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# Vaccines and Cell-Mediated Immunity

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**INTRODUCTION** ..... 371
**CELLULAR IMMUNITY TO INTRACELLULAR PARASITES** ..... 372
**ROLE OF "VIRULENCE" FACTORS IN THE DEVELOPMENT OF CELLULAR IMMUNITY** ..... 373
**TRANSFER OF ACQUIRED RESISTANCE FROM VACCINATED TO NORMAL RECIPIENTS** ..... 375
**MEDIATORS OF CELLULAR IMMUNITY** ..... 376
**Mononuclear Phagocytes** ..... 376
**"Activated" Macrophages** ..... 378
**T-Lymphocytes** ..... 381
**RELATIONSHIP OF DTH TO CELL-MEDIATED IMMUNITY** ..... 387
**INDUCTION OF CELL-MEDIATED IMMUNITY WITH VARIOUS TYPES OF VACCINE** ..... 388
**Living Vaccines** ..... 388
**Inactivated Whole Cells** ..... 389
**Cell Components** ..... 390
**ROLE OF ADJUVANTS IN THE DEVELOPMENT OF CELL-MEDIATED IMMUNITY** ..... 392
**LITERATURE CITED** ..... 394

## INTRODUCTION

The mechanism by which the normal host overcomes a microbial infection and the reasons for the accelerated secondary response following reinfection with the same microorganism has intrigued microbiologists, immunologists, and epidemiologists for almost a century. The two primary arms of the host defense system were first described about the turn of this century, but the manner of their interaction with one another and with the parasite during the development of acquired resistance by the host continues to be a matter of some controversy (125, 159, 164, 194, 247). The classical roles for specific antitoxic, opsonic, and bactericidal antibodies in preventing many microbial infections present an intellectually satisfying explanation for the humoral response almost invariably seen in the convalescing host (195), but attempts to explain antimicrobial resistance solely in terms of such immunoglobulin-mediated host responses (213) now seem too simplistic (53).

Circulating specific immunoglobulins undoubtedly do protect the host effectively against many infectious agents, especially those caused by the toxigenic microorganisms, some of the viruses, and the obligate extracellular parasites such as *Diplococcus pneumoniae* (75) and *Pasteurella multocida* (17, 58). However, there is no indication that humoral factors alone can prevent such diseases as listeriosis, tuberculosis, brucellosis, or salmonellosis, in either actively or passively protected animals (152, 154, 165, 264). The inability of passively introduced antibody to protect mice against salmonellosis has been ascribed to the use of insufficient amounts of hyperimmune serum (127). However, enumeration of the systemic bacterial population in such animals indicates that almost as much *growth* occurs within the tissues of the serum-protected animal as in the controls, despite the fact that the survival rates for the former are significantly increased (49). Such serum treatment increased the early inactivation of the challenge population 10- to 100-fold (50, 115) but then, despite the continuing presence of bactericidal antibodies in the circulation (135), the survivors multiplied extensively in vivo so that the systemic population eventually reached near lethal proportions (32, 50), with an ongoing bacteremia (135, 165) and an extensive degree of multiplication by the pathogenic organisms within the host's phagocytic cells (32, 67). The elimination of this systemic population requires the presence of activated or "angry" macrophages within the infected tissues, and in the passively immunized host these must be produced de novo, as a result of the challenge infection itself (161). The specific humoral factors in the circulation contribute very little to this later intracellular bactericidal process (102). Largely as a result of this type of study, few immunologists would now espouse a purely humoral mechanism to explain acquired antibacterial resistance, although it is interesting to

note that the immunological literature still contains numerous references to the induction of "immunity" and "immune responses" in an experimental context in which "humoral antibody production," "plaque-forming cell counts," or "blood clearance indices" might perhaps be more properly used.

The present review will be primarily concerned with those aspects of cellular resistance involved in the innate and acquired antimicrobial activity of the activated macrophage. The immunobiology of T- and B-lymphocytes and their interactions during the development of cellular hypersensitivity by the appropriately immunized host constitute one of the most exciting areas in contemporary immunology, but this aspect has been the subject of several recent reviews (30, 189, 194) and will not be dealt with in great detail in the present paper.

## CELLULAR IMMUNITY TO INTRACELLULAR PARASITES

Much of our present knowledge regarding cell-mediated immunity stems from the pioneering studies of Lurie (146) carried out in tuberculous rabbits. In a series of classic experiments, he demonstrated the need for activated or "immune" macrophages if the growth of virulent tubercle bacilli was to be inhibited in vivo (145). Such active cellular resistance was associated with the development of a tuberculin skin hypersensitivity by the host (153, 164); both of these responses seem to require the presence of specifically sensitized T-lymphocytes (158). At first, this close association of the two cellular responses to the mycobacterial antigens seemed to place the tubercle bacillus in a unique position as an infectious agent (154), but it is now realized that many other microorganisms can induce specific states of delayed skin hypersensitivity in the infected host (159). The term "facultative intracellular parasite" has been coined to describe such organisms, all of which are able to multiply actively within normal host phagocytes (239). Highly virulent strains of *Mycobacterium tuberculosis*, *Brucella abortus*, *Salmonella typhimurium*, and many other organisms are able to multiply freely within the phagocytic cells of the normal host and may eventually kill the involved phagocytes (167, 218). Inactivation of such organisms requires the entry of "activated" or "angry" macrophages into the lesion; the generation of this specialized population of mononuclear cells on the part of the infected host involves a process referred to as "cell-mediated immunity" (157).

The above discussion should not be taken to exclude an important role for specific humoral factors in the overall defense of the host. There has been a tendency in the past to allot a predominating role to either humoral or cellular factors, often to the virtual exclusion of the other arm of the defense system (53). As a result, a long-standing debate has developed in the literature regarding the *relative* importance of humoral versus cell-mediated defenses in protection against many of these diseases (125, 211, 247). Much of this controversy stems from the fact that the contribution made by the two defense systems can vary extensively depending upon the strain of organism, the host, and the experimental conditions selected by the individual investigator for a particular study (11, 32, 126). For instance, some investigators routinely use an intraperitoneal route of challenge (125) and may even add gastric mucin to increase the virulence of the challenge organism (234), whereas others recommend an intravenous (32), an intragastric (51, 62), or a subcutaneous (57) route of challenge. At the same time, "immunity" may be assessed in terms of an increased survival rate by the vaccinated group (73, 80, 132, 199), an increase in mean survival time (208, 212, 267), or a change in the size of the median lethal challenge dose (114, 169). Many of these experimental protocols indicate a degree of protection without giving a clue as to the precise nature of the defense mechanism(s) involved. This may be particularly important when nonspecific resistance factors are also involved (209). For instance, the increased rate of phagocytosis seen in the lipopolysaccharide-stimulated mouse peritoneal cavity may result in sufficient inactivation of a lethal challenge dose of unrelated microorganisms to permit the host to survive. However, the actual defense mechanism involved may not depend upon the specificity of the antigenic groups in the stimulating preparation (46, 212).

As a result of numerous in vivo studies, it seems that the best way to study the immune response in a vaccinated animal is to compare the rate of growth or inactivation of a sublethal challenge population of bacilli in test and control groups throughout the entire infectious period (67, 165, 248). Serial bacterial counts carried out on liver and spleen homogenates prepared from intravenously (165) or orally challenged mice (51) have demonstrated unequivocally that the effectively vaccinated host can express an accelerated antibacterial response to the challenge population from the time of infection (69). This will ideally be expressed as an immediate sharp decline in the

bacterial counts in the liver and spleen following challenge. Thus, to be fully effective, a vaccine must not only increase the early inactivation of the challenge population in the tissues but there must also be an enhanced active antibacterial response capable of eliminating the entire bacterial population from the tissues. Only in this way can a clinical attack of the disease be completely aborted. Thus, any rational study of the role of vaccination in the prevention of infections caused by intracellular microbial parasites must be primarily concerned with the development and maintenance of this state of cell-mediated immunity by the host (160).

## ROLE OF "VIRULENCE" FACTORS IN THE DEVELOPMENT OF CELLULAR IMMUNITY

Virulence seems to depend primarily upon the rate of growth by a given strain of organism within the tissues of the susceptible host (38, 49, 53). Despite considerable effort, no direct correlation has yet been shown to exist between the endotoxic content of the cell walls of various salmonellae and their virulence for experimental animals (69, 147, 168, 178, 210, 249). Although most virulent salmonellae are antigenically smooth and the transition from smooth to rough is associated with a sharp loss of virulence (206), the reasons for this effect are still not clear. Immunochemical studies of the lipopolysaccharides of smooth and rough salmonellae indicate structural changes (144), but there is no explanation as to how these result in a loss of virulence, since endotoxins isolated from rough organisms are still highly toxic for the host (38). Endotoxins have a variety of potent pharmacological effects in the intact animal (176), but it is not clear what role this toxicity plays in the pathogenesis of the resulting disease (206). In fact, the mechanism by which virulent Salmonellae actually kill the host is still not clear; death does not seem to be due to a simple endotoxemia since the total population of virulent *S. typhimurium* or *S. enteritidis* within the carcasses of moribund mice seldom exceeds $10^9$ viable bacilli, and perhaps 50 times this number of heat-killed bacilli are needed to kill the animal. Furthermore, BCG-infected mice are exquisitely sensitive to endotoxic challenge (240), but seem to be very little more susceptible to living *S. typhimurium* challenge than normal mice (122). There is considerable evidence that actively infected individuals become tolerant to the endotoxins of the causative organism (109, 184), but still the host succumbs to the infectious agent once the viable population reaches $10^8$ to $10^9$ bacilli in the liver and spleen (117).

Despite these largely incompatible data, much of the rationale underlying recent studies of the immunochemistry of the surface antigens of the *Enterobacteriaceae* and the role of the corresponding humoral antibodies in the expression of immunity to mouse typhoid fever is predicated upon the assumption that the smooth somatic and/or capsular antigens are primarily involved in the pathogenesis of enteric disease. The mechanism by which the smooth somatic antigen antagonizes the normal host defenses is still unclear (206). The long-chain smooth lipopolysaccharides may well limit the access of lysosomal enzymes to the underlying structural cell wall elements, so that bacteriolysis is slowed or prevented. Such a postulate is almost certainly naive, however, since quantitative differences are known to exist between the median lethal dose values for fully smooth salmonellae bearing different smooth somatic antigens (169). However, within a single group, the amount of protective surface antigen in strains of high and low virulence would have to play an important role in slowing or preventing intracellular digestion of the former. Such a thesis would be compatible with reports that dead *Salmonella* vaccines prepared from highly virulent strains (especially when grown in vivo; 192) may be more protective than those prepared from attenuated organisms grown in vitro (11, 126). However, such a direct relationship between the amount of smooth somatic antigen on the *Salmonella* cell wall and the immunogenicity of that organism cannot explain the ability of many antigenically rough salmonellae to protect mice and guinea pigs against challenge with the fully smooth parental strain (104, 229). In at least one case, this seems to be due to the ability of a gal-E deficient mutant to synthesize the smooth somatic antigen when growing in an intracellular environment (104). Other rough strains may be able somehow to persist in vivo long enough to induce sufficient cellular immunity to nonspecifically protect the host against the later challenge with the smooth parental strain (105, 131). Where growth data show that the rough strains cannot survive in vivo or when heat-killed suspensions of these organisms are used, all protective value disappears (24, 104, 229). On balance, the above findings seem to be consistent with the thesis that the lipopolysaccharide-containing cell wall antigens are *not* directly involved in the development of cell-mediated immunity (64).

Even closely related smooth strains of

*Salmonella* may differ enormously in their virulence for mice (67, 100) and for chickens (129, 228, 233), despite close similarities in the lipopolysaccharide content of the organisms (47). Growth rates determined in vivo for a number of group D salmonellae in mice and chickens differ enormously, and it is this parameter of the host-parasite interaction which seems to provide the most accurate index of the virulence of the organism for a given experimental host (38, 53). Blood clearance rates, sensitivity or resistance to the bactericidal action of specific antibodies in vitro, and percent inactivation by normal peritoneal macrophages (32) may bear little relationship to the virulence of the organism in the naturally infected host. If this overall growth rate by the organism within the reticuloendothelial organs of the host is the ultimate controlling factor for *Salmonella* virulence, then the nature of the limiting metabolic, nutritional, or transport steps responsible for these differences is still not at all clear (114, 138) and further work in this area is clearly needed.

