector cells in host resistance to the infection, it is tempting to associate their relative antibacterial activities as expressions of genetic variations of the host.

Native resistance to salmonellosis in mice is regulated by several genes. The *Ity* gene on chromosome 1 appears to exert a primary control in most of the inbred mouse strains (98). It affects the net growth rate of virulent *S. typhimurium* in the liver and spleen during the infection (41, 97), but not the growth of some strains of low virulence (7, 86, 118). Mice homozygous for the Ity^s allele (C57BL/6 and BALB/c) develop a rapidly fatal disease when challenged with virulent strains of *S. typhimurium*. However, those with the dominant Ity^r allele have varying degrees of resistance to the pathogen. In many of these studies, one parameter to show the relative host resistance to infection is the rate of bacterial growth in the liver or spleen or both, with the implication of genetic expression through antibacterial actions of the resident macrophages (88). With in vitro experiments, it remains

unclear whether the gene regulates the relative rate of intracellular killing or growth of the pathogen (10, 64, 123–125), while the rate of phagocytosis is not appreciably different. Monolayers of Kupffer cells from resistant *Ity*^{r} mice also resist infection with virulent *S. typhimurium* better than those from susceptible *Ity*^{s} mice (36). Consistent with the evidence of genetic control of macrophages, resident peritoneal macrophages from *Ity*^{r} mice exhibit a greater bactericidal capacity against both *S. typhimurium* and certain other extracellular bacteria than those from *Ity*^{s} mice (65). However, when one examines the data, even with a subsequent bacterial growth after an initial intracellular killing, there is no net increase of the bacterial population as a result of the intracellular sojourn (64). These studies suggest that antibacterial functions of macrophages are governed by the *Ity* gene, but not through the mediations of the T cells (10, 87), nor does the *Ity* gene control acquired immunity.

Two other mutant alleles, the lipopolysaccharide-unresponsive gene *Lps*^{d} on chromosome 4 and the X-linked immunodeficiency gene *xid*, confer susceptibility on the C3H/HeJ and CBA/N mouse strains, respectively (89, 90, 92). Mice with homozygous *Lps*^{d} are both unresponsive to the endotoxic effects of lipopolysaccharide and susceptible to *S. typhimurium* infections. The latter is presumed to be due to a defective function of macrophages. The hypersusceptibility of mice homozygous for *xid* is believed to be due to their inadequate humoral response to the pathogen in the late phase of the infection. Using *S. typhimurium* strains of low (84) or intermediate (42) virulence, the longer persistence of, or, alternatively interpreted, the late emerging resistance to, the bacterial growth within the liver and spleen in the susceptible mice in comparison with the resistant ones is believed to be significantly influenced by the *H-2* gene of the major histocompatibility complex. A gene distinct from *Ity*, *Lps*, and *xid* and not linked to *H-2* is also found to be responsible for susceptibility in the late phase of infection in DBA/2 and C57L mice, both of which are *Ity*^{r} (91). At least the difference in the rates of bacterial clearance in the late phase of infection appears to be under polygenic control (84). On the other hand, there appears to be no difference in the antibody response to vaccination between the resistant A and the susceptible C57BL/6 mice, as determined by the reduced rate of bacterial growth in the spleen of recipient mice after they received a passive transfer of immune serum from either the resistant or the susceptible mice (85).

As mentioned in the preceding section, Eisenstein and Sultzer (26) attributed the discrepancies in the protective efficacies of nonliving vaccines to the genetic diversity of mouse strains used by different investigators. They believed that nonliving vaccines are effective in inherently resistant mice, while attenuated vaccines are required to protect the hypersusceptible ones against challenges, and they associated this relationship with the genetic expression of the antibacterial function of macrophages.

A SYSTEMATIC STUDY ON PATHOGENESIS AND IMMUNITY IN MURINE SALMONELLOSIS

The brief review of the complex literature is necessarily limited to the theme of pathogenesis and immunity in experimental salmonellosis. If pathogenesis is viewed as the invasive property of bacteria to produce a progressive disease, it is evident that the pathogenesis of murine salmonellosis has never been clearly understood beyond a traditional belief of *Salmonella* being a facultative intracellular

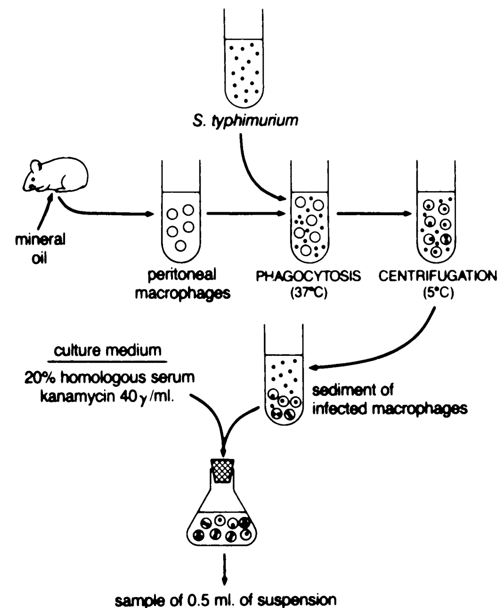

FIG. 1. Procedure for cultivation of peritoneal macrophages derived from guinea pigs and mice and infected with salmonellae.

parasite. The early literature contains supportive evidence of humoral immunity as an effective mechanism in acquired resistance to the infection. In retrospect, the concepts of intracellular killing of salmonellae aided by antiserum and the role of cytophilic antibodies as promoted by Rowley and his associates (50, 51, 103, 119, 131) deserve a critical evaluation. Unfortunately, somewhere along the way, the emphasis on host resistance was shifted to cell-mediated immunity, which has dominated the literature in the past two decades. This may have been related to the sudden recognition of the role of T lymphocytes in immune responses and their functional activities on macrophages. It is not surprising to find that most of the current publications on this subject refer to the macrophages as the effector cells of host resistance in salmonellosis.

In spite of my background training in experimental tuberculosis (43, 44), I stumbled into research on murine salmonellosis, persuaded by the textbook classification of *Salmonella* as a facultative intracellular parasite, though fortuitously not bound by a bias toward the preeminent role of macrophages in host resistance to the infection. The purpose of the following dissertation is to present a stepwise analysis on the pathogenesis and immunity of this experimental model as I understand it through a sequence of systematic investigations. It is certain to be considered by some as a nontraditional (or perhaps heretic) approach to the subject.

Fate of *Salmonella* in Murine Macrophages as Determined by Cell Culture Experiments

In the mid-1960s, the two schools of thought on humoral versus cellular immunity in murine salmonellosis began to take shape, and the fate of intracellular salmonellae was yet to be clearly defined. I began my investigations with the intention of showing the growth of virulent *S. typhimurium* within normal guinea pig macrophages. As discussed in an earlier section, the cell culture method can provide reliable observations if it is meticulously designed to achieve a quantitative recovery of intracellular bacteria.

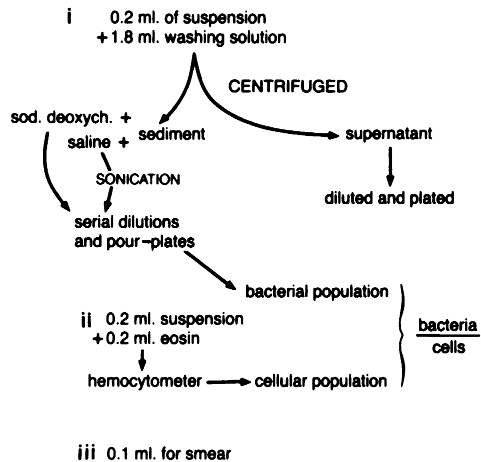

FIG. 2. Procedure for quantitative recovery of salmonellae within cultured macrophages at sampling intervals (0.5-ml cell suspension). In earlier studies, the infected cells were disintegrated by SDC, but sonic treatment was found to be more effective for the recovery of viable intracellular bacteria.

