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Christopher R. Lissner

Christopher R. Lissner
Department of Microbiology
Uniformed Services University
of the Health Sciences

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HÉBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

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WALTER REED ARMY MEDICAL CENTER
NAVAL HOSPITAL, BETHESDA
MALCOLM GROW AIR FORCE MEDICAL CENTER
WILFORD HALL FORCE MEDICAL CENTER

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Name of Candidate: LCDR Christopher R. Lissner, MSC, USN
Doctor of Philosophy Degree
July 20, 1984

Thesis and Abstract Approved:

Randall K. Holmes Committee Chairperson	20 July 1984 Date
Ronald C. Peterson Committee Member	20 July 1984 Date
Stefanie N. Vogel Committee Member	20 July 84 Date
Susan D. Langreth Committee Member	20 July 1984 Date
Alison D O'Brien Committee Member	20 July, 1984 Date

ABSTRACT

Title of Dissertation: Genetic Control of the Innate Resistance of Mice to *Salmonella typhimurium*: Expression of the *Ity* Gene in Peritoneal Macrophages Isolated *In Vitro*

Christopher Roland Lissner, M.S.; Candidate, Doctor of Philosophy, 1984

Dissertation directed by: Alison D. O'Brien, Ph.D., Associate Professor, Department of Microbiology

The mouse Chromosome 1 locus *Ity* regulates the extent to which *Salmonella typhimurium* replicates within the reticuloendothelial cell system (RES) during the first days of infection. If mice are homozygous for the Ity^s susceptibility allele, the gram negative bacterium undergoes rapid net multiplication in the liver and spleen, a severe secondary bacteremia ensues, and mice die of a typhoid fever-like disease by day 10 of infection. Animals that are homozygous or heterozygous for the resistance allele, Ity^r, control net bacterial replication and survive the first phase of murine typhoid. Indirect studies have implicated the resident macrophage as the effector cell for regulation of early *in vivo* salmonellae growth. To verify this supposition and to evaluate the phenotypic expression of *Ity*, an *in vitro* assay was developed to compare the fate of S. typhimurium within Ity^r and Ity^s macrophages. Resident peritoneal macrophages were used from inbred Ity^r and Ity^s mice and from *Ity* congenic mice. With these mice and

through the use of radiolabeled S. typhimurium, indirect immunofluorescence antibody (IFA), light microscopy, transmission electron microscopy (T.E.M.), and avirulent non-replicating mutants of the bacterium the following conclusions were reached. The phagocytosis of S. typhimurium by Ity^r and Ity^s was the same. At the ultrastructural level both Ity^r and Ity^s macrophages phagocytized the bacterium into phagosomes, which subsequently fused with lysosomes to form phagolysosomes. After 24 hours of infection there were more cell-associated S. typhimurium in Ity^s peritoneal macrophages than in Ity^r cells. Ity^r macrophages killed intracellular salmonellae more efficiently than did Ity^s macrophages. These studies demonstrated directly that Ity is expressed by the macrophage and showed for the first time with Ity congenic mice that the basis for differential net growth of virulent S. typhimurium in Ity^r and Ity^s macrophages is a variation in the degree of bacterial kill.

The in vitro assay was used to infect macrophages from Ity congenic mice with mouse avirulent gram negative and gram positive bacteria. Expression of Ity phenotype was shown to be a non-specific differential trait of murine macrophages and was shown not to be restricted to infection with Salmonella species. The in vitro assay was used to determine the Ity phenotype of introgressively backcrossed mice which were to be utilized in the construction (breeding) of coisogenic Ity mouse strains. A discussion which addresses the possible mechanisms by which the Ity gene product or function may mediate microbicidal activity of murine resident macrophages and additional experimental approaches proposed to specifically identify this product or function is presented.

GENETIC CONTROL OF THE INNATE RESISTANCE OF MICE TO *SALMONELLA TYPHIMURIUM*: EXPRESSION OF THE *ITY* GENE IN PERITONEAL MACROPHAGES ISOLATED *IN VITRO*

by

Christopher Roland Lissner, M.S.

Dissertation submitted to
the Faculty of the Department of Microbiology
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1984

DEDICATION

One may set upon oneself a task of great sacrifice and commitment. To attain the goal is to succeed. To find oneself accompanied throughout the arduous course is one of those rare moments of understanding what it is to be human and to be loved without qualification or measure. I therefore dedicate this body of work to my devoted companion of fifteen years, my wife Patricia Weisbeck Lissner. Without this most special of nature's creatures, I surely would have stumbled and fallen from the course. Her love, her aid, her smile, and her gentle, unselfish heart have sustained me through many challenges, this work being but one among many. My words are inadequate to describe Patricia or to acknowledge her share of and contribution to my life. I will offer the most beautiful reflection of her love for me and mine for her, our daughter Margaret Joanne, as the testament of my devotion and gratitude to Patricia.

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INTRODUCTION

Significance

Natural or innate resistance to a microbe may be defined as the capacity of a host species either to prevent the disease normally caused by the microbe or to attentuate the pathological effects of the microbe in the absence of any previously acquired specific immunity (O'Brien, 1981). Investigation of the genetic control of innate resistance to a pathogenic microbe is important because results obtained from such research may eventually be applied to control of infectious diseases among human families and populations which display enhanced susceptibility to that agent. Furthermore, such investigation may help elucidate specific natural host defense mechanisms at the molecular level.

In human populations there are differences in susceptibility to infectious diseases. The genetic mechanisms for such differential susceptibilities are understood in only a very few diseases: one such disease is Plasmodium falciparium malaria (Bradley, 1980). For other infectious diseases, such as Yersinia arthritis and Salmonella arthritis, susceptibility and linkage to particular major histocompatibility (HLA) haplotypes have been shown (Benacerraf and Unanue, 1979). In contrast to these specific examples, evidence for genetic control of

resistance in humans to most other microbes is less well documented and is based primarily on observations of differential severity or incidence of disease between two population groups (Rosenstriech, 1980; Rosenstreich, et al., 1982a.).

Inbred Mice as Genetic Models

The restrictions inherent in the direct experimental investigation of innate and acquired resistance to infection in humans can be circumvented by the use of animal models. One such model is the laboratory mouse, Mus musculus. This species has been particularly valuable in the identification of specific genetic loci that influence the innate resistance of the host to infection by specific pathogens. The value of this species as a model of infectious disease extends to acquired (specific immunological) resistance as well. The basis for its success in the investigation of the relationship of host genetics and innate and acquired resistance is founded on the extensive data defining its genetic makeup (Mouse in Biomedical Research, The, 1981) and on its differential susceptibility to human pathogens, which include representatives of all major groups: protozoa, helminths, fungi, bacteria, and viruses (Rosenstreich et al., 1982b.; Skamene and Kongshavn, 1980).

The identification and mapping of resistance genes has been achieved through the creation of inbred

strains of mice. An inbred mouse strain is derived from 20 or more generations of brother/sister matings and is homozygous at all genetic loci of the nineteen chromosomal pairs of Mus musculus. With such inbred strains it is possible to determine the phenotype of each strain for resistance or susceptibility to a particular pathogen. The classification (scoring) of phenotype is based on the nature of the disease caused by the pathogen. For example, if the disease leads to death, one may use lethality (usually the 50 percent lethal dose [LD_{50}]) to define the phenotype. Alternatively, the phenotype may be defined by the extent of multiplication or persistance of the organism in the murine tissues or by the development or severity of a particular lesion. If prototype resistant and susceptible strains can be delineated they can be used to characterize the gene or genes which differentiate the strains (O'Brien, 1981). Interstrain matings and progeny crosses can be used to determine: whether genetic expression of resistance or susceptibility is dominant, recessive, intermediate, or sex-linked; the number of genes involved; the chromosomal location of the gene(s). Dominance or recessiveness is ascertained by the phenotype of F1 progeny (all heterozygous). Sex linkage (gene carried on the X chromosome) is deduced by the phenotype of F1 male and female progeny obtained from either resistant or susceptible female parents. Single gene expression is detected by the ratio of resistant to susceptible mice in backcross (F1 to parent) and F2 progeny. As an example, if

resistance is dominant one would expect a 1:1 ratio among the progeny of F1 mice backcrossed to a susceptible parent and a 3:1 ratio (resistant to susceptible) among F2 progeny.

Several approaches can be used to map a murine resistance gene to a particular chromosome. Linkage analysis may be employed in which the association of resistance with previously mapped genetic traits may be followed in various backcross progeny. An association of resistance to the major histocompatibility locus, H-2 on Chromosome 17, can be assessed by the use of H-2 congeneic resistant strains (a congeneic strain is syngeneic for all chromosomal pairs except for a defined segment of one chromosomal pair [Flaherty, 1981]). It is of interest to note that none of the murine genes known to control the early responses to a variety of bacteria and parasites have been shown to be H-2 linked (O'Brien, 1981). Gene mapping may also be approached by the use of recombinant inbred strains. These strains are produced by inbreeding F2 progeny derived from two inbred progenitor strains. Each of these strains contains a random assortment of permanently segregated genes from the original progenitor strains (Taylor, 1980). By making a set of recombinant inbred strains from resistant and susceptible progenitors, the chromosomal location of a resistance gene can be mapped by its linkage to a marker expressed by some of the recombinant inbred strains. The application of the methods described here has shown that the innate susceptibility of

mice to the causitive agent of murine typhoid fever, Salmonella typhimurium, is under genetic control. (Rosenstreich, 1982b.; O'Brien et al., 1983).

Human Typhoid Fever

Typhoid fever is a serious systemic disease of humans. Untreated cases of typhoid fever have a mortality of about 10 percent; thus, the disease remains an important public health concern. The causitive agent of human typhoid fever is Salmonella typhi. The disease is acquired by the ingestion of food or water contaminated with this bacterium (Warren and Hornick, 1979). The only natural host for S. typhi is man and, with the exception of the chimpanzee, no other animal species develops, after ingestion of S. typhi, a disease which simulates typhoid fever (Hornick et al. 1970). The clinical manifestations of typhoid fever are those of an acute febrile illness. After an incubation period of 7 to 14 days the infected individual may experience malaise, anorexia, and headache. These signs are usually followed by fever, which rises in a step-like fashion, to an average temperature of 104° F. (Morgan, 1965). During the first week of illness the individual is usually prostrate and may have diarrhea or constipation. As the second week of illness is entered abdominal tenderness, enlargement of the spleen, and continued fever are observed. Other manifestations may include the appearance of rose spots, cough with

bronchitis, and, in severe cases, delirium (Morgan, 1965). These symptoms may persist for 3 to 4 weeks following onset of clinical disease, after which, a gradual defervesence occurs and convalescence is entered. Relapses occur in about 10 percent of affected individuals (Morgan, 1965). Two to five percent of typhoid cases become fecal excretors or carriers (Mims, 1982). The bacteria colonize scarred, avascular areas of the gall bladder from which they are shed, via the bile, into the feces. Women are three times more likely than men to become carriers (Hornick et al., 1970); this may be due to the greater extent and prevelance of gall bladder damage in female typhoid cases (Mims, 1982).

During typhoid fever S. typhi can be routinely isolated from blood, feces, and urine. The organism may also be isolated from the bone marrow, spleen, gall bladder, and rose spots. In fatal cases postmortem findings include ulceration of the small intestines, especially in the area of Peyer's patches, hyperplasia of the intestinal lymphoid tissue, splenomagly, and focal necrosis of the liver (Morgan, 1965).

Murine Typhoid

The investigation of the pathogenesis of human typhoid has been hindered by the necessity of using volunteers or chimpanzees as models. In 1928 Orskov and Moltke (Orskov and Moltke, 1928) showed that mice orally infected

with S. typhimurium developed a systemic disease which closely paralled human typhoid fever. Murine typhoid has been used extensively as a model of S. typhi infection in humans. It is of interest to note that S. typhimurium may cause gastroenteritis and, rarely, septicemia in humans and that S. typhi is naturally nonpathogenic for mice (Carter and Collins, 1974a; Collins and Carter, 1978; O'Brien, 1982a). S. typhimurium and certain strains of Salmonella enteritidis are facultative intracellular pathogens of mice (Collins, 1974). These salmonellae produce in the murine host a systemic infection and septicemia. Regardless of the route of inoculation the bacteria take up residence in the reticuloendothelial system (RES), particularly in the spleen and in the liver where they multiply in macrophages. If this intracellular multiplication is not controlled tissue damage, secondary bacteremia with further dissemination of bacteria to other organs, and death occur (Hobson, 1957a.; Stuart, 1970).

The survival of the murine host is a function of several factors. These factors include the route of inoculation, the size of the inoculum, the virulence of the Salmonella strain, the immune status of the host, and the genetic constitution of the mouse strain. For the purpose of summarizing the course of murine typhoid as an analogue of human typhoid, examples have been chosen that represent the pathogenesis of virulent salmonellae administered at or above the inoculum size at which one half of infected mice are observed to survive and one half of infected mice are

observed to die, i.e. at the LD_{50}.

Hobson (Hobson, 1957b.) intravenously infected white mice with a mouse virulent S. typhimurium strain. The rate of bacterial clearance from the blood and the growth of bacteria in the spleen, liver, and kidney were followed. S. typhimurium was rapidly cleared over a 5 hour period from the blood. The number of blood-borne bacteria remained constant over the next 19 hours and then increased rapidly. The increase indicated a secondary bacteremia. Enumeration of viable S. typhimurium in various organs showed a rapid uptake of salmonellae in the liver and spleen, followed by a steady decline in the number of organ-associated bacteria. Thereafter, a logarithmic increase of bacterial numbers in the liver and spleen occurred. This increase chronologically paralleled the increase seen in the blood. In a more detailed study Collins (Collins, 1969a.) used three different parenteral routes, intravenous (i.v.), intraperitoneal (i.p.), and subcutaneous (s.c.), to challenge outbred mice. The results of this study showed that regardless of the type of parenteral inoculation used, virulent S. enteritidis strain 5694 left the site of inoculation via hematogenous and lymphatic spread and were taken up by the phagocytes of the liver and spleen. In these RES organs the bacteria underwent rapid net multiplication. Net multiplication, as used herein, is the increase in the number of viable bacteria. It reflects a positive balance between bacterial replication and bacterial death. Uptake of S. enteritidis

by phagocytes in the liver and spleen occurred within minutes of an i.v. or i.p. challenge. When the s.c. route was used a period of 24 to 36 hours passed before salmonellae were recoverable in splenic or hepatic tissues.

The natural portal of entry to establish a salmonellae infection in mice is by oral challenge. Collins (Collins, 1972) orally infected CD-1 and C57BL/6 mice with S. enteriditis strain 5694 and quantified the number of salmonellae in the walls of the stomach, small intestine, cecum, large intestine, and in the corresponding intestinal contents. Most of the bacteria in the oral inoculum were rapidly inactivated in the gut; after 24 hours less than 1 percent of the infecting dose was present in the intestine. Nevertheless, this small percentage of surviving salmonellae was sufficent to seed the liver and spleen; by 48 hours after oral challenge salmonellae were detected in these RES organs. The bacteria then underwent net multiplication and, subsequently, a systemic disease ensued. Carter and Collins (Carter and Collins, 1974b.) utilized a dye injection technique, in addition to enumeration of viable bacteria, to trace the course of S. enteritidis strain 5694 systemic invasion after oral challenge. The bacteria in the challenge inoculum were observed to move quickly (within 1 hour) through the gut and to infect the lower intestinal tract. By 6 hours less than 1 percent of the inoculum could be recovered from the entire gut. As in the

previous study, bacteria were not recoverable from the liver and spleen until 48 hours after challenge. The primary site of bacterial penetration was found to be the mucosa of the distal ileum. From this site the bacteria spread through the Peyer's patches of the ileum to the distal mesenteric lymph nodes. The salmonellae gained access to the liver and spleen from these lymph nodes.

Pathological findings in mice dying of murine typhoid resemble findings observed, post mortem, in fatal cases of human typhoid fever. The liver initially shows many granulomatous nodules which contain aggregates of macrophages, polymorphonuclear neutrophils, and some lymphocytes. The cellular composition of the nodules changes as the infection progresses and, eventually, consists primarily of macrophages and lymphocytes. These mononuclear cells actively push their way into hepatic cells (Stuart, 1970). The liver cells develop cytoplasmic inclusions which consist of swollen mitochondria and lipid droplets. The spleen is enlarged and shows hypercellularity and congestion which may lead to a severe depletion of lymphoid cells (Stuart, 1970). Necrosis of the liver and, to a lesser extent, of the spleen occurs (Stuart, 1970). The mesenteric lymph nodes show hyperplasia and an increase in the number of mononuclear phagocytes (Stuart, 1970).

As may be the case in human typhoid fever, a carrier state may occur in murine typhoid. The primary anatomical locations of persisting salmonellae are the

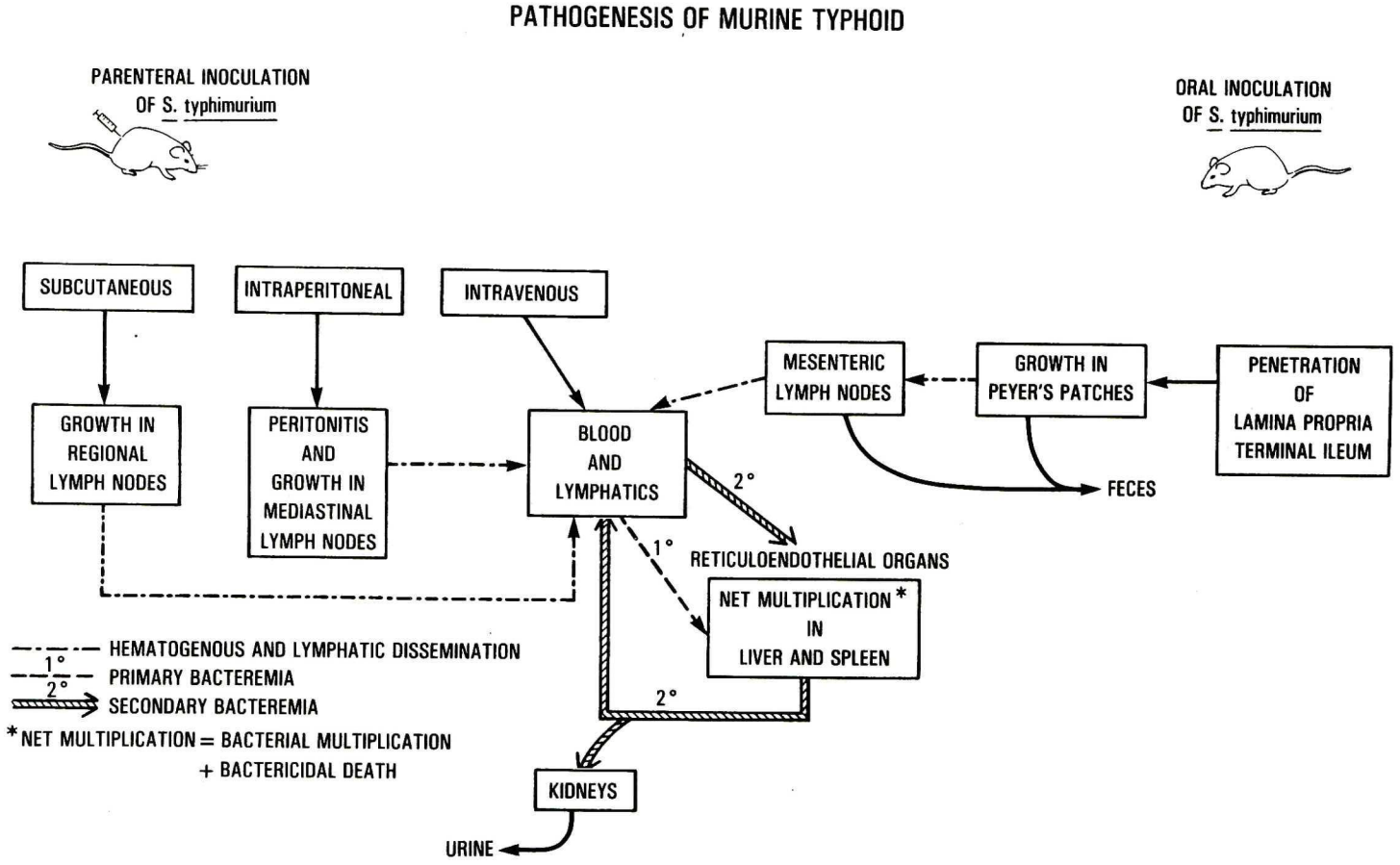
liver, spleen, and kidney. Hobson (Hobson, 1957a.) intraperitoneally infected white mice with a small number of virulent S. typhimurium. Two courses of disease were observed. Net multiplication of salmonellae occurred in the liver and spleen of all the mice. One half of the mice were unable to control this multiplication, which resulted in bacterial counts of 1×10^8 in the liver and spleen and in death of these animals within 14 days. Mice that survived controlled early bacterial multiplication and, after 14 days, progressively diminished the number of salmonellae in the liver and spleen. Fifty-six days after the infection S. typhimurium was recovered from the liver and spleen of 80 percent of the survivors. Hobson noted that these chronically infected mice appeared to reach an equilibrium with the parasite.

The murine typhoid model has furthered our insight into the host-facultative intracellular parasite relationship. This model, depicted in Figure 1, has provided a system for addressing questions concerning the acquired and innate resistance of animal hosts to salmonellae. In particular, the knowledge gained from this model has extended our understanding of human typhoid fever.

Acquired Immunity in Murine Typhoid

Host immune defense mechanisms in murine typhoid have been studied extensively: for reviews see Stuart (Stuart, 1970), Roantree (Roantree, 1967), and Eisenstein

Figure 1.


PATHOGENESIS OF MURINE TYPHOID
PARENTERAL INOCULATION OF S. typhimurium
ORAL INOCULATION OF S. typhimurium
SUBCUTANEOUS
INTRAPERITONEAL
INTRAVENOUS
GROWTH IN REGIONAL LYMPH NODES
PERITONITIS AND GROWTH IN MEDIASTINAL LYMPH NODES
BLOOD AND LYMPHATICS
MESENTERIC LYMPH NODES
GROWTH IN PEYER'S PATCHES
PENETRATION OF LAMINA PROPRIA TERMINAL ILEUM
FECES
2°
1°
RETICULOENDOTHELIAL ORGANS
NET MULTIPLICATION * IN LIVER AND SPLEEN
2°
KIDNEYS
URINE
HEMATOGENOUS AND LYMPHATIC DISSEMINATION
1° PRIMARY BACTEREMIA
2° SECONDARY BACTEREMIA
*NET MULTIPLICATION = BACTERIAL MULTIPLICATION + BACTERICIDAL DEATH

and Sultzer (Eisenstein and Sultzer, 1983). The investigation of acquired/immunological resistance to S. typhimurium infection has followed two paths: humoral immunity resulting from the ability of the host to produce pathogen-specific protective antibodies (immunoglobulins) and cellular immunity mediated by the host's pathogen-specific stimulation of broadly enhanced microbicidal activity of host macrophages. As Eisenstein and Sultzer point out (Eisenstein and Sultzer, 1983), delineation of the relative protective contributions of both humoral and cellular immunity in the murine typhoid model is of value, not only for the resolution of this complex host-parasite relationship, but, for the kind of integrative insight necessary for development of safe, efficacious human vaccines for S. typhi.