Many of the "protection" studies carried out with killed *Salmonella* vaccines have been primarily concerned with the toxigenic responses by the challenged host, or with changes in the rate of blood clearance or intracellular inactivation of the challenge population by peritoneal cells taken from vaccinated mice (15, 179). Relatively few studies have concentrated on the level of antimicrobial resistance (53, 56), and many of these have used the intraperitoneal route of infection (32, 125, 208), despite problems associated with the interpretation of such data (32). This has resulted in some semantic problems regarding the use of the term "protection" (211). Obviously, the survival of 100% of a group of challenged, vaccinated mice in the face of 100% mortality in the normal controls represents a very real degree of protection (17). However, it is more difficult to assess the biological significance of a 50% increase in overall survival, regardless of the fact that statistical significance may have been demonstrated. This is especially the case when a unique inoculation method or some form of prior immunosuppression must be used to enhance the virulence of the challenge organism (126, 171, 181). In this context, increases in the mean time to death may have very little relevance if the majority of both vaccinated and control groups eventually succumb to the challenge infection (270). In an attempt to obviate this type of criticism, we have used serial bacterial enumeration of the in vivo bacterial populations to compare the fate of a sublethal challenge population in the vaccinated and control animals (165). Under such circumstances, death or survival of the host no longer constitutes a usable parameter of the host response; the development of a protective acquired resistance can only be inferred from the presence of a significant shift in the systemic bacterial growth rate at an earlier stage of the challenge infection. Such an accelerated antibacterial response must be sufficient to prevent completely the development of a clinical attack of the disease (32, 50), a criterion widely used in field trials of human typhoid vaccines (77). The effective vaccine will so sensitize the host that an accelerated recall of the earlier induced cell-mediated immunity will occur almost immediately following re-exposure to the parasite. The time required to regenerate this type of progressive antibacterial response is perhaps one of the most important parameters of the immune reaction (48). The above discussion should not be taken to mean that mortality data are of no further use; death or survival should, however, be combined with an assessment of the bacterial growth rate in vivo and the demonstration of a cell-mediated type of immune response. In this way, the most comprehensive assessment of the immunogenicity of different vaccine strains may be made. This situation can perhaps best be illustrated in the following experiments.

Groups of specific pathogen-free CD-1 mice were infected with sublethal doses of the highly virulent *S. enteritidis* 5694 (intravenous $LD_{50} = 5 \times 10^3$ organisms), the mouse attenuated *S. gallinarum* 9240 (intravenous $LD_{50} = 8 \times 10^6$), and the avirulent strain *S. pullorum* 223 (intravenous $LD_{50} > 10^8$). The *S. enteritidis* population increased almost 1,000-fold over the first 4-day period of the infection, whereas the *S. gallinarum* population remained relatively constant and the *S. pullorum* population declined sharply from the time of inoculation (Fig. 1). Challenge of these three groups of "vaccinated" mice with a lethal dose of a drug-resistant strain of *S. enteritidis* $SM^R$ (intravenous $LD_{50} = 10^4$ organisms) clearly indicated that both the *S. enteritidis*- and *S. gallinarum*-vaccinated mice were fully protected against the challenge infection. There was, however, no progressive inactivation of the *S. enteritidis* $SM^R$ population in the *S. pullorum*-vaccinated mice, at least over the first 7-day period (Fig. 1). However, from the number of deaths observed up until day 7 (Table 1), the *S. pullorum*-vaccinated mice seemed to be better protected from the lethal effects of the challenge than the normal controls. By day 14, this "protection" had van-

ished, constituting only a small increase in the mean time to death, with both groups of mice showing a mortality near 100%. Protection may be made to appear greater (at least so far as mortality is concerned) by using smaller challenge doses (1 to 2 $LD_{50}$) or a strain with reduced virulence (54), or by the use of alternative inoculation routes (57). However, the object of any vaccination regimen should be the complete protection of 100% of the vaccinees against a biologically realistic challenge infection, using the most virulent organism available, given by a natural route of inoculation (61). Under such conditions, the live *S. pullorum* vaccine clearly fails dramatically (67).

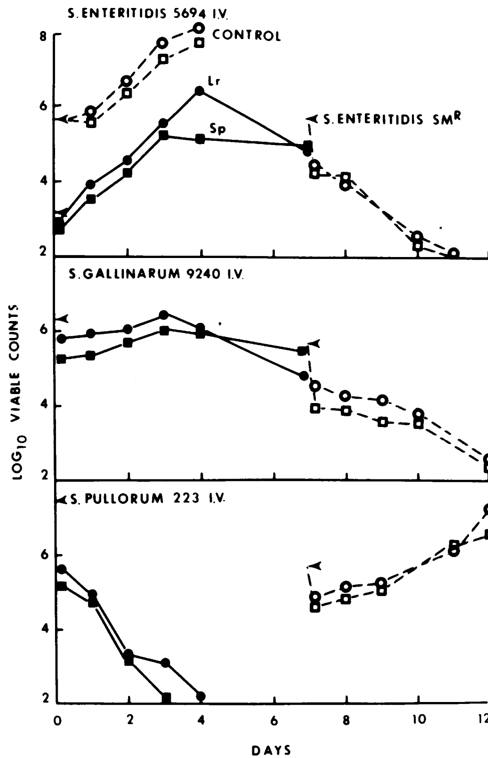


FIG. 1. *Growth of S. enteritidis 5694 (top), S. gallinarum 9240 (middle), and S. pullorum 223 (bottom) in specific pathogen-free CD-1 mice after intravenous inoculation. Lr, liver; Sp, spleen. The broken lines refer to the growth of a challenge inoculum of S. enteritidis SM$^R$ injected intravenously on day 7 of the primary infection. The arrows indicate the size of the inocula.*

## TRANSFER OF ACQUIRED RESISTANCE FROM VACCINATED TO NORMAL RECIPIENTS

It has been postulated that live salmonellae induce the production of cytophilic antibodies which then mediate the resulting immune response (125). If this were so, then the lack of immunogenicity shown by the live *S. pullorum* vaccine could be due to its inability to induce the production of such antibodies. The possibility that specific cytophilic antibodies provide the arming mechanism for the immune macrophage has been explored experimentally, with variable results (107, 213). Although there is no question that specific anti-*Salmonella* immunoglobulins can be recovered from the surface membranes of peritoneal macrophages harvested from immune mice (213), the critical involvement of cytophilic antibodies in the expression of immunity to the naturally acquired disease is still open to doubt (35, 247). If these specific antibodies are adsorbed onto the cell surface from the serum, then it should be possible to protect mice passively with large enough doses of hyperimmune mouse serum (210). Such transfer studies are important since they constitute the only definitive method for determining the real role of humoral versus cellular factors in the expression of anti-*Salmonella* immunity in a fashion analogous to that reported earlier for *Listeria monocytogenes* (155), *B. abortus* (118), and *M. tuberculosis*

TABLE 1. *Mortality data for CD-1 mice infected intravenously with S. enteritidis, S. gallinarum, or S. pullorum*[a]

| Vaccine | Challenge | Time in days | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 14 | 21 | 28 |
| *S. enteritidis* | — | | 1 | | | | 2 | | 2 | 2 | 2 | 2/10[b] |
| *S. gallinarum* | — | 1 | | | | | 1 | | | | | 1/10 |
| *S. pullorum* | — | | | | | | | | | | | 0/10 |
| *S. enteritidis* | *S. enteritidis* SM$^R$ | | | | | | | | | | | 0/10 |
| *S. gallinarum* | *S. enteritidis* SM$^R$ | | | | | | | | | 1 | 1 | 1/10 |
| *S. pullorum* | *S. enteritidis* SM$^R$ | | 1 | 1 | 3 | 3 | 5 | 6 | 8 | 9 | 9 | 10/10 |
| — | *S. enteritidis* SM$^R$ | 1 | 2 | 4 | 4 | 4 | 6 | 9 | 9 | 10 | 10 | 10/10 |

[a] Animals from each group were superinfected with *S. enteritidis* SM$^R$ on day 7 of the primary infection.
[b] Dead/total.

infections (141). Normal CD-1 mice were injected with 0.25 ml of hyperimmune mouse serum (50) at the time of challenge with $8 \times 10^3$ *S. enteritidis* cells (2 $LD_{50}$). Two more injections of 0.25 ml of serum were given intravenously on days 1 and 2 of the infection. The resultant growth curves are shown in Fig. 2. There was an increase in blood clearance and an enhanced early inactivation of the challenge population in the serum-treated mice, but by 24 h growth of the surviving bacteria occurred in vivo at the same rate as in the controls, despite the further serum injections (49). The passive serum treatment had a definite protective value in terms of overall survival (Fig. 2), but was not able to prevent the extensive growth of the challenge organism in vivo, despite the fact that the original serum donors were highly resistant to such a challenge (49). The inadequacy of the response to the serum treatment is further highlighted by the behavior of the superinfecting *S. enteritidis* $SM^R$ population injected 7 days after the primary challenge (Fig. 2). The immediate and progressive decline in viability by both the liver and spleen drug-resistant populations, in the absence of any initial growth period, is one of the main characteristics of the cell-mediated immune response (64).

Normal mice were adoptively immunized with $2 \times 10^8$ filtered spleen cells (155) prepared from actively immune donors used as a source of the hyperimmune serum. The mice showed an accelerated immune response 24 h after challenge with the live *S. enteritidis* $SM^R$ (Fig. 3). Although the level of immunity was inferior to that observed in the original spleen cell donors, it was clearly superior to that seen in the serum-protected animals (Fig. 2). Small numbers ($10^3$ to $5 \times 10^3$) of viable salmonellae were also transferred to the recipients with the filtered spleen cell suspension, and these host-adapted bacteria were paradoxically able to multiply in vivo, even in the face of an immune response against the drug-resistant challenge population. However, the transferred bacterial population was too small to induce significant levels of immunity de novo during the short period of the transfer experiment. The general conclusion to be drawn from both in vivo and in vitro experiments of this type (99, 215–217) must be that specific humoral factors make an important contribution during the early stages of the immune response, but that virulent strains of *Salmonella* then become established in the lymphoreticular organs of the host and that the rapidly dividing bacteria seem to be protected from the further bactericidal action of circulating serum factors (102, 222, 227). It is only the emerging cellular immune response developed against the infecting population itself which is finally able to eliminate the parasite from the tissues (160).