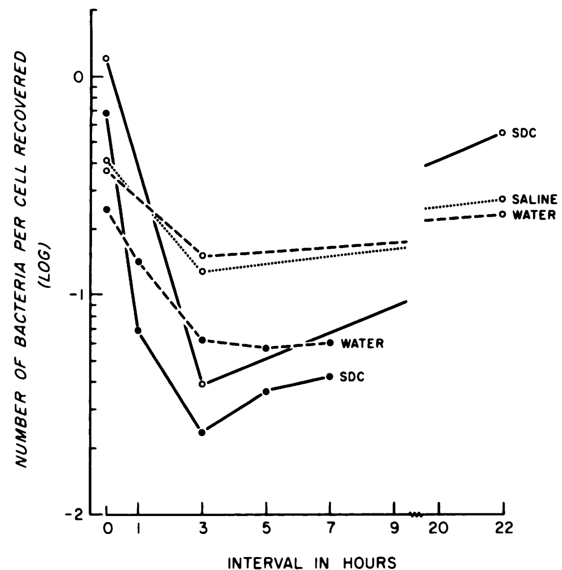

FIG. 3. Comparative recovery of intracellular salmonellae, using SDC, saline, or water. Peritoneal macrophages from guinea pigs were infected with *S. typhimurium*. At each sampling interval, an equal amount of samples from the infected cell culture was treated with SDC, saline, or water. Two sets of experiments were done, as identified by the closed and open circles. Reproduced from the *Journal of Bacteriology* (49) with permission of the publisher.

Figure 1 shows the scheme used for culturing peritoneal exudative macrophages infected with *S. typhimurium* (49). The organisms were cultivated in tryptic soy broth (Difco Laboratories) for 6 h in a rotating drum, washed in saline, and optically adjusted to a suitable concentration of viable bacteria. Peritoneal exudates containing >70% macrophages were harvested from guinea pigs 3 to 5 days after an i.p. inoculation of mineral oil. Appropriate volumes of exudate cells and bacteria were mixed in a screw-capped tube and rotated in a drum at 37°C for 15 min to effect the infection of the cells. The mixture was diluted in chilled Hanks solution and centrifuged to sediment the infected cells. A suspended cell culture was then maintained in a 25-ml, silicone-coated Erlenmeyer flask placed in a shaking water bath. The culture medium contained 20% homologous serum and 40 μg of kanamycin per ml to inhibit the extracellular bacterial population. Although the infected cell population would continue to decline, such a cell culture could easily be maintained for up to 30 h.

Figure 2 shows the basic procedure with which samples of the infected cells were taken at intervals to assay the viable intracellular bacteria (49). A 0.5-ml portion of sample was removed from the culture flask and treated in the following manner. (i) A 0.2-ml portion was placed into a 15-ml centrifuge tube containing 1.8 ml of Hanks solution and centrifuged to sediment the infected cells. (ii) A 0.2-ml portion was mixed with an equal amount of eosin Y for viable leukocyte population count, using a hemacytometer. (iii) The remaining sample could be used to make a stained smear.

From the centrifuged portion of the sample, the supernatant fluid was diluted and plated on tryptic soy agar (Difco) to verify the proper inhibition of the extracellular bacterial population. The sedimented leukocytes were lysed to release their content of viable bacteria, which was determined by colony counts on tryptic soy agar plates. Thus, a ratio of bacteria/cells is derived from the total viable bacterial population and the viable cellular population in a 0.2-ml sample volume. The phagocytic index in the infected cell culture is defined as the number of bacteria per cell at the beginning of the cell culture, i.e., 0 h. The fate of the intracellular bacterial population is determined by the quantitative recovery of viable salmonellae within the cultured leukocytes at designated intervals and is presented as a changing ratio of the bacterial/cells to the phagocytic index at each interval.

The critical part of this cell culture procedure depends on the proper quantitative recovery of viable intracellular bacteria. SDC was used successfully to disintegrate cultured macrophages for the recovery of tubercle bacilli (43) and is an ingredient in selective salmonella-shigella agar (Difco) for the isolation of *Salmonella* spp. We had first tested a 0.5% solution of SDC and found no effect on the viability of *S. typhimurium* during a 10-min exposure. Figure 3 shows a comparison of SDC, distilled water, and saline in their relative abilities to lyse identical samples of infected cells for the quantitative recovery of salmonellae, using the scheme shown in Fig. 2 (49). As seen by microscopic observation, neither water nor saline could disrupt the cultured leukocytes beyond damaging their ability to reject the vital stain eosin Y. Therefore, it is reasonable that, at 0 h of sampling, SDC consistently released more intracellular bacteria than either water or saline from an equal amount of cell samples. However, between the sampling intervals of 1 and 7 h, the ratio of bacteria/cell in each sample was closely identical when water and saline were used, but more intracellular bacteria were recovered with either water or saline than with SDC at each sampling time. One possible explanation of the latter observation was offered at the time. When the intracellular salmonellae are exposed to the digestive enzymes of the host cells, the bacterial cell surface is probably damaged first. As these damaged bacteria are released and exposed to the SDC, the bacterial cell membrane is probably lysed similarly to the mammalian cell membrane. Hence, these damaged bacteria do not generate colonies on agar plates. In contrast, they could possibly regenerate to form colonies if their host cells were not lysed by water or saline.

Two strains of *S. typhimurium* are used in our laboratory. The virulent strain SR-11 has a 50% lethal dose (LD_{50}) of $<10^2$ bacteria by an i.p. injection into Swiss-Webster mice, and the avirulent strain RIA has an LD_{50} of approximately

10^6 organisms. In our first set of experiments (49), SDC was used for the recovery of *S. typhimurium* in cultured macrophages of guinea pigs. There was a rapid initial intracellular destruction of the virulent salmonellae within the first 3 h of infection, followed by a logarithmic increase in the bacterial population from 3 to 21 h. In contrast, a substantially greater portion of the avirulent bacteria was killed in the first 4 h, followed by a parallel rate of increase as seen in the virulent strain. It would appear that those organisms surviving the initial killing by the host cells are capable of subsequent intracellular replication. However, it was also noted that, after 28 h of cultivation, the final intracellular bacterial population of the virulent strain reached only 30% of that initially phagocytized. This raises the question as to whether the intracellular environment is really favorable for the virulent *S. typhimurium*. Accordingly, the efficiency with which extracellular bacteria are ingested by the host cells might be a crucial factor in host resistance. By using guinea pig macrophages in the same cell culture procedure, the phagocytic index was found to be inversely related to the virulence of *S. typhimurium* (130). Thus, the SR-11 strain harvested from in vivo growth is more virulent in animals as well as more resistant to phagocytosis by guinea pig macrophages than the same strain grown in tryptic soy broth. The latter organism is in turn more resistant to phagocytosis than the avirulent RIA strain. Conversely, the presence of antiserum significantly increases the phagocytic index of the virulent organisms (130).

We failed to realize in our preliminary test that, when *S. typhimurium* is exposed to a 0.5% solution of SDC, about 60% of the organisms are immediately killed but the viability of the survivors is unaffected. Similarly, when a suspension of the organism is plated on salmonella-shigella agar, <50% will form colonies. This may explain why the disruption of infected cells with SDC produces fewer intracellular bacteria than that with either water or saline, as mentioned earlier. A sonication procedure was later developed which could disintegrate host cells without interfering with the viability of salmonellae (45). Figure 4 compares the two methods of recovery of intracellular bacteria. With sonication, a continuous decline of the virulent *S. typhimurium* SR-11 was seen within guinea pig macrophages. In comparison, the quantitative recovery of salmonellae with SDC yielded substantially fewer organisms. The initial killing and subsequent increase in the intracellular bacteria thus appear to be an artifact of the sensitivity of the organism to the detergent. In all subsequent investigations, we have used sonication for the quantitative recovery of intracellular salmonellae. With this method, the ingested *S. typhimurium* SR-11 is destroyed continuously within peritoneal exudative macrophages of mice over an 8-h period (72).