Reports of anti-Salmonella immunity provided by killed vaccines have emphasized the role of humoral immunity as an important acquired host defense mechanism (Roantree, 1967; Eisenstein and Sultzer, 1983). The extent of protection afforded mice given these vaccines is dependent on many parameters: the method of vaccine preparation, the strain of bacterium, the route of challenge, the strain of mice, and the criteria for categorizing effective immunity (Eisenstein and Sultzer, 1983).

Jenkin and Rowley (Jenkin and Rowley, 1965) compared killed vaccines of S. typhimurium C5 to a live vaccine of the attenuated C5 strain M206. Swiss-

Webster mice that had been vaccinated with ethyl-alcohol or acetone killed vaccines had a mortality 28 days after challenge of approximately 30 percent, compared to a 10 percent mortality for mice vaccinated with M206, and a 90 percent mortality for mice vaccinated with a heat killed (boiled) vaccine. Herzberg et al. (Herzberg et al., 1972) demonstrated that white Swiss mice could be protected, based on mortality at 21 days post challenge, by vaccines of S. typhimurium strains Suc LL and Sr-11 prepared by desoxycholate extraction or by heat killing. From the growth kinetics of the bacteria in the spleen and liver of immunized mice, these authors concluded that killed vaccines protected the host by the induction of an immune state which reduced the initial challenge size and thus provided the host with enough time for cellular immunity to develop. The contention of Herzberg et al. was consistent with the earlier in vitro observations by Jenkin et al. (Jenkin et al., 1964). These studies showed that 14 days after live M206 vaccination the peritoneal macrophages from vaccinated mice were better able to kill S. typhimurium C5 than were peritoneal macrophages from unvaccinated controls, whether or not the virulent salmonellae were opsonized in normal or immune serum. However, the opsonization with immune serum imparted enhanced bactericidal activity to non-immune macrophages. If the same kind of experiment was performed at 4 months post vaccination different results were obtained. At this later time enhanced killing of S. typhimurium by immune

macrophages was achieved only if the bacteria were opsonized in immune serum.

The demonstration of protection by killed Salmonella vaccines and of the enhancement by antibody of macrophage bactericidal activity raised the question of the nature of the antigen(s) against which protective antibody was formed. Topley (Topley, 1929) showed that protection conferred by heat-killed, formalin-preserved, whole Salmonella vaccines was due to antibody against the somatic or "O"-antigens. Ornellas et al. (Ornellas et al., 1970), Eisenstein (Eisenstein, 1975), and Lyman et al. (Lyman et al., 1979) further demonstrated the specificity and importance of the anti-somatic antigen response of the murine host.

The use of bacterial genetics to construct hybrid strains that express particular Salmonella O-antigens has also shown that a specific humoral response is an important expression of acquired resistance. Diena et al. (Diena et al., 1973) constructed hybrid strains of S. typhimurium that expressed the somatic (9,12) and capsular (Vi) antigens of an S. typhi donor. These hybrids were as mouse virulent as the parental S. typhimurium strain (somatic antigens 4, 5, and 12). Killed hybrid strain vaccines protected mice from hybrid strain challenge, but not from parental strain challenge. Kiefer et al. (Kiefer et al., 1976) made an S. typhimurium-E. coli hybrid strain that had the O-antigens of the Salmonella donor. Both an acetone-killed and a live vaccine of the hybrid strain protected

with S. typhimurium. These investigators did note that the live vaccine had better protective efficiency than the killed preparation.

A final example of the role acquired humoral immunity may play in the pathogenesis of murine typhoid is provided by the work of Hochadel and Keller (Hochadel and Keller, 1977). The passive transfer of B-lymphocytes or T-lymphocytes from S. typhimurium-sensitized donor mice into non-immune recipient mice allowed these investigators to ascertain the specific protective capacity of each kind of lymphocyte population. By the eighth day after an S. typhimurium i.p. challenge, mice that had received B-lymphocytes, as compared to T-lymphocyte recipients or to controls, showed a significant decrease in the number of salmonellae in the blood, liver, and spleen. The 10 day survival rate for B-lymphocyte recipients was three times greater than that for T-lymphocyte recipients (65 percent vs. 21 percent). The conclusion drawn from these results was that the humoral response, while not totally protective, did play an important part in the early suppression of a systemic Salmonella infection.

The basis for favoring cellular immunity as the primary acquired defense mechanism in murine typhoid has its foundations in the observations that vaccines prepared from killed S. typhimurium, as well as the passive transfer of immune sera, provide little or no protection to mice challenged with virulent strains of the bacterium (Hobson, 1957c.; Ushiba et al., 1959). Mitsuhashi et al.

(Mitsuhashi et al., 1961) demonstrated in vitro that glycogen-induced murine peritoneal macrophages from mice previously vaccinated with a live attenuated strain of S. enteriditis were able to inhibit the intracellular replication of virulent S. enteriditis. Macrophages from uninfected mice or mice vaccinated with a killed virulent strain preparation were not able to control the intracelluar multiplication of virulent S. enteriditis. The studies of G. B. Mackaness, R. V. Blanden, and F. M. Collins during the late 1960's provided convincing evidence for the importance of acquired cellular immunity. Mackaness et al. (Mackaness et al., 1966) used two virulent strains of S. typhimurium, C5S (streptomycin sensitive) and C5R (streptomycin resistant), to show that outbred Swiss-Webster mice that had survived an initial i.v. infection with C5S and then challenged with C5R 14 days or later were capable of eliminating C5R from the liver and spleen within 96 hours. These animals still had significant numbers of the original strain C5S in these RES organs. Blanden et al. (Blanden et al., 1966) showed that the cell responsible for this rapid clearance of a second salmonellae challenge was probably the macrophage. Peritoneal macrophages from Swiss-Webster mice that survived infection with either S. typhimurium C5S or Listeria monocytogenes (L. monocytogenes, when given i.v. to mice, behaves as a facultative intracellular parasite multiplying in macrophages of the RES [Mackaness, 1962] and produces in mice an acquired cellular resistance which

cross protects against unrelated bacteria, e.g. Brucella abortus and Mycobacterium tuberculosis [Mackaness, 1964]) had greater bactericidal activity when removed from the animal and tested in vitro against C5R than did peritoneal macrophages from uninfected controls. In vivo studies (Blanden et al. 1966) showed that the level of acquired resistance to S. typhiumurium or to L. monocytogenes i.v. challenge was directly related to the number of bacteria that had been used as live vaccines. Mice that were vaccinated with large inocula remained residually (chronically) infected and were more resistant to reinfection than were animals vaccinated with lower doses of bacteria. No acquired S. typhimurium resistance was observed when heat-killed homologous vaccines were used. In summary, these studies support the hypotheses that cellular immunity is the primary acquired defense mechanism in murine typhoid and that the enhancement of the microbicidal activity of macrophages is of major importance in acquired resistance in murine typhoid.

Collins et al. (Collins et al., 1966) strengthened the hypothesis that the enhanced bactericidal activity of macrophages in the liver and spleen of mice vaccinated with living salmonellae was an acquired cellular immune response. The use of Salmonella species of antigenic similarity, but of different virulence, enabled these investigators to demonstrate that only when mice received live vaccines of the virulent species S. enteritidis strain Se795 or S. gallinarium were they able to control the

replication of S. enteritidis strain Se795 in the liver and spleen and were able to prevent secondary bacteremia. Conversely, when live S. pullorum, which does not persist systemically beyond the fourth day of inoculation, was used as a vaccine or when alcohol-killed vaccines, prepared from any of these three Salmonella species, were used, mice challenged subsequently with S. enteritidis strain Se795 showed rapid multiplication of this virulent strain in the liver and spleen and developed significant secondary bacteremia. Such in vivo results may have been due to a more efficacious humoral response arising from better antigenic stimulation by the persistence of the virulent Salmonella species. The possiblity of such a mechanism for acquired immunity was tested by S. enteritidis challenge of mice chronically infected with an antigenically dissimilar Salmonella species, S. montevideo. These mice were better able to control S. enteritidis RES replication compared to non-chronically infected control mice. Collins et al. (Collins et al., 1966) concluded that in murine typhoid the ability of a particular species or a strain of Salmonella to persist in the tissues of the host was the primary requisite for the development of an effective, specific cellular immunity expressed by a nonspecific enhancement of macrophage bactericidal activity.

The conclusion of Collins et al. (Collins et al., 1966) was consistent with the development and the expression of acquired cellular immunity demonstrated for other intracellular pathogens: L. monocytogenes, B. abortus, and

Mycobacterium bovis, BCG (Mackaness, 1964). The host cell required for the enhancement of macrophage microbicidal activity was later shown to be the pathogen/antigen-specific thymus-derived lymphocyte, T-lymphocyte (Mackaness, 1969; Mackaness, 1971; North, 1975). These experimental observations further underscore the contribution of acquired cellular immunity in the pathogenesis of murine typhoid.

Experimental evidence suggests that both humoral and cellular acquired immunities are important host defense mechanisms directed against the facultative intracellular agent of murine typhoid. From a holistic point of view, the sequence of host responses to S. typhimurium embraces the whole of immunology. Recent work by Akeda et al. (Akeda et al., 1981), consistent with this holistic viewpoint, emphasizes the central effector role which macrophages play throughout the course of murine typhoid. In the early phase resident macrophages are able to phagocytize and to kill a significant proportion of an S. typhimurium challenge. In the later phase the cell mediated enhanced bactericidal activity of macrophages works in concert with humorally enhanced phagocytic activity of macrophages to eliminate the parasite from the tissues of the host. Although both arms of the immune system are essential to effectively combat this systemic pathogen, it is the genetically determined innate resistance of the murine host that permits the host sufficient time to develop protective immune mechanisms.

Innate Resistance to Murine Typhoid

As defined earlier, natural or innate resistance to a microbe is the capacity of a host, in the absence of any previously acquired specific immunity, either to prevent an infectious disease or to attenuate the microbe. Mice exhibit different degrees of genetically determined innate susceptibility/resistance to murine typhoid (O'Brien et al., 1983). Schott was the first to demonstrate this differential susceptibility (Schott, 1932). He assessed, by scoring the number of mice that died over a 21 day period, the susceptibility of several strains of laboratory mice that had received 5 X 10^4 S. aertrycke (typhimurium) intraperitoneally. This investigator then selectively bred 21 day survivors through 6 generations. Such breeding resulted in new strains of mice that exhibited reduced susceptibility. This enhanced resistance was not sex-linked or associated with animals of a particular age, nor was there any indication of passive transfer of resistance from mothers to progeny (Schott, 1932).

Webster (Webster, 1933a.) extended the early study of inherited resistance to murine typhoid. Unlike Schott, Webster bred mice that had not been previously infected. Susceptibility or resistance of breeding pairs was determined by challenging F1 progeny with an S. enteritidis strain. The S. enteriditis susceptibility of parental mice was inferred from the susceptibility of infected F1 mice.

With parental susceptibility determined, F1 mice that had never been exposed to S. enteritidis were then used for further selective breeding. Webster (Webster, 1933a.) developed mouse strains which, after an oral challenge of 5 X 10^6 S. enteritidis, exhibited mortality rates as high as 97 percent (susceptible) and as low as 15 percent (resistant). These mortality rates were quite stable as indicated by no change in their values when susceptibles were given 5 X 10^4 S. enteritidis or when resistants were given 5 X 10^7 S. enteritidis. Crosses of resistant and susceptible strains resulted in F1 progeny of a Salmonella resistant phenotype. Backcross analysis of such F1 mice to the parental strains and determination of F2 progeny phenotype indicated that the resistance trait was dominant and not sex-linked. Resistant and susceptible strains were then compared for differences in weight, fertility, and S. enteritidis susceptibility by various routes of inoculation (Webster, 1933b.). No differences in weight or fertility were noted between the strains. Moreover, regardless of the route of inoculation (oral, s.c., i.p., or i.v.), susceptible strains had more salmonellae in the blood, spleen, and feces, died sooner ($\leq$ 14 days), and had greater mortality rates than did resistant strains.

Although the studies described above were consistent with a genetic/inheritable model for innate resistance or susceptibility to murine typhoid, this concept was not unanimously accepted. An alternative explanation was that transmission of immunity from females to progency in utero

might be the mode of resistance transfer. However, experiments of Gowen and Schott (Gowen and Schott, 1933) conclusively showed that innate resistance of mice to S. typhimurium was an inheritable, genetic trait: thus corroborating the earlier work of Webster. These workers used a double mating technique to reach this conclusion. A Salmonella susceptible Silver strain female was mated in the same estrus to a Salmonella resistant White male and to a Silver male. Within the same litter of F1 progeny mice could be identified as homozygous (Silver/Silver) or heterozygous (Silver/White) by color coat. All homozygous progeny were susceptible to S. typhimurium; whereas, the heterozygous progeny were resistant to S. typhimurium.

Webster (Webster, 1937) continued to refine mouse strains so that by 1937 he had developed three phenotypically stable strains characterized by their innate susceptibility or resistance to S. enteritidis and to St. Louis encephalitis virus. These strains were designated: BSVS (bacteria susceptible virus susceptible), BRVS (bacteria resistant virus susceptible), and BSVR (bacteria susceptible virus resistant). By crossing these susceptible and resistant strains and testing F1, F2, F3, and backcross progeny, Webster was able to demonstrate that resistance to S. enteritidis and to St. Louis enchephalitis virus were inherited independently as single traits and that resistance was dominant to susceptibility.

Oakberg (Oakberg, 1946) carried out histopathological and bacteriological studies of several mouse

strains which exhibited different degrees of susceptibility to salmonellae. The i.p. route with a challenge dose of 2 X 10^5 S. typhimurium was used. The severity of liver and spleen damage was more extensive in susceptible strains. Salmonellae were visible in macrophages of the liver and spleen 4 days after infection. The initial number of intracellular bacteria was not different among resistant and susceptible strains. This finding suggested that RES uptake was comparable in resistant and susceptible strains. As the infection proceeded, however, macrophages of susceptible strains contained more salmonellae than did macrophages of resistant strains, and there was a greater degree, as judged microscopically, of bacterial degeneration in macrophages of resistant strains. Oakberg concluded that the genetic expression of host resistance to murine typhoid was directly reflected in the attenuation of RES organ pathology and in the enhancement of bacterial destruction by host macrophages.

Murine Genes which Determine Susceptibility to Salmonella typhimurium

Inbred mouse strains exhibit differential susceptibility to the etiological agent of murine typhoid S. typhimurium (and certain strains of S. enteritidis) (O'Brien et al., 1983). The availability of such inbred strains makes it possible to analyze genetically determined innate susceptibility of mice to murine typhoid. Through

such analyses three distinct genes that regulate the dose-dependent response to Salmonella challenge have been identified and the chromosomal location of each has been determined. These genes are designated Ity, Lps, and xid (O'Brien et al., 1983a.; Rosenstreich et al., 1982b.). The characterization of how each of these genes is expressed has greatly enhanced both in vivo and in vitro studies of the host-parasite relationship in the pathogenesis of murine typhoid. With these genetically defined models it has been possible to delineate temporal phases of the disease and to probe specific cellular and humoral components of the defense mechanisms of the host.

The expression of the Ity gene by murine macrophages is the subject of these studies. For the purpose of providing a general background to the genetic control of murine susceptibility to Salmonella typhimurium, brief overviews of the Ity gene and the other Salmonella response genes will be given. These will be followed by a detailed discussion of the identification and expression of the Ity gene.

The first Salmonella response gene to be identified in inbred mice was named Ity for immunity to S. typhimurium (Plant and Glynn, 1977). This gene is located on mouse Chromosome 1 (Plant and Glynn, 1979). Strains of mice that are innately resistant, Ity^r, (Table 1) are able to control the early net multiplication of S. typhimurium in the liver and spleen (Plant and Glynn, 1976), have parenteral LD_{50}s of $\geq 10^3$ (O'Brien et al.,

Table 1. RESPONSES OF SOME INBRED MOUSE STRAINS TO *SALMONELLA TYPHIMURIUM* INFECTION [a].

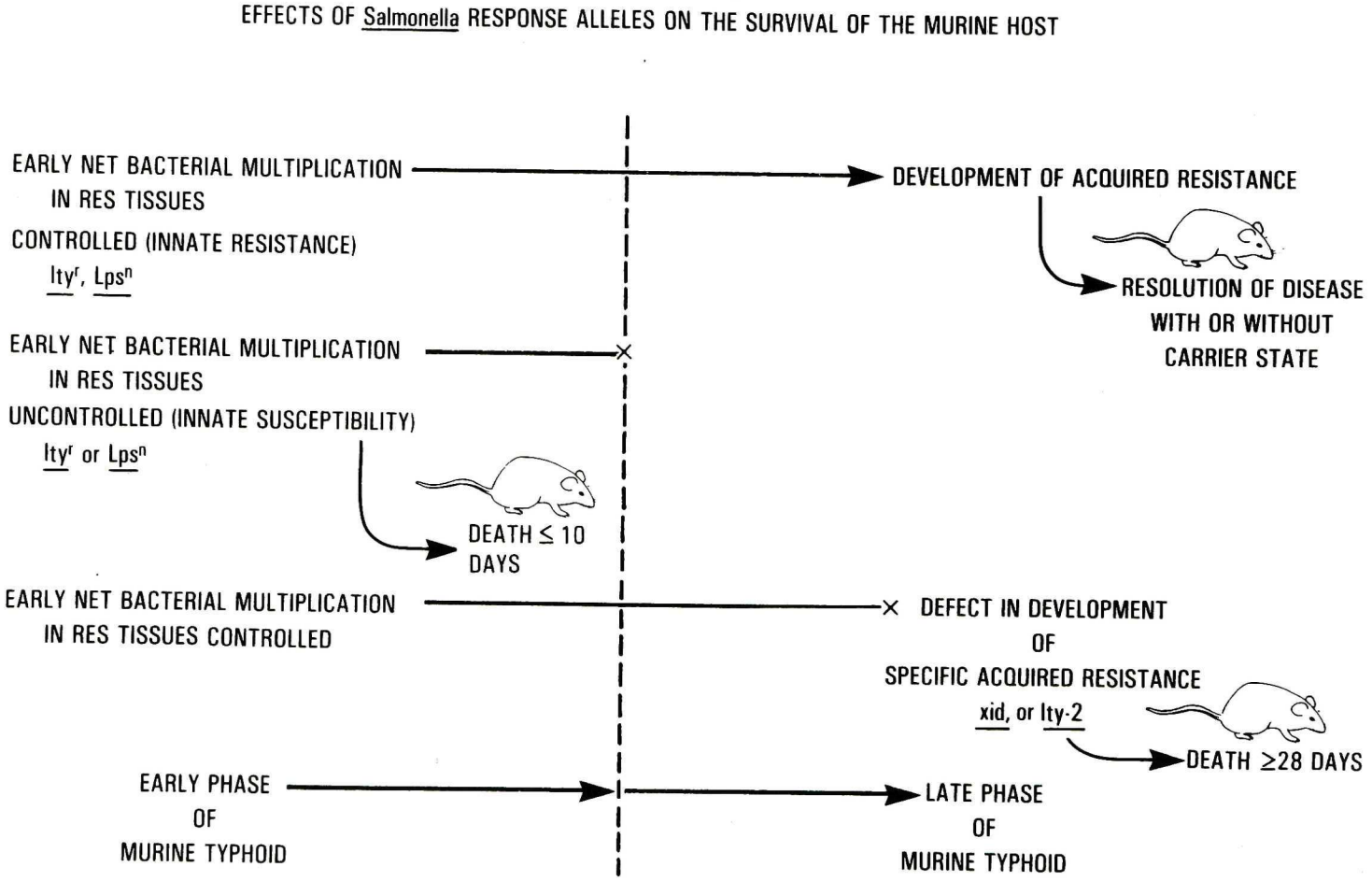
STRAIN	INITIAL NET *S. typhimurium* MULTIPLICATION [b]. AFTER PARENTERAL OR ORAL INOCULATION (GENOTYPE)	PHENOTYPE	TIME DURING DISEASE [c]. WHEN SUSCEPTIBLE PHENOTYPE IS EXPRESSED
A/J BRVR C3H/HeN CBA/Ca C.D2*Ity*^{r} SWR/J	SLOW (*Ity*^{r})	RESISTANT	
BALB/cπ BSVS C57BL/6	RAPID (*Ity*^{s})	SUSCEPTIBLE	EARLY
C3H/HeJ	RAPID (*Lps*^{d})	SUSCEPTIBLE	EARLY
CBA/N (CBA/N × DBA/2N)♂	SLOW (*xid*)	SUSCEPTIBLE	LATE
DBA/2J C57L/J	SLOW (*Ity-2*^{s})	SUSCEPTIBLE	LATE
C3Heb/FeJ	RAPID (?)	SUSCEPTIBLE	EARLY

a. After O'Brien *et al.*, 1983.
b. Net bacterial multiplication in liver and/or spleen; rapid - ≥ 1.0 log increase/day reaching ≥ 10^8 *S. typhimurium* at time of death, slow - ≤ 0.5 log increase/day of *S. typhimurium*
c. Early ≤ 10 days; late ≥ 28 days

1980a.), and develop an infection of the RES which may eventually clear or may enter a chronic carrier state. Strains of mice that are innately susceptible, Ity^s, (Table 1) cannot control the early net multiplication of S. typhimurium in the RES. These strains have parenteral LD_{50}s of $\leq 2 \times 10^1$ (O'Brien et al., 1979a.). If a challenge of 1 LD_{50} for an Ity^r strain is used to infect an Ity^s strain, bacterial counts logarithmically reach $\geq 10^8$ in the RES. This uncontrolled net multiplication invariably leads to the death of the animal within 10 days of inoculation (O'Brien et al., 1983). The rapid expression of the susceptible allele of the Ity gene is indicative of the key role of this locus in determining the outcome of S. typhimurium infection. Additionally, the temporal nature of Ity expression demarcates the initial or early phase of murine typhoid fever (Figure 2).

The Lps gene is a second locus which determines the innate susceptibility of mice to S. typhimurium. Watson et al. (Watson et al., 1978) mapped this gene to the middle of mouse Chromosome 4. Mouse strains that carry the normal allele Lps^n are biologically responsive to low doses of the lipopolysaccharide (LPS) or endotoxin of gram negative bacteria; whereas, mice that are homozygous for the defective allele Lps^d are hyporesponsive to LPS (Vogel et al., 1981). The Lps^d allele is a mutation confined to three inbred strains: C3H/HeJ (Watson et al., 1978), C57BL/10CR (McAdam and Ryan, 1978), and C57BL/10ScN (Vogel et al., 1979). C3H/HeJ mice are susceptible to S.

Figure 2.