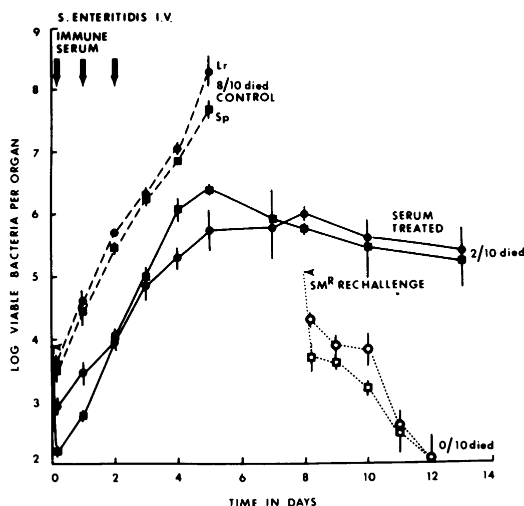


FIG. 2. *Growth of S. enteritidis 5694 in mice passively immunized with three doses of 0.25 ml of hyperimmune serum (solid lines) or normal mouse serum (broken lines) injected intravenously daily for 3 days. The dotted lines refer to the behavior of an intravenous inoculum of S. enteritidis $SM^R$ injected into the hyperimmune serum-treated mice on day 8 of the primary challenge infection. The figures on the graph refer to the number of deaths per 10 mice over the 14-day period of the study.*

## MEDIATORS OF CELLULAR IMMUNITY

### Mononuclear Phagocytes

Normal mouse peritoneal macrophages are known to inactivate a considerable portion of an infecting *Salmonella* population, often within minutes (32), but, if the challenge organism is highly virulent, the survivors of this initial inactivation phase begin to multiply and may eventually overwhelm the normal host defenses. Presumably the same is true for organisms leaving the gut and passing through the draining lymph nodes to reach the fixed phagocytes of the liver and spleen. There the organisms multiply extensively and the systemic population eventually assumes lethal proportions. The more attenuated the challenge organism, the smaller this secondary systemic growth phase tends to be (55). Furthermore, many of the bacilli penetrating the gut mucosa will be inactivated by the normal host phagocytes within the lamina propria (241). As a result, the size of the systemic bacterial population will be greatly reduced and a

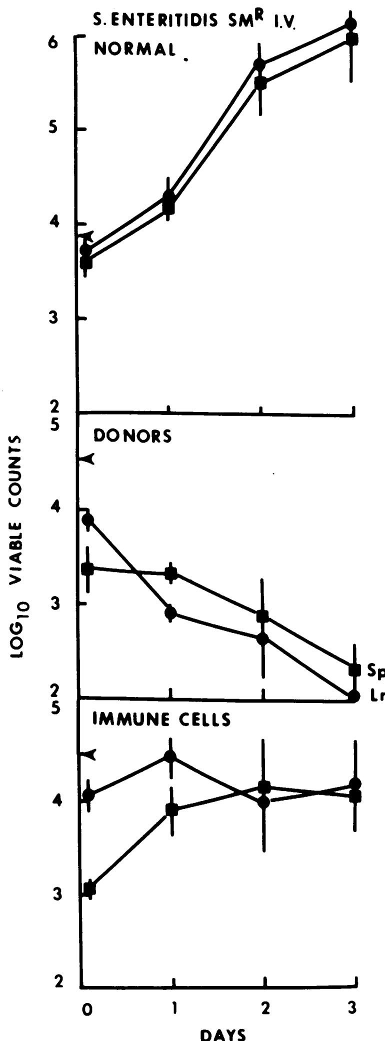


FIG. 3. *Adoptive transfer of anti-Salmonella immunity to normal CD-1 mice infused with $10^8$ filtered spleen cells from S. gallinarum-vaccinated donors. The growth behavior of the S. enteritidis SM$^R$ challenge injected 24 h after the immune spleen cells (bottom) or normal spleen cells (top) can be compared with the same challenge inoculum injected into a group of mice used as the source of immune spleen cells (middle).*

sublethal infection is more likely to result (54). On the other hand, a completely avirulent organism will be wholly inactivated within the lamina propria and should not gain entry to the more peripheral tissues at all (55). Thus, "protection" can vary considerably depending upon the dose and the virulence of the challenge organism. By such manipulations, a killed *Salmonella* vaccine can give rise to apparently widely differing levels of protection, depending upon the experimental conditions selected (54). The fact remains, however, that a challenge with a highly virulent *Salmonella* strain can only be controlled effectively by a cell-mediated type of immune response (56).

Parenteral infection of normal mice and guinea pigs with a variety of intracellular parasites has been criticized on the grounds that the route of inoculation is an artificial one and the protection data may bear little relevance to the naturally acquired disease (6, 133). Recent studies have been designed to mimic the naturally acquired infection as closely as possible (6, 260). However, oral inoculation of normal mice with enteric pathogens is associated with a number of technical difficulties (174), and starvation, bicarbonate treatment, massive oral pretreatment with antibiotics, or intraperitoneal opium injections (to inhibit normal gut mobility) have all been used to increase host susceptibility to the challenge infection. Presumably, such pretreatments permit a local concentration of viable salmonellae adjacent to the gut mucosa, thus resulting in focal infections within the lamina propria of the small intestine (241). Because of the nonbiological nature of such pretreatments, the resulting infection data still bear little more relevance to the naturally acquired disease than the earlier intraperitoneal challenges.

As a result of these technical difficulties, very few investigators have studied the progress of *Salmonella* infections within the normal undisturbed intestinal tract (61). Such studies are possible with B6D2 hybrid mice, which are 100 to 1,000 times more sensitive to salmonellosis than CD-1 mice (55). When animals were infected intragastrically with $10^6$ viable *S. enteritidis* 5694 cells (40), within hours small numbers of salmonellae appeared in the Peyer's patches associated with the distal ileum. Later, viable organisms were also isolated from the draining mesenteric lymph node. No other Peyer's patches seemed to be involved at this stage of the infection (62), but within 48 h an extensive systemic infection had become established (61). The subsequent amount of growth in the livers and spleens of these mice was little different from that reported earlier for sublethally infected mice which had received an intravenous challenge (49, 67). The immune mechanism involved in combating both types of experimental infection seemed to be very similar (50, 51), although the evolution of the immune response in the orally infected animals was noticeably slower than in the intravenous group, and this was related to

the slower rate of spread by the oral infection to the liver and spleen (41, 62).

Regardless of the route by which the virulent *Salmonella* challenge population enters the tissues, extensive growth will occur within many of the unstimulated macrophages, but once these cells become activated or "angry" they are able to inactivate even the most virulent bacilli (156). The nature of the changes in lysosomal activity associated with this ability are probably both quantitative and qualitative in nature, although we still know relatively little about the actual bactericidal mechanisms involved (29). The activated cells are certainly larger, with an increased number of cytoplasmic granules (156), are able to spread on glass surfaces faster (78, 185), and can engulf and inactivate foreign particles at an enhanced rate compared with normal cells (153). As a result of these changes, both specific and nonspecific antibacterial activity is markedly increased (72, 214). In the context of the present discussion, the specific aspects of these changes are of primary importance, but some workers have chosen to emphasize the nonspecific antibacterial activity of these cells (271). Although it is true that activated macrophages can kill unrelated microorganisms (33, 60) at a faster rate than normal (considerable experimental advantage has accrued from this property; 31), the fact remains that specific inactivation rates are nearly always quantitatively the greater. As an example, macrophages harvested from *L. monocytogenes*-infected mice take up and inactivate *Listeria* cells far more rapidly than is the case for BCG-stimulated cells (238). Furthermore, only the specific organism can induce an anamestic recall of the earlier cellular immunity (111, 152); heterologous organisms cannot achieve this effect (48). Thus, the widely believed misconception that cellular immunity is essentially nonspecific in nature (14) arose from an uncritical evaluation of the inactivation of adventitious organisms also present in the infected tissue. This latter effect must not be allowed to detract from the specific nature of the induction mechanism or the fact that only the original infecting strain can trigger the production of more activated cells. Once the primary infecting organism has been eliminated from the tissues, the number of specifically activated cells tends to decrease sharply (186). The antigens involved are highly specific and may not be present even in strains from the same *Salmonella* "O" group (48, 64). The sensitized host retains an immunological "memory" for these sensitizing antigens which will enable it to mount the enhanced immune response against the same organism months or even years later (154, 161). However, it is not clear whether this *Salmonella* "memory" is due to a persisting population of specifically sensitized small lymphocytes analogous to that described for humoral antibody production (124) or to the persistence of antigen following the establishment of a carrier state (117), or even to an unsuspected latent infection (149). The latter two possibilities cannot be discounted since detection of small numbers of bacilli in the tissues can be extremely difficult (148). Thus, the problems associated with the elucidation of immunological memory under the present circumstances are considerable, but the mechanism(s) involved is clearly fundamental to our understanding of this extremely important recall phenomenon and this work should be continued.

## "Activated" Macrophages

"Activated" macrophages are normally induced by the interaction of immunocompetent T-lymphocytes and blood-borne monocytes as a result of the release of specific sensitizing antigens by the actively multiplying bacterial cells within the tissues. This process has been the subject of several recent reviews (79, 158, 161, 189). However, there are other experimental means of achieving lymphoreticular stimulation. The majority of killed bacterial vaccines have little or no ability to induce cellular activation (31, 66), although endotoxins, polyinosinic-polycytidylic acid, corticosteroids, and *Bordetella pertussis* or *Corynebacterium parvum* are known to increase greatly the bactericidal activity of spleen and liver macrophages (91). *C. parvum* has been used successfully in the treatment of some tumors, in both humans and experimental animals (232), and increases host resistance to a number of microbial infections (2). This latter effect is somewhat unexpected since *C. parvum* is known to inhibit some T-cell functions (221) and anti-*Salmonella* immunity seems to be a T-cell-mediated host response. It was therefore of some interest to see whether *C. parvum*-treatment quantitatively affected the growth of *S. enteritidis* in vivo. Groups of normal CD-1 mice were injected intravenously with 700, 350, 175, or 88 μg of heat-killed *C. parvum*, and 10 days later they were challenged intravenously with $5 \times 10^3$ viable *S. enteritidis* cells (1 $LD_{50}$). The growth of the challenge organism in both the livers and spleens of the *C. parvum*-treated mice (Fig. 4) should be compared with that seen in untreated animals (Fig. 1). The cellular responses in the *C. parvum*-treated mice slowed the growth of *S.*

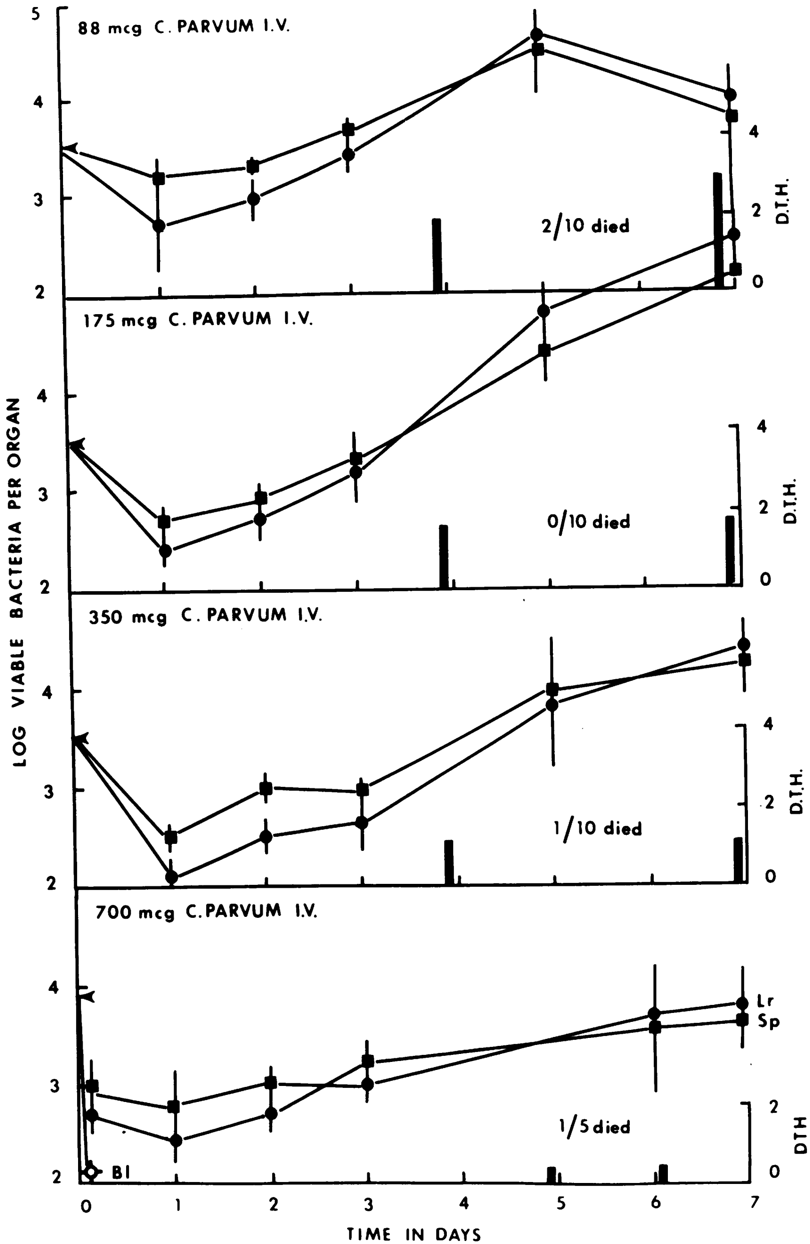


FIG. 4. *Effect of increasing amounts of heat-killed C. parvum vaccine injected intravenously into CD-1 mice 7 days prior to their intravenous challenge with 3 × 10³ to 5 × 10³ viable S. enteritidis 5694 cells. Mice receiving the 88-µg dose and the normal controls gave almost identical growth curves. The histograms refer to the increase in foot thickness 24 h after injection of 5 µg of Salmonella test antigen into one hind foot, compared with the contralateral foot which received only saline. An increase of 2 units (0.2 mm) or more was significant at the 1% level.*

*enteritidis* in both the liver and spleen, but there was no antibacterial immune response and there was an almost complete absence of DTH to the *Salmonella* test antigen (71). However, mice receiving 88 µg or less of *C. parvum* did develop significant levels of DTH, and there was a late cell-mediated immune response against the *S. enteritidis* infection (Fig. 4).