Taken collectively, we believe our cell culture procedure for the determination of the fate of intracellular salmonellae to be more reliable than procedures described above in the literature review.

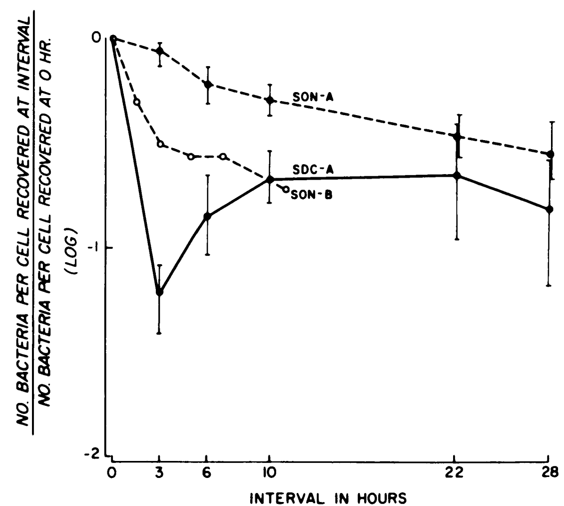

FIG. 4. Fate of *S. typhimurium* in macrophages of guinea pigs as determined by the quantitative recovery of intracellular bacteria, using SDC or sonic treatment (SON). In the first set of experiments (SDC-A and SON-A), an equal amount of samples from the infected cell culture was treated either with SDC or by sonication at each interval. The phagocytic indices were both 0.5, but the subsequent patterns of intracellular killing of salmonellae appear different. In the second set of experiments (SON-B), the cell samples were sonicated and the phagocytic index was 0.3. Cross-bars at intervals represent the range of data from which the average was taken. Reproduced from *Infection and Immunity* (45) with permission of the publisher.

Antibacterial Cellular Immunity in Murine Salmonellosis

The question of whether macrophages from a previously infected host possess enhanced antibacterial activities in murine salmonellosis was assessed with the cell culture procedure (72). Mice immunized i.p. with avirulent *S. typhimurium* were used as donors of immune macrophages. At the time of harvesting the peritoneal exudative macrophages, a sample was plated on tryptic soy agar, on which a few colonies usually appeared. This indicated the continuous presence of antigens in the peritoneal cavity of the immunized mice. The immune cells were also trypsinized before infection to avoid clumping. The fate of virulent *S. typhimurium* was compared within normal and immune macrophages cultured in normal serum. No statistical difference in the rate of intracellular killing of the pathogen is seen within these two cell populations. Even when the infected immune cells were cultured in immune serum, the rate of intracellular killing is unchanged.

Similarly, guinea pigs were immunized intracutaneously with virulent *S. typhimurium*, followed by a booster i.p. injection (45). Both normal and immune macrophages were cultured overnight in the presence of normal serum before they were infected with virulent salmonellae to prevent clumping of the immune cells. The rates of intracellular killing of the bacteria were identical among the normal macrophages cultured in normal serum and immune macrophages cultured in either normal or immune serum over an 8-h period.

In line with the prevalent concept of the antibacterial action of macrophages being mediated by lymphokines from immunologically committed T lymphocytes (13, 35), another approach to detect the expression of cellular immunity was taken (73). Peripheral lymphocytes were isolated from guinea pigs previously infected with the virulent *S. typhimurium* and stimulated in vitro with the protein antigens of *S. typhimurium*. The supernatant from the immune lymphocyte culture contained the migration inhibition factor, as shown by its ability to inhibit the migration of normal guinea pig macrophages in vitro. However, when one-half of the peritoneal exudative macrophages from a normal guinea pig were first cultured overnight in this supernatant, washed, infected with the virulent *S. typhimurium*, and again returned to the same culture medium containing the supernatant, their bactericidal action during a 6-h period of observation was

not altered when compared with that of the other half of the infected macrophages cultured in control medium.

In a preliminary study with immune macrophages, we noted that they tend to clump together when infected with *S. typhimurium*. This interferes with the proper enumeration of the cell population in a sample. We proposed that this phenomenon is due to the presence of cytophilic antibodies on the surface of the immune cells and can be circumvented by treating the immune macrophages with trypsin or by culturing the cells overnight in the presence of normal serum before they are to be infected. The presence of specific cytophilic antibodies on the immune macrophages of mice and guinea pigs was confirmed by inactivating the cells with merthiolate and then demonstrating their agglutination with both H and O antigens of *S. typhimurium*, but not with O antigens of other species of *Salmonella* or *Escherichia coli* (45, 72). Furthermore, cytophilic antibodies can be eluted from immune macrophages by incubation in the absence of immune serum or passively transferred onto normal macrophages by incubation in the presence of immune serum (45). The presence of cytophilic antibodies on immune macrophages appears to represent an expression of antibacterial cellular immunity. They enable the macrophages to be specifically attracted toward the pathogen, to trap them by clumping around them, and to engulf them by opsonization.

It appears that macrophages derived from the infected guinea pigs and mice are not endowed with an enhanced capacity to digest the intracellular *S. typhimurium*, nor are they more active in phagocytizing the bacteria unless coated with cytophilic antibodies. Under our experimental conditions, there is also no evidence that the antibacterial functions of the macrophages are altered by mediators from antigen-stimulated immune lymphocytes. Perhaps the more pertinent point is that the innate bactericidal action of macrophages can no longer be further escalated, nor is cellular immunity (as expressed by an enhanced bactericidal action) necessarily an essential component of acquired resistance in murine salmonellosis. Once *S. typhimurium* is engulfed by the macrophages, it faces a hostile environment. In cell culture experiments, a small residual intracellular bacterial population usually remains. Whether these survivors will later proceed to propagate is an inevitable question. More realistically, while these survivors stay dormant, the extracellular bacterial population is likely to be expanding logarithmically and reaching a lethal proportion.

Effect of Antibiotics on Intracellular Salmonellae

In our cell culture experiments, kanamycin was incorporated into the culture medium to ensure that the fate of intracellular bacteria was not influenced by the uncontrolled growth of extracellular bacteria. The observed intracellular killing of *S. typhimurium* makes it difficult to exclude the possibility that the antibiotic might penetrate the host cells and kill the bacteria. The antibody-sensitive *S. enteritidis* E-100 was used to infect guinea pig macrophages in cell culture (100). The medium contained 30% specific antiserum with or without kanamycin. The intracellular and extracellular bacterial populations were followed. Antiserum alone is bactericidal to the extracellular salmonellae for the first 2 to 4 h, during which there is a concomitant reduction of the intracellular bacteria. As the extracellular organisms begin to grow, the intracellular bacterial population increases. If kanamycin is added initially to inhibit the extracellular bacteria, the intracellular bacterial population continues to decline. However, if the kanamycin is removed by replacing the culture medium, the extracellular bacteria will multiply and the intracellular bacterial population will also increase. It would appear that the increase in the intracellular bacteria is associated with the continuous phagocytosis of extracellular bacteria, as observed by others (53). Thus, a continuous destruction of intracellular bacteria will occur as long as the extracellular bacteria are controlled, and it is unlikely to be the result of penetration or retention of kanamycin in the host cells. Rather, there is an innate capacity of the normal host macrophages to digest virulent salmonellae.