EFFECTS OF Salmonella RESPONSE ALLELES ON THE SURVIVAL OF THE MURINE HOST
EARLY NET BACTERIAL MULTIPLICATION IN RES TISSUES CONTROLLED (INNATE RESISTANCE) Ity^r, Lps^n
DEVELOPMENT OF ACQUIRED RESISTANCE
RESOLUTION OF DISEASE WITH OR WITHOUT CARRIER STATE
EARLY NET BACTERIAL MULTIPLICATION IN RES TISSUES UNCONTROLLED (INNATE SUSCEPTIBILITY) Ity^r or Lps^n
DEATH ≤ 10 DAYS
EARLY NET BACTERIAL MULTIPLICATION IN RES TISSUES CONTROLLED
DEFECT IN DEVELOPMENT OF SPECIFIC ACQUIRED RESISTANCE xid, or Ity-2
DEATH ≥28 DAYS
EARLY PHASE OF MURINE TYPHOID
LATE PHASE OF MURINE TYPHOID

typhimurium (Table 1). This strain has a parenteral LD_{50} of < 10, is unable to control the early net multiplication of salmonellae in the spleen, and dies within 10 days of infection (Robson and Vas, 1972; von Jeney et al., 1977; O'Brien et al., 1980a.). C3H/HeJ mice phenotypically resemble the early phase murine typhoid susceptible Ity^s mouse strains. However, if C3H/HeJ mice are crossed with an Ity^s strain the F1 progeny are phenotypically Salmonella resistant. The demonstration of gene complementation in the heterozygous F1 progeny (O'Brien et al., 1980a.) showed that the Ity gene and the Salmonella susceptibility locus of C3H/HeJ mice are separate and distinct genetic loci. Backcross linkage analysis (O'Brien et al., 1980a.) showed that the early phase, highly Salmonella susceptible trait of the C3H/HeJ strain mapped to the same or a closely linked locus as the Lps^d allele. O'Brien (O'Brien et al., 1982a.) suggested that the early deaths of C3H/HeJ mice, that follow S. typhimurium challenge, reflect a failure of Lps^d macrophages to limit the early net growth of salmonellae in the liver and spleen. Two lines of indirect evidence supported this hypothesis: 1., the transfer of syngeneic C3H/HeN (Ity^r, Lps^n) bone marrow cells to irradiated C3H/HeJ (Ity^r, Lps^d) recipients increased the 10 day LD_{50} from < 10 to 8 X 10^2; 2., the pre-infection administration of live Mycobacterium bovis (BCG), a potent macrophage activator, to C3H/HeJ mice decreased the rate of net multiplication of S. typhimurium in the RES and

extended the mean time to death from (10 days to 20 days. When the rates of blood clearance of S. typhimurium by C3H/HeN and by C3H/HeJ mice were compared no differences were observed. The observation that BCG treatment could extend the time of survival, but not ultimately prevent the death of C3H/HeJ mice, suggested to these authors that this mouse strain might have defects in other cell types required for effective host defense during the late phase of murine typhoid (O'Brien et al., 1982a.).

An X chromosome locus also affects the outcome of S. typhimurium infection in mice. Mice homozygous for the recessive mutant allele, designated xid, include the inbred CBA/N strain and F1 male progency derived from CBA/N females. These xid animals have certain B-lymphocyte functional abnormalities (low serum IgM levels, poor antibody responses to some T-lymphocyte independent antigens) (Scher, 1981). Macrophage and T-lymphocyte functions appear to be normal in these mice (Scher, 1981). CBA/N mice and F1 male mice of CBA/N females are susceptible to S. typhimurium (Table 1), and this susceptibility is inherited in an X-linked recessive fashion (O'Brien et al., 1979a.). Unlike Ity^s or Lps^d inbred strains, xid mice die in the late phase ( ) 10 days) of murine typhoid, have a LD_{50} of 2×10^3 at day 10 and a LD_{50} of 2×10^1 at day 28, and have normal early phase macrophage effector function in the RES (O'Brien et al., 1979a.; O'Brien et al., 1981).

Several experimental observations by O'Brien et

al. (O'Brien et al., 1981b.) offer a mechanism of xid conferred S. typhimurium susceptibility: 1., xid mice control the early net multiplication of salmonellae in the RES; 2., crosses of CBA/N females with males of a Salmonella resistant strain produce an F1 progeny in which both male and female mice exhibit similar delayed-type hypersensitivity to S. typhimurium antigens; 3., xid mice made a delayed and diminished IgG anti-Salmonella antibody response to killed S. typhimurium (Metcalf and O'Brien, 1981); 4., the resistance of xid F1 male mice to S. typhimurium is significantly increased by lethal irradiation and reconstitition with either (CBA/N X DBA/2N) F1 female or (DBA/2N X CBA/N) F1 male bone marrow or by passive transfer of immune (CBA/N X DBA/2N) F1 female serum. The protective substance in immune serum is in the gamma globulin fraction and can be removed by adsorption with whole S. typhimurium cells. Taken together these findings (O'Brien et al., 1979a.; Metcalf and O'Brien 1981; O'Brien et al., 1981b.) suggest that the S. typhimurium late phase susceptibility encoded by the xid allele is primarily a consequence of a defective antibody response to the agent of murine typhoid fever.

The identification of three genetic loci that determine the innate susceptibility of inbred mouse strains to murine typhoid illustrates the polygenic expression of host defense mechanisms against a facultative intracellular parasite. In vivo analyses of the expression of these genes have shown that murine typhoid may be divided into at

least two critical periods: an early phase (≤ 10 days post infection), represented by the Ity and Lps genes, in which initial control of net multiplication of salmonellae in the RES is essential; and a late phase (≥ 14 days post infection), represented by the xid allele, in which the ability to mount an efficacious humoral immune response may determine long term survival.

The prediction by several investigators (Hormache, 1979a.; Hormache, 1979b.; O'Brien et al., 1980b.; O'Brien et al., 1982b; Plant, 1983) that other murine Salmonella response genes exist has been recently established. The demonstration by Eisenstein et al. (Eisenstein et al., 1982) that LPS hyporesponsiveness and early phase S. typhimurium susceptibility could be dissociated in the C3HeB/FeJ strain (Table 1) and the initial genetic analysis of this strain by O'Brien and Rosentreich (O'Brien and Rosenstreich, 1983) have indicated that another genetic locus, perhaps closely linked to Lps (O'Brien and Rosenstreich, 1983), influences early phase Salmonella susceptibility. O'Brien et al. (O'Brien et al., 1984) have also identified another major Salmonella resistance gene, tentatively designated Ity-2. This gene is first expressed during a transitional period between the early and late phases (10 to 14 days post infection) of murine typhoid. The susceptible allele is expressed by the DBA/2 and C57L mouse strains (Table 1), is inherited as a single autosomal recessive trait, and does not show linkage to any of the other known Salmonella resistance loci.

As the list of *Salmonella* resistance genes grows so do the possibilities for further characterizations of *in vivo* host defense mechanisms and for the analyses *in vitro* of specific host defense components. This latter course of investigation, which was pursued in the studies described herein of the *Ity* gene, may lead to the characterization of specific gene expression at the cellular level and eventually to the identification at the molecular level of those gene products or regulatory functions which define the innate resistance or susceptibility of a host species to a particular microbe.

The *Ity* Gene

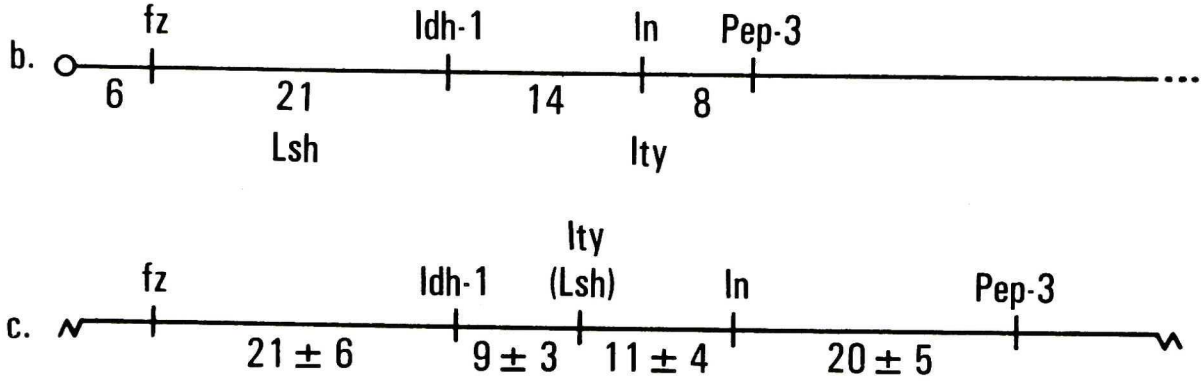
Plant and Glynn (Plant and Glynn, 1976) extended the earlier work of Robson and Vas (Robson and Vas, 1972) by examining eight inbred strains of mice for innate susceptibility to *S. typhimurium* strain C5. Strains that were scored as innately resistant were those that had s.c. LD_{50}s of $> 10^5$ *S. typhimurium*: these were CBA, A/JAX, C3H/He, and DBA/2. Strains that were scored as innately susceptible had s.c. LD_{50}s of < 10 *S. typhimurium*: these were BALB/c, C57BL/10, B10.D2, and DBA/1. Susceptible strains were unable to control the early logarithmic growth of salmonellae in the liver and spleen and died between the fourteenth and sixteenth day of infection with bacterial counts of $\geq 10^8$ in the liver and the spleen. Resistant strains were better able to control the multiplication of

salmonellae in the liver and spleen (viable salmonellae counts not exceeding 10^4 per organ). Furthermore, by the fourteenth day of infection the number of viable salmonellae in these RES tissues of resistant mice began to decline. Examination of F1, F2, and parental backcross generations of CBA and BALB/c mice showed that resistance was inherited as a single, dominant autosomal trait. No linkage of Salmonella resistance or susceptibility to the H-2 locus was observed. Plant and Glynn (Plant and Glynn, 1977) designated the Salmonella-response locus Ity for resistance to S. typhimurium (the resistant allele Ity^r, the susceptible allele Ity^s). The Ity locus was subsequently mapped by Plant and Glynn (Plant and Glynn, 1979) to mouse Chromosome 1. This mapping was accomplished by a series of backcross linkage analyses using known chromosomal markers.

O'Brien et al. (O'Brien et al., 1980c.) confirmed that the Ity locus was located on Chromosome 1. These investigators evaluated the patterns of Salmonella typhimurium strain TML (Gianella et al., 1971) susceptibility of 48 recombinant inbred mouse strains. The data of O'Brien et al. (O'Brien et al., 1980c.) also indicated that the Ity locus was distinct but closely linked to the Chromosome 1 locus Lsh, which had been shown to regulate the extent of the early phase (2-4 weeks after i.v. inoculation) multiplication of amastigotes of Leishmania donavani in the livers of mice (Bradley, 1974; Bradley and Kirkley, 1977; Bradley, 1977; Bradley et al.

1979). The conclusion of O'Brien et al. (O'Brien et al., 1980c.) that the Ity and Lsh loci were not identical was based on two findings. The first of these was the discordant responses to S. typhimurium and to L. donavani in 3 of the 48 recombinant inbred strains. The second finding was that the mapping studies of Bradley et al. (Bradley et al., 1979) and of Plant and Glynn (Plant and Glynn, 1979) placed the two loci, Ity and Lsh, on opposite sides of the Idh-1 (isocitrate dehydrogenase) Chromosome 1 marker (Figure 3). However, a subsequent collaborative study by Plant et al. (Plant et al., 1982) showed that after retyping the 3 discordant recombinant inbred strains (O'Brien et al., 1980c.), that had been designated Ity^r/Lsh^s (1 strain) and Ity^s/Lsh^r (2 strains), these strains were in fact all Ity^s/Lsh^s. Backcross generations from these three stains of mice further substantiated the retyping designations. Plant et al. (Plant et al., 1982) concluded, "... there is therefore no clear evidence to demonstrate that Ity and Lsh are not the same genetic locus." This conclusion was later corroborated by the work of Taylor and O'Brien (Taylor and O'Brien, 1982) (Figure 3). This work, employing a five point backcross linkage analysis, showed that Ity and, therefore, Lsh mapped to a position distal to Idh-1 and proximal to the color coat marker leaden, ln. These mapping studies taken together indicate that either Ity and Lsh are so closely linked as to be indistinguishable, or that Ity and Lsh are, in fact, the same gene.

Figure 3.[a.]



a. Taylor and O'Brien, 1982; with permission
b. Standard linkage map of mous Chromosome 1 with approximate positions of Lsh and Ity from previous reports (Bradley et al., 1977; Plant and Glynn, 1979).
c. Map showing the position of Ity relative to other markers. Distances between markers are expressed in centimorgans.

The tight chromosomal linkage or identical chromosomal position of the Ity and Lsh genes was further documented by the genetic analysis of another intracellular pathogen of the reticuloendothelial system. Gros et al. (Gros et al., 1981) examined inbred mouse strains for innate susceptibility to Mycobacterium bovis (BCG). Two distinct patterns of early phase (3 weeks after i.v. inoculation of 10^4 bacilli) response were observed. C57BL/6J, BALB/c, DBA/1J, and B10.A mouse strains by the third week of infection had significantly higher viable counts of BCG in the spleen than did A/J, C3H/HeCr, and DBA/2J mouse strains. Mendelian analysis of the putative resistant and susceptible strains demonstrated that the rate of early phase growth of M. bovis (BCG) was under the control of a single, dominant autosomal locus. Gros et al. (Gros et al., 1981) named this locus Bcg. Skamene et al. (Skamene et al., 1982) examined 14 inbred and 38 recombinant inbred mouse strains for the inheritance of the resistant Bcg^r allele and for the inheritance of the susceptible Bcg^s allele. The inheritance of both the resistant and the susceptible Bcg alleles was concordant with the inheritance of the resistant and susceptible alleles of both Lsh and Ity. Skamene et al. (Skamene et al., 1982) concluded that the Lsh, Ity, and Bcg loci were either a tightly linked complex on Chromosome 1 or identical genes on this chromosome. The resistant phenotype of a specially constructed mouse strain BALB/c.DBA/2 $Idh\text{-}1^b\text{-}Ity^r\text{-}Pep\text{-}3^b$, developed by M. Potter (Potter et

al., 1983), and congenic with the BALB/cn strain except for the 30 centimorgan segment Idh-1---Pep3 of Chromosome 1 from a DBA/2N progenitor strain, has given further evidence for the close linkage or identity of the Lsh, Ity, and Bcg genes. All of these congenic mice are resistant in vivo to L. donavani, S. typhimurium, and M. bovis (BCG).

Most recently, the inheritance of the innate resistance or susceptibility of inbred mouse strains to Mycobacterium lepraemurium, the rodent leprosy bacillus, has been examined by Brown et al. (Brown et al., 1982). After an i.v. challenge of 10^7 M. lepraemurium (it should be noted that regardless of the strain of mouse M. lepraemurium is uniformly lethal for mice) strains A/J, CBA/Ca, and C3H/He had both longer mean times to death (165 days vs. 136 days) and increased bacterial doubling times in the bone marrow than did the strains C57BL/6, BALB/c, and B10.D2. Phenotypic analysis of F1 generations derived from these three resistant and three susceptible strains demonstrated that innate resistance to M. lepraemurium was inherited as a single dominant autosomal trait. Brown et al. (Brown et al., 1982) pointed out that their results were consistent with the hypothesis that in inbred mouse strains the resistance/susceptibility to M. lepraemurium followed the Lsh-Ity-Bcg pattern.

An intriguing aspect of the genetic and pathogenic studies of inbred mouse strains is the effect of a single gene or tightly linked complex of genes on Chromosome 1 regulating the early innate susceptibility of mice to a

taxonomically diverse group of microorganisms. S. typhimurium is a natural pathogen of mice, is a facultative intracellular parasite (Collins, 1974), and is lethal at doses dependent on the genetic constitution of the murine host. L. donavani is not a natural pathogen of mice, is an obligate intracellular parasite, and, while not lethal for the murine host, its intracellular replication is under the genetic control of the murine host (Bradley, 1977). M. bovis (BCG) is not a natural pathogen of mice, is a facultative intracellular parasite, and its early replication is regulated by the genetic constitution of the murine host. M. lepraemurium is a natural pathogen of mice and is an obligate intracellular parasite. The growth rate of M. lepraemurium in the early phase of infection is regulated by the genetic constitution of the host (Brown et al., 1982). In spite of the taxonomic diversity of these microorganisms, a characteristic they share is their replication either obligately in macrophages or facultatively in macrophages. The gene or genes of Chromosome 1, Ity, Lsh, Bcg, that determine the early innate susceptibility of mice to S. typhimurium, L. donavani, M. bovis (BCG) respectively, and probably M. lepraemurium, is(are) strongly implicated as a locus or loci that regulate(s) the growth of these intracellular pathogens in murine macrophages. It is in this mononuclear phagocyte of the reticuloendothelial system that the mechanism of the Lsh-Ity-Bcg locus may play its key role in determining the early innate susceptibility of the murine

host.

The histopathological studies of Oakberg (Oakberg, 1946) suggested that the *in vivo* ability of macrophages to destroy *S. typhimurium* had a genetic basis. Hormaeche (Hormaeche, 1979c.) demonstrated that the early phase growth kinetics of *S. typhimurium* strain C5 in the liver and spleen of lethally irradiated (800 Rads) mice were phenotypically determined by the innate resistance or susceptibility of the mouse strain used as the donor of bone marrow graft cells and not determined by the *Ity* phenotype of the reconstituted recipient mouse strain. Although these bone marrow grafts contained the stem cells for several cell linages, including lymphocytes and macrophages, Hormaeche (Hormaeche, 1979b.) postulated that, because expression of susceptibility could be discerned by 48 hours after infection, it seemed possible that early net growth rate was under resident macrophage control. This postulate was strenthened by the early phase growth kinetics of *S. typhimurium* in lethally irradiated BALB/c Ity^s and A/J Ity^r mice (Hormaeche *et al.*, 1983). Irradiation had little effect on the growth of *S. typhimurium* in the liver and spleen during the first through fifth day of infection, in either irradiated Ity^s or Ity^r strains compared to non-irradiated control mice. The radioresistance of the early phase regulation of *S. typhimurium* net replication in the liver and spleen suggested to Hormaeche *et al.* (Hormaeche *et al.*, 1983) that the regulatory mechanism did not require cell

proliferation, was not an inducible acquired immune response, and was consistent with an effector function of resident macrophages.

O'Brien et al. (O'Brien et al., 1979b.) used silica, an agent shown to be selectively toxic for macrophages (Kessel et al., 1963), to evaluate the role of macrophages in the expression of early innate susceptibility to S. typhimurium. Female F1 (CBA/N X DBA/2N) Ity^r mice were intravenously administered silica prior to i.p. challenge with 5 X 10^2 S. typhimurium strain TML. The growth kinetics of salmonellae in the spleen of silica treated mice phenotypically resembled that of an Ity^s strain, i.e., viable bacterial counts of $\geq$ 1 X 10^6 per spleen by the fifth day of infection vs. $\leq$ 5 X 10^4 per spleen in untreated control mice. Additionally, the i.p. LD_{50} of silica treated mice was reduced from 1 X 10^3 to $< 10^1$. The conversion to the Ity^s phenotype could be prevented if the lysosomal stablizing agent PVNO (poly-2-vinylpyridene-N-oxide) was administered concurrently with silica. The results of O'Brien et al. (O'Brien et al., 1979) clearly showed the importance of resident macrophages in the control of S. typhimurium replication during the early phase of murine typhoid.

The regulation of early phase multiplication of S. typhimurium has been shown to be independent of T-lymphocyte function and to be determined by the Ity phenotype of the murine host. O'Brien and Metcalf (O'Brien and Metcalf, 1982) intravenously challenged CD-1, CD-1 nu/+

(euthymic) and nude CD-1 nu/nu (athymic) outbred Ity^r mice with S. typhimurium strain TML. The number of viable salmonellae in the liver and spleen was determined over 24 days. No difference was seen among these mice until the fourteenth day of infection. After this time the athymic mice began to have higher bacterial counts, began dying on the seventeenth day of infection, and had all died by the twenty-fourth day of infection. Similar findings were reported by Hormaeche et al. (Hormaeche et al., 1983), who used euthymic and athymic inbred CBA Ity^r and BALB/c Ity^s strains to show that Ity phenotype was independent of T-lymphocyte function. Several investigators have used in vitro systems for studying the contribution of macrophages to the host parasite relationship in the pathogenesis of murine typhoid (Furness, 1958; Furness and Ferreira, 1959; Geltzer and Suter, 1959; Rowley and Whitby, 1959; Jenkin and Benacerraf, 1960; Mitsuhashi et al., 1961; Morello and Baker 1965; Blanden et al., 1966). These studies addressed the phagocytic and bactericidal activities of macrophages obtained from non-immune, as well as from immunized mice, and the basis of differential virulence among different species and strains of Salmonella ingested by macrophages. The first in vitro study which was designed to evaluate the expression of genetically determined Salmmonella susceptibility by macrophages was that of Maier and Oels (Maier and Oels, 1972). These investigators used the inbred BSVS and BRVR mouse strains of Webster (Webster, 1933a.) as sources for resident

peritoneal macrophages. Glass adherent macrophages were infected *in vitro* with normal mouse serum-opsonized *S. typhimurium* strain SR-11. After a 1 hour infection period the number of bacteria per macrophage on Giemsa-stained glass coverslips was determined. No difference, as judged by this microscopic procedure, was noted between BSVS and BRVR peritoneal macrophages. The bactericidal activities of BSVS and of BRVR peritoneal macrophages were compared after the i.p. injection of *S. typhimurium* into the peritoneal cavity and collection of resident cells after 15 minutes of *in vivo* opsonization. Adherence of resident cells to glass petri dishes was followed by washing away unphagocytized salmonellae and non-adherent peritoneal cells. Viable counts of *S. typhimurium* were determined over a 2 hour period in lysates of infected macrophages. The results of this short term *in vitro* bactericidal assay showed that BRVR peritoneal macrophages killed ingested salmonellae more quickly and to a greater degree than did BSVS peritoneal macrophages. Maier and Oels (Maier and Oels, 1972) concluded that the innate resistance to *S. typhimurium* of BRVR mice could be explained by the greater ability of this strain's macrophages to kill ingested *Salmonella*.

In 1981, the year in which the work presented herein was begun, Blumenstock and Jann (Blumenstock and Jann, 1981) used a 90 minute *in vitro* assay to evauluate the phagocytic and bactericidal activities of resident peritoneal macrophages from C57BL/6 *Ity*^{s} and from C3Hf

Ity^r mice. After a 5 minute infection period, the number of viable *S. typhimurium* in lysates of adherent macrophages was determined. Additionally, the chemiluminescence emission of infected macrophages was quantified. Blumenstock and Jann (Blumenstock and Jann, 1981) divided the killing of *S. typhimurium* into two phases: early phase killing, within 5 minutes of infection, which was similar for both mouse strains' macrophages, and late phase killing, within 15 to 30 minutes of infection, which was enhanced in C3Hf macrophages. The early phase was that period in which salmonellae and macrophages made initial contact. The late phase was that period in which salmonellae were internalized within macrophages. The chemiluminescence responses of *Salmonella*-infected macrophages from *Ity*^{s} and *Ity*^{r} mice revealed no differences in the generation of bactericidal, reduced oxygen intermediates (Klebanoff, 1980).