*C. parvum* mobilizes the production of large

numbers of monocytes within the spleens and livers of intravenously treated mice. Protection against the lethal effects of the *S. enteritidis* challenge could thus have been due to the increased early nonspecific inactivation of the challenge inoculum by the stimulated macrophages (91). There was a 10-fold increase in splenic weight in the *C. parvum*-treated mice but a 50% decrease in cellular proliferation (measured as tritiated thymidine [$^3$H-TdR] incorporation by the dividing spleen cells; 190) due to the *S. enteritidis* infection, compared with that seen in the control animals (Fig. 5). Thus, in terms of tritium uptake per unit weight of splenic tissue, the $^3$H-deoxyribonucleic acid (DNA) content for the *C. parvum*-treated *S. enteritidis*-infected spleens was less than 25% of the corresponding control values, and this difference was quite significant ($P < 0.02$). The peak $^3$H-DNA value in the spleens of the control mice (day 5) coincided with the peak DTH response, as well as with the appearance of the immune response. Although the *C. parvum*-stimulated spleen $^3$H-DNA content reached a lesser peak on day 5, this was not associated with either DTH or a cell-mediated type of immunity, but seemed to be caused by a rapidly expanding hematogenous population within the splenic tissue. Histologically, these *Salmonella*-infected, *C. parvum*-treated spleens contained very few lymphocyte-like cells, but the lesions consisted of enormous aggregations of mononuclear cells seen in large, poorly organized granulomata throughout the

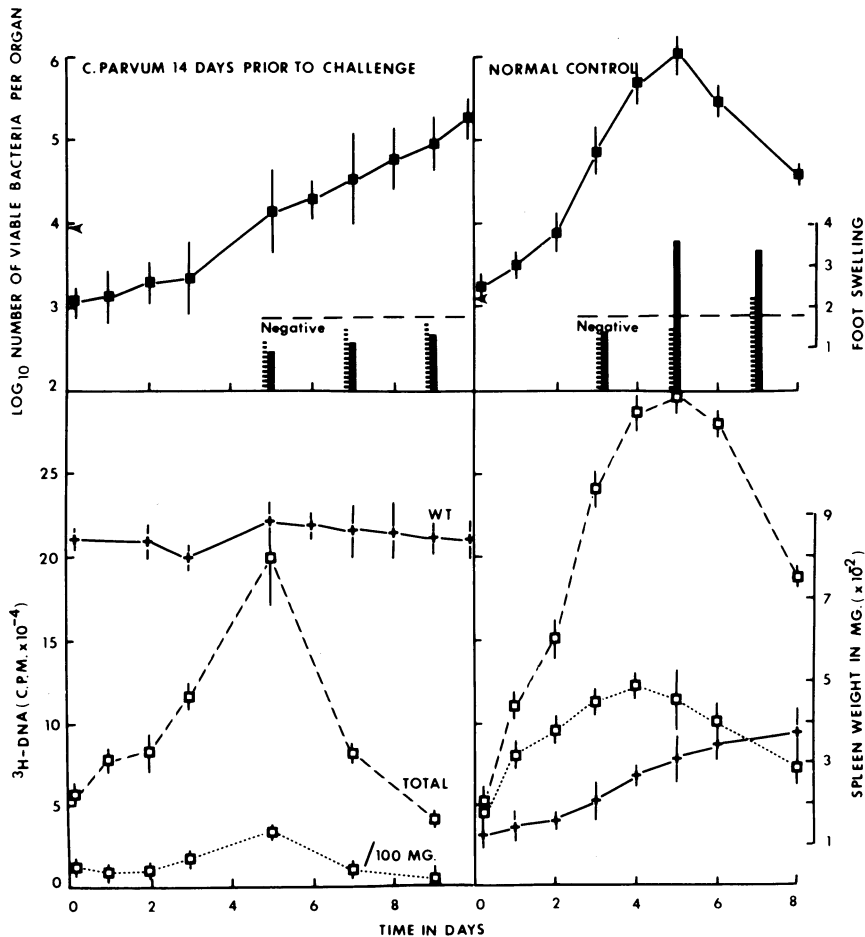


FIG. 5. *Effect of 700 µg of heat-killed C. parvum intravenously on growth of S. enteritidis 5694 in the spleen following intravenous inoculation 14 days later. The normal control curve is shown on the right. The broken histogram refers to the 3-h foot swelling, and the solid bar, the 24-h footpad swelling. The bottom curves refer to the uptake of $^3$H-TdR by the spleen cells, either per whole spleen (broken lines) or per 100 mg of splenic tissue (dotted lines). The weight of the spleen (mg) is represented by the solid lines.*

infected tissue. On the other hand, sections prepared from control spleens on day 5 contained large numbers of lymphoid cells, as well as many mature macrophages organized within well-defined lymphoid follicles. As the infection progressed further, these granulomas tended to resolve. Presumably, it was the massive mononuclear infiltrates seen in the *C. parvum*-stimulated spleens and livers which resulted in the depressed rate of growth shown by the infecting population (Fig. 4). The reduced T-cell reactivity seen in these mice (note the virtual absence of DTH in Fig. 5) was also associated with an inability on the part of the infected host to mount a cell-mediated immune response analogous to that seen in the controls about day 5.

The corresponding host responses to massive intravenous doses of heat-killed *M. tuberculosis* (a nonspecific stimulus), *S. enteritidis* (specific stimulus), or *Bordetella pertussis* (histamine-sensitizing stimulus) were quite different from that seen with *C. parvum*. The *Salmonella* growth curves shown in Fig. 6 indicate that the stimulated mice were still able to mount a normal immune response to the *Salmonella* challenge. Both the heat-killed $H_{37}R_v$ and *S. enteritidis* suspensions increased host resistance to the lethal effects of the virulent *Salmonella* challenge (Fig. 7), but much of this protection was due to the increased early inactivation of the challenge population seen in these mice. Near normal growth rates and a cell-mediated immune response developed in all of these mice by day 5 or 6 (Fig. 6). Only the *B. pertussis*-treated mice showed an enhanced susceptibility to the lethal effects of the *Salmonella* challenge (Fig. 7), and the mortality data correlate with an enhanced rate of growth seen in this group of mice. This effect was presumably due to the histamine-sensitizing action of the *Bordetella* suspension (177). Thus, the present data seem to be in complete agreement with earlier reports that the *C. parvum* effect is almost uniquely limited to this (and a few related anaerobic) species of *Corynebacterium* (3).

There was still the possibility that *C. parvum* was acting as an immunological adjuvant (123) which increased the humoral response to *Salmonella* antigens introduced with the challenge inoculum. Attempts to demonstrate specific *Salmonella* hemagglutinins in the sera of *C. parvum*-treated mice, both before and after *Salmonella* challenge, were largely unsuccessful (71). When mice were pretreated with a mixture of 350 μg of heat-killed *C. parvum* and 200 μg of dead *S. enteritidis*, the growth curves obtained after virulent challenge (Fig. 8) make it clear that the mixture was no more effective in inducing resistance to the *S. enteritidis* challenge than was *C. parvum* alone. Heat-killed *S. enteritidis* injected without the *C. parvum* was little better than the unvaccinated controls (Fig. 8). Thus, the characteristic slowed type of growth shown by the *S. enteritidis* challenge population in the livers and spleens of the *C. parvum*-treated mice was not due to any adjuvant action on the *Salmonella* antigens by the *C. parvum*, at least so far as conventional humoral responses were concerned.

### T-Lymphocytes

The striking advances in our recent understanding of the intereactions between immunocompetent T-lymphocytes and mononuclear phagocytes in the expression of cell-mediated immunity to intracellular parasites (162, 189) represent one of the most exciting areas in immunology today. The role of the thymus in the expression of immunological competence and cell-mediated immunity has been the subject of numerous recent reviews and needs little further discussion here (79, 175, 189). However, it is interesting to note that, although DTH was early established as a T-cell-dependent reaction (12, 266), it has only been in the last few years that this dependence has been extended to include cell-mediated immunity to intracellular parasites (150, 188, 242). The demonstration that adoptive transfer of immunity to *Listeria* challenge can be achieved with anti-theta sensitive lymphocytes (139) confirms the involvement of immunocompetent T-lymphocytes in the expression of antimicrobial immunity deduced from earlier studies using antilymphocyte serum (166) and the antimitotic drug vinblastine (244). Such findings have now been extended to include antituberculous immunity (188, 238). It is interesting in this regard that the depressed state of cellular resistance seen in the T-cell depleted host (THXB) was capitalized upon by Rees (200) for increasing yields of *M. leprae* in the mouse footpad (224) several years before the involvement of T-lymphocytes in leprosy immunity had been formally demonstrated (106).

Although T-lymphocytes are essential for the expression of immunocompetence, they are unable alone to express immunity when adoptively transferred to heavily irradiated recipients (244). Both cell-mediated immunity and DTH were observed only when nonsensitized macrophages or normal bone marrow cells were also introduced along with

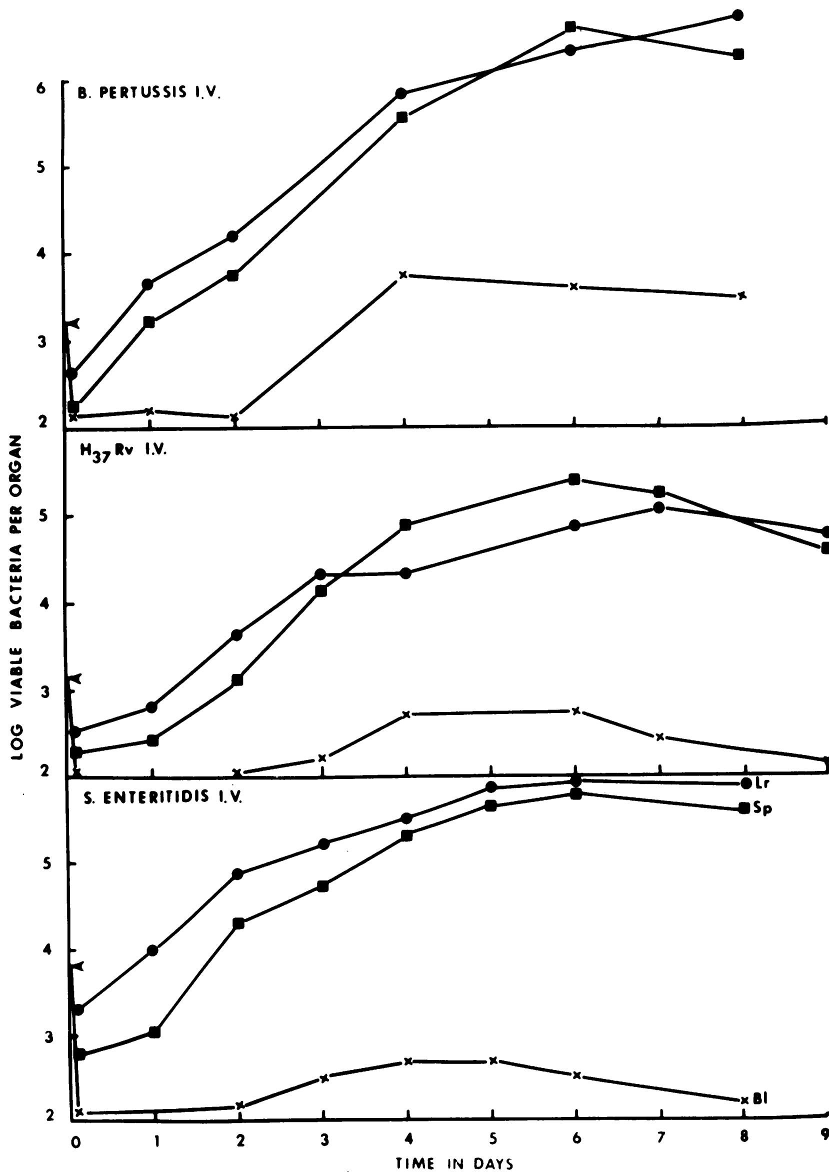


FIG. 6. *Effect of 700 μg of heat-killed S. enteritidis 5694 (bottom), M. tuberculosis H37Rv (middle), or B. pertussis (top) vaccine injected 7 days prior to intravenous challenge of the mice with 2 × 10³ to 6 × 10³ viable S. enteritidis 5694 cells. Lr, liver; Sp, spleen; Bl, blood.*

the T-lymphocytes into the irradiated recipient (143, 255). This double cellular requirement is consistent with the known radiosensitivity of macrophage precursors within normal bone marrow (254); normal macrophages are also essential for the expression of skin or footpad DTH in sublethally irradiated mice and guinea pigs (255).