Correlations of In Vitro Data with Infected Animals and Effect of Vaccination

Our in vitro studies show an inherent ability of macrophages from guinea pigs and mice to destroy virulent *S. typhimurium* and *S. enteritidis* and an expression of cellular immunity in the nature of cytophilic antibodies rather than that of an altered antibacterial function of immune macrophages. On this basis, the pathogenicity of virulent salmonellae is dependent on their resistance to ingestion by host phagocytes; conversely, acquired immunity of the host is related to the cytophilic and opsonic natures of antibodies, which facilitate the transfer of extracellular bacteria to an adverse intracellular environment. Several experiments were designed to see whether these findings could be used to explain the infectious process in animals.

Cutaneous lesions generated by a pathogen could be used as an experimental model to study host-parasite interactions (66). Guinea pigs were injected intracutaneously with *S. typhimurium*, and development of the lesions was followed (48). The area of expanding inflammation can be used to determine the relative virulence of the pathogen and, conversely, the resistance of the host. It has been known that virulent *S. typhimurium* can be rendered more virulent by repeated passages in mice. The lesions produced by the virulent SR-11 strain harvested from infected mice are significantly larger than those produced by the same number of this organism grown in tryptic soy broth. The latter organism in turn generates larger lesions than the avirulent RIA strain. The relative virulence of these three organisms is directly associated with their relative resistance to phagocytosis as seen by the in vitro assay (130). The greater ability to resist phagocytosis by host cells allows the invading salmonellae to propagate extracellularly and thereby produces a larger lesion. The opsonizing effect of antiserum added to the bacterial inoculum reduces the ability of the virulent salmonellae to generate as large a lesion as the same inoculum mixed with normal serum. In primary lesions induced by 10^6 bacteria of strain SR-11, the inflammation reaches its maximum in 5 to 7 days and then subsides at the end of 2 weeks. The lesions are characterized by central hemorrhagic necrosis, followed by ulceration. When these guinea pigs are rechallenged either 2 or 4 weeks after the primary infection, the development of the secondary lesions appears different. The area of lesions is significantly smaller and reaches its peak 1 or 2 days earlier and usually with minimal or no necrotic ulceration. The characteristics of the secondary lesions suggest the development of both acquired resistance and DTH against subsequent challenge. The elicitation of DTH in these infected animals can be confirmed by the in vitro migration inhibition factor test, using peritoneal exudate cells cultured in the presence of protein antigens of *S. typhimurium* (73). The most revealing observation from this series of experiments was the gross appearance of a necrotic abscess in the primary lesions, which was later found by

tissue biopsy to be composed primarily of polymorphs in the early stage of the lesions. It gave us the first indication of the absence of macrophages in the early stage of salmonellosis.

The outbred Swiss-Webster RFW mice used to be highly susceptible to virulent *S. typhimurium* SR-11, with an LD_{50} of approximately 10 organisms by i.p. inoculation (72). If they were vaccinated with the live avirulent RIA strain, they could survive a challenge of 10^7 virulent salmonellae. A single i.p. vaccination with 10^6 or 10^7 heat-killed avirulent or virulent salmonellae protected these mice against a subsequent challenge of 10^3 virulent organisms 3 weeks later (72). In subsequent studies, two doses of 10^8 heat-killed virulent salmonellae given i.p. 3 weeks apart provided protection in all animals challenged 10 days later with 10^5 virulent organisms and to 70% of those challenged with 10^6 organisms (82). The inability of the killed vaccine to induce DTH was confirmed by the absence of footpad swelling when mice were injected with the protein antigens and by the absence of granulomatous lesions in the early stage of the infection (47, 82). Also, a nonviable vaccine, prepared by sonication of heat-killed virulent salmonellae and consisting primarily of Triton-insoluble fragments of the bacterial envelope, proved to be equally effective in the highly susceptible C3H/HeN mice (LD_{50}, $<10^2$) against a subsequent infection with 10^5 and 10^6 pathogens (47). In a further study, the lipopolysaccharide of *S. typhimurium* SR-11 was extracted with phenol-water and delipidated by alkaline hydrolysis to remove its endotoxic effect. Both the lipopolysaccharide and the detoxified preparation provided significant protection to C3H/HeN mice against challenges (H. F. Ding, I. Nakoneczna, and H. S. Hsu, J. Med. Microbiol., in press). Collectively, therefore, the nonviable vaccines are highly effective, although not as fully protective as the live attenuated vaccine. The immunogenic property of the nonviable vaccines appears to be associated with the O antigens of *S. typhimurium*. Along this line, antisera from both guinea pigs and mice can provide passive immunity against a low-dose i.p. challenge of 10 bacteria. However, as the challenging doses are increased to 10^2 and 10^3 bacteria, the protective efficacy of the antisera is proportionately reduced (72). This is consistent with the proposed opsonic nature of antibodies in host immunity.

Role of DTH in Murine Salmonellosis

Inflammation is a common denominator of tissue response in host resistance to infections and in DTH. The accelerated inflammatory reaction seen in the secondary cutaneous lesions induced by virulent *S. typhimurium* in guinea pigs is clearly a manifestation of DTH to bacterial antigens during the infection (48). The expression of DTH to bacterial protein antigens can be demonstrated by the migration inhibition factor test, using guinea pig peritoneal cells (73). Although allergic sensitivities are usually viewed as an injurious tissue reaction in the host, the enhanced inflammation mediated by DTH in the secondary cutaneous lesions appears to have exerted a beneficial effect on acquired resistance to the infection. To test the latter hypothesis, Swiss-Webster RFW mice were vaccinated by an i.p. injection of *M. bovis* BCG and challenged 4 to 10 weeks later with an i.p. injection of virulent *S. typhimurium* (46). A DTH reaction in the vaccinated mice was elicited by a simultaneous injection of purified protein derivatives of tuberculin along with the pathogen. The tuberculin sensitivity prolongs the survival time and protects 70% of the mice challenged with 10^4 bacteria from an otherwise fatal disease within 12 days. In contrast, BCG vaccination alone raises the survival rate to about 36%. When the infective dose was increased to 10^6 bacteria, the protective effect of the tuberculin reaction was reduced, although there remained a statistically significant improvement in the survival distribution of the challenged mice as compared with those receiving BCG vaccination alone. Cytological examinations of the peritoneal washings from mice undergoing a tuberculin reaction showed a quantitative increase in the total leukocyte population as well as in the percentage of the phagocytic cell population. The accelerated influx of phagocytic cells into the site of infection induced by DTH is believed to have contributed to the enhanced host resistance to salmonellosis. On this basis, it would be reasonable to imply that the elicitation of a DTH response at the site of salmonella infection would accelerate the accumulation of macrophages armed with cytophilic antibodies and thus enhance the specific resistance of the host.

Histopathology of Murine Salmonellosis

Virulent *S. typhimurium* can be isolated from the blood within 2 h after an i.p. inoculation of 10^3 bacteria into highly susceptible mice. This indicates a rapid hematogenous dissemination of the pathogen, leading to a systemic infection. To follow the histopathological development of salmonellosis, the susceptible Swiss-Webster RFW mice were injected i.p. with a small (10^2 or 10^3) dose of the virulent SR-11 organism (81). They were sacrificed daily beginning on day 3 after infection, and tissue samples were embedded in paraffin. Detailed descriptions on the histopathology of the disease have been given in previous publications (81, 82).