Specific Aims

In vivo findings strongly suggested that the *Ity* locus influences the rate of net multiplication of *S. typhimurium* within macrophages. The *in vitro* results of Maier and Oels (Maier and Oels, 1972) and of Blumenstock and Jann (Blumenstock and Jann, 1981) indicated that macrophages, isolated *in vitro*, might express the *Ity* phenotype of the intact animal. These *in vitro* studies, however, were of relatively short duration (both $\leq$ 2

hours), did not provide a means of preventing any extracellular bacterial replication, did not use a controlled opsonization technique nor a fixed ratio of bacteria:macrophages per infection (Maier and Oels, 1972), and did not differentiate between intracellular and extracellular bacteria in total viable bacterial counts of macrophage lysates (Blumenstock and Jann, 1981).

To test the premise that the expression of the Ity gene in vivo is a macrophage related function, it was necessary to circumvent the difficulty of examining specific macrophage effector function(s) in the intact animal. Therefore, the goal of these studies was to develop an in vitro assay in which macrophages could be infected with S. typhimurium and by which the interaction of the host cell with the parasite could be evaluated under well controlled experimental conditions. With this goal, the following specific aims were drawn:

I. Develop an in vitro assay to examine the effector function of murine resident peritoneal macrophages infected with a mouse virulent strain of S. typhimurium, and devise the means for:
   - A. following the bacterium-macrophage interaction for at least 24 hours
   - B. infecting macrophages in a standardized fashion
   - C. controlling bacterial extracellular replication
   - D. quantifying the number of viable intra-

cellular bacteria in relation to the number of macrophages present at the time of quantification

E. determing the viability of infected macrophages at any time point

II. Use the *in vitro* assay to assess the expression of innate *S. typhimurium* susceptibility/resistance by resident peritoneal macrophages from inbred mouse strains of known *Ity* phenotype

III. Use *Ity* congenic mouse strains to definitively show that differential expression of innate *S. typhimurium* susceptibility/resistance by macrophages *in vitro* is attributable to the *Ity* gene

IV. Demonstrate whether the differential expression of the *Ity* gene is due to a difference in the uptake of *S. typhimurium* between Ity^r and Ity^s macrophages, by the application of these methods

A. use of radiolabeled *S. typhimurium*

B. indirect immunofluorescence microscopy

C. light microscopy

V. Use the *in vitro* assay to determine the *Ity* phenotype of mice which will subsequently be used in the construction (breeding) of coisogenic *Ity* mouse strains

VI. Examine the intracellular fate/location of virulent *S. typhimurium* in resident peritoneal macrophages from *Ity* congenic mouse strains by transmission electron microscopy.

VII. Examine the nature of the differential expression of

the *Ity* gene by infecting *Ity*^{r} and *Ity*^{s} resident peritoneal macrophages with strains of *Salmonella typhimurium* which are avirulent for mice *in vivo*

VIII. Use the *in vitro* assay to compare the bacterium-macrophage interaction of *Ity* congenic mouse strains infected with gram negative bacteria other than *Salmonella typhimurium* or with gram positive bacteria

METHODS AND MATERIALS

Mice

SWR/J, C57BL/6J, C3H/HeJ, C3Heb/FeJ, and C57L/J mice were supplied by the Jackson Laboratory, Bar Harbor, ME. The C3H/HeNCr1Br (C3H/HeN) mice were obtained from the Division of Research Sciences of the National Institutes of Health, Bethesda, MD. Male and female (CBA/N X DBA/2N) and male (DBA/2N X CBA/N) mice were obtained from Dominion Laboratories, Dublin, VA. Dr. M. Potter kindly provided the BALB/c.DBA/2 Idh-1^{b}-Ity^{r}-Pep-3^{b} (C.D2Ity^{r}), BALB/cn, DBA/2N, and F1 (DBA/2N X BALB/cn) mice: these mice were bred under N.C.I. Contract # 1-CB-25584. In designations of F1 mice the 1st strain shown is the female parent. Although the C.D2Ity^{r} mice have only been introgressively backcrossed to N7, they appear, by tests of various chromosomal markers, to be congenic with BALB/cn mice and differ from this later strain by a 30 centimorgan Chromosome 1 segment derived from DBA/2N mice. Thus C.D2Ity^{r} mice, unlike the Ity^{s} BALB/cn congenic strain, are resistant to *in vivo* infection with *S. typhimurium*, as well as *M. bovis* strain BCG and *L. donovani* (Potter *et al.*, 1983).

Both male and female mice of each strain were used throughout these studies, unless sex-linked *Salmonella*-susceptibility was to be examined. Mice were 6 to 14 weeks of age. The animals were kept on a 12 hour light-12 hour

dark cycle and were permitted food and water ad libitum.

Macrophage Culture Media

Basal medium for all macrophage culture preparations was RPMI-1640 with glutamine (Flow Laboratories, McLean, VA) buffered with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid, Flow Laboratories). For some steps, this basal medium was supplemented with 10% heat inactivated (56°C, 30 minutes) fetal bovine serum (FBS, Flow Laboratories) and 100 µg/ml gentamicin sulfate (Gibco, Grand Island, NY): this supplemented medium was designated adherence medium. The medium used to maintain adherent macrophages (maintenance medium) consisted of basal medium supplemented with 5% FBS and 5 µg/ml gentamicin sulfate. Basal medium or Dulbecco's phosphate buffered saline (PBS), pH 7.4, was used to wash all adherent macrophages. For macrophage counting procedures (described below) PBS was made without $CaCl_2$ or $MgCl_2$ (Ca^{++},Mg^{++}-free PBS).

Bacteriological Methods

The following strains of S. typhimurium were used in these studies: the human isolate TML (Gianella et al., 1971), its temperature sensitive mutant TML/TS27 (Swanson and O'Brien, 1983), and the mouse avirulent aroA$^-$ strain SL 3235 (Hoiseth and Stocker, 1981). Additionally, the

human virulent Quailes strain of S. typhi (Hornick et al., 1970; O'Brien, 1982), the nonvirulent HS strain of Eschericia coli (Dupont et al., 1971), a human isolate of a coagulase positive Staphyloccus aureus (kindly provided by the Microbiology Section, Naval Hospital, Bethesda, MD), and the non-toxigenic strain C7 of Corynbacterium diphtheriae (Barksdale and Pappenheimer, 1954; kindly provided by Drs. R. K. Holmes and L. Russell) were used.

Bacteria were routinely grown from a single isolated colony to a stationary phase culture by overnight incubation in Penassay broth (Difco, Detroit, MI) (when C. diphtheriae strain C7 was grown, Penassay broth was supplemented with 5% FBS) in a shaking water bath at 37° C (25° C for TML/TS27). The number of viable bacteria in such cultures, as well as all assay samples, was determined by appropriate dilution in sterile 0.85% NaCl (normal saline) and by subsequent quantitative plate count on Trypticase Soy Agar (Difco). From such overnight cultures bacteria for all further manipulations were diluted in normal saline to the desired number of viable bacteria.

To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of gentamicin for S. typhimurium strains TML and SL 3235, S. typhi strain QS, E. coli strain HS, and C. diphtheriae strain C7, two-fold dilutions of the antibiotic were made in basal medium containing 5% FBS. Bacteria from overnight Penassay cultures were diluted in normal saline to give a final concentration of 2×10^5 bacteria per ml when added

to each antibiotic dilution tube (done in duplicate) and to antibiotic-free control tubes. All tubes were incubated overnight at 37° C in 5% CO_2 and 95% air. The MIC was taken as the lowest concentration of gentamicin which permitted no visible turbidity; whereas, the MBC was considered the lowest concentration of gentamicin that prevented the growth of any bacterial colonies on subculture. The MIC and MBC were 4.5 µg/ml and 4.5 µg/ml, for S. typhimurium strain TML; 1.5 µg/ml and 3.0 µg/ml for S. typhimurium strain SL 3235; ≤ 0.75 µg/ml and ≤ 0.75 µg/ml for S. typhi strain QS; ≤ 1.5 µg/ml and ≤ 1.5 µg/ml for E. coli strain HS; ≤ 0.75 µg/ml and ≤ 0.75 µg/ml for S. aureus; and ≤ 0.75 µg/ml and 1.5 µg/ml for C. diphtheriae strain C7. From these results a gentamicin concentration of 5.0 µg/ml was selected for incorporation into maintenance medium because it was considered to be the lowest antibiotic concentration that would consistently prevent extracellular replication of S. typhimurium strain TML in infected macrophage cultures.

To follow the kinetics of gentamicin kill of S. typhimurium strain TML, viable salmonellae from an overnight Penassay culture were added to basal medium with 5% FBS. Duplicate samples were taken for plate count and then gentamicin was added to give a final concentration of 5.0 µg/ml. Immediately following the addition of antibiotic, duplicate samples for plate count were taken, and then the medium was dispensed in 500 µl aliquots into 16 mm wells of a 24 well plastic culture dish (Costar, Cambridge, MA).

The dish was placed in a humidified incubator at 37° with 5% CO_2 and 95% air. Thereafter, the number of viable salmonellae in each of two wells was determined by plate count per each time interval.

The bacteria used to infect macrophages of each mouse strain were suspended in a filter (0.45 μm pore size) -sterilized solution of basal medium that contained 10% normal mouse serum obtained from that strain of mice from which peritoneal cells were explanted. The final concentration of bacteria in the solution was adjusted to the desired ratio of infecting bacteria to macrophages. To completely suspend the bacteria in the 10% normal mouse serum solution, the solution was lightly vortexed for 15 seconds and then rotated in a gyrorotary water bath at 45 rpm, 37° for 30 minutes. This latter period was termed "the opsonization period."

In order to study the uptake of S. typhimurium by resident peritoneal macrophages, S. typhimurium strain TML was radiolabled by incorporation of ^{3}H-leucine. After overnight incubation at 37° C, minimal medium M-9 (Miller, 1972) cultures of S. typhimurium were diluted in fresh M-9 medium and incubated at 37° C until a concentration of approximately 1 X 10^9 bacteria per ml, determined spectrophotometrically at 600 nm, was reached. These salmonellae cultures were diluted 10-fold in fresh M-9 medium with 10 or 20 μCuries/ml of ^{3}H-leucine (Amersham, Arlington Heights, IL). These cultures were incubated for 1 hour at 37° C to allow for uptake of the

^{3}H-leucine by the salmonellae. Free ^{3}H-leucine was separated from radiolabeled bacteria by centrifugation at 1000 X G at 4° C for 20 minutes, decanting off the supernatant and resuspending the bacterial pellet in cold, 4° C, PBS. This washing procedure was repeated three times and followed by a final resuspension of the bacteria in PBS. Radiolabeled S. typhimurium were used in the same manner as unlabeled S. typhimurium to prepare the 10% normal mouse serum solutions used to infect macrophages. The specific activities (counts per minute [cpm]/ml ÷ colony forming units [cfu]/ml) of radiolabeled S. typhimurium in 10% normal mouse serum solutions were 0.01 to 0.02.

Preparation of Resident Peritoneal Macrophages

Mice were sacrificed by cervical dislocation or by exsanguination from the retroorbital plexus. The peritoneum of each animal was exposed, cleansed with 70% ethanol, and injected with 3 ml of cold adherence medium. After a vigorous 1 minute massage of the fluid-distended peritoneal cavity, the resident cells were collected by aspiration of the lavage fluid through a 26 guage hypodermic needle into a 3 ml plastic syringe. Lavage fluids from 10 to 25 mice were pooled and the resident cells harvested by centrifugation for 15 minutes at 150 X G, 4° C. Next, the cells were gently resuspended in adherence medium and the number of macrophages determined

in a hemocytometer. Viability of the cells was checked by trypan blue dye exclusion (0.05% in normal saline) and was typically found to be ≥ 95%.

Two methods of seeding macrophages were used during these studies:

Method 1 - Between 2 X 10^5 and 4 X 10^5 macrophages were seeded into each well of a 24 well, 16 mm, plastic culture dish (Costar) which had been held briefly in steam rising from a beaker of boiling distilled H_2O to reduce static electric surface charge (K. English and S. Vogel, personal communication). An additional 500 µl of adherence medium was then added to each well, and dishes were vigorously rotated to obtain homogenous cell suspensions. The culture dishes were placed in a humidified 37° C incubator (5% CO_2, 95% air atmosphere) for 4 to 6 hours to permit the macrophages within the suspensions to adhere to the plastic wells.

Method 2 - Between 4 X 10^5 and 5 X 10^5 macrophages were seeded as 200 µl droplets onto the center of each well of a 24 well, 16 mm, plastic culture dish (Costar), pre-steamed as in Method 1. After a 90 minute adherence period at room temperature, an additional 500µl of adherence medium was added over the underlying layer of adherent cells in each well. The dishes were placed in a humidified 37° C incubator (5% CO_2, 95% air atomsphere) for 4 hours. This method produced more uniform cell monolayers and (as described below) a higher yield of adherent resident peritoneal macrophages

than did Method 1.

After seeding and adherence of macrophages, the medium and nonaderent cells were aspirated and the adherent cells in each well overlaid with 500 µl of basal medium containing 10% FBS. The dishes were then returned to the incubator and cultured overnight.

After the overnight incubation period in basal medium with 10% FBS and one basal medium wash, the average number of adherent peritoneal macrophages per well was determined for each of several mouse strains. This was done by counting in a hemocytometer the number of macrophages released from the plastic wells following treatment with a 10 mg/ml lidocaine (with preservative, Butler, Fredricksburg, VA) (Rabinovitch and DeStefano, 1975; Rabinovitch and DeStefano, 1976) with 50 µg/ml gentamicin in Ca^{++}, Mg^{++}-free PBS solution for 4 hours at 37° C. The results indicated that between 22% to 33%, for Method 1, and 74% to 94%, for Method 2, of the total number of macrophages present in the original seeding suspension was retained on the plastic surface after overnight incubation; these percentages were independent of mouse strain. From these findings the number of macrophages per well at the time of bacterial infection was taken as 30%, Method 1, and 80%, Method 2, of the number used to seed the culture wells. These standardized values were required to calculate the number of bacteria to be added to each well to approximate the desired ratio of bacteria to macrophages.

Preparation of Thioglycollate-Elicited Peritoneal Macrophages

Mice that were to serve as sources of thioglycolate-elicited peritoneal macrophages were injected intraperitoneally with 3 ml of Fluid Thioglycollate Medium (BBL, Microbiological Systems, Cockeysville, MD). After 96 hours the peritoneal exudate cells were collected in two ways: 1., mice were sacrificed by cervical dislocation, and the peritoneal exudate cells were collected and processed in the same manner as were resident peritoneal cells; 2., if the *in vitro* infection assay was to be used to assess the *Ity* phenotype of mice, 3 ml of adherence medium was injected into the peritoneal cavity. The fluid contents of the peritoneal cavity were immediately collected by tapping the peritoneum with an 18 gauge hypodermic needle. The peritoneal exudate was collected directly into a 50 ml polypropylene conical tube (Corning Glass, Corning, NY) which contained enough heparin (Eli Lilly & Co, Indianapolis, IN) to give a final approximate concentration of 150 U per ml of adherence medium. The exudate was centrifuged for 15 minutes at 150 X G, 4° C. The cell pellet was gently resuspended in and washed with adherence medium. After a second centrifugation the peritoneal exudate cells were resuspended in adherence medium and processed in the same manner as were resident peritoneal cells.

In Vitro Assay for Salmonella typhimurium Infection of Peritoneal Macrophages

Adherent macrophages in culture dish wells were washed once with basal medium and then exposed to 500 µl of normal mouse serum-suspended S. typhiumurium. To permit phagocytosis of the bacteria by the macrophages, culture dishes were then placed in a 37° C, humidified incubator with an atmosphere of 5% CO_2 and 95% air for 50 minutes. The actual number of normal serum-suspended bacteria used to infect macrophages of each mouse strain was verified by plate count.

After the 50 minute phagocytosis (infection) period all macrophages were washed 3 times with PBS to remove nonadherent bacteria. For those wells that were sampled at 0 hour after infection, the numbers of S. typhimurium present in the last wash and in macrophage lysates (prepared as described below and designated cell lysate t_0) were determined by plate count. Those wells that contained macrophages to be sampled later were overlaid with 500 µl of maintenance medium. At specified times, thereafter, maintenance medium over the infected cells was sampled for plate count and then aspirated from the wells. The macrophages were then washed 3 times with PBS. The final PBS wash was removed from the wells and replaced with 500 µl of macrophage lysing solution (Hsu and Radcliffe, 1968), 0.5% sodium desoxycholate (Difco) in sterile normal saline. Each lysate was vigorously stirred

with the tip of a sterile 1.0 ml plastic pipet and withdrawn from the well for plate count. That the macrophage lysing solution did not kill salmonellae was ascertained by a comparison of the viability of 0.5% sodium desoxycholate-suspended S. typhimurium with the viability of saline-suspended salmonellae at room temperature over a 90 minute period. To verify that the bacteria recovered from infected macrophages were unaffected by 0.5% sodium desoxycholate, an assay was performed in which Salmonella-infected macrophages were lysed as usual or disrupted by exposure for 15 minutes to 0.01% bovine serum albumin in sterile, distilled H_2O, a modification of the method of Leijh et al. (Leijh et al., 1979).

To determine the number and viability of adherent macrophages present at each sample time, macrophages infected with S. typhimurium were washed 3 times with PBS and then overlaid with 500 µl of 10 mg/ml lidocaine with 50 µg/ml gentamicin and 0.05% trypan blue in Ca^{++},Mg^{++}-free PBS. The viability of adherent macrophages was assesed using an inverted stage microscope to score at least 200 cells for trypan blue exclusion in situ. The number of macrophages released after a 4 hour, 37° C exposure to the lidocaine solution was determined by hemocytometer count and then was used to calculate the average number of bacteria (obtained from the time-matched infected macrophage lysates) per macrophage counted. In addition, the Lowry (Lowry et al., 1951) assay was used to determine the protein concentration of macrophage lysates

in some experiments.

When other species of bacteria, i.e., S. typhi strain QS, E. coli strain HS, S. aureus, and C. diphtheriae strain C7, were used to infect resident peritoneal macrophages, the in vitro assay for S. typhimurium was used without modification (When gram positive bacteria were used to infect macrophages, macrophage-lysates were prepared by disruption in 0.01% BSA in distilled H_2O). In those experiments using thioglycollate-elicited macrophages, the source of serum for opsonizing bacteria was from normal mice of the same strain as that used for thioglycolate elicitation.

Assessment by Radioimmunoassay (RIA) of the Gentamicin Concentration in Salmonella typhimurium-Infected Macrophages

To determine if gentamicin was taken up by cultured resident peritoneal macrophages, the in vitro assay for S. typhimurium strain TML infection of resident peritoneal macrophages was performed with the following modifications. 2.5×10^6 resident peritoneal macrophages from BALB/cn (Ity^s) and 2×10^6 resident peritoneal macrophages from SWR/J (Ity^r) mice were seeded per each 35 mm well of a 6 well plastic culture dish (Costar). Macrophages were cultured as described above except fluid volumes in wells were increased to 2 ml to compensate for the larger surface area. For each strain, macrophages were

collected at 4 hours and at 24 hours (triplicate samples) after Salmonella infection or after a sham infection (10% normal mouse serum solution without bacteria), and the cells were prepared by washing the macrophages 3 times with PBS, overlaying the cells with cold, 4° C, Ca^{++},Mg^{++}-free PBS, and carefully scrapping the cells off the plastic with a rubber policeman. Macrophage suspensions were transferred to chilled, 4° C, glass tubes and the cell number determined as described above. Macrophages were then completely lysed by 3 minutes of intermittent (15 seconds on, 10 seconds off) sonic oscillation at 50 watts (Sonifier Cell Disrupter Model W185 with microtip probe; Heat Systems Ultrasonic Inc., Plainview, NY). This prolonged period of sonic disruption was not required to lyse macrophages, but was used as a safety precaution to render the samples bacteriologically sterile. Solutions of basal medium with 5% FBS that contained known amounts of gentamicin were also subjected to this sonic disruption procedure. An aliquot of each macrophage lysate was assayed for protein concentration, and the remainder of each sample was frozen and sent to a commercial laboratory (Litton Bionetics Medical Laboratories, Kensington, MD) for evaluation of the gentamicin concentration by RIA (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA).

Fixation and Staining of Macrophage Preparations for Light Microscopy

Specimens examined by light microscopy were: Cytospin-2 (Shandon Southern, Sewickley, PA) preparations of fluid suspensions of macrophages or cultured adherent macrophages on the surfaces of glass (Ernest Fullam Inc., Schenectady, NY) or plastic "Thermanox" (Miles Scientific, Naperville, IL), 13 mm circular coverslips. These specimens were stained with the Diff-Quick histochemical stain according to the instructions provided by the manufacturer of the kit (American Scientific Products, McGraw Park, IL), except that all macrophage specimens were fixed for 20 minutes in absolute methanol (Fisher Scientific Company, Fair Lawn, NJ) instead of the fixative provided by the manufacturer. Such a fixation step resulted in better preservation of cell morphology, better differential uptake of the staining solutions, and better visualization of intracellular bacteria compared to the commercial fixative. Light microscopy was carried out using an Olympus BH-2 microscope with a PM-10AD photomicrograph system, 35 mm C-35D camera with a 2.5X drop in lens (Olympus Optical Co. LTC., Tokyo).

Phagocytosis of Latex Beads

At various times during the in vitro assay the capacity of Salmonella-infected macrophages to ingest latex beads was evaluated and compared to uninfected macrophages. Medium was aspirated from culture dish wells

that contained macrophages cultured on 13 mm glass coverslips. Five hundred microliters of PBS was added to each well and immediately removed by aspiration and without the vigorous rotation of the culture dish routinely done at all other macrophage culture washings. Five hundred microliters of maintenance medium containing latex beads (1.10 diameter uniform particles, approximately 1 X 10^8 particles per ml, Dow Chemical Co., Indianapolis, IN) diluted either 1/1000 or 1/5000 (as required by the experimental protocol) was added to each well. The culture dishes were incubated for 30 minutes at 37° in 5% CO_2 and 95% air, the maintenance medium with latex beads was aspirated from the wells, and 500 µg of PBS was added to each well. The PBS was aspirated from the wells without culture dish rotation, and 500 µl of basal medium with 5 ug/ml of gentamicin was then added to each well. The culture dishes were returned to the incubator for 15 minutes. After this incubation the medium was removed from the wells and 500 µg of PBS were added to each well and then immediately removed by aspiration. Macrophages were fixed on the glass coverslips with absolute methanol, as described previously. After fixation coverslips were washed to remove non-ingested latex particles. The washing procedure consisted of three separate additions of 300 µl of PBS per well, followed by vigorous rotation of the culture dishes on a mini-orbital shaker (Bellco Glass Inc., Vineland, NJ) for 1 minute at a rotation setting of 6.5. Washed coverslips were removed from the culture dish wells

and stained for light microscopy. For each time point, 100 to 200 macrophages on triplicate coverslips were examined and the average number of latex particles ingested per macrophage was calculated (for numerical scoring, macro- which contained) 6 latex particles were assigned a value of 7).