Animals subject to thymectomy and irradiation during adult life may still retain some T-cell function, presumably due to small numbers of T-lymphocytes transferred to the THXB host with the normal bone marrow cells used during the reconstitution (81). In any T-cell depletion regimen, a percentage of the mice die during the first week or so after irradiation, despite the use of an antibiotic umbrella to reduce systemic infections. Presumably those animals which have been completely depleted of T-cells can no longer resist invasion by their own normal gut flora, a common source of potentially lethal infections

in individuals suffering from radiation sickness (26). If anti-*Salmonella* immunity is a predominantly cell-mediated response, then the T-cell-depleted mouse should be incapable of resisting even a modest challenge with an attenuated strain of *S. enteritidis* Se 795 (intravenous $LD_{50}$ in C57BL mice = $10^4$ viable organisms). However, after such a challenge, the systemic *Salmonella* population actually grew at a slower rate in the THXB mice than it did in the irradiated reconstituted controls (Fig. 9). There was, however, no sign of an immune response to the systemic infection and none of the mice developed any specific DTH, although there was some Arthus (3 h) reactivity. Some of the THXB mice eventually died as a result of this slowed continuing infection, but the survivors eventually exhibited low levels of delayed footpad sensitivity, suggesting that some cellular immunity had been developed against the parasite. The sham-thymectomized controls behaved essentially the same as the normal controls. There is a superficial similarity between the *Salmonella* growth curves seen in the THXB mice (Fig. 9) and those for the *C. parvum*-treated animals (Fig. 4). This may indicate a common cellular defect in both groups of animals, but the evidence is still only suggestive and further study on this point will clearly be necessary.

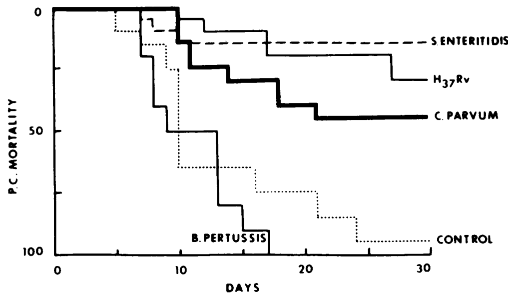


FIG. 7. *Mortality curves for mice pretreated with heat-killed S. enteritidis, M. tuberculosis H37Rv, B. pertussis, or C. parvum, or with saline, 7 days prior to intravenous challenge with 2 × $10^4$ viable S. enteritidis cells (2 to 5 $LD_{50}$).*

Infection of THXB mice with the attenuated strain of *Mycobacterium bovis* (BCG) also yielded interesting results. BCG normally gives rise to a self-limiting infection with the development of tuberculin hypersensitivity and an antimycobacterial resistance in 10 to 14 days (65). However, when $10^7$ viable BCG were inoculated into THXB mice, a severe mycobacteriosis developed with a 95% mortality over a 100-day period (Fig. 10). Death of uninoculated THXB mice was less than 10% over the same period, and none of the normal BCG-infected mice died as a result of the infection. Growth of

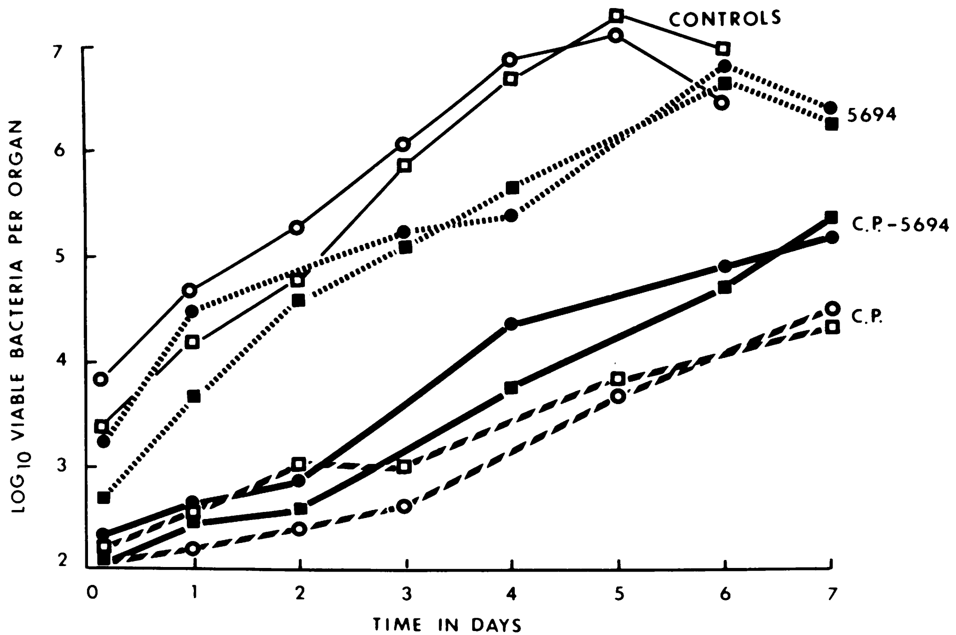


FIG. 8. *Growth of $10^4$ viable S. enteritidis 5694 cells injected intravenously into mice pretreated with 350 µg of C. parvum alone (C.P.), 350 µg of C. parvum + 200 µg of heat-killed S. enteritidis 5694 (C.P.-5694), heat-killed S. enteritidis 5694 alone (5694), or saline only (controls). Circles, liver; squares, spleen.*

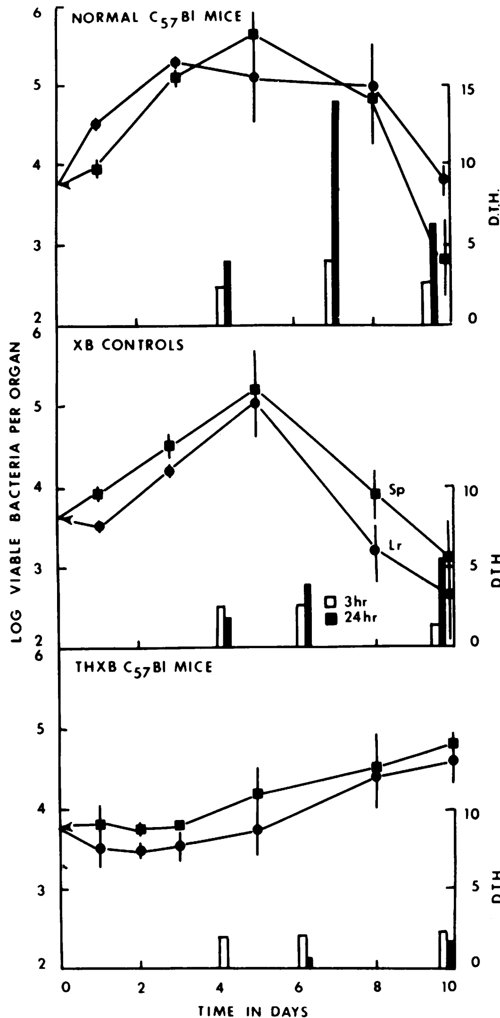


FIG. 9. *Growth of S. enteritidis Se 795 in normal C57Bl mice (top), sham-thymectomized lethally irradiated, bone marrow-reconstituted (XB) controls (center), or adult thymectomized, irradiated, bone marrow-reconstituted (THXB) mice (bottom) after intravenous challenge. The open histograms refer to the 3-h footpad swelling, and the solid bars, to the 24-h swelling after injection of 1 µg of Salmonella test antigen.*

the BCG population in the lung, liver, spleen, bone marrow, and a variety of lymph nodes continued unchecked for 30 days, but then a slow decline in viability occurred until day 90, which coincided with the mean survival time for these animals (Fig. 10). A lung bacterial population of 2 × $10^6$ viable bacilli (seen in the THXB animals at this time) did not cause any apparent distress in normal mice (65), and it was not clear how the continued presence of this number of bacteria in the lung could have been responsible for the deaths observed. If death of the THXB mice was not due to a runaway systemic infection analogous to that seen in miliary tuberculosis, then the most likely explanation would seem to be an aberration in the cellular response in the T-cell depleted host in the face of the ongoing infection. An attempt was therefore made to follow the cellular changes occurring within the lungs and spleens of the BCG-infected animals by means of pulse labeling with $^3$H-TdR (188). North (187) showed that the amount of splenic $^3$H-TdR incorporation by normal mice increased sharply following a tuberculous challenge, to a peak coinciding with the emergence of the T-cell-mediated immune response on the part of the host. This finding was confirmed in the present study, since by day 20 the BCG curves had turned down and the uptake of label by the spleen was four times the preinfection value (Fig. 11). This peak was partly due to the doubling in the spleen weight over this time, but even when the counts were corrected to a per unit weight basis the rate of incorporation of $^3$H-TdR was still double the normal values. This enhanced incorporation rate then fell towards preinfection levels over the next 40 to 50 days. The amount of incorporation by the uninfected lung cells was only 10% of that shown by the spleen (Fig.

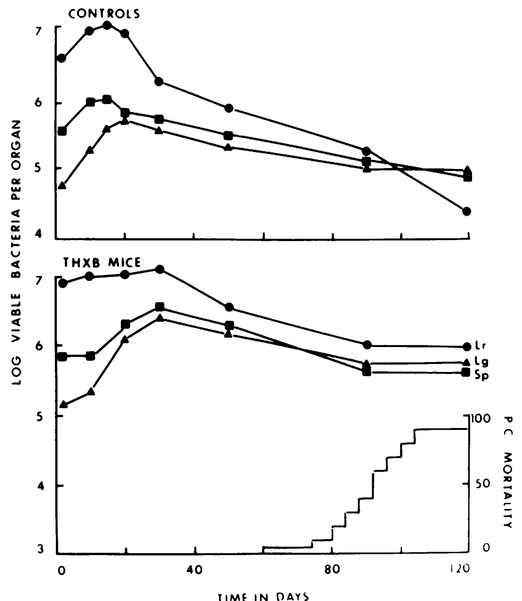


FIG. 10. *Growth of M. bovis (BCG) in normal control mice (top) or in THXB mice (bottom). Lr, liver; Sp, spleen; Lg, lung. The mortality curve refers to the number of deaths in the BCG-infected THXB mice compared with no deaths observed in the uninfected THXB animals.*

11), but this value also increased at least threefold, to a maximum about day 20, followed by a slow steady decline to preinfection values by day 65.