In the primary infection, no gross changes were seen in various organs except for splenomegaly, and numerous grey-white lesions became visible in the liver and spleen as the disease progressed. Primary lesions began typically as multiple microscopic acute abscesses, with the infiltration of predominantly polymorphs in the liver and spleen from day 4 on. They became enlarged and more numerous with the appearance of well-developed microabscesses composed almost entirely of polymorphs. Acute vasculitis was also prominent in the liver and spleen. From day 6 on, peripheral infiltration of mononuclear cells began to appear in the expanding lesions, while polymorphs were disintegrating in the central area. The gradual transformation of microabscesses into granulomas was usually obvious by days 6 and 7 and was clearly associated with the manifestation of DTH to the bacterial antigens. The enlarging lesions often became confluent. With such a low dose of infection, the mice usually died within 12 days. Around day 9, most of the lesions seen in the surviving animals consisted of granulomas, many of which exhibited a central necrosis. In the terminal stage of the disease, multiple infarcts appeared predominantly in the liver as a result of the occlusion of small hepatic vessels by masses of mononuclear cells, suggestive of an intense allergic reaction to the rapidly expanding bacterial antigens.

When mice were vaccinated with the avirulent RIA strain and challenged i.p. with 4×10^5 virulent *S. typhimurium*, no histopathological lesions were found in the liver and spleen up to 12 days after infection. This would suggest that the organisms were effectively eliminated at the site of inoculation by acquired immunity, retarding their hematogenous dissemination. Subsequently, 10^6 virulent salmonellae were directly introduced i.v. to initiate a systemic infection. Over 90% of the mice survived the challenge and showed no gross

changes in the organs except for occasional mild splenomegaly at the time of sacrifice. Multiple microscopic nodular lesions were seen in the liver and spleen as early as 3 days after infection. These secondary lesions were characterized as typical granulomas composed almost entirely of macrophages with a peripheral rim of lymphocytes. By day 5, a few larger granulomas may show minute central necrosis with polymorphs. However, the lesions became smaller, discrete, and nonconfluent in subsequent days. The healing process proceeded with the regression of cellular exudation and replacement of lesions with regenerated parenchymal cells. The early appearance of granulomatous lesions in the secondary infection was clearly a manifestation of DTH to the bacterial antigens, which, in concert with acquired resistance, endowed the host with an accelerated capability to terminate the infectious process before the pathogen gained a foothold in the host tissues.

In contrast, when the highly susceptible mice were vaccinated with heat-killed *S. typhimurium* (82) or their sonicated fragments (47) and challenged with virulent *S. typhimurium* as described above, the development of histopathological lesions in the protected mice began with an early formation of microscopic abscesses identical to those seen in the primary lesions. Among the control mice infected i.p. with 10^5 ($>10^3\times$ LD_{50}) virulent salmonellae, scattered acute microabscesses appeared in the liver and spleen as early as 2 days after infection. The lesions rapidly increased in size and number, became confluent, and spread across the tissues of these organs by day 4. About this time, extensive areas of necrosis and infiltration of mononuclear cells into some lesions became apparent. The mice began to die. Among the surviving ones autopsied after day 5, the tissue lesions consisted of a mixture of acute frequently confluent abscesses, an increasing number of granulomas, and numerous microinfarcts, resulting in a rapidly invasive destruction of the involved organs. By comparison, gross examinations of the vaccinated and similarly infected mice revealed all organs to be normal with the exception of a slight splenomegaly. A few isolated microabscesses started to emerge in the liver about 4 days after infection. Between 7 and 10 days, limited numbers of minute necrotic foci appeared in the liver, spleen, and occasionally the lymph nodes. Acute lesions began to transform into granulomas, with the appearance of peripheral infiltration of mononuclear cells displacing the polymorphs. The most significant feature seen in these lesions was that they usually remained small in size and number, with rare confluence. From week 3 on, only a few residual microscopic lesions, mostly in the form of minute granulomas, were found in the liver and spleen. Regeneration of normal tissue ensued. If the vaccinated mice were challenged with 10^6 virulent salmonellae, the development of the infectious process was accelerated in comparison to that of mice infected with 10-fold-fewer bacteria. Early microabscesses appeared in 3 days, and transformation into granulomas began by 6 days after infection. However, the sequence of events remained unchanged in these protected animals. A pertinent observation was that blood cultures taken from the vaccinated and infected mice at the time of sacrifice were almost consistently negative, indicating the absence of septicemia among these animals, presumably because of the effective containment of the pathogen by an enhanced phagocytosis of the opsonized bacteria.

From the comparative histopathological studies above, the most striking revelation is the conspicuous absence of macrophages in the early stage of the primary infection. It raises the question of whether macrophages play a significant role in the pathogenesis of salmonellosis and whether salmonellae are truly facultative intracellular bacteria. Rather, the early appearance of microabscesses in the primary infection is reminiscent of the pathogenesis of the obligate extracellular *Streptococcus pneumoniae*. This contention is consistent with the in vitro observation of a direct correlation between the antiphagocytic property and the virulence of *S. typhimurium* (130). The rapid extracellular proliferation of salmonellae would instigate an acute and progressive inflammation leading to the destructive injury of host tissues. In contrast, nonviable vaccines induce only a humoral immune response but not the DTH, which can be confirmed by the failure of salmonella protein antigens to elicit a typical footpad swelling in the vaccinated mice (19, 82). With these vaccinated mice, the early influx of polymorphs, aided by the opsonic antibodies, can effectively eliminate the invading pathogen and succeeds in containing the infection at an early stage and allowing time for the host to develop its own complete immunity in the form of anamnestic humoral response and DTH. The delayed appearance of initial lesions in the organs and the slow transformation into granulomas reflect the efficient localization of the pathogen and the reduction of bacterial antigens for the induction of DTH. In contrast, mice vaccinated with the avirulent salmonellae are endowed with both arms of the immune response at the time of challenge, as evidenced by the early appearance of granulomatous lesions. The prompt influx of antiserum and antibody-coated macrophage into the lesions is more vigorous in terminating the invading organisms at the site of entry.

It is apparent that our histopathological study has been highly instructive toward our understanding of the infectious process in murine salmonellosis. Regrettably, this line of approach has not been fully appreciated and utilized in current investigations of host-parasite interactions. With the paraffin-embedding method, tissue sections are cut at a thickness of approximately 5 to 7 μm. Bacteria are not seen in these preparations. Currently, tissue specimens are being embedded in JB-4 Plus plastic resin (Polysciences, Inc.), which makes it possible to cut sections at 2-μm thickness. Using Wright's stain, bacteria can be visualized both intracellularly and extracellularly in the tissue specimens. But as emphasized previously, the viability of the stained organisms cannot be ascertained. Nevertheless, continued investigations with this method will prove productive especially on the cell-cell interactions in the histopathology of the infectious process.

Location of Bacterial Proliferation In Vivo in Salmonellosis

With the persistent controversy of whether virulent salmonellae can replicate within host phagocytes, it became desirable to pursue a definitive investigation to resolve this issue. We thought that a more reliable approach would be to examine the tissues of the infected animals directly by electron microscopy (EM). This is necessarily a tedious undertaking, but it affords the most direct way to observe the fate of the pathogen at the site of infection and eliminates the criticisms inherent with in vitro techniques.