Uptake of Radiolabeled Salmonella typhimurium Strain TML by Resident Peritoneal Macrophages

S. typhimurium strain TML was radiolabeled by ^{3}H-leucine incorporation, as previously described, and suspended, as described above, in 10% homologous normal mouse serum. The number of peritoneal macrophages seeded per 35 mm well of a 6 well plastic culture dish (Costar) for these analyses was approximately 2 X 10^{6}. Radio- activity associated with macrophage lysates of peritoneal cells was measured by counting 200 µl of lysate in 9 ml of Ready-Solv liquid scintillant (Beckman Instruments Inc., Fullerton, CA) using a model LS7500 counter (Beckman).

Immunofluorescence Microscopy of Salmonella-Infected Macrophages

Resident peritoneal macrophages were seeded onto 13 mm glass coverslips and infected with S. typhimurium strain TML or with S. typhimurium strain TML/TS27, as described previously. For immunofluorescence studies the

coverslips were washed 3 times with 500 µg of PBS and stained, as described below, by the indirect fluorescent antibody (IFA) technique. Macrophages, either unfixed or methanol-fixed, were overlaid for 30 minutes at 37° C in a humidified chamber with rabbit anti-TML serum, which had been prepared against whole acetone-killed and dried salmonellae as described by Metcalf and O'Brien (Metcalf and O'Brien, 1981). Following two 1 ml PBS washes fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit 7S globulin (GIBCO) diluted 1/20 in PBS was overlaid for 30 minutes at 37° C in the humidified chamber. After two additional 1 ml PBS washes, IFA-stained coverslips were mounted in 10% PBS buffered-glycerol and examined using a Zeiss Photomicroscope III (Carl Zeiss Inc., New York, NY), equipped with a 50 watt mercury lamp, a 450-490 nm excitation filter, and a 520 nm barrier filter. One to two hundred macrophages per coverslip were examined, and the number of fluorescent bacteria per macrophage was recorded. In all experiments coverslips were evaluated in triplicate. Because immunoglobulin cannot permeate intact cell membranes (Johnson et al., 1978), the bacteria counted from unfixed macrophages were considered extracellular (cell surface-associated), while bacteria from methanol-fixed macrophages were scored as a total of intracellular and extracellular bacteria (Kihlstrom, 1977). Data are presented as intracellular bacteria per macrophage and were calculated by subtracting fluorescent,

extracellular bacteria from total fluorescent bacteria.

Electron Microscopy of Salmonella-Infected and Uninfected Resident Peritoneal Macrophages

Resident peritoneal macrophages from C.D2*Ity*^{r} and from BALB/cn mice were seeded (Method 2) onto 13 mm Thermanox coverslips in 16 mm, 24 well plastic culture dishes. *S. typhimurium* strain TML was used to infect macrophage cultures. Sham-infected macrophage cultures were included as uninfected controls. At various time points of the *in vitro* assay, macrophage cultures were fixed and embedded for electron microscopy directly on the coverslips: the fixation and embedding procedure is a modification of the method of Langreth *et al.* (Langreth *et al.*, 1983), as detailed below. At each time point macrophage cultures were prefixed for 10 minutes at room temperature in 1 ml per well of 0.2% (v/v) glutaraldehyde (Polysciences, Inc., Warrington, PA) in cacodylate buffer (pH 7.4, 0.1 M Na cacodylate buffer [Amend, Irvington, NJ] containing 0.12 M sucrose and 0.02 mM $CaCl_2$). Prefixation for some macrophage cultures was performed immediately after the aspiration of overlying medium and for other macrophage cultures was performed after 3 PBS washes. After prefixation coverslips were transferred to 35 mm plastic petri dishes and were fixed for 1 hour at room temperature in 2.0% glutaraldehyde in cacodylate buffer. Following three 5 minute washes with cacodylate

buffer, macrophages were postfixed for 1 hour at 4° C in 1.5 % O_sO_4 (Electron Microscopy Sciences, Fort Washington, PA) in cacodylate buffer. The coverslips were washed (5 minute duration) once with cacodylate buffer and once with distilled H_2O. Macrophages were then stained with 0.5% aqueous uranyl acetate (Ernst Fullam) for 1 hour at room temperature. After one 5 minute distilled H_2O wash the macrophages were dehydrated through a graded series (50%, 70%, 95%, and 100%) of ethanol; each ethanol solution was left over the coverslips for 5 minutes, and the 100% ethanol step was repeated three times. The coverslips were quickly transferred to aluminum foil pans and were put through two 5 minute changes of propylene oxide (Polysciences, Inc.). The coverslips were then overlaid with a 1:1 mixture of Epon 812 (Electron Microscopy Sciences) and propylene oxide and left for 12-16 hours at room temperature. The Epon-propylene oxide infiltration mixture was poured from each foil pan and fresh Epon added for final embedding and polymerization at 60° for 48 hours.

Polymerized Epon wafers were removed from the foil pans and the coverslips peeled away, which left the macrophages behind in the embedding medium. Small semi-rectangular pieces were cut with a jigsaw from areas of the Epon which contained macrophages. These pieces were mounted on blank Epon blocks and sectioned (60-90 nm thick sections) with a diamond knife on a MT 5000 ultramicrotome (Sorvall, Dupont, Newtown, CT). Sections were picked up on 400 mesh

copper grids without support films (Ernst Fullam). The sections were stained with 5.0% aqueous uranyl acetate for 1 hour at room temperature and with 0.3-0.5% Pb-citrate (Ernst Fullam) in 0.1 N NaOH for 30 seconds at room temperature. Stained sections were examined using a JEOL 100 CX electron microscope (JEOL Ltd., Tokyo) operating at 60 kv.

Statistical Analysis

Statistical analysis was performed using Student's two-tailed t-test for independent means and a one-way analysis of variance (ANOVA) for independent means from more than two sample populations. Data are expressed as the mean ± 2 standard errors of the mean. Attained levels of significance, p values, were considered statistically significant when $p < 0.05$.

RESULTS

Configuration of the In Vitro Salmonella Infection Assay

I. Assessment of the cellular composition of resident adherent macrophages at the time of infection

The methods used for peritoneal lavage and for peritoneal lavage fluid collection yielded from 8 X 10^5 to 1 X 10^6 resident peritoneal macrophages per mouse. This range did not vary among the several mouse strains examined. Table 2 shows the percentages of specific cell types present in resident peritoneal cell suspensions from seven of these mouse strains. The percentages of macrophages and of lymphocytes are expressed both in relationship to the total number of peritoneal cells counted and to the total number of white cells counted. Each strain represents the pooled peritoneal cells collected and resuspended from at least 10 mice. The relative percentages of macrophages to lymphocytes is in agreement with percentages expected from the non-stimulated murine peritoneal cavity (Daems, 1980).

To determine the number of plastic-adherent resident peritoneal macrophages that would be present at the time of S. typhimurium infection, a solution that contained the cationic anesthetic lidocaine (10.0 mg/ml lidocaine with 0.05% trypan blue and 50.0 µg/ml gentamicin

Table 2. CELL TYPES PRESENT IN RESIDENT PERITONEAL CELL SUSPENSIONS [a].

Mouse Strain (n)[b].	Macrophages and Monocytes[c].	Lymphocytes	Polymorphonuclear Neutrophils	Basophils	Red Blood Cells
SWR/J (10)	61.8 [75.4][d].	17.4 [20.0][e].	0.6	2.2	18.0
C3H/HeJ (15)	60.0 [68.8]	24.0 [27.5]	0.8	2.4	12.8
C3H/HeN (15)	48.4 [67.4]	20.2 [28.1]	1.2	2.0	28.2
C57BL/6J (15)	54.0 [69.2]	21.0 [26.9]	1.0	2.0	22.0
DBA/2N (10)	59.6 [74.9]	17.8 [22.4]	0.8	1.4	20.4
BALB/cᵑ (10)	59.0 [69.2]	24.0 [28.2]	1.0	1.2	16.8
C.D2Ity^r (10)	58.0 [72.1]	20.0 [24.9]	1.0	1.4	19.6

a. Resident peritoneal cells from several mouse strains were collected and resuspended in adherence medium. An aliquot of each strain's peritoneal cell suspension was used to prepare cytospin specimens for differential staining. A total of 500 cells per strain was examined microscopically. The percentage of each cell type in the total cells counted is shown.
b. (n) The number of mice of each strain pooled for each peritoneal lavage fluid.
c. It should be noted that it is technically difficult to defferentiate large lymphocytes from monocytes in differential microscopic preparations.
d. [ ] The percentage of macrophages and monocytes in the total number of white cells, counted.
e. [ ] The percentage of lymphocytes in the total number of white cells, counted.

in Ca^{++}, Mg^{++}-free PBS) was used to release macrophages from culture dish wells. The efficiency of this lidocaine solution and the optimal incubation time for harvesting cultured resident peritoneal macrophages after exposure to the anesthetic were determined in the following study. Resident peritoneal macrophages from SWR/J (Ity^r) and BALB/c (Ity^s) mice were seeded at 2.6 X 10^5 macrophages per well. The macrophage cultures were processed (Method 1) up to the point of Salmonella infection. At that time, the macrophage cultures were overlaid with 500 µl/well of the lidocaine solution, and incubated at 37° C (5% CO_2 and 95% air). The number of macrophages released from the plastic, after exposure to the lidocaine solution for 1, 4, or 24 hours, was then determined. In addition, the viability of all macrophage cultures was evaluated by scoring, within the first 5 minutes after lidocaine solution exposure, at least 200 cells per well for trypan blue exclusion. The average number of macrophages released per well at the different incubation times is shown in Table 3. One-way ANOVA showed no statistically significant ($p < 0.05$) difference in the number of BALB/cn macrophages counted per well at t_o and a statistically insignificant ($p = 0.05$) difference in the number of SWR/J macrophages counted per well at t_o. Because the number of released SWR/J macrophages at 4 hours and at 24 hours of incubation with the lidocaine solution was higher than at 1 hour, it appeared that the macrophages of the SWR/J strain may have been relatively more adherent at 1 hour than those of the

Table 3. EFFECT OF INCUBATION TIME ON THE NUMBER OF LIDOCAINE-RELEASED RESIDENT PERITONEAL MACROPHAGES [a].

Time of Incubation	SWR/J[b]	BALB/cπ
1 hour	77,667 ± 7,512	65,333 ± 23,842
4 hours	104,000 ± 15,144	71,000 ± 15,875
24 hours	113,677 ± 22,696	62,000 ± 15,100

a. Resident peritoneal macrophages from SWR/J and from BALB/cπ mice were seeded into 16 mm culture dish wells. After the overnight incubation and basal medium wash, 500 µl/well of a 10 mg/ml lidocaine with 50 µg/ml gentamicin and 0.05% trypan blue in Ca++,Mg++-free PBS solution was added to all macrophage cultures. The number of lidocaine released adherent macrophages per well was determined after 1, 4, and 24 hours of 37° C incubation.

b. Data for each mouse strain is presented as the average number of released macrophages ± 2 standard errors of the mean, n = 3.

BALB/cᵞ strain. The viability of the macrophages of both mouse strains was ≥ 95% by trypan blue exclusion. Based on the statistical analyses and the observation that macrophages which have ingested particles become more adherent than macrophages that have not ingested particles (Nathan, 1981), a 4 hour incubation period of adherent macrophages with the anesthetic solution was selected for use in all subsequent experiments.

The average percentage of the original number of macrophages seeded per well and adherent at the time of Salmonella infection was determined, as described above: for Method 1, data obtained using C3H/HeN, C57BL/6J, DBA/2N, BALB/cπ , C.D2Ity^r and F1 (DBA/2N X BALB/cπ) mouse strains were combined, and for Method 2, results obtained using C3H/HeN, C3H/HeJ, C57BL/6J, BALB/cπ, and F1 (DBA/N X DBA/2N) mouse strains were combined. The average percentage for Method 1 was 27.5 + 5.3% (mean ± standard deviation, n = 12) and for Method 2 was 85.4 ± 6% (n = 15). The Method 1 results were in agreement with reported percentages (25-33%) of adherent resident peritoneal macrophages from initial seeding suspensions (Herscowitz and Cole, 1981). Method 2 is original to this work, and no previously reported values were available for comparison.

From the results of these studies, the following parameters and methods were configured into the in vitro Salmonella-macrophage infection assay: 1., The percentage of adherent macrophages at the time of Salmonella infection would be taken as 30% (Method 1) or 80% (Method 2) of the

total number of macrophages seeded per cultured dish well. From these adherence value(s) the bacteria to macrophage infection ratio would be estimated. 2., The viability of Salmonella-infected and uninfected macrophage cultures would be determined by trypan blue exclusion in situ. 3., The lidocaine releasing solution technique would be used to count the number of adherent infected macrophages at the times of macrophage lysate preparation: the macrophage counts would be used in the assay to calculate a relative index of S. typhimurium infection, i.e., the number of cell-associated bacteria in macrophage lysates ÷ the number of adherent macrophages present at the time of macrophage lysate preparation.

II. Assesment of the survival of S. typhimurium in the macrophage lysing solution

To completely disrupt Salmonella-infected mouse peritoneal macrophages, the method of Hsu and Radcliffe (Hsu and Radcliffe, 1968) was used in the in vitro S. typhimurium infection assay. To establish that the 0.5% Na-desoxycholate in sterile normal saline lysing solution did not, by itself, diminish the viability of salmonellae, S. typhimurium strain TML was exposed, at room temperature, to the lysing solution or to normal sterile saline. The results of this exposure are presented in Table 4. Only at 0 minutes, the time of mixing of the S. typhimurium inocula with the different solutions, was there a statistically

Table 4. SURVIVAL OF S. TYPHIMURIUM IN 0.5% Na-DESOXYCHOLATE IN NORMAL SALINE [a].

Time of Exposure[b].	Normal Saline[c].	Normal Saline + 0.5% Na-Desoxycholate
0	49,500 ± 1,000	40,000 ± 3,999
5	48,500 ± 7,000	40,500 ± 9,000
15	54,500 ± 5,001	50,000 ± 0
30	50,000 ± 8,000	42,500 ± 5,001
60	48,600 ± 2,800	46,600 ± 6,001
90	55,000 ± 3,000	59,000 ± 21,000

a. Samples of duplicate suspensions of 5.0×10^4 S. typhimurium strain TML in normal saline or in 0.5% Na-desoxycholate in normal saline were removed at the times indicated, and the number of colony forming units were determined. All solutions were held at room temperature.
b. Time in minutes.
c. Colony forming units of S. typhimurium expressed as the average + 2 standard errors of the mean, n = 2.

significant ($p < 0.05$) difference between the normal saline controls and the 0.5% Na-desoxycholate lysing solution. From 5 minutes to 90 minutes of exposure of salmonellae to the lysing solution there were no statistically significant ($p > 0.05$) differences. The initial difference at 0 minutes was interpreted as a function of mixing the salmonellae inocula with the test solutions. These results indicated that exposure of S. typhimurium strain TML to a 0.5% Na-desoxycholate in normal saline solution for as long as 90 minutes did not diminish the viability of the bacteria.

III. Evaluation of the kinetics of killing of S. typhimurium by gentamicin suspended in macrophage culture medium

To demonstrate in vitro a differential, intracellular expression of the Ity^r and the Ity^s alleles by S. typhimurium-infected resident peritoneal macrophages, it was necessary to control any extracellular bacterial multiplication. Preliminary attempts to accomplish this by frequent washing of infected macrophages and by culture medium replacement proved inadequate. The aminoglycoside antibiotic (Davis, 1980) gentamicin was, therefore, incorporated into the infected macrophage culture medium (maintenance medium). The concentration of antibiotic required to prevent any replication of S. typhimurium strain TML was based upon the MBC (as described in Methods

and Materials). Gentamicin has been shown to be bactericidal for species of Enterobacteriaceae regardless of the growth phase of the bacterial culture (Rubenis et al., 1963). The validity of these previous findings was tested for S. typhimurium strain TML exposed to gentamicin under the conditions used in the in vitro macrophage assay. Approximately 1 X 10^5 S. typhimurium strain TML, from a stationary phase Penassay broth culture, was added to a solution of basal medium with 5% FBS, and gentamicin was then added to a final concentration of 5.0 µg/ml. This final solution was dispensed into the 16 mm wells of a culture dish and incubated at 37° C with 5% CO_2 and 95% air. The number of viable salmonellae in duplicate samples of the medium solution prior to and immediately following the addition of the antibiotic and from culture dish wells at various times of incubation was determined by plate count. The results of this procedure are shown in Figure 4. The number of viable S. typhimurium was observed to drop from the initial count by nearly 1.5 logs within 15 minutes of incubation with the gentamicin solution and by 3.3 logs after 30 minutes of antibiotic exposure. The number of viable salmonellae at each time point thereafter, over a 48 hour period, was below the sensitivity of the plate count procedure ($\leq$ 5 cfu). This rapid and sustained killing of S. typhimurium was considered to be sufficient to prevent any significant extracellular replication of bacteria-infected macrophage cultures.

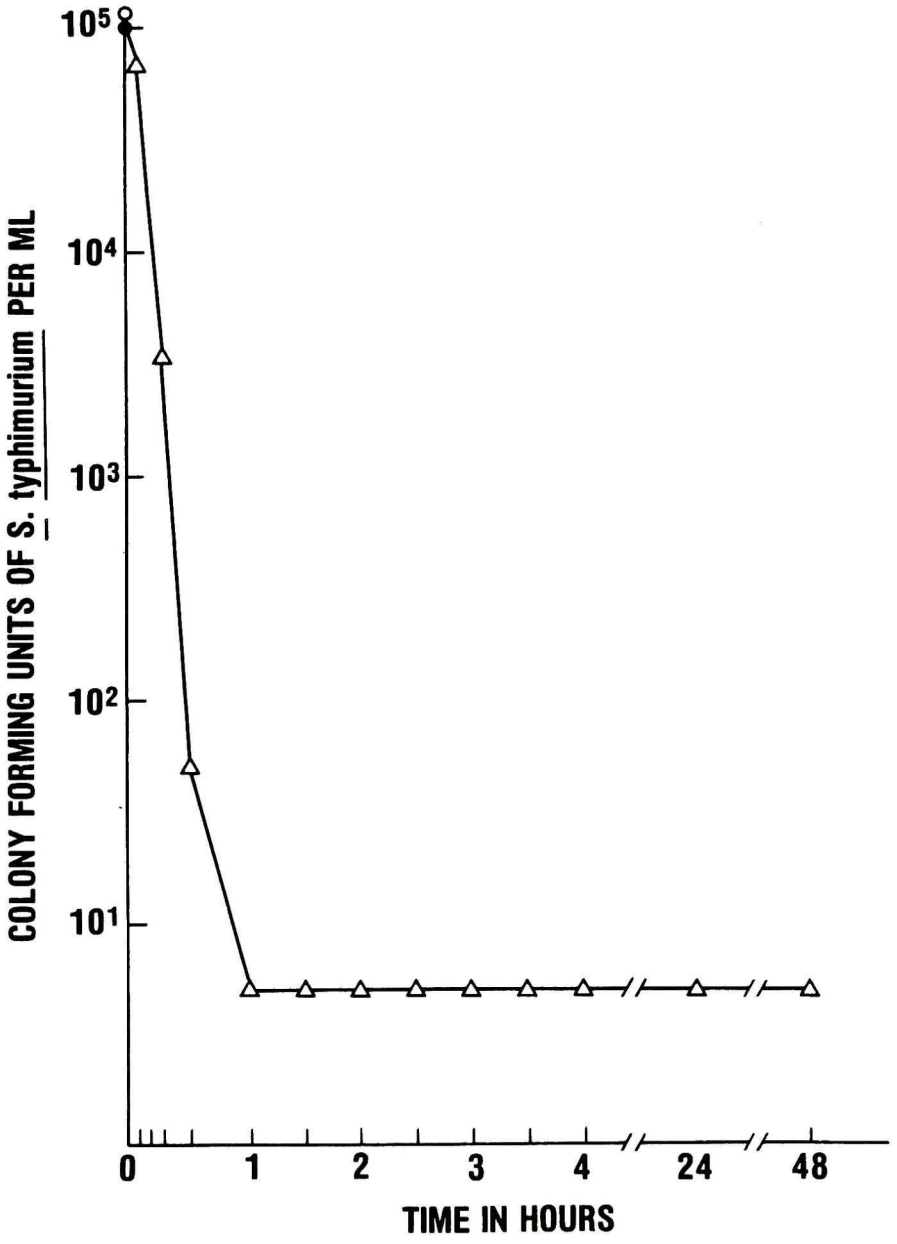


Figure 4. The bactericidal activity of 5.0 ug/ml of gentamicin on S. typhimurium strain TML, suspended in basal medium + 5% FBS. o average of duplicate viable counts prior to antibiotic addition; • average of duplicate viable counts immediately following antibiotic addition; ▵ average of the viable counts from duplicate dulture dish wells. From 1 hour through 45 hours of incubation the number of colony forming units was < 5.

IV. Establishment of conditions for the opsonization of S. typhimurium, the time of infection of peritoneal macrophages, and the ratio of infecting salmonellae to peritoneal macrophages

Previous in vitro studies had used several different sources and concentrations of serum for opsonizing salmonellae prior to infecting murine mononuclear phagocytes. Some of these experimental modalites were: 10% heat inactivated fetal calf serum (Furness, 1956: Furness and Ferreira, 1959), heat inactivated normal or anti-Salmonella mouse serum (Rowley and Whitby, 1959), 29% normal or anti-Salmonella rabbit serum (used for in vitro infection of rabbit macrophages) (Gelzer and Suter, 1959), 2% normal or anti-Salmonella serum (Mitshuhashi et al., 1960), 50% normal or anti-Salmonella serum (Jenkin and Benacerraf, 1960), 20% normal horse serum and 20% anti-Salmonella rabbit serum (Morello and Baker, 1965), 20% normal and anti-Salmonella mouse serum (Blanden et al., 1965), 17% normal guinea pig serum (used for in vitro infection of guinea pig macrophages) (Hsu and Radcliffe, 1968), 10% normal mouse serum or 10% rabbit anti-Salmonella serum (Maier and Oels, 1972), 20% normal mouse serum or 13% anti-Salmonella mouse serum (Blumenstock and Jann, 1981), and 20% normal or anti-Salmonella mouse serum (Tomita et al., 1981). The time intervals for opsonization in these studies ranged from 30 minutes (Blumenstock and Jann, 1981) to 90 minutes (Furness, 1956).