The DNA synthesis by spleen cells was somewhat more complex in the THXB mice. There was an initial sharp drop in $^3$H-TdR uptake by the spleen after infection (Fig. 12) followed by a return to about preinfection levels by day 20. The incorporation curve then remained at about this level until day 50 when a later minor peak was observed (Fig. 12). This peak seemed to correspond to one seen about this time also in the controls (Fig. 11), but its significance is still

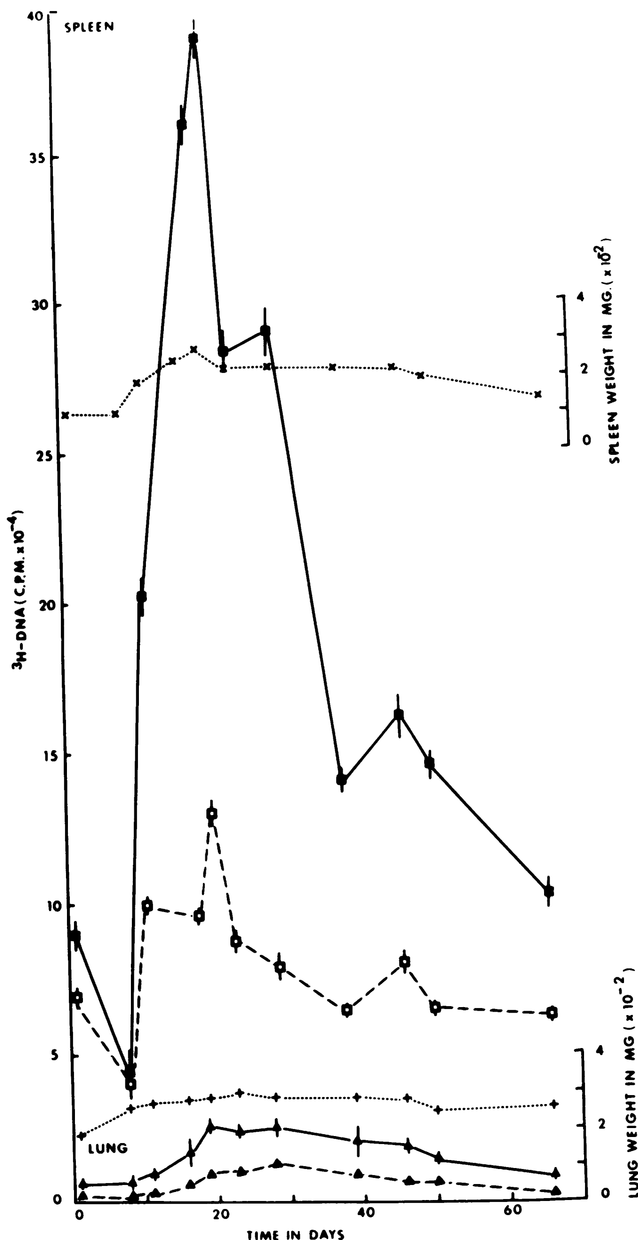


FIG. 11. *Incorporation of $^3$H-TdR by spleen cells (squares) and lung cells (triangles) after intravenous inoculation of normal mice with 2 × 10$^6$ BCG Montreal cells. The solid lines represent total incorporation per organ, and the broken lines, the rate per 100 mg (wet weight) of tissue. The dotted lines refer to the respective organ weights.*

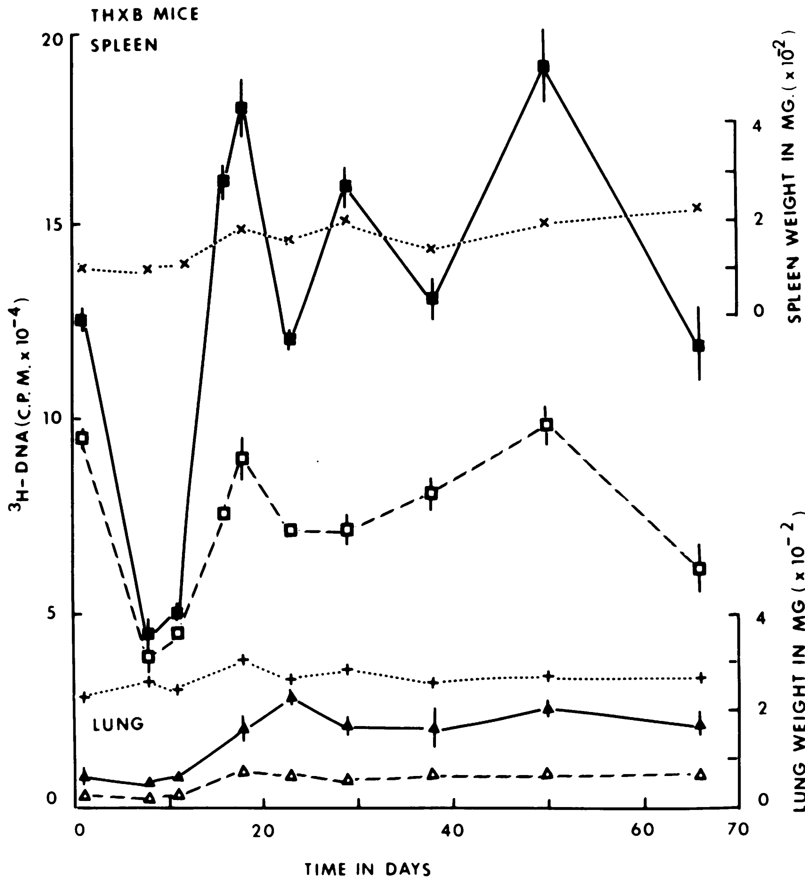


FIG. 12. *Incorporation of $^3$H-TdR by spleen cells (squares) and lung cells (triangles) in BCG-infected THXB mice (see legend to Fig. 11 for further details).*

not clear. T-cell depletion eliminated the large splenic peak seen about day 20 in the controls, and this was presumably responsible for the almost complete absence of tuberculin hypersensitivity in these mice (63, 191). On the other hand, T-cell depletion had little effect on the early lung incorporation of $^3$H-TdR, and as the infection progressed so the rate of incorporation of label also increased slowly; by day 50 it was three times that seen in the controls. Significantly, this was the time when the infected THXB mice were beginning to die.

Histologically, the lungs of the THXB mice showed many nonresolving granulomas which contained very few lymphocyte-like cells. There were large numbers of macrophages present, many of which had incorporated label. The cellular density within the lung tissue slowly increased until the organ completely lost its buoyancy in water, suggesting that many of the animals were dying as a result of pulmonary insufficiency (63). A similar picture has been described in normal mice lethally infected with a highly virulent strain of tubercle bacillus (108). It is evident that the enormous influx of mononuclear cells into the lung was unable to eliminate the attenuated mycobacteria from the tissues, and the resulting, fruitless tissue response to the infection eventually killed the host. It was only when anti-theta-sensitive lymphocytes were present within the lesion that specifically activated macrophages could be produced in sufficient numbers to induce an antibacterial response which then resulted in a rapid resolution of the lesions within the lung and elsewhere (188).

The BCG-infected THXB mice demonstrated a high degree of tuberculin anergy (63) rather analogous to that shown by lepromatous leprosy patients to the lepromin skin test antigen (36, 106). This latter state could be overcome, at least in some cases, by the injection of specific transfer factor (37) or by multiple injections of normal human blood leukocytes (142). In both

cases, the return of hypersensitivity was associated with a dramatic regression of the clinical disease, and this study constitutes a very clear demonstration of the practical advantages which may follow the direct application of immunological theory to clinical practice.

## RELATIONSHIP OF DTH TO CELL-MEDIATED IMMUNITY

Most facultative intracellular parasites induce a state of delayed skin hypersensitivity to one or more microbial antigens in the infected host. Skin sensitivity usually appears about the time when cell-mediated immunity is also first observed (65, 164), although there may be some quantitative differences in the size of the response seen in mice of varying susceptibility to the infection (198). On the other hand, killed vaccines induce humoral rather than cellular responses (34, 50), and the level of acquired resistance to the naturally acquired disease is much lower (64, 264). There has been an ongoing debate for some years regarding the exact nature of the relationship between specific DTH and acquired antimicrobial resistance (153, 154, 181). At least some of this controversy stems from the fact that tuberculin hypersensitivity can be detected in the apparent absence of antituberculous resistance and vice versa (65, 164). This may occur partly because of difficulties in demonstrating antituberculous immunity in BCG-vaccinated animals (56) and partly because peripheral tuberculin sensitivity is not always a valid index of cellular hypersensitivity (65, 265). Large doses of viable tubercle bacilli may desensitize the host to tuberculin without affecting its ability to mount an effective antituberculous response (56, 159). Spleen cells taken from such an anergic host will still adoptively sensitize normal animals, indicating the presence of tuberculin-sensitive lymphocytes within the heavily infected spleen (65). Because of this fact, purified protein derivative unresponsiveness, especially in heavily vaccinated or infected mice, should not be taken as *prima facie* evidence for separate cellular mechanisms for DTH and antituberculous immunity (180).

Different strains of mycobacteria differ widely in the level of cross-resistance that they can induce against a subsequent challenge with a virulent strain of *M. tuberculosis* (52). The immunogenicity of the vaccinating organism seems to depend more upon its ability to persist within the normal host tissues than on the closeness of any phylogenetic relationship existing between the vaccine and challenge strains. The size of the immune response also varies depending upon the virulence of the vaccinating strain since this will determine the amount of growth which occurs in vivo after inoculation (59). This is even the case with different substrains of BCG which can vary extensively in their in vitro (83) and in vivo (68, 197) behavior. Commercially available preparations of BCG may vary extensively in their immunogenicity, both for humans and for experimental animals (28, 84). These vary from the maximal responses shown by BCG Pasteur (163, 225) to a virtually complete lack of immunogenicity shown by a streptomycin-resistant strain (68) of BCG Tice (BCG $SM^R$). If a large enough vaccinating dose of the latter is used, even this nonreplicating *Mycobacterium* may become immunogenic, provided that the viable population is large enough to persist in vivo for a period of several weeks (52, 56). The poor immunogenicity of BCG $SM^R$ has been ascribed to the rapid decline in viability seen when $10^6$ viable bacilli are introduced into normal mice (70). A second explanation for this phenomenon however, could be that one or more critical sensitizing antigens had been lost from the cell walls of the drug-resistant mutant. This possibility was rendered very unlikely by the finding that heat-killed BCG $SM^R$ induced an equally effective level of antituberculous immunity in mice as the parent BCG Tice, provided that two doses of the vaccine were used and the organism was suspended in Freund's adjuvant. There was a 100-fold difference in the lung and spleen bacterial counts 14 days after a virulent Erdman challenge in both groups of vaccinated mice compared with the controls (70), and this was highly significant ($P < 0.01$). Thus, the lack of immunogenicity shown by the living BCG $SM^R$ vaccine did not seem to be due to a loss of sensitizing antigens but was rather due to the rapid decline in viability of both liver and spleen populations below an arbitrarily designated sensitizing threshold value. Weekly injections of $10^6$ viable BCG $SM^R$ or a single intravenous dose of $10^8$ viable bacilli elevated the viable populations above this threshold, which seemed to be about $10^5$ viable organisms for the liver. This residual viable population was sufficient to trigger the necessary cell-mediated immune response by the host. Heat inactivation of the $10^8$ BCG $SM^R$ population destroyed this protective value unless the cells were first incorporated into Freund's adjuvant (66, 70).

Claims have been made that some inactivated antituberculous vaccines can induce immune responses equivalent to that observed with live BCG preparations (204, 226). How-

ever, quantitative comparisons of the cellular responses to the live and dead vaccines (using the highly sensitive indicator strain *L. monocytogenes;* 37) indicate that the response to the former is invariably superior (66). On the other hand, the host response to inactivated preparations presented in an appropriate oil-based adjuvant often outlasts that achieved with the living preparation (8,203), and this fact alone more than justifies the continued search for improved inactivated immunogens for use in the prevention of tuberculosis, enteric fever, gastroenteritis, brucellosis, and perhaps some urogenital infections.