Inbred susceptible C3H/HeN mice were injected i.p. with 2×10^7 *S. typhimurium* SR-11 to induce a rapid and intense inflammatory response (33, 34). Peritoneal washings were harvested at intervals and examined by EM. The viability of bacteria is based on their morphological integrity as exemplified by the extracellular organisms. Polymorphs began to arrive as early as 1.5 h after infection. There appeared to be

a rapid destruction of the ingested bacteria within both polymorphs and macrophages. The degenerative changes in salmonellae occur in two possible sequences: it might begin with an enlarging central electron-lucent area in the cytoplasm and peripheral condensation of cytoplasmic granules, followed by the disruption of bacterial envelope and the disintegration of cellular structure; or it might start with an irregular and discontinuous bacterial envelope with the compression of the bacterium and the diffuse condensation of cytoplasmic granules. Many bacteria were enclosed within a membrane-bound phagolysosome as traditionally described, while some were internalized in direct contact with the host cytoplasm. The overwhelming majority of the intracellular bacteria observed from 1.5 h on were in some stage of degeneration, while very few intact intracellular organisms were seen over a period of up to 72 h after infection. Both polymorphs and macrophages are actively phagocytizing bacteria, and at times macrophages are found to have engulfed bacteria as well as a polymorph, which has in turn ingested its share of bacteria. Bacterial multiplication took place in the extracellular locations of the peritoneal cavity, as evidenced by intact organisms undergoing division. Similar observations of intracellular killing and extracellular multiplication of virulent salmonellae were documented in the i.p. infection of the genetically resistant A/J mice (60a). On this basis, there appears to be no appreciable difference in the ability of genetically resistant and susceptible host phagocytes to destroy virulent salmonellae. When the virulent *S. typhimurium* is preopsonized with antiserum before its injection into the C3H/HeN mice (34), enhanced phagocytosis of the bacteria is evident, making the destructive events within the phagocytes even more easily observed as early as 1.5 h after infection. This supports the view that, once the salmonellae are ingested, they are rapidly destroyed.

Genetically susceptible C57BL/6 mice were also injected i.p. with 2×10^7 virulent *S. typhimurium* to effect a rapid dissemination of the pathogen throughout the liver and spleen (61, 129). This would facilitate the search for bacteria in the tissues by EM at the early stage of the infectious process and prior to the onset of acquired immunity, which would otherwise complicate the host cell-parasite interactions. Tissue samples were obtained at 2 and 3 days after infection. Consistent with the observations in the peritoneal washings, rapid infiltration of polymorphs and macrophages was evident in the liver and spleen. They appeared to be leukocytes of hematogenous origin and were engaged in an active process of digesting salmonellae. This is contrary to a common implication that only resident macrophages are involved in these organs during the infectious process. In fact, the majority of the macrophages seen in the liver do not resemble the typical Kupffer cells. The prominent sites for massive bacterial proliferation are in the extracellular locations of sinusoids and among tissue debris of lesions (Fig. 5). The most unexpected finding is the bacterial invasion of the hepatocytes, which appear to be a safe haven for salmonella propagation and away from the attack by infiltrating phagocytes. The active bacterial replication in the extracellular locations and within hepatocytes can be visualized by the presence of morphologically intact organisms, some of which are in the process of division.

There are certain limitations in the use of EM. It is a descriptive method to provide visual evidence of the host-parasite interactions in the infected tissues. Since only a minute area of the tissue can be examined at a time and each tissue specimen represents only a plane of sectioning across the host cells amounting to <1% of their spherical volume, it does not lend itself to any quantitative study on the fate of intracellular versus extracellular bacteria. In spite of a relatively large infective dose given to initiate the disease, it apparently did not overwhelm or paralyze the host response to infection, as implied by the avid antibacterial actions of both the polymorphs and macrophages even up to the terminal stage of the disease. On rare occasions, structurally intact bacteria are seen among disintegrating ones within phagocytes. They may be recently ingested organisms prior to digestion, organisms capable of intracellular survival, or even the progeny from intracellular multiplication. The EM technique is unable to distinguish among these possibilities. On the other hand, the presence of disintegrating salmonellae and their residual antigens can be identified within inflammatory polymorphs and macrophages by using an immunogold labeling technique in EM (60). The latter method can in fact trace the dynamic process of intracellular destruction, as evidenced by the progressive degeneration of bacteria to their residual antigens without any recognizable morphological structure (Fig. 6).

Composite View of Pathogenesis and Immunity in Murine Salmonellosis and Its Perspectives with Data from the Literature

From a review of the literature and our own investigations, it is apparent that each experimental method has its limitations and none is likely to satisfy all critics. Nevertheless, in our stepwise analysis involving multiple approaches in this project, a logical understanding of the pathogenesis in murine salmonellosis may be proposed. Our early experiments with cell cultures together with recent EM evidence from the infected tissues have credibly established the destruction of virulent *S. typhimurium* within host polymorphs and macrophages. This places the primary site of bacterial proliferation in the extracellular locations of host tissues, which can now be documented by EM evidence and supported by the observation of a direct correlation between the resistance to phagocytosis and the virulence of the pathogen. On this basis, when virulent salmonellae are introduced into the animals, either in the peritoneal cavity or in RES organs, those that escape phagocytosis by the resident phagocytes and, more significantly, by the influx of inflammatory cells will replicate rapidly in the extracellular locations and disseminate into a systemic infection. Even if a minority of the ingested pathogens manage to survive the intracellular killing, their contribution to the total proliferating population of bacteria in the host is likely to be negligible. Hepatocytes (and possibly other parenchymal cells), which are usually not considered to be phagocytic, appear to provide a safe haven for the propagation of virulent salmonellae and to protect them from the hostile cellular and humoral elements of the host. The early tissue response to the initial bacterial challenge is manifested as acute inflammation. The progressive accumulation of polymorphs at this stage, as revealed in histopathological studies, is unable to contain the rapid growth and spread of the organisms due to their antiphagocytic property, but instead leads to the progressive destruction of host tissues. The distinct absence of macrophages disputes their role at least in the primary stage of infection. In the highly susceptible mice, a fatal disease is inevitable as the host is overwhelmed by the rapid, logarithmic increase of the pathogen and their accompanying endotoxic effect, on the one hand, and by the corresponding invasive tissue pathology, on the other. The subsequent