With these previous studies in mind and with the specific aim of paralleling, *in vitro*, the early innate *in vivo* interaction of macrophages of the murine host with *S. typhimurium*, a 10% normal mouse serum in basal medium solution was chosen for opsonizing *Salmonella*. Thirty minutes at 37° C were selected as the time and the temperature of opsonization. Because normal mouse serum does not kill *S. typhimurium* (Muschel and Muto, 1956) and because the LPS of *Salmonella* can activate complement via the alternative pathway (Liang-Takasaki *et al.*, 1982; Liang-Takasaki *et al.*, 1983), it was anticipated that this opsonization method would not affect the viability of the salmonellae inocula, but would facilitate phagocytosis of salmonellae by murine resident peritoneal macrophages.

An infection period of 50 minutes (time of exposure of opsonized salmonellae to macrophages) was initially selected for use in the studies presented here. This selection represented a compromise between the lengths of infection periods previously reported in the literature (5 to 90 minutes).

To ascertain the efficacy of both the opsonization method and the infection time interval and to determine what infection ratio of *S. typhimurium* to peritoneal macrophages would permit a clear *in vitro* differentiation between the Ity^r and Ity^s phenotypes, resident peritoneal macrophages from C3H/HeN (Ity^r) and from C57BL/6J (Ity^s) mice were collected and cultured (Method 1). Macrophages of each mouse strain were infected for 50

minutes at 37° C with three different concentrations of S. typhimurium strain TML suspended in 10% normal mouse serum. The final infection ratios of bacteria to macrophages were approximately 3:1, 30:1, and 300:1. Macrophage lysates were then prepared in each experiment at t_o and at 24 hours post infection, and the number of colony forming units of S. typhimurium in macrophage lysates quantified. The number of culture dish wells required to examine each mouse strain at three different infection ratios necessitated performing two separate experiments. Interpretation of these preliminary findings was, therefore, specific for intra-strain differences and inferentially general for inter-strain differences. The results of these in vitro infections are shown in Table 5.

C3H/HeN macrophages infected at a ratio of 30:1 showed a statistically significant ($p < 0.05$) decline in the number of viable S. typhimurium in macrophage lysates. At the ratio of 3:1 no significant ($p > 0.05$) difference was found, and at the ratio of 300:1 a statistically significant ($p < 0.05$) increase of 1.3 fold in the number of viable S. typhimurium was observed. Although no other times within the t_o to 24 hours interval were sampled, the data obtained suggested that at the infection ratios of 3:1 and 30:1 the resistant Ity^r phenotype of the C3H/HeN strain could be discerned. The significant increase at 24 hours with the ratio of 300:1 may reflect an inoculum in excess of the bacterial concentration at which C3H/HeN macrophages are able to control the net multiplication

Table 5. EFFECT OF DIFFERENT INFECTION RATIOS ON THE NUMBER OF S. TYPHIMURIUM IN RESIDENT PERITONEAL MACROPHAGE LYSATES [a].

Experiment	Strain	Infection Ratio	Time[b]	S. typhimurium cfu/well[c]
1	C3H/HeN	3:1	0	275 ± 150
		3:1	24	103 ± 26
		30:1	0	3,125 ± 530
		30:1	24	1,875 ± 689*
		300:1	0	46,125 ± 3,591
		300:1	24	60,000 ± 7,000*
2	C57BL/6J	3:1	0	138 ± 48
		3:1	24	115 ± 65
		30:1	0	1,688 ± 394
		30:1	24	3,563 ± 823*
		300:1	0	27,000 ± 18,628
		300:1	24	23,125 ± 2,529

a. Resident peritoneal macrophages from C3H/HeN (Ity^r), Experiment 1, and from C57BL/6J (Ity^s), Experiment 2, mouse strains were infected in vitro at three different infection ratios (bacteria: macrophages) with S. typhimurium strain TML.

b. Time in hours after the 50 minute infection period at which macrophage lysates were prepared and the number of viable cell-associated salmonellae determined.

c. The average number of viable cell-associated S. typhimurium per well ± 2 standard errors of the mean, n = 4.

* Significantly ($p < 0.05$) different, by Student's two-tailed t-test from corresponding 0 hour value.

of S. typhimurium: a situation analogous to the capacity to kill an Ity^r animal with a dose of S. typhimurium in excess of the LD_{50}.

C57BL/6J macrophages infected at a ratio of 30:1 showed a statistically significant ($p < 0.05$) increase of 2.1 fold in the number of viable S. typhimurium in macrophage lysates. At the ratios of 3:1 and of 300:1 no statistically significant ($p > 0.05$) difference in the number of viable salmonellae was observed. The significant rise at the ratio of 30:1 suggested that the susceptible Ity^s phenotype was discernable at this infection ratio. Light microscopy done in parallel with lysate preparations provided a possible explanation for the anomalous observation of no difference in the number of viable salmonellae in C57BL/6J macrophage lysates at the infection ratio of 300:1. Macrophage cultures on glass coverslips stained after 24 hours of infection showed only rare intact macrophages surrounded by "ghost-like" destroyed cells. This finding was consistent with a scenario in which the uncontrolled replication of S. typhimurium within Ity^s macrophages led to a physical disruption of these cells and to the consequent exposure of intracellular salmonellae to the gentamicin in the maintenance medium. Such an exposure could artifactually lower the number of viable cell-associated Salmonella. From these and the aforementioned results, it was concluded that the following parameters would be used in the initial in vitro macrophage assay experiments: opsonization of S.

typhimurium in 10% normal mouse serum; a 50 minute infection period; a bacteria to macrophage ratio of 10:1 (based upon a value within the 3:1 to 30:1 infection ratios tested).

V. Implementation of the in vitro assay to monitor the fate of S. typhimurium in resident peritoneal macrophages

Based on the results of the developmental experiments described above, resident peritoneal macrophages from C57BL/6J (Ity^S) mice were cultured (Method 1) and infected in vitro with S. typhimurium strain TML. Macrophage lysates were prepared at t_o and at 4 and 24 hours post infection and the average number of adherent macrophages and percentage of viable macrophages per well at each time of macrophage lysate preparation were determined. The results of this experiment are shown in Figure 5.

During the first 4 hours of the in vitro infection a 90% decrease ($p < 0.05$) occurred in the number of viable cell-associated S. typhimurium. Over the next 20 hours of the infection, the number of viable cell-associated S. typhimurium increased 5.7 fold ($p < 0.05$). The viability of infected macrophages was $\geq$ 95% at all time points. These results indicated that the Ity^S macrophages were capable of killing a significant proportion of the initial number of S. typhimurium taken up at t_o, and that,

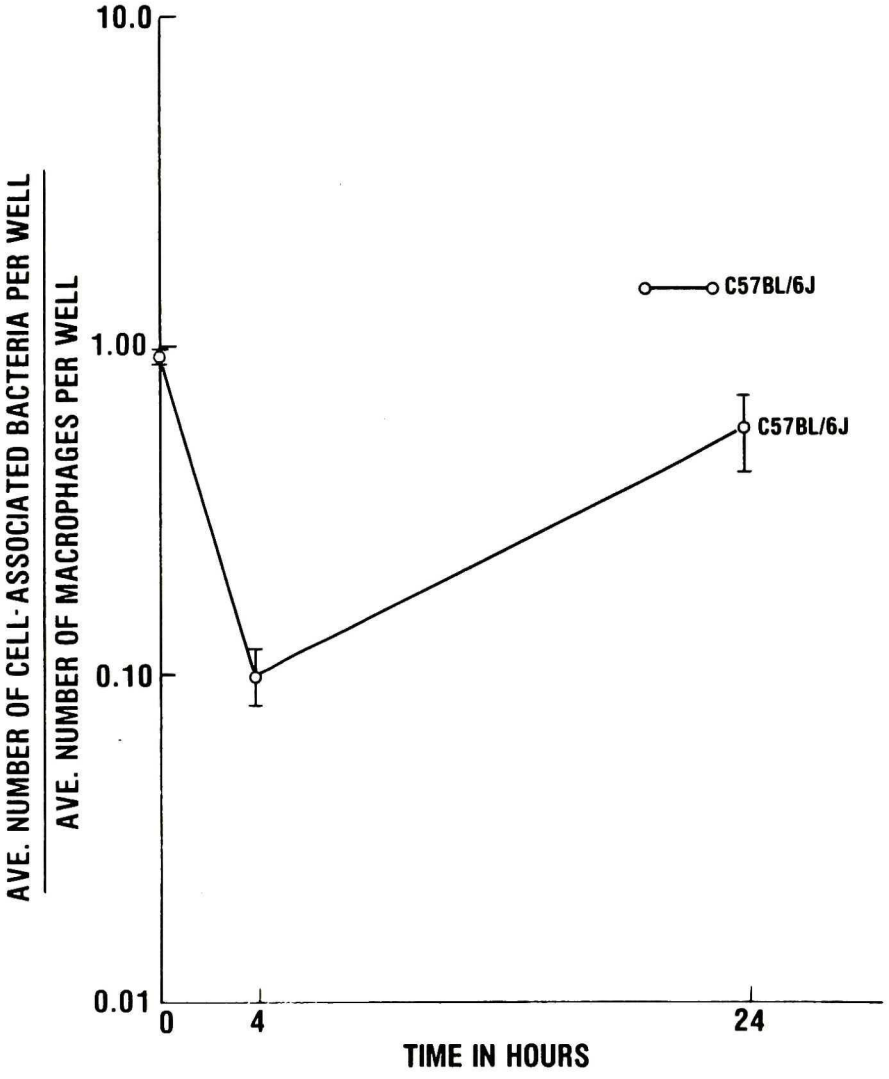


Figure 5. Resident peritoneal macrophages from C57BL/6J mice were infected in vitro with S. typhimurium strain TML. Macrophage lysates were prepared at 0, 4, and 24 hours after infection and the number of viable salmonellae determined. The results are expressed as the number of viable cell-associated bacteria ÷ the number of macrophages counted. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

after 4 hours of infection, the intracellular salmonellae were able to replicate. Furthermore, the *in vitro* assay parameters used in the configuration of this experiment made it possible to establish an *S. typhimurium* infection of murine resident peritoneal macrophages and to quantitatively follow, over a 24 hour period, the fate (bacterium-macrophage interaction) of intracellular *S. typhimurium*. The determination of the number of adherent macrophages at the time of lysate preparation was an effective index of the *in vitro S. typhimurium* infection.

VI. Examination of the effect of the 0.5% Na-desoxycholate macrophage lysing solution on the viability of *S. typhimurium* released from the disrupted phagocytes

The 0.5% Na-desoxycholate in normal saline solution, used to prepare macrophage lysates, was shown (Table 4) to have no effect on the viability of *S. typhimurium* strain TML suspended in this solution. However, Hsu and Mayo had reported that this lysing solution artificially lowered the number of viable salmonellae early in the *in vitro* infection of guinea pig macrophages (Hsu and Mayo, 1973). To assess whether the number of viable cell-associated salmonellae recovered from infected macrophages was affected by the Na-desoxycholate lysing solution, an *in vitro* assay was performed in which *Salmonella*-infected resident peritoneal macrophages from

SWR/J (Ity^r) and BALB/cn (Ity^s) mice were lysed as usual or by exposure, for 15 minutes, to a 0.01% bovine serum albumin (BSA) in sterile distilled H_2O solution, a modification of the method of Leijh et al. (Leijh et al., 1979). A preliminary experiment (data not shown) had demonstrated that there was no difference, over a 90 minute observation period, between the viable counts of S. typhimurium strain TML suspended in normal saline or suspended in 0.01% BSA in H_2O. The effects of the two macrophage disrupting solutions on the number of viable cell-associated S. typhimurium per µg of macrophage lysate protein (protein determinations were based on values obtained by Lowry analysis of Na-desoxycholate lysates) are compared in Figure 6.

The average number of viable salmonellae in Na-desoxycholate lysates of SWR/J macrophages at t_o was 1.9 times higher than that for 0.01% BSA lysates ($p < 0.05$). At 90 minutes and at 4.5 hours after infection no statistically significant ($p > 0.05$) differences were found between SWR/J macrophages lysates prepared by either of the two lysing techniques. At t_o and at 90 minutes and 4.5 hours after infection no statistically significant ($p > 0.05$) differences were found among the average number of viable cell-associated salmonellae in BALB/cn macrophage lysates prepared by either of the two lysing techniques.

These results obtained by comparing the two lysing techniques showed that, with the exception of the t_o point for SWR/J cells at which the desoxycholate values

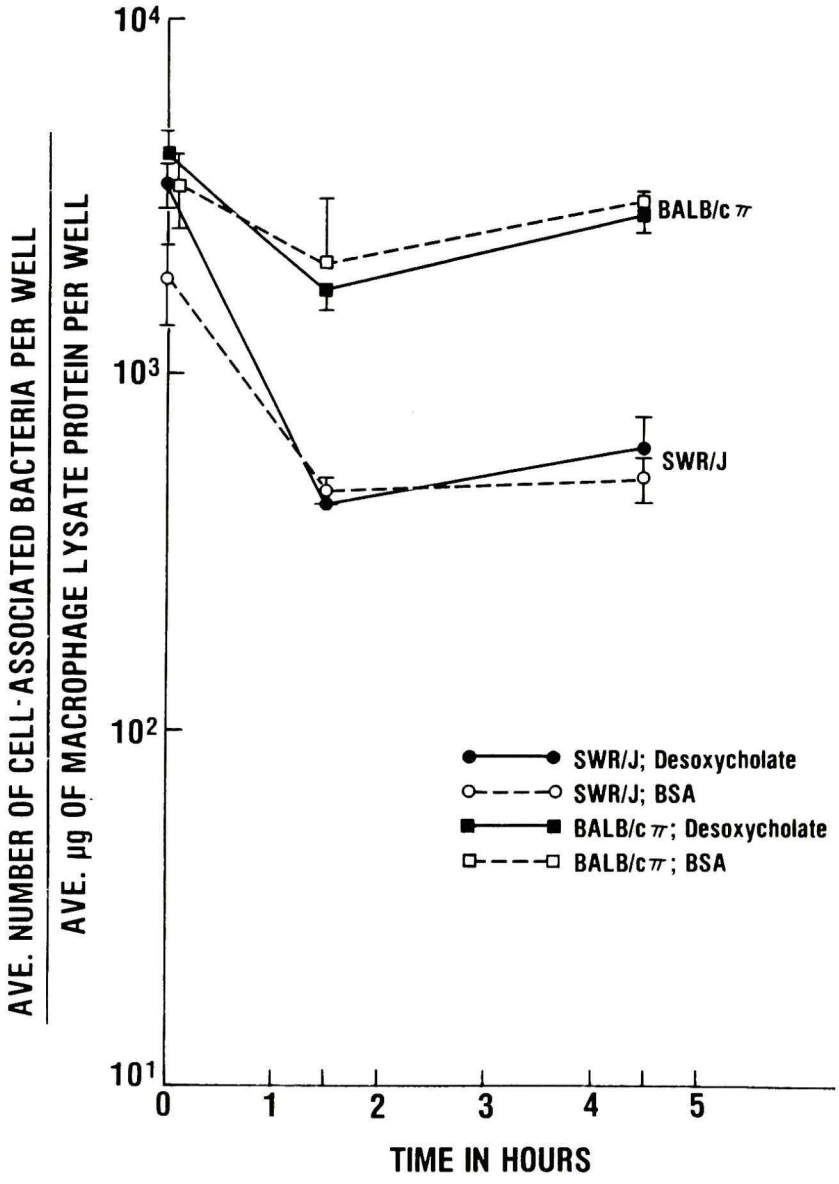


Figure 6. Resident peritoneal macrophages from SWR/J and from BALB/cπ mice were infected in vitro with S. typhimurium strain TML. The number of viable cell-associated salmonellae per µg of macrophage lysate protein was determined after lysate preparation with either 0.5% Na-desoxycholate or 0.01% BSA in H_2O at 0, 90 minutes, and 4.5 hours after infection. The results are expressed as the number of cell-associated bacteria ÷ the µg of macrophage lysate protein. Each point represents the average of 3 culture dish wells ± the standard deviation.

were actually higher, the two lysing techniques gave statistically indistinguishable bacterial counts. These results indicated that in our hands the rapid and efficient Na-desoxycholate lysing technique (Hsu and Radcliffe, 1968) was as valid as a more "gentle" method of macrophage disruption.

VII. Comparison of gentamicin concentrations in lysates of *S. typhimurium*-infected and uninfected macrophages

Because other investigators had found that the aminoglycoside antibiotic streptomycin could be taken up by murine macrophages cultured for several days (Bonventre and Imhoff, 1970) and, hence, could potentially affect the enumeration of viable intracellular salmonellae (Jenkin and Benacerraf, 1960; Morello and Baker, 1965), it was necessary to examine macrophage lysates, prepared in the *in vitro* assay, for the presence of gentamicin. The ideal method to detect uptake of gentamicin by cultured macrophages would have been to use radiolabeled gentamicin; however, during the course of these studies such a preparation was not commercially available. Therefore, a less direct radio-immunoassay (as described in Methods and Materials) was used to detect gentamicin in sonicates of infected and uninfected SWR/J (Ity^r) and BALB/cπ (Ity^s) resident peritoneal macrophages. The concentration of gentamicin, as quantified by RIA (detection limit 0.1 μg/ml), in SWR/J and BALB/cπ

macrophage sonicates at 4 hours and at 24 hours after S. typhimurium strain TML infection was ≤ 0.1 μg per 1 X 10^5 disrupted cells, ≤ 4.0 X 10^{-3} μg/μg of sonicate protein, or based on a single macrophage volume of 5.0 X 10^{-9} ml (Bonventre and Imhoff, 1970) ≤ 2.0 X 10^{-3} μg/macrophage. The same values were obtained for uninfected SWR/J and BALB/cn macrophage sonicates. Because the MBC of gentamicin for S. typhimurium strain TML was found to be 4.5 μg/ml, it was concluded that the gentamicin used to control extracellular replication in the infected macrophage cultures was probably not affecting Ity expression.

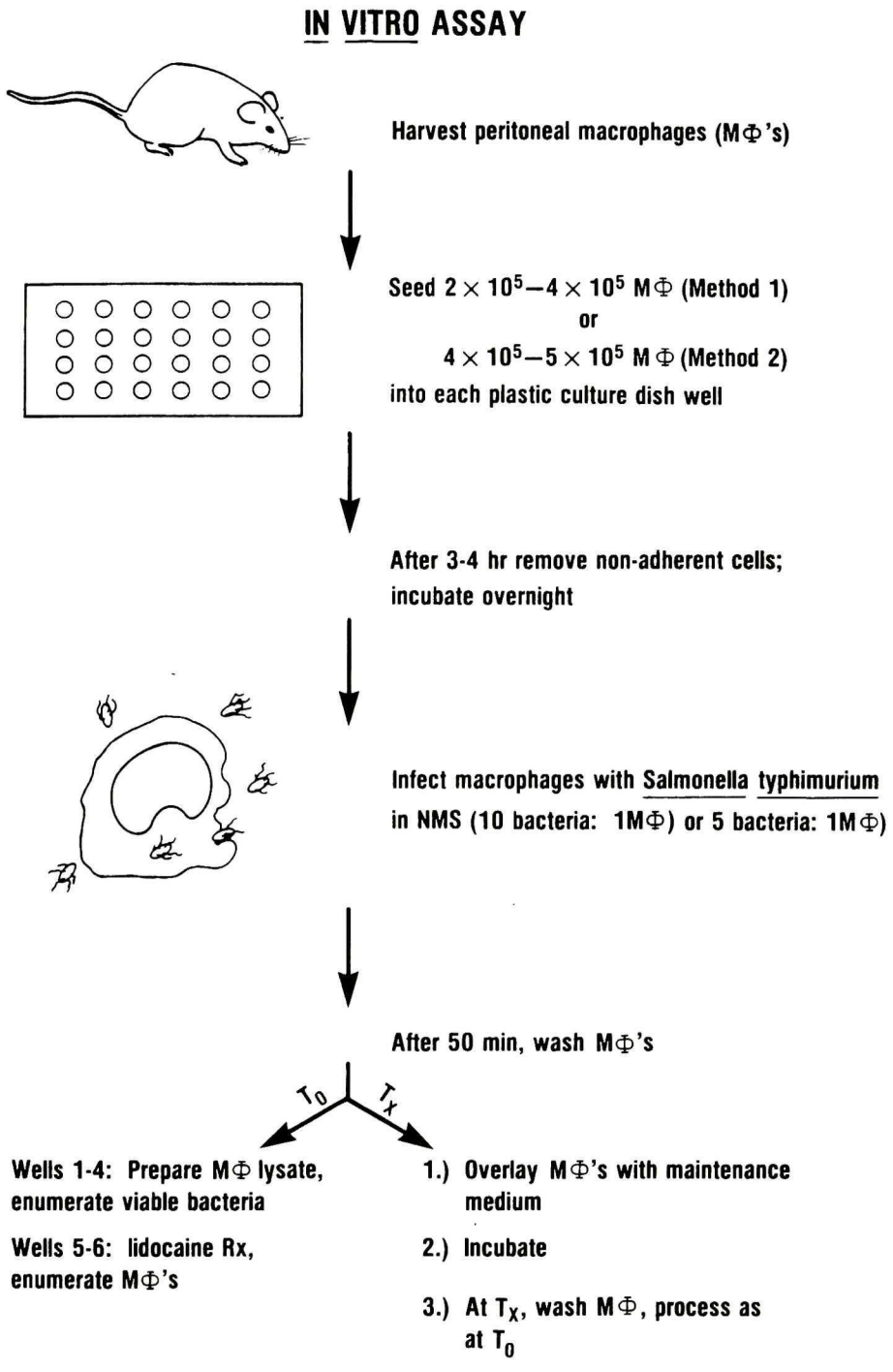
VIII. Establishment of the final configuration and parameters of the in vitro Salmonella-macrophage infection assay

From the results of preliminary experiments, such as those described above, the final configuration and parameters of the in vitro macrophage infection assay were established. The in vitro assay is diagramatically depicted in Figure 7.

Fate of Salmonella Typhimurium in Resident Peritoneal Macrophages of Ity^r and Ity^s Mice

The average number of viable S. typhimurium strain TML per resident peritoneal macrophage, cultured by Method

Figure 7.