## INDUCTION OF CELL-MEDIATED IMMUNITY WITH VARIOUS TYPES OF VACCINE

### Living Vaccines

Although it is generally agreed that live attenuated vaccines induce a highly effective type of immune response (53, 116, 248, 264), this type of preparation is not yet commercially available for the prevention of enteric disease in humans. Several experimental vaccines have been developed recently (4, 76, 85) but are still in the initial stages of assessment in human populations (86). Inactivated vaccines against enteric fever and cholera (16, 77, 98) may increase resistance to the naturally acquired disease, but enthusiastic claims for their efficacy must be evaluated in the light of the predominantly IgG and IgM antibody responses which they induce (27, 43). In the experimental system, neither live nor killed *S. enteritidis* vaccines can completely *prevent* the passage of challenge organisms across the intact intestinal mucosa (57, 61), although they may delay the appearance of systemic disease for as much as 72 h (62). The live vaccine has the great advantage that entry of the challenge inoculum into the tissues induces an accelerated recall of the earlier cell-mediated immunity which is able to control the further growth of the organism before the infection can assume clinically significant proportions (61). Killed vaccines are quite unable to achieve this type of response, and although all of the vaccinated mice may survive the challenge, viable counts carried out on the liver and spleen make it quite clear that they have all suffered a severe clinical attack of mouse typhoid fever. This apparently poor level of experimental protection seems to be at variance with the large body of typhoid fever field trial data (13, 112, 113, 119) showing significant protection in appropriately vaccinated human populations. However, it should be remembered that such trials merely indicate a significant *decrease in the number of cases of clinical typhoid fever* per 100,000 vaccinated individuals compared with that observed for a similar group given a suitable placebo (119, 136). Both groups of individuals will usually be exposed to a relatively low level of endemic infection which will result in a number of clinical cases of typhoid fever in both, but with a *reduced* number of patients requiring hospitalization in the vaccinated group. Unless the overall incidence of endemic disease is very low (136), complete protection will not be achieved with a killed vaccine. Controlled experimental studies using human volunteers indicate that the incidence of disease and the apparent level of protection against a standardized oral dose of virulent *S. typhi* Ty2 varies with the size of the challenge inoculum (120). An inoculum of more than $10^7$ viable bacilli will overwhelm any immunity induced by previous exposure to killed typhoid vaccine. This is also consistent with the clinical finding that typhoid fever may occur within weeks of administration of a booster dose of typhoid vaccine (9) and even in recently convalescent individuals (172). As it stands at present, the statistics obtained from these human trials make it clear that the killed typhoid vaccine is better than nothing in an endemic situation (234), but that the development of more effective attenuated living vaccines for human use is a matter of some urgency, especially in light of the recent severe outbreaks of typhoid fever due to drug-resistant strains of *S. typhi* of unusual virulence for humans (258).

*Salmonella* "carriers" have a high degree of resistance to re-infection, and this state usually lasts as long as the primary infection persists in vivo (48, 116, 117). However, not all live salmonellae are able to induce solid resistance against reinfection. For instance, *S. pullorum* has no immunogenic value against a subsequent challenge with the antigenically related *S. enteritidis* $SM^R$ (Fig. 1). On the other hand, the antigenically related chicken pathogen (*S. gallinarum*) is highly protective under these circumstances (67). Although there are a number of minor biochemical, physiological, and antigenic differences between the two avian pathogens (47), they are not sufficient to explain the total lack of immunogenicity shown by the live *S. pullorum* vaccine in mice (57, 64). Recent studies from this laboratory indicate that the inability of *S. pullorum* to establish a persisting bacterial infection in the mouse is somehow associated with this phenomenon (Fig. 1). In the day-old chick, on the other hand,

both strains of *S. gallinarum-pullorum* multiply extensively in the liver and spleen (Collins, unpublished data), and at 5 weeks a high degree of resistance to reinfection can still be demonstrated in both groups of birds (233). This response appears to be cellular rather than humoral in nature (228), although there is little doubt that specific antibodies are also involved (129). Smith (228) reported good protection in chickens receiving a living but not a killed *S. gallinarum* vaccine prior to oral challenge. Similarly, in our own studies, live but not dead *S. gallinarum* vaccines increased resistance in challenged mice (64) and in chickens receiving an intravenous challenge with the virulent *S. gallinarum* 9240 (Collins, unpublished data). Anti-0-9 and 0-12 antibody titers were elevated in the sera of both groups of vaccinated animals, but no correlation could be shown between blood clearance values for *S. gallinarum-pullorum* in either mice or chickens and the level of virulence demonstrated by the infecting organism for the corresponding host (53). Although these data do not exclude the involvement of opsonic antibodies in host protection (especially with regard to the behavior of these fowl pathogens early in the infection; 130), there seems little doubt that the overall defense against systemic salmonellosis in both mice and chickens depends upon a cell-mediated immune mechanism (53, 162).

*S. pullorum* induces an excellent humoral response in vaccinated mice, and this is also seen from the immediate (3 h) type of hypersensitivity developed following the footpad injection of a test antigen isolated from this organism (64). There was little or no delayed hypersensitivity to the *Salmonella* sensitin (57) despite its known presence in *S. pullorum* culture filtrates. Apparently the critical antigen(s) in *S. pullorum* is inactivated very quickly in vivo so that the inability of the organism to multiply in the mouse tissues results in an inadequate antigenic stimulus to the host defenses. As a result, no DTH is developed even when repeated daily doses of viable *S. pullorum* are administered to artificially maintain a sensitizing population of organisms. Apparently, the sensitizing population within the liver and spleen must remain in a metabolically active state (although it may not be necessary for the organisms to divide during this time; 89, 90) in order to induce the necessary cellular response. The *S. pullorum* population does not do so (Fig. 1) and the vaccine is therefore nonimmunogenic for mice. The reason for this exquisite sensitivity to the bactericidal action of normal mouse phagocytes is still a mystery. Rough salmonellae are known to be inactivated very rapidly by normal phagocytes (210), but *S. pullorum* is antigenically smooth (47) and seems to resist blood clearance as effectively as the highly immunogenic *S. gallinarum* (53). Subtle differences obviously exist between the cell wall structure of *S. gallinarum* and that of *S. pullorum*, and these differences presumably render the latter more sensitive to the bactericidal action of the lysosomal enzymes of the normal mouse macrophage but not apparently to the corresponding chicken phagocyte. Clearly, we have a lot more to learn about this fascinating process.

### Inactivated Whole Cells

Assessment of the immunogenicity of different killed vaccines often depends upon the criteria used to measure the resulting "protection" expressed by the vaccinated host. The mere demonstration of survival may not give any clue as to the true nature of the immune mechanism involved, and extrapolation of protection data from one host species to another may be quite misleading (53). As an example of the latter, the widely accepted tenet that acetone-killed typhoid vaccines are superior in their protective value to heat-killed preparations (95) may be true for mice, but not for humans (136). The mild chemical inactivation of typhoid vaccines was initiated after the demonstration by Felix that a surface "Vi" antigen was present in most virulent strains of *S. typhi* (94) and that this antigen was "lost" after heating, together with most of the mouse protective value of the vaccine (93). Although the interpretation of some of his protection data was quickly disputed (193), the importance of the Vi antigen as an immunogen in human typhoid vaccines was widely accepted, and the chemical method of inactivation for typhoid and TAB vaccines has been retained ever since (10, 234, 236). This is somewhat ironic in view of the repeated demonstration that purified Vi antigen is quite heat stable (237), and can, in fact, withstand boiling water temperatures. Boiling merely releases the intact Vi antigen from the cell surface, a fact that has been used repeatedly in its in vitro assay (262). Heat inactivation of the typhoid vaccine releases the Vi antigen into the liquid phase from which it will, of course, be lost if the cell suspension is washed prior to use. There is in fact very little quantitative data in the literature to indicate that purified Vi antigen has any protective value whatsoever in humans (120, 173), although there is no question that it is important in the mouse protection test when the intraperi-

toneal route of challenge is used (19, 95, 101). The mouse is not naturally susceptible to *S. typhi* infection, and death will occur only when massive inocula are injected directly into the peritoneal cavity (62), frequently with the addition of hog gastric mucin to inhibit phagocytosis (234, 235). In the peritoneal cavity of the vaccinated mouse, anti-Vi antibodies do play an important protective role (128, 263). However, the validity of the intraperitoneally infected mouse as a model of human typhoid fever has been disputed by a number of workers (88, 193) and at present, evidence for a protective role for Vi antigen in human typhoid fever vaccines is not strong (43). However, despite this caveat, claims for increased protection following the use of acetone-killed vaccines continue to appear in the literature (15, 126), although a careful examination also reveals a number of reports that heated phenol-killed suspensions can be just as protective (61, 69, 82, 113).

Claims for increased immunogenicity by acetone-killed suspensions of *S. typhimurium* due to the preservation of a heat-labile "virulence" antigen 0-5 (126) should be interpreted in the light of later reports that this antigen is also quite heat stable (44, 134). In fact, the whole question of the role of somatic antigens in the development of host resistance is still not at all clear (111), especially with respect to orally infected individuals (51, 61). The opsonic and bactericidal antibody titers induced by parenteral *Salmonella* vaccines (mainly IgG and IgM immunoglobulins; 43, 207, 243) show little correlation with the level of acquired resistance to the naturally acquired disease (27, 62, 110). Even when the immunizing regimen is adapted so as to induce a maximal IgA response (87, 98, 243), the level of protection against an oral *S. enteritidis* challenge is still relatively low (61), and it is not presently clear just how these secretory IgA immunoglobulins protect the host against this pathogen at the mucosal level (62).

### Cell Components

*Salmonella* cell walls have not been tested extensively for their immunogenicity in mice (39), although excellent specific protection has been claimed with *Listeria* walls (137) and *Mycobacterium* cell walls (202). The latter induce both tuberculin hypersensitivity and a considerable level of antituberculous resistance, especially against an aerogenic challenge infection with virulent *M. tuberculosis* $H_{37}R_v$. To be protective, the purified walls must be mixed into a paste with a small amount of mineral oil and then injected into mice intravenously as an oil-in-water emulsion (8, 205). This type of vaccine has been used extensively in experimental studies of antituberculous immunity in mice (204) and monkeys (203), but so far it has not been used clinically in humans. Recent studies indicate that BCG cell walls also have significant antitumor activity (273), and it may well be that this antituberculous reagent will play an important role in the future, not against the specific infectious agent for which it was developed, but rather as part of the developing immunological armamentarium to be used against some human cancers.

Almost every mycobacterial product so far tested has some antituberculous activity (18, 217), especially when incorporated into a mineral oil carrier, although many of such products may then be too toxic for human use. One mycobacterial cell product which has received extensive study is the ribosomal antigen isolated from *M. tuberculosis* $H_{37}R_a$ by Youmans (267). Analogous preparations have also been made from *S. typhimurium* by Venneman and Bigley (253) and from *Staphylococcus aureus* by Winston and Berry (261). Vaccinated mice showed increased resistance to challenge following intraperitoneal or subcutaneous injections of a ribonucleic acid (RNA)-containing component of the cell contents (253). Some protection has also been achieved by use of a synthetic nucleotide preparation (269). Studies with these vaccines raise a number of important theoretical immunological considerations, since Youmans (272) has repeatedly reported excellent antituberculous resistance in the absence of detectable levels of tuberculin hypersensitivity. This last point has been disputed, however (18, 205). Some of the controversy regarding the immunogenicity and/or allergenicity of ribosomal and cell wall vaccines may well stem from technical considerations involved in the demonstration of peripheral hypersensitivity in heavily infected mice (63). Furthermore, there is some question as to whether the different experimental protocols used actually measure the same parameters of the host response. For instance, Youmans employs a large intravenous challenge inoculum which kills more than 90% of the controls within 30 days (270). Protection is then expressed as the percentage increase in survival at day 30 in a similarly challenged prevaccinated group. This protocol in effect demonstrates an increased mean survival by the vaccinated animals since most of the mice will die eventually as a result of the infection. Under these conditions, "protection" may be more antitoxic than antibacterial in nature, involving humoral and nonspecific factors (250, 271).

Other investigators use a small aerogenic challenge in the vaccinated host and compare the amount of growth and tubercle formation in the lungs of the sublethally infected test and control animals (23, 204). This method may also be recommended on the grounds that it bears a closer relationship to the natural human infection (260).