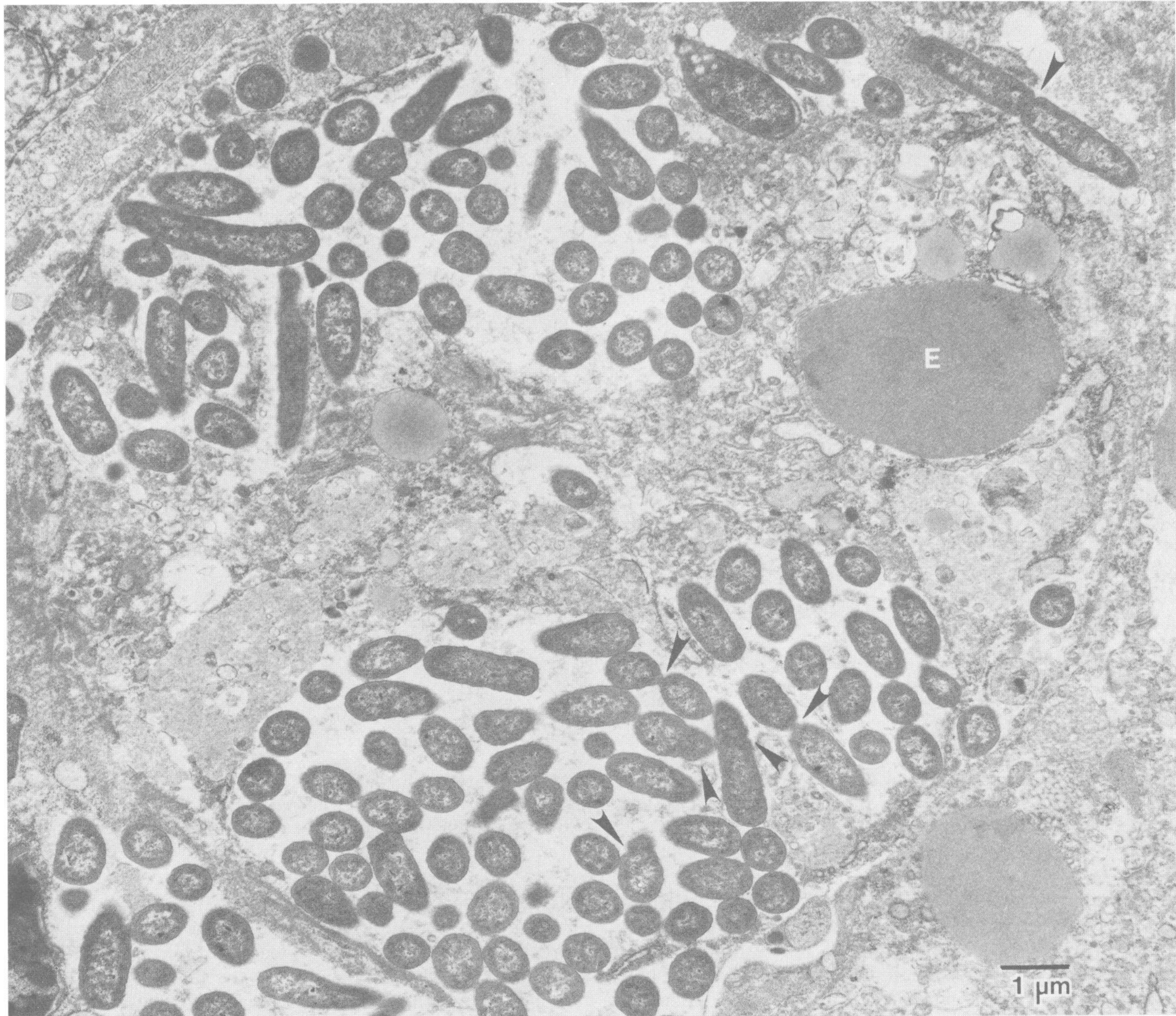

FIG. 5. Extensive proliferation of salmonellae among cellular debris of a lesion in the liver of an infected mouse. Some organisms are undergoing division (arrowheads). E, Erythrocyte. EM preparation by F. R. Lin, X. M. Wang, and V. R. Mumaw.

transformation into granulomatous lesions, associated with the development of DTH, is no longer capable of reversing the course of a fatal infection.

In contrast, as the infection ensues in the natively resistant mice, the host appears to be better able to retard the initial invasion of the pathogen while it develops both humoral and cell-mediated immunity. Antibodies directed primarily to the bacterial O antigens exert their protective functions by their opsonic, cytophilic, and agglutinating properties, all of which collaborate to facilitate the removal of extracellular salmonellae into a detrimental intracellular environment of the phagocytes. The concomitant induction of DTH to the bacterial protein antigens is manifested in the transformation of primary lesions, characterized as microabscesses, into granulomatous lesions. Aside from its injurious effect on the tissues, the enhanced inflammation of DTH also exerts its beneficial role by accelerating the influx of cellular and humoral elements into the site of infection. Thus, the synergistic functions of the immune response will enable the host to overcome the infection as long as the challenging dose is not overwhelming. The precise mechanisms through which the genetically resistant mice are better able to survive an infectious challenge than their susceptible counterparts are yet to be elucidated.

If the avirulent salmonellae are inoculated into the host, their slower growth rate and their greater susceptibility to phagocytosis would retard the extracellular bacterial propagation in the early stage. The animal is likely to survive the challenge and acquire a lasting immunity. In the secondary infection, the prompt appearance of granulomas reflects the elicitation of DTH and amasses the macrophages into the site of infection. Although our in vitro data do not support the expression of acquired cellular immunity in the form of enhanced intracellular killing of salmonellae, the early accumulation of macrophages in the secondary lesions effectively terminates the infection and reveals the activated functions of macrophages, presumably due to the presence of cytophilic antibodies.

In essence, therefore, a solid acquired immunity in murine salmonellosis, as established by a previous infection or an attenuated vaccine, is a synergistic manifestation of the innate capacity of polymorphs and macrophages to destroy

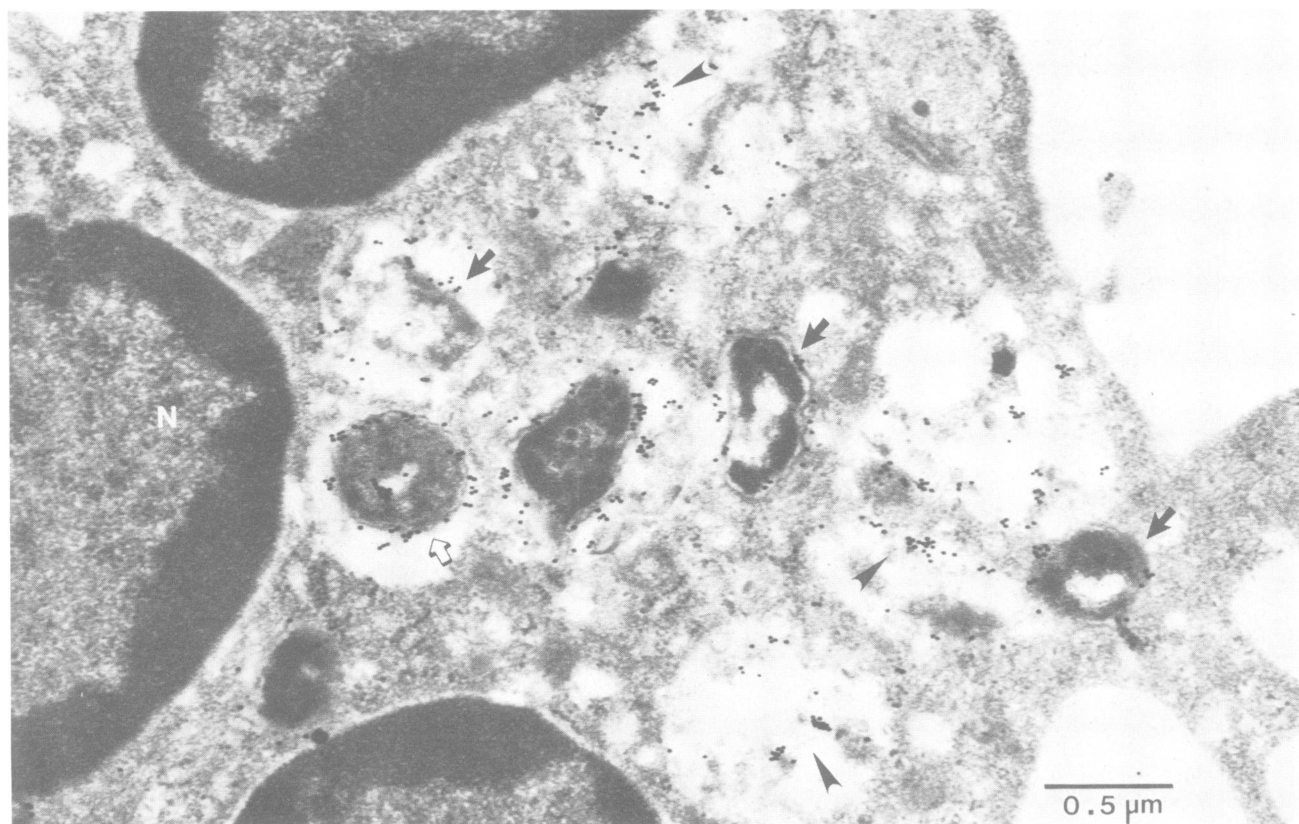

FIG. 6. Immunogold labeling of salmonella antigens within the cytoplasm of a polymorphonuclear leukocyte. One organism appears to be still morphologically intact (open arrow), while others are disintegrating (closed arrows). Areas with residual salmonella antigens can be identified in the absence of obvious bacterial structures (arrowheads). N, Nucleus of the polymorph. EM preparation by F. R. Lin and V. R. Mumaw.

the ingested salmonellae, the activated antibacterial functions of macrophages mediated by cytophilic antibodies, the opsonic and agglutinating actions of antiserum, and the enhanced inflammation associated with DTH. We have long promoted such a proposition (72, 73, 81, 82) since the integral components of acquired immunity have all been identified by our in vitro experiments. On the other hand, the nonviable vaccines, in their ability to induce specific antibodies against the O antigens of *S. typhimurium*, can also confer in the host a highly effective, though not a complete, protection against subsequent challenges. Such a humoral immunity is manifested most efficiently through its opsonic function, as evidenced in vivo by the successful suppression of bacterial proliferation through the early formation of acute abscesses in the RES organs. The rapidly progressive and fatal infection seen in the highly susceptible mice challenged with 10^2 or 10^3 virulent salmonellae indicates an unusually high rate of bacterial replication in the host tissues. Whether mice immunized with nonviable vaccines can survive a challenge depends on the size of the infective dose and their initial ability to inactivate a sufficient portion of it. The infection itself will serve as a booster immunization to accelerate the humoral response as well as the induction and elicitation of DTH. A favorable outcome is attained by suppressing the early propagation and systemic dissemination of the extracellular bacteria and by allowing time for the host to develop its own solid immunity to the infection. Such is the fascinating balance in the host-parasite interactions.