IN VITRO ASSAY
Harvest peritoneal macrophages (MΦ's)
Seed 2 × 10⁵—4 × 10⁵ MΦ (Method 1)
or
4 × 10⁵—5 × 10⁵ MΦ (Method 2)
into each plastic culture dish well
After 3-4 hr remove non-adherent cells;
incubate overnight
Infect macrophages with Salmonella typhimurium
in NMS (10 bacteria: 1MΦ) or 5 bacteria: 1MΦ)
After 50 min, wash MΦ's
T0
Tx
1.) Wells 1-4: Prepare MΦ lysate,
enumerate viable bacteria
2.) Wells 5-6: lidocaine Rx,
enumerate MΦ's
1.) Overlay MΦ's with maintenance
medium
2.) Incubate
3.) At Tx, wash MΦ, process as
at T0

1, from two Ity^r and two Ity^s mouse strains was determined at 0, 4, and at 24 hours after infection. The results of this experiment are shown in Figure 8. During the first 4 hours of infection macrophages from all strains showed a decline in bacterial counts compared to t_o. Although the magnitude of this initial decline in the number of viable S. typhimurium varied with the experiment, bacterial counts at 4 hours were usually $\leq$ 40% of t_o. Thus, both Ity^r and Ity^s resident peritoneal macrophages were able to kill virulent S. typhimurium. Over the next 20 hours of infection viable counts of salmonellae in Ity^r peritoneal macrophages remained unchanged (C3H/HeN) or continued to decline (SWR/J); whereas, in Ity^s (BALB/cn and C57BL/6J) peritoneal macrophages the number of viable salmonellae increased significantly ($p < 0.05$) relative to 4 hours. The number of salmonellae present in culture fluids at t_o was generally 10% to 20% of the t_o lysate values; but, at all time points thereafter, this number dropped to $\leq$ 2% of lysate bacterial counts. The viability of both Ity^r and Ity^s macrophages was $\geq$ 95% throughout the in vitro assay as assessed by the exclusion of 0.05% trypan blue in the lidocaine solution used to release macrophages from the plastic.

Expression of Ity Phenotype In Vitro by BALB/cn and C.D2 Ity^r Macrophages

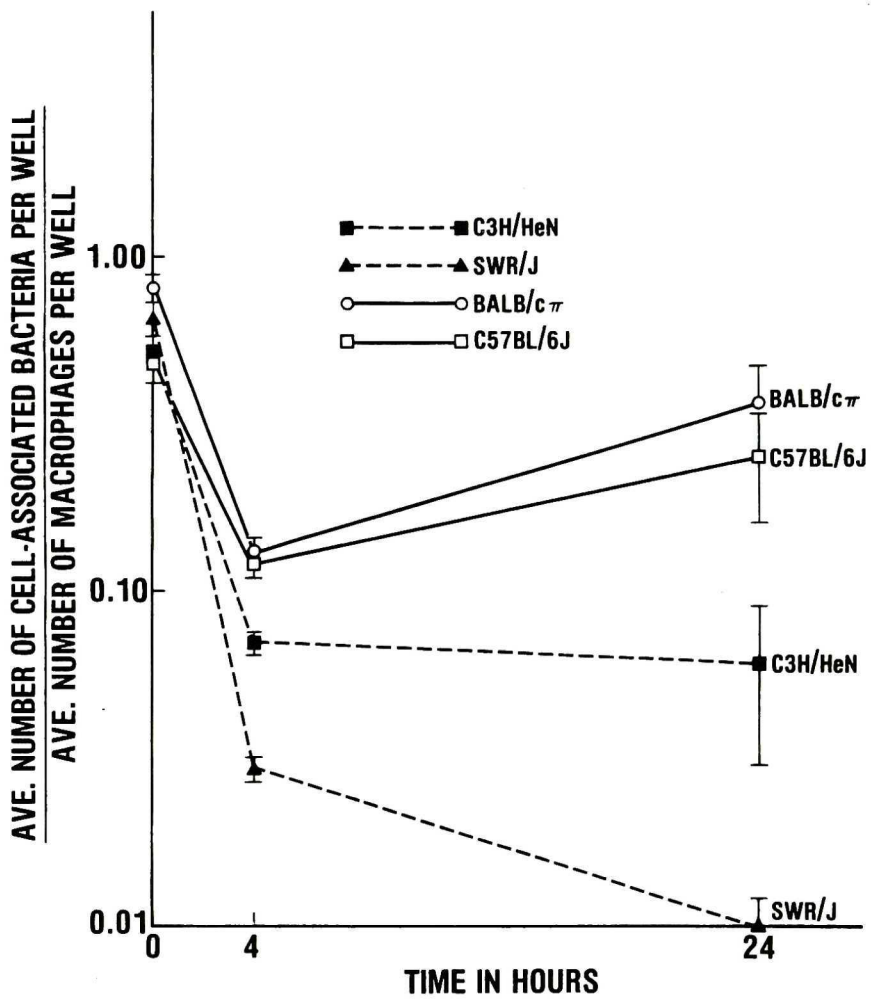


Figure 8. Resident peritoneal macrophages from Ity^r and Ity^s mice were infected *in vitro* with *S. typhimurium* strain TML. Macrophage lysates were prepared at 0, 4, and 24 hours after infection and the number of viable salmonellae was determined. The ordinate is a logarithmic scale. The results are expressed as the number of viable cell-associated bacteria ÷ the number of macrophages counted. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean. (from Lissner *et al.*, 1983)

I. Comparison of Method 1 and Method 2

To verify that the differential growth of S. typhimurium in macrophages from Ity^r and Ity^s mice was, in fact, due to differences only in the expression of Ity, the assay was performed with resident peritoneal macrophages, cultured by Method 1, from BALB/cπ and from congenic C.D2Ity^r mice. A typical experiment, in which a 1 hour time point was included to further characterize the initial phase of the in vitro infection, is shown in Figure 9. The same patterns of viable counts of S. typhimurium strain TML that distinguished inbred mouse strains of different Ity phenotypes were seen in mice congenic at the Ity locus. At t_o the ratio of the average number of viable salmonellae per macrophage for BALB/cπ to C.D2Ity^r was 1.2 ($p < 0.05$). At 1 hour there was no difference between the two strains ($p > 0.05$). By 4 hours the strains had significantly ($p < 0.05$) diverged from one another with an approximately 2 fold higher number of bacteria in BALB/cπ macrophages. Over the next 20 hours of infection the viable counts of S. typhimurium in BALB/cπ macrophages continued to rise significantly ($p < 0.05$). In contrast, the number of bacteria in C.D2Ity^r macrophages remained statistically unchanged ($p > 0.05$) from 4 hours to 24 hours. At the 24 hour time point the ratio of BALB/cπ to C.D2Ity^r viable salmonellae counts had risen to 5 fold higher in the Ity^s strain ($p < 0.05$).

The same experiment was performed with BALB/cπ and

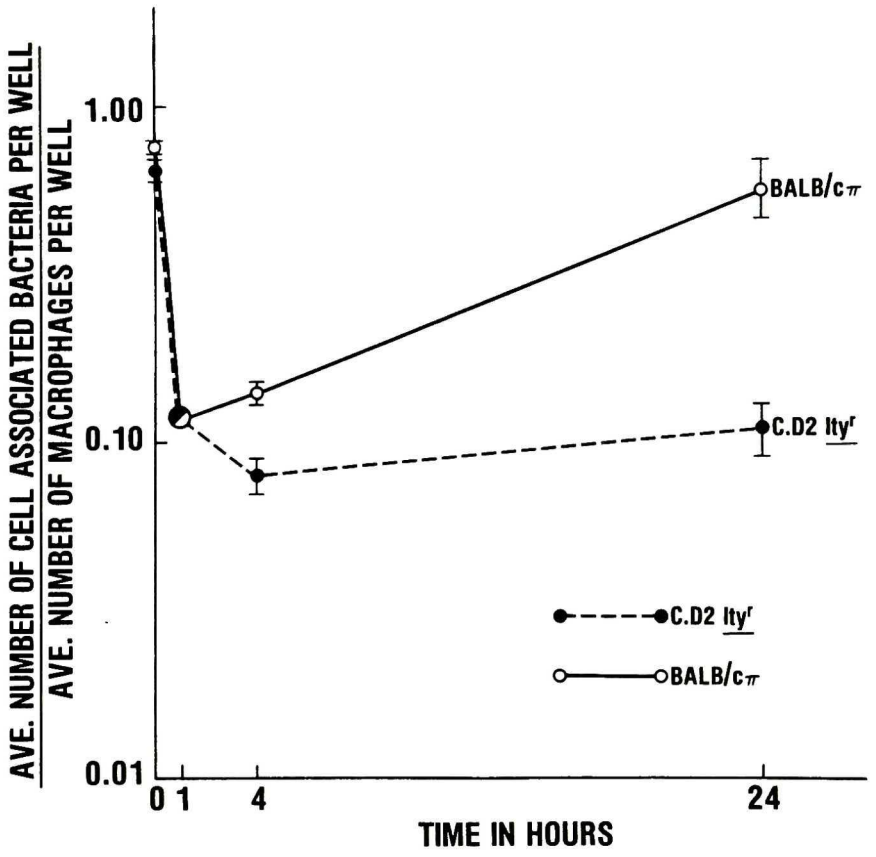


Figure 9. Resident peritoneal macrophages from congenic C.D2Ity^r and from BALB/cπ mice were infected in vitro with S. typhimurium strain TML and the number of viable bacteria in macrophage lysates prepared at 0, 1, 4, and 24 hours after infection was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean. (from Lissner et al., 1983).

congenic C.D2Ity^r macrophages seeded using Method 2 and infected with S. typhimurium strain TML. Based upon preliminary experiments (data not shown) an infection ratio of 5:1 was found to give "t_o" viable cell-associated bacterial values that approximated those obtained with Method 1 macrophage cultures. The results of this experiment are depicted in Figure 10. The expression of Ity phenotype was clearly discernable by 24 hours of S. typhimurium infection, which confirmed the result obtained with Method 1. Furthermore, the expression of Ity phenotype was evident whether the number of cell-associated bacteria were indexed to macrophage counts (Figure 10) or indexed to protein content of macrophage lysates (Figure 11).

An in vitro assay was performed to compare the intracellular fate of S. typhimurium in macrophages from the progenitors of the congenic mouse strain, i.e., BALB/cn and DBA/2N mice. Resident peritoneal macrophages were cultured (Method 2) and infected with S. typhimurium strain TML. The results of this experiment are shown in Figure 12. By 24 hours the mouse strains could be ranked by the number of viable intracellulr salmonellae: BALB/cn > DBA/2N > C.D2Ity^r. The findings with DBA/2N macrophages were intriguing. Although by the number of cell-associated salmonellae the DBA/2N strain was phenotypically Ity^r in relation to the BALB/cn (Ity^s) progenitor strain, the DBA/2N strain appeared by this index of infection to be less efficient than the congenic C.D2Ity^r strain in

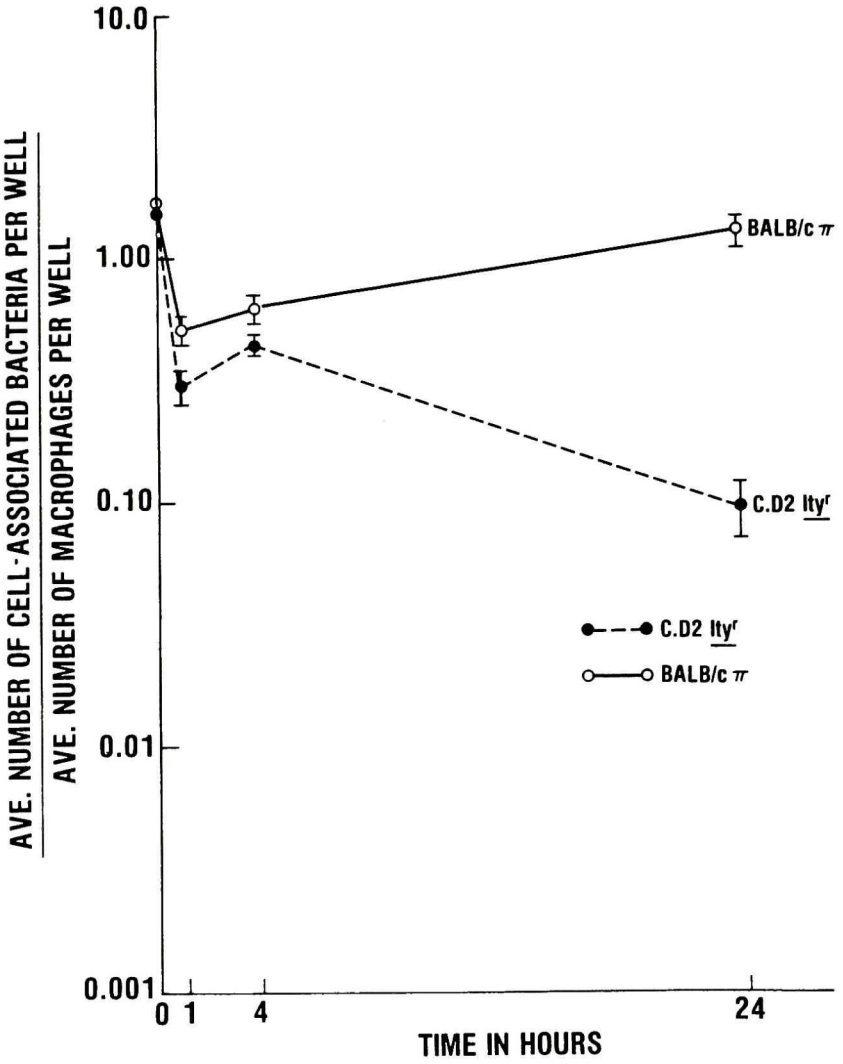


Figure 10. Resident peritoneal macrophages from congenic C.D2Ity^r and from BALB/cπ mice were infected in vitro with S. typhimurium strain TML, and the number of viable bacteria in macrophage lysates prepared at 0, 1, 4, and 24 hours after infection was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

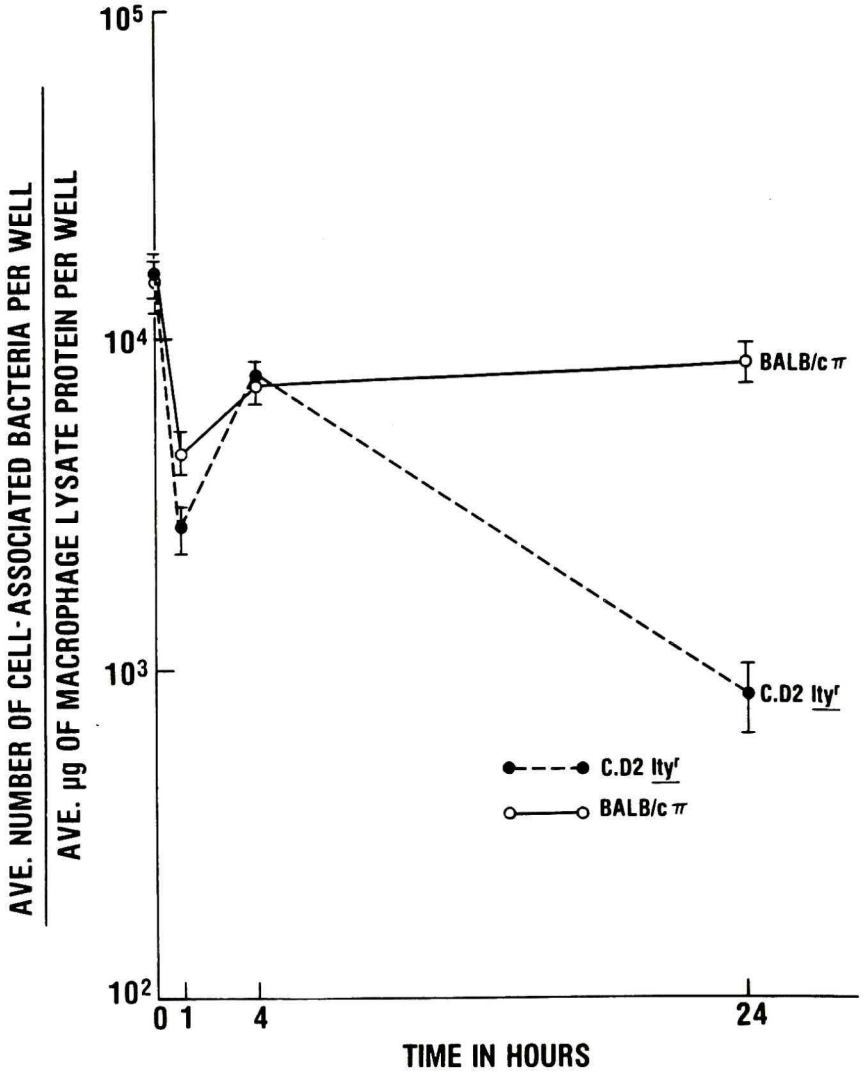


Figure 11. Resident peritoneal macrophages from congenic C.D2Ity^r and from BALB/cπ mice were infected *in vitro* with *S. typhimurium* strain TML. Macrophage lysates were prepared at 0, 1, 4, and 24 hours after infection and the number of viable salmonellae was determined. The results are expressed as the number of viable cell-associated bacteria ÷ the µg of macrophage lysate protein. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

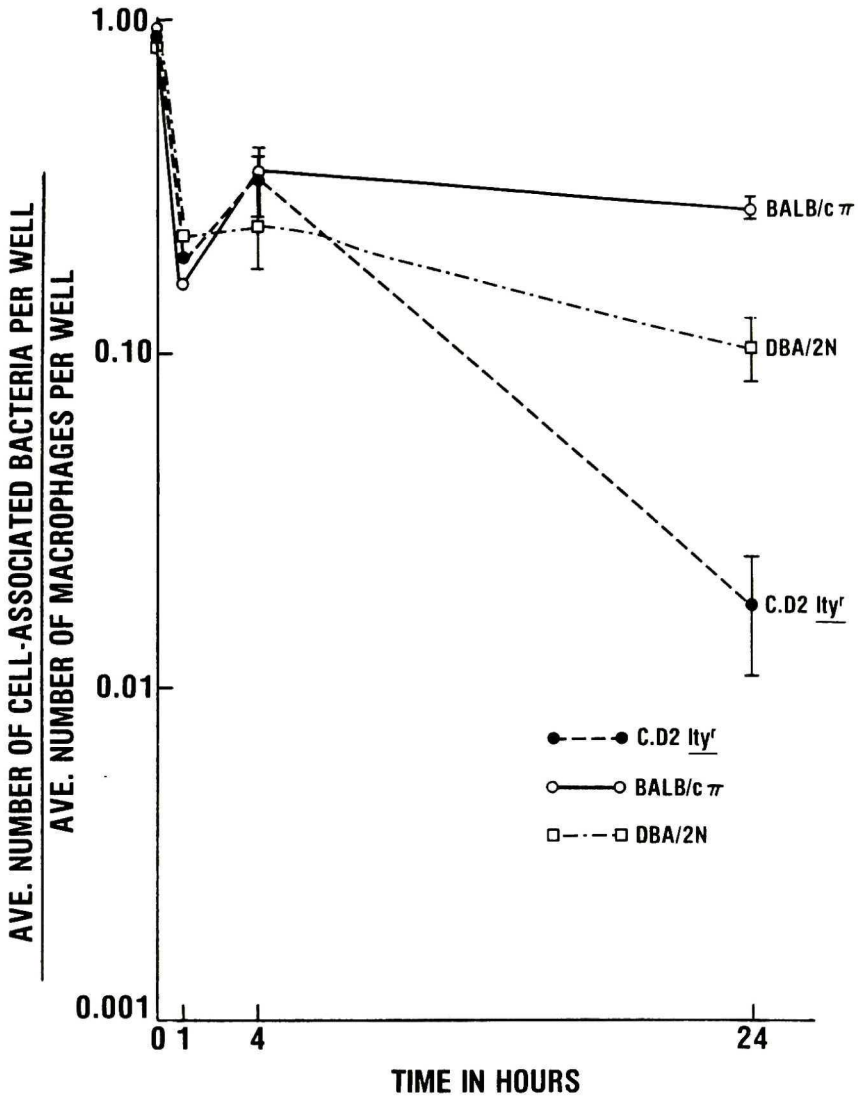


Figure 12. Resident peritoneal macrophages from BALB/c , DBA/2N, and congenic C.D2Ity^r mice were infected in vitro with S. typhimurium strain TML, and the number of viable bacteria in macrophage lysates prepared at 0, 1, 4, and 24 hours after infection was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells. Bars represent ± 2 standard errors of the mean.

lowering the number of viable salmonellae.