In the corresponding studies using ribosomal antigens isolated from *S. typhimurium*, host resistance to virulent challenge has also been reported to be significantly increased (251). Unfortunately, the most commonly used test protocols employ an intraperitoneal route of infection in which antibodies developed against contaminating cell wall antigens might be expected to exert a maximal protective effect (32). As a result, there has been considerable debate regarding the nature of the protective moiety in these ribosomal vaccines. Smith and Bigley (230) suggested that the active principle may be a cellular protein, or perhaps contaminating somatic antigens carried mechanically on the RNA moiety. The latter has no antigenic properties per se but merely acts as an immunological adjuvant. This last possibility gains further credence from the report by Houchins and Wright (121) that a specific immunogen can be isolated from their ribosomal antigen prepared from *S. typhimurium* and that this material seems to be a high-molecular-weight glycoprotein or mucopolysaccharide. The RNA portion in their preparation did not seem to be directly involved in the host response. This would also be consistent with the findings of Venneman and Bigley (253) that the immunogenicity of their ribosomal antigen was not destroyed by ribonuclease treatment. This seems to be in direct contrast to the sensitivity shown by the mycobacterial ribosomal antigens to this enzyme (268). On balance, it seems possible that the RNA present in this type of vaccine may function mainly as a carrier which perhaps amplifies the humoral response to the traces of immunogen carried on it. This conclusion would be compatible with the fact that ribosomal vaccines without an adjuvant can also protect mice (250). A second possibility would be that the RNA nonspecifically increases host resistance to the challenge organism, since synthetic polyinosinic-polycytidylic acid has recently been shown to increase resistance to listeriosis (191) and to staphylococcal infections (257). The ribosomal particles may also increase macrophage activation since carbon clearance rates are increased and both *Listeria* and *Staphylococcus* are inactivated more readily by ribosome-stimulated mouse macrophages (201).

Claims have been made that resistance induced with adjuvant-containing ribosomal vaccines can be adoptively transferred to normal recipients with peritoneal exudate cells but not with immune serum (252). Smith and Bigley (231) also reported that mice immunized with such a preparation developed a specific state of DTH to the ribosomal antigens. This last finding is particularly important if a close relationship exists between DTH and cell-mediated resistance to salmonellosis (53, 164, 183). However, the exact nature of the immunogen injected may be less important than its composition following processing by the phagocytic cells surrounding an infectious lesion (196). The only thing that is known about such immunogens is that they are not lipopolysaccharide in nature (64). With very few exceptions (74, 103), delayed hypersensitivity has been demonstrated only against protein antigens (246) and polypeptides (45, 219). It is perhaps unfortunate that the protein component of endotoxin, as well as other soluble protein components of the bacterial cell, have received such scant attention in the past as possible immunogens (20). It is known that soluble proteins are released into the culture medium from actively growing cells (245). Presumably many of these proteins are extracellular enzymes involved in the digestion of macromolecules within the culture medium. At present, we know very little about their antigenic properties or functions within an intracellular environment (21, 22). Enzymes such as hyaluronidase and collagenase have been studied extensively for their disease-enhancing properties, but there is no reason to suppose that some of these proteins may not also act as immunogens within the infected host (170).

In vitro cultures of *Salmonella* release a great deal of antigenic material into the medium as the culture passes into the stationary phase and an increasing number of cells begin to autolyze (64). However, the sensitins isolated from such culture filtrates are normally heavily contaminated with endotoxin and nucleic acid. In the actively infected host, the logarithmically multiplying organisms are all in a metabolically active state and would release any critical sensitins to the surrounding phagocytes as a slow, steady stimulus. It is known that living organisms in the tissues must be in direct contact with the host's inflammatory cells, since enclosure of the organisms in a peritoneal diffusion chamber induces a humoral rather than a cellular immune response (5). The sensitizing antigen(s) released from the actively growing organism into the surrounding tissues

somehow induces a predominantly cellular type of hypersensitivity (196). It is only with the evolution of the cellular immune response that large numbers of bacilli will be destroyed, presumably releasing large amounts of lipopolysaccharide and nucleoprotein into the tissues, and it is then that the characteristic antibody response begins to develop (64, 110, 133).

Cellular proliferation within the infectious lesion itself, and in the draining lymph nodes (62, 163) and the liver and spleen (187, 190), may peak at different times, depending upon the route of infection involved (41). As a rule, the maximal systemic immune response correlates best with the peak in tritiated thymidine incorporation by the spleen cells (186–188). This also coincides with the time when the DTH response reaches a maximum (158, 191). This peak cellular division usually declines again with time, and this is often associated with a slowed rate of inactivation by the remaining bacterial population in vivo (56). Eventually a stable balance will be struck between the inhibitory effect of the host defenses and the rate of bacterial multiplication in vivo, and a more or less permanent carrier state evolves (117). The sequence of cellular events during the infection and convalescence of the host has perhaps been most thoroughly investigated in mice suffering from listeriosis (151, 155). After a peak in bacterial counts on day 2 or 3, the level of cellular immunity increases to a maximum about day 6 and then declines again but remains above normal for as long as the stimulating infection persists in vivo. Incorporation rates for the liver and spleen also reach a peak about day 6 and then rapidly decline towards normal values again (186). Adoptive transfer studies indicate that cellular immunity is proportional to the number of actively dividing T-cells within the spleen (190). The systemic *Listeria* population will be eliminated completely within 10 to 12 days, and the number of specifically sensitized T-cells also falls precipitously. By 4 weeks, the host's cellular defenses will have essentially returned to normal, and by 4 months the host will again be fully susceptible to this infectious agent (155). Other bacteria tend to persist in vivo for longer periods, and, even after the organisms have apparently been completely eliminated from the liver and spleen, isolated abscesses may be detected in other organs, such as the lung or kidney. For instance, viable *S. enteritidis* can be recovered from the kidneys of intravenously infected rats up to 12 months after the infectious agent has been apparently eliminated from the liver and spleen (256). Such a residual (frequently latent) infection may have a considerable survival value for the host by ensuring that an accelerated immune response occurs each time the individual is reinfected. This may be particularly important for subjects living in an endemic disease area.

Latent carrier states seem to be particularly important in the case of *M. tuberculosis* (18) and *Corynebacterium kutcheri* (92) infections where the infectious agent may persist in vivo throughout the life of the individual (149). This probably explains the continuing tuberculin sensitivity seen in many patients who have made an apparently complete recovery from active tuberculosis (56). The bacilli within the latent focus of infection leak small amounts of sensitizing antigen(s) into the host tissues, resulting in the continuing presence of specifically sensitized T-lymphocytes within the draining lymph node. The presence of these cells within the tissues probably represents a sufficient defensive "edge" to ensure an accelerated immune response at any time when the individual is reinfected with the parasite. The resulting infection will then be limited to subclinical proportions. Such immunity will never be absolute, however, since a large enough dose of highly virulent organisms may induce a second attack of clinical disease before the host defenses can be mobilized sufficiently. Similarly, if the host defenses are temporarily reduced by dietary or environmental factors (140), or by prior immunosuppressive therapy (220), then a second attack of the disease will very likely occur. At present we know little about the interaction of many of these variables on the host-parasite relationships involved in the maintenance of acquired resistance, and further investigation of the relevant parameters is clearly warranted.

## ROLE OF ADJUVANTS IN THE DEVELOPMENT OF CELL-MEDIATED IMMUNITY

Freund first described the striking enhancement to the immunogenicity of antigens suspended in a water-in-oil emulsion containing whole mycobacterial cells (96). The reagent is referred to as Freund's complete adjuvant. The whole mycobacteria may be replaced by BCG cell walls, by the Wax D component of the mycobacterial lipid fraction and even, in some circumstances, by large quantities of gram-negative lipopolysaccharide (7, 223, 259). Multiple injections of Freund's complete adjuvant into normal mice induce considerable levels of tuberculin hypersensitivity and an enhanced antituberculous resistance (66, 70). Although the

reason is still not clear, the addition of extraneous protein antigens to the emulsion (97) results in the induction of a state of delayed hypersensitivity to an added antigen(s). Nelson and Mildenhall (183) reported observing some DTH in mice receiving a soluble protein incorporated into Freund's incomplete adjuvant. In the present study, no cellular hypersensitivity could be detected when killed whole *Salmonella* cells were presented to the host in this vehicle (57). This apparent conflict may be resolved in terms of the differing antigens used in the two studies. The mosaic of polysaccharide antigens present on the *Salmonella* cell wall surface will induce strong humoral responses resulting in Arthus- or immediate-type hypersensitivity which could easily mask or suppress any delayed component of the host response (62). In studies carried out in this laboratory, the addition of 200 μg of killed salmonellae to Freund's complete adjuvant increased the level of anti-*Salmonella* immunity against both intravenous (54) and oral challenges (57) with virulent *S. enteritidis*. At the highest level of killed *S. enteritidis* tested (500 μg of whole cells), the immune response to the adjuvant-carried vaccine was equivalent to that seen previously only in mice receiving a living attenuated vaccine (54). On the other hand, when the same amount of bacterial antigen was introduced into the tissues in Freund's incomplete adjuvant, only an increased humoral but no cellular response could be observed (62). The level of specific resistance to the virulent *S. enteritidis* challenge was then little better than that seen in controls receiving Freund's adjuvant alone.

The mode of action of the mycobacterial component of Freund's complete adjuvant is still unclear. The presence of the mycobacterial cells in the oil droplets (23) apparently increases the cellular response in the tissues surrounding the antigen depot (25). This somehow results in a delayed type of hypersensitivity, in addition to the enhanced antibody production normally seen when the water-in-oil emulsion is used (96). Such a response could not be observed when the whole mycobacteria (or purified cell walls) were suspended in the aqueous phase of the emulsion (204, 205). The tissue response to Freund's *complete* adjuvant is particularly severe, so that this type of preparation cannot presently be used in humans (16). In an attempt to overcome this toxicity, a water-soluble adjuvant has been isolated from mycobacterial cell walls which has been claimed to be able to induce high levels of DTH to added protein antigens without being highly irritant to the tissues (1, 42). Although such a reagent undoubtedly increases the humoral response to the added antigens, the data presented must be interpreted very carefully, in the light of possible Arthus or Jones-Mote type of reactions (182), and, in general, claims for marked adjuvant action in the absence of a severe granulomatous tissue response should be regarded with some reservation at present.

Mycobacterial enriched water-in-oil emulsions are the only practical alternative to living infections in the induction of a specific cellular response to the microbial antigens. Living BCG induces an almost pure T-cell response on the part of the infected host (159), but salmonellae tend to induce a mixed B- and T-cell reaction. At the time the immune response to live *S. enteritidis* first becomes apparent (day 4 to 5), this humoral (B-cell) response is still too low for ready detection (61, 64). On the other hand, dead *S. enteritidis* (or *S. pullorum*) induces a pure humoral response in the vaccinated mouse (67). As was shown in Fig. 1, even living *S. pullorum* cells were unable to induce a cell-mediated immune response, and this organism appeared to be completely nonimmunogenic for mice. However, when heat-killed suspensions of *S. pullorum* (200 μg) were suspended in Freund's complete adjuvant and two weekly injections were given subcutaneously, the cellular immune response to the *S. pullorum* antigens was almost as good as that achieved with the corresponding *S. enteritidis* preparations (57). Protection tests using decreasing amounts of the two bacterial suspensions indicated that some quantitative antigenic differences existed between them (61), but this did not seem to be sufficient per se to account for the total lack of immunogenicity shown by the live *S. pullorum* vaccine (Fig. 1). The nature of the qualitative and quantitative cellular changes which occur as the antigens are released by the live salmonellae in vivo are clearly very different depending upon the strain and the circumstances under which the antigen is introduced into the tissues.

Unravelling the cellular interactions involved in these two contrasting host responses constitutes one of the most provocative and intriguing problems in comtemporary immunology. Since the host-parasite relationships involved in the early phases of a systemic microbial infection probably play a crucial role in determining the ultimate fate of the host, a better understanding of the relevant cellular interactions occurring in vivo will not only influence the development of improved immunizing procedures, but may well have important implications in the control of several human infections for which there is

presently no effective prophylaxis, and even for the immunotherapy of some human cancers.

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