This proposition of the pathogenesis and immunity in murine salmonellosis, deduced from collective experimental data, is clearly at variance with the traditional definition of *Salmonella* as a facultative intracellular parasite and with the presumed role of macrophages as effector cells in host resistance. However, in view of the lack of reliable experimental support to show the multiplication of virulent salmonellae within phagocytes, there appears to be no compelling reason to retain that traditional classification, unless its restrictive meaning is modified to include the ability of the organism to proliferate within hepatocytes and possibly other nonphagocytic parenchymal cells. Along this line, it is tempting to postulate that the pathogen may remain in a symbiotic relationship with the hepatocytes and thus establish a carrier state, contributing to the source of salmonellae in the gallbladder. The most persuasive evidence against a major role of macrophages, particularly in the early stage of salmonellosis, is their prominent absence at the site of primary infection.

The experimental techniques used both in vivo and in vitro to promote macrophages as the effector cells require scrutiny. Our histopathological and EM observations fail to support the assumption that the bacterial growth in the liver and spleen is primarily confined within the resident macrophages. The popular belief that the clearance of bacteria from the circulation by the RES is attributed to the fixed macrophages is surely inconsistent with the basic understanding of histopathology in the infectious process. The organisms can just as well be sequestered in the sinusoids of the RES organs, as now documented by EM. An equally plausible interpretation for the suppressed bacterial growth in the liver and spleen of the immune host is that the

extracellular salmonellae are more effectively reduced by the bactericidal action of inflammatory phagocytes mediated by opsonic antibodies. There appears to be no indisputable experimental evidence to support the expression of activated macrophages in acquired immunity as an enhanced bactericidal action against salmonellae. Recent publications comparing the survival of virulent salmonellae within macrophages of different sources rely on SDC treatment to disrupt cultured macrophages for the recovery of intracellular bacteria (12, 36, 64). Regardless of whether these investigators had first determined the possible injurious effect of SDC on their salmonella cultures, as we reported in 1973, the characteristics of intracellular survival curves seen in these publications appear comparable to our data (45). Namely, there is a rapid initial killing of intracellular salmonellae, followed by a period of increase. In comparison, our data (Fig. 4) show that sonication of the cultured cells can recover more intracellular salmonellae than SDC treatment of the same amount of samples and it results in an observation of continuous intracellular killing. Thus, data on the fate of intracellular salmonellae based on the lysis of cultured macrophages with SDC must be considered inconclusive at best. The more pertinent issue is that these data from cell culture experiments show very little or no net increase in the intracellular bacteria over the initially phagocytized population during a period of up to 28 h. In the meantime, there will no doubt be a rapid, logarithmic increase in the extracellular bacterial population in vivo. In its proper perspective, the current emphasis on interactions between salmonellae and macrophages appears to be of theoretical interest rather than a priority in the understanding of the pathogenesis of the disease. On the other hand, in light of the innate bactericidal capacity of host phagocytes, one can reasonably expect the virulence of the pathogen to be directly related to its resistance to phagocytosis (59, 109, 120, 130). This is, by nature, to accommodate the extracellular bacterial proliferation.

Our observation on the efficacy of nonviable vaccines in protecting the highly susceptible mice challenged by the i.p. route is also contrary to the hypothesis that only genetically resistant mice can be protected by nonviable vaccines (26). This may be related to the regimen of vaccination in our experiments, in which the susceptible mice were injected i.p. with two doses of nonviable vaccines 3 weeks apart and challenged 7 to 10 days thereafter. Alternatively, we found that mice vaccinated twice in a 6-day interval and challenged 3 weeks later gain a relatively poor protection. In the former case, the anamnestic response to the booster immunization at the end of 3 weeks after the primary immunization appears to have raised the antibody level significantly to augment the host resistance (Xu et al., manuscript submitted). Some investigators question the relevance of i.p. infection in our experimental protocol, since peritonitis is not a feature in the early stage of murine salmonellosis (8, 99). With the highly susceptible mice, bacteremia occurs within 2 h after an i.p. inoculation of 10^3 virulent *S. typhimurium*, indicating a prompt hematogenous dissemination of the organism. One must also concede that the peritoneal cavity does provide certain advantages in analyzing the relative role of cellular and humoral factors in infection and in allowing the pathogen some degree of freedom to replicate locally without being rapidly removed by the RES as in the case of an i.v. inoculation. The latter contention could explain why the LD_{50} is generally lower with an i.p. than with an i.v. route of challenge.

DTH is customarily regarded as injurious to host tissues in the infectious process, but it can also have a beneficial manifestation, as we have demonstrated here in murine salmonellosis. The enhanced inflammation associated with DTH accelerates the influx of macrophages armed with cytophilic antibodies to the site of infection and facilitates the elimination of the pathogen. An analogous view has been advanced by Campbell, hypothesizing that antigen-specific T cells could enhance the recruitment of early inflammatory phagocytes and hence the resistance of the host against listeriosis (14), although she did not specifically relate these functions to DTH as we did (46).

In our present understanding, the host-parasite relationship in murine salmonellosis is uniquely different from the classical models of pneumococcal infection and tuberculosis at both extremes of the spectrum of bacterial infections (35). One is caused by an obligate extracellular pathogen and dependent on humoral immunity, while the other is caused by a facultative intracellular pathogen and associated primarily with cellular immunity and DTH. Murine salmonellosis is now placed in the middle of the two, as *S. typhimurium* behaves more like an obligate extracellular pathogen and acquired immunity to the disease is manifested by the synergistic actions of circulating antibodies, macrophages with cytophilic antibodies, and DTH. The ability of *Salmonella* to propagate within nonphagocytic parenchymal cells may qualify it as an intracellular parasite.

CONCLUDING REMARKS

In this review, I have attempted to (i) summarize the opposing views on pathogenesis and immunity in murine salmonellosis; (ii) raise the issue that the prevailing view is not necessarily based on sound experimental evidence and the discrepancies are largely the results of improper interpretations of data; (iii) provide the background information which could help the reader to identify the source of controversies; and (iv) describe an alternate understanding on the subject based on our systematic analysis of this bacterial disease.

The pressing question is whether the original classification of *Salmonella* as a facultative intracellular parasite (114) and the popular assertion of cellular immunity (17) should remain firmly grounded as the articles of faith in experimental salmonellosis. Alternatively, it appears that we have formulated a coherent explanation of the basic mechanisms of pathogenesis and immunity in murine salmonellosis. Its unique complexity offers a challenging experimental model for investigations of the genetic alterations in the virulence of the pathogen, the cellular and molecular mechanisms of acquired immunity, the preparation of vaccines, and the genetic basis for host resistance among inbred animals. May we begin to reassess the fundamental issues and move on to the applications of this experimental model?

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