II. Comparison during the 50 minute infection period of the number of viable cell-associated S. typhimurium in BALB/cn and in congenic C.D2Ity^r macrophages

To examine the effect of Ity phenotype on phagocytosis of S. typhimurium during the 50 minute infection period of the in vitro assay, resident peritoneal macrophages from BALB/cn and from C.D2Ity^r mice were cultured (Method 2) and infected with S. typhimurium strain TML. The in vitro assay was performed as usual, except that infected macrophages were washed prior to lysate preparation with chilled, 4° C, PBS. The number of viable salmonellae in macrophage lysates was determined after 5, 15, 25, 35, and 50 minutes of infection. The results of this experiment are shown in Figure 13 and Figure 14. At 15 minutes and at 25 minutes there was a significant ($p < 0.05$) difference in the number of cell-associated salmonellae per macrophage counted between the Ity^s and Ity^r macrophages (Figure 13). When each strain was considered individually, BALB/cn macrophages appeared to reach a plateau by 25 minutes with no further significant ($p > 0.05$) increase in the number of viable salmonellae between the 25 to 50 minute interval. C.D2Ity^r macrophages reached a plateau by 15 minutes; however, a significant ($p < 0.05$) increase in the number of viable salmonellae did occur during the 35 to 50 minute

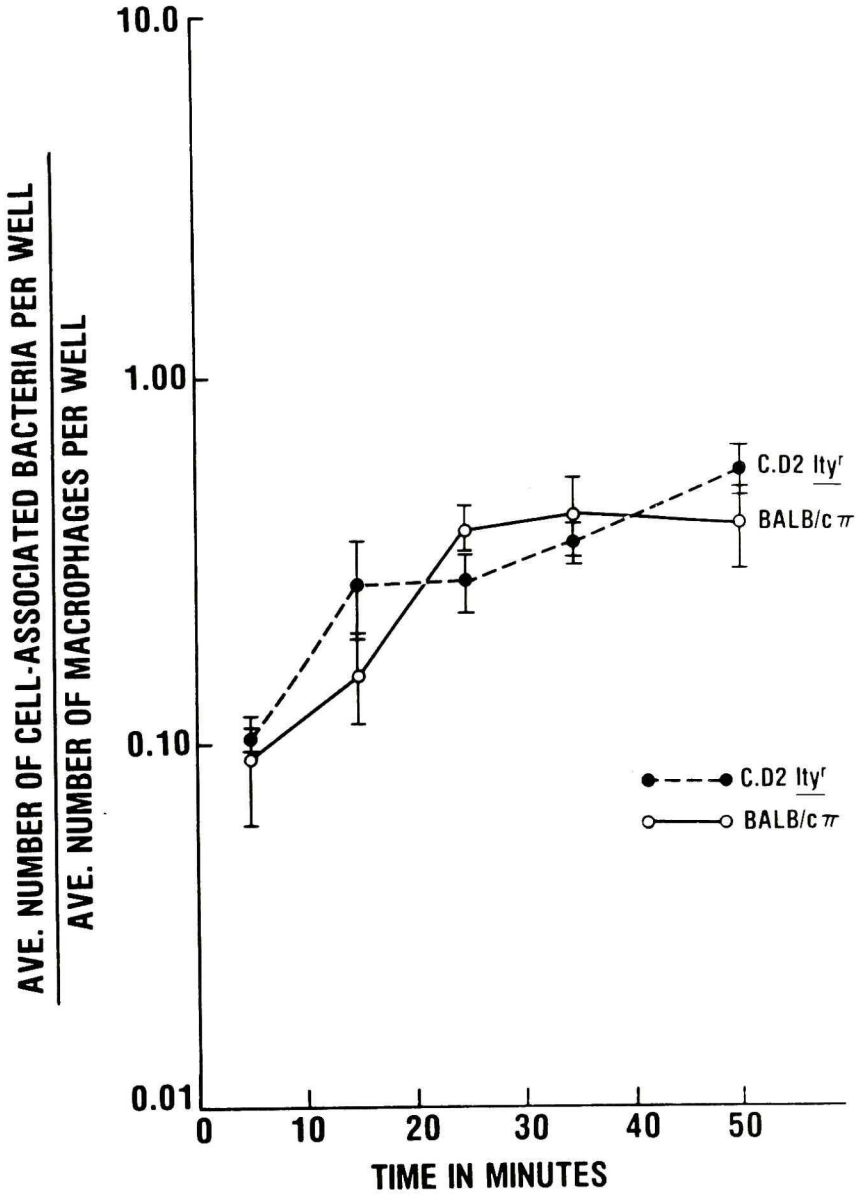


Figure 13. Resident peritoneal macrophages from congenic C.D2Ity^r and from BALB/cπ mice were infected in vitro with S. typhimurium strain TML, and the number of viable bacteria in macrophage lysates prepared after 5, 15, 25, 35 and 50 minutes of infection was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

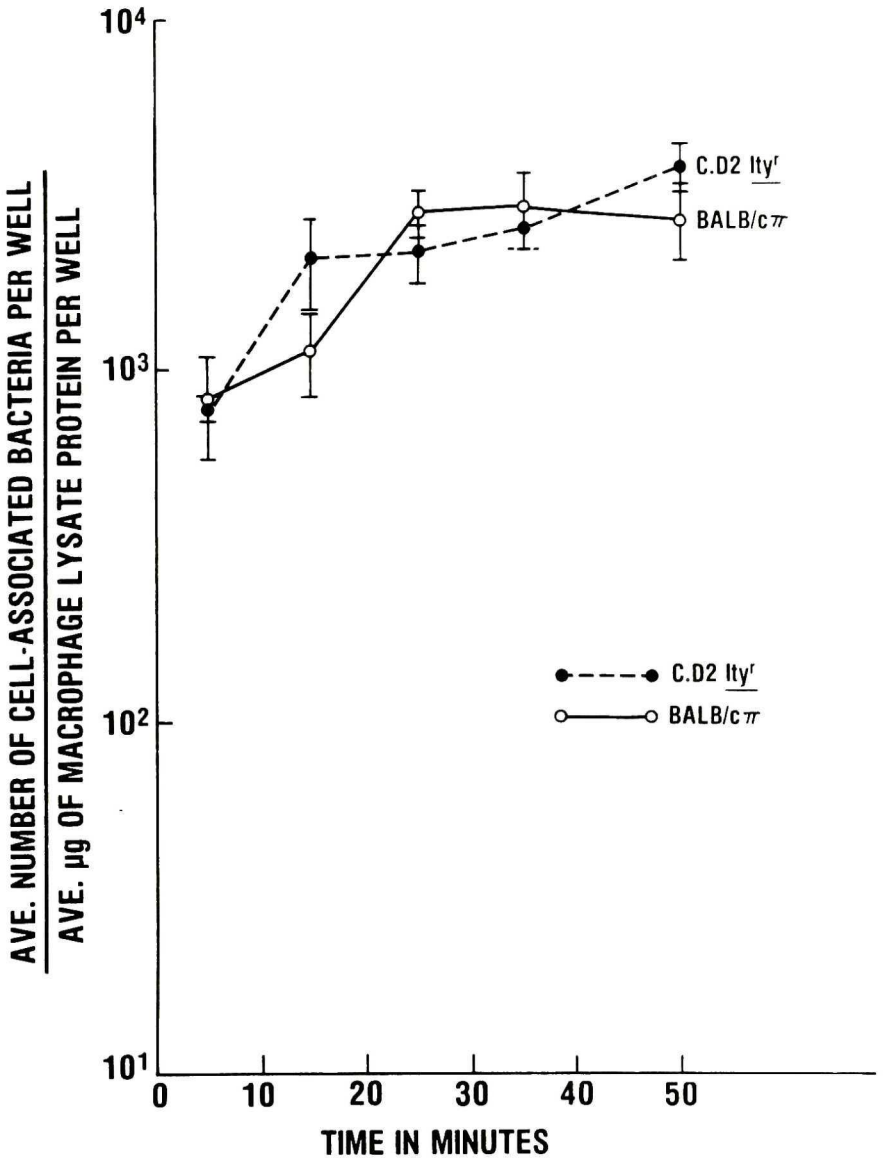


Figure 14. Resident peritoneal macrophages from congenic C.D2Ity^r and from BALB/cπ mice were infected in vitro with S. typhimurium strain TML, and the number of viable bacteria in macrophage lysates prepared after 5, 15, 25, 35 and 50 minutes of infection was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

interval. It should be noted that when the index of infection was the number of viable salmonellae per μg of lysate protein (Figure 14) rather than per the number of adherent macrophages (Figure 13), there was no significant ($p > 0.05$) difference at any time point between the Ity^s and Ity^r macrophages.

The results obtained in this experiment suggested that phagocytosis of S. typhimurium was similar, if not identical, for BALB/cn and congenic C.D2Ity^r resident peritoneal macrophages. However, only viable salmonellae could be enumerated by the approach used in this experiment. To address the question of uptake of total salmonellae (live + dead/damaged) at the end of the 50 minute infection period, experiments were designed that would measure total bacteria associated with Ity^s and Ity^r macrophages.

III. Uptake of S. typhimurium by Ity^r and Ity^s resident peritoneal macrophages

Two procedures were used to compare the uptake (phagocytosis) of S. typhimurium by Ity^r and by Ity^s macrophages. First, S. typhimurium strain TML was radiolabeled by ^{3}H-leucine incorporation (as described in Methods and Materials), suspended in 10% homologous normal mouse serum, and used to infect in vitro SWR/J (Ity^r) and BALB/cn (Ity^s) resident peritoneal macrophages (Table 6) and congenic C.D2Ity^r and BALB/cn resident

Table 6.

a. Resident peritoneal macrophages from mice of different strains were exposed to S. typhimurium strain TML which had been radiolabeled by ^{3}H-leucine incorporation and suspended in 10% homologous normal mouse serum. After the standard 50 minute phagocytosis period, lysates were prepared from these macrophages and the cell-associated radioactivity measured.

b. Mean $\pm$ 2 standard errors of counts per minute per macrophage, n = number of replicate samples.

c. Mean $\pm$ 2 standard errors of counts per minute per ug of lysate protein, n = number of replicate samples.

d. NS - not statistically significantly ($p > 0.05$) different.

Table 6. UPTAKE OF RADIOLABELED S. TYPHIMURIUM BY MOUSE RESIDENT PERITONEAL MACROPHAGES IN VITRO a.

EXPERIMENT	STRAIN	CPM/MACROPHAGE[b]		CPM/UG[c]	
1	SWR/J	.032 ± .006 (n = 4)	NS[d]	254.1 ± 20.8 (n = 4)	NS
	BALB/c πr	.036 ± .004 (n = 4)		265.3 ± 38.8 (n = 4)	
2	C.D2 lty[r]	.012 ± .003 (n = 4)	NS	84.4 ± 20.1 (n = 4)	NS
	BALB/c πr	.011 ± .001 (n = 4)		87.8 ± 6.5 (n = 4)	

peritoneal macrophages (Table 6). All macrophage cultures were prepared by Method 1 and were seeded at approximately 2 X 10^6 per 35 mm culture dish well. Macrophage lysates from each mouse strain were prepared at t_o and the cell-associated radioactivity measured. No statistically significant ($p > 0.05$) difference in cell-associated radioactivity was found between SWR/J and BALB/cn macrophages (Table 6: Experiment 1) or between congenic C.D2Ity^r and BALB/cn mice (Table 6: Experiment 2). In the second procedure used to compare the uptake of S. typhimurium by Ity^r and by Ity^s macrophages, resident peritoneal macrophages from congenic C.D2Ity^r and from BALB/cn mice were cultured (Method 2) on glass coverslips and were infected with S. typhimurium strain TML or with the avirulent temperature sensitive mutant S. typhimurium strain TML/TS27 (Swanson and O'Brien, 1983). These infected macrophage cultures were examined at t_o by an indirect immunofluorescence assay (IFA) (as described in Methods and Materials). For each S. typhimurium strain the number of fluorescent intracellular salmonellae per infected macrophage at t_o was statisticaly the same ($p > 0.05$) for C.D2Ity^r macrophages and BALB/cn macrophages; the average values for TML-infected macrophages ± 2 standard errors of the mean, n=4, were 1.45 ± 0.09 (C.D2Ity^r) and 1.43 ± 0.08 (BALB/cn); whereas, the average values for TML/TS27-infected macrophages ± 2 standard errors of the mean, n=3, were 1.57 ± 0.08 (C.D2Ity^r) and 1.69 ± 0.13 (BALB/cn). These findings

strongly suggested that the *in vitro* expression of Ity phenotype is not due to a difference in the initial uptake of the bacterium.

IV. Effect of *S. typhimurium* infection on the number of adherent macrophages present at the time of macrophage lysate preparation

To evaluate how infection with *S. typhimurium* might affect the number of adherent macrophages present at the time of lysate preparation, resident peritoneal macrophages from BALB/cn and from congenic C.D2Ity^r mice were cultured (Method 2) and infected with *S. typhimurium* strain TML. Sham-infected macrophage cultures from each strain served as uninfected controls. The number of adherent macrophages, from each type of culture, released after the standard lidocaine treatment, was determined at 0, 1, 4, 12, and 24 hours of the *in vitro* assay. Additionally, the infection of these Ity^s and Ity^r macrophages was monitored by preparing macrophage lysates in quadruplicate from wells infected at the same time as the wells to be used for quantifying infected macrophages.

The ratio of the average number of cell-associated salmonellae per macrophage for BALB/cn to C.D2Ity^r macrophage lysates was: 1.4 ($p > 0.05$) at t_o; 1.2 ($p > 0.05$) at 1 hour; 2.4 ($p < 0.05$) at 4 hours; 3.7 ($p < 0.05$) at 12 hours; 12.3 ($p < 0.05$) at 24 hours. These results indicated that by viable counts of cell-associated

salmonellae the infection had proceeded as in previous experiments; i.e., by 24 hours there were significantly more S. typhimurium in the Ity^s macrophages.

The numbers of adherent macrophages counted from lidocaine-treated infected and uninfected BALB/cn and C.D2Ity^r cultures are shown in Figure 15. At the 1 hour time point both Salmonella-infected and uninfected Ity^s and Ity^r macrophage cultures lost approximately 25% of the adherent cells counted at t_o. This loss appears to be attributable to the physical effect of the additional PBS washes. At all time points after 1 hour no further significant loss of adherent cells was observed for infected C.D2Ity^r, uninfected C.D2Ity^r, or uninfected BALB/cn macrophage cultures. However, infected BALB/cn macrophage cultures at 4 hours lost an additional 20% of adherent cells; so that, by this time point approximately 50% of the number of macrophages counted at t_o remained adherent. From 4 hours through 24 hours no further loss of adherent, infected BALB/cn macrophages occurred. In each of the aforementioned experiments all adherent macrophages for all conditions were $\geq$ 95% viable throughout the 24 hour assay. Because intact non-adherent cells could be seen microscopically prior to PBS washing, it appeared that the additional loss of adherent, infected Ity^s macrophages was not the result of cell death and/or lysis, but, rather the result of a detachment phenomenon directly related to S. typhimurium infection and the Ity^s phenotype (a similar finding has been reported for L. donovani-infected

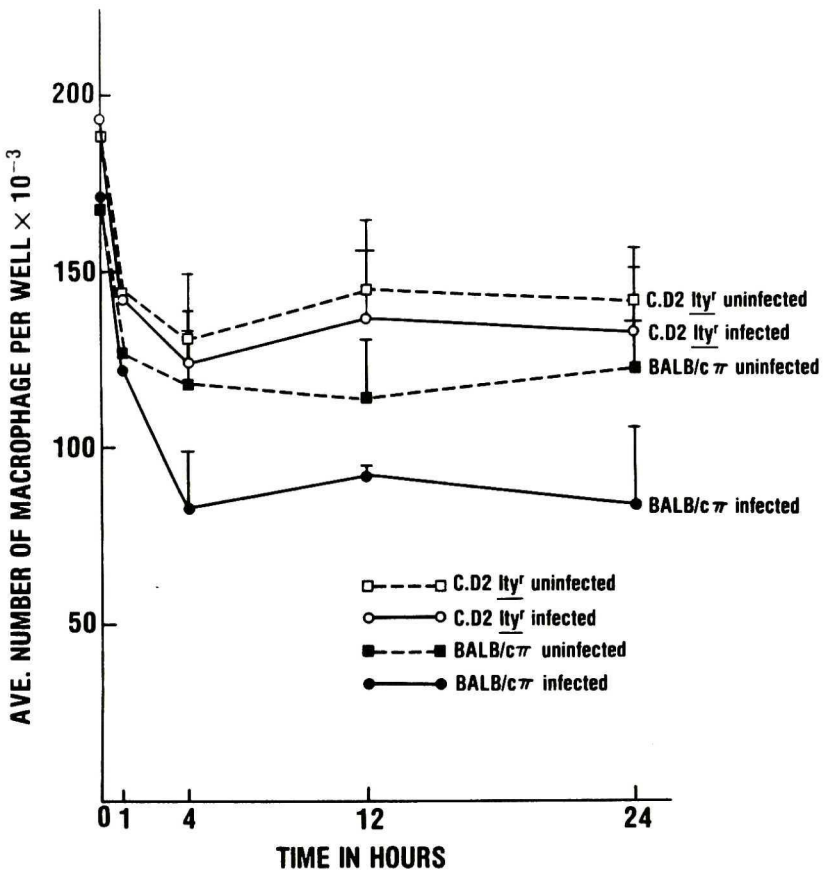


Figure 15. Resident peritoneal macrophages from congenic C.D2Ity^r and from BALB/cπ mice were infected in vitro with S. typhimurium strain TML or were sham-infected (uninfected). The number of adherent macrophages at 0, 1, 4, 12, and 24 hours after infection was determined by the lidocaine treatment. Each point represents the average number of adherent macrophages ± 2 standard errors of the mean, n = 3.

Lsh^s liver macrophages [Croker et al., 1984]). The implication of these findings was that the number of viable cell-associated salmonellae in Ity^s macrophage lysates may be conservative if the detached Ity^s macrophages represent a more heavily infected population. Moreover, evaluation of detached ("fluid- phase"), infected macrophage populations might further differentiate the Ity^s and congenic Ity^r phenotypes.

V. Ingestion of latex particles by S. typhimurium-infected and uninfected BALB/cn and congenic C.D2Ity^r macrophages

The inclusion of 0.05% trypan blue in the lidocaine solution was an effective and convenient method to determine the viability of macrophage cultures prior to lysate preparation or macrophage quantification. The in situ method gave results identical to results obtained (data not shown) when infected Ity^r and Ity^s coverslip macrophage cultures were removed from culture dish wells, inverted onto drops of either the standard lidocaine solution or a 0.05% trypan blue in normal saline solution, and examined microscopically for viability. To further test the validity of the standard method of macrophage viability assessment, another parameter of macrophage function, the ingestion of latex particles was used.

Resident peritoneal macrophages from BALB/cn and congenic C.D2Ity^r mice were cultured on glass coverslips

(Method 2) and infected *in vitro* with *S. typhimurium* strain TML. At t_o and at 1, 4, and 24 hours of the *in vitro* assay, infected and uninfected macrophage cultures were exposed to a 1/1000 dilution of latex particles (as described in Methods and Materials) (a preliminary study using a 1/5000 dilution had resulted in only 50% of uninfected macrophages ingesting latex under similar experimental conditions; a 1/1000 dilution was used to enhance latex ingestion [S. Vogel, personal communication]). After the ingestion period macrophages were immediately fixed in absolute methanol, vigorously washed by mechanical rotation to remove non-ingested and glass adherent particles, and stained for light microscopy. The results of this study are given in Table 7 and representative photomicrographs of infected macrophages after latex ingestion are shown in Figures 16, 17, and 18.

At all time points examined, the percentage of both infected and uninfected *Ity*^{s} and *Ity*^{r} macrophages capable of ingesting one or more latex particles closely paralleled the percentage of trypan blue excluding cells routinely observed, i.e., $\geq$ 95%. The respective percentages of *Ity*^{s} and *Ity*^{r} macrophages which contained intracellular salmonellae, compared to the respective percentages of *Ity*^{s} and *Ity*^{r} macrophages which had ingested latex particles and contained intracellular salmonellae, clearly indicated that infected macrophages were still functionally capable of latex particle

Table 7.

a. Resident peritoneal macrophages from congenic C.D2*Ity*^{r} and BALB/c*n* mice were cultured on glass coverslips. Some of these cultures were infected *in vitro* with *S. typhimurium* strain TML, while others were sham-infected. At various time points both infected and uninfected macrophages were tested for the ability to ingest latex particles.

b. Time in hours after infection at which latex particles were given.

c. The percentage of 100 macrophages per coverslip that had ingested 1 or more latex particles. The data is presented as the average $\pm$ 2 standard errors of the mean; n = 3 for infected, n = 2 for uninfected.

d. The percentage of 100 macrophages per coverslip which had 1 or more intracellular bacteria. The data is presented as in c. above.

e. The percentage of macrophages with both ingested latex particles and intracellular bacteria. This value was calculated by subtracting the percentage of macrophages with intracellular bacteria but without ingested latex particles from the respective values in d. above.

Table 7. LATEX PARTICLE INGESTION BY S. TYPHIMURIUM INFECTED AND UNINFECTED MACROPHAGES FROM CONGENIC C.D2Ity^r AND BALB/cπ MICE [a].

MOUSE STRAIN	TIME[b]	CONDITION	% OF MACROPHAGES[c] WITH LATEX PARTICLES	% OF MACROPHAGES[d] WITH S. typhimurium	% OF MACROPHAGES[e] WITH LATEX + S. typhimurium
C.D2 Ity^r	0	uninfected	87 ± 11	–	–
BALB/cπ	0	uninfected	91 ± 8	–	–
C.D2 Ity^r	0	infected	92 ± 1	46 ± 5	44
BALB/cπ	0	infected	91 ± 7	45 ± 6	42
C.D2 Ity^r	1	uninfected	92 ± 4	–	–
BALB/cπ	1	uninfected	93 ± 3	–	–
C.D2 Ity^r	1	infected	94 ± 1	40 ± 2	39
BALB/cπ	1	infected	91 ± 3	43 ± 2	41
C.D2 Ity^r	4	uninfected	94 ± 1	–	–
BALB/cπ	4	uninfected	95 ± 1	–	–
C.D2 Ity^r	4	infected	95 ± 3	22 ± 3	21
BALB/cπ	4	infected	98 ± 1	32 ± 3	31
C.D2 Ity^r	24	uninfected	96 ± 3	–	–
BALB/cπ	24	uninfected	97 ± 0	–	–
C.D2 Ity^r	24	infected	95 ± 2	15 ± 2	15
BALB/cπ	24	infected	96 ± 5	32 ± 12	31

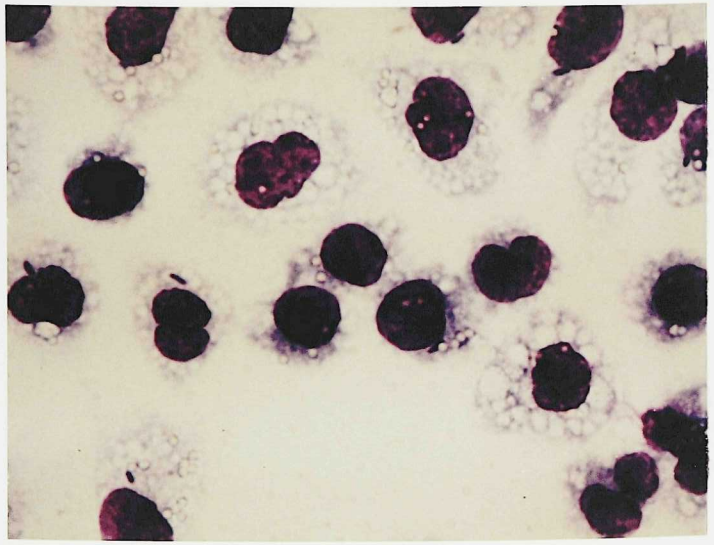


Figure 16a. Resident peritoneal macrophages from congenic C.D2Ity^{r} mice were infected *in vitro* with *S. typhimurium* strain TML. At 0, 4, and 24 hours after infection, latex particle ingestion by infected macrophages was assessed. Figure 16a. is representative of latex particle ingestion at 0 hours; cells examined at 100 X. Magnification shown = 975 X.

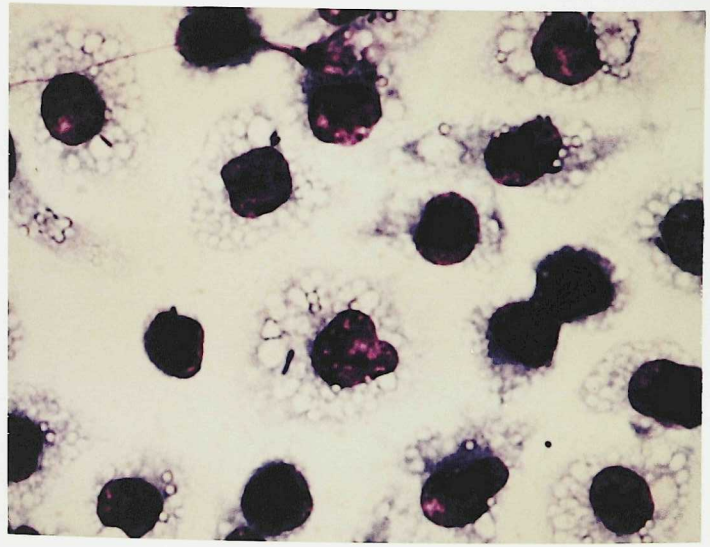


Figure 16b . Resident peritoneal macrophages from BALB/cπ mice were infected *in vitro* with *S. typhimurium* strain TML. At 0, 4, and 24 hours after infection, latex particle ingestion by infected macrophages was assessed. Figure 16b. is representative of latex particle ingestion at 0 hours; cells examined at 100 X. Magnification shown = 975 X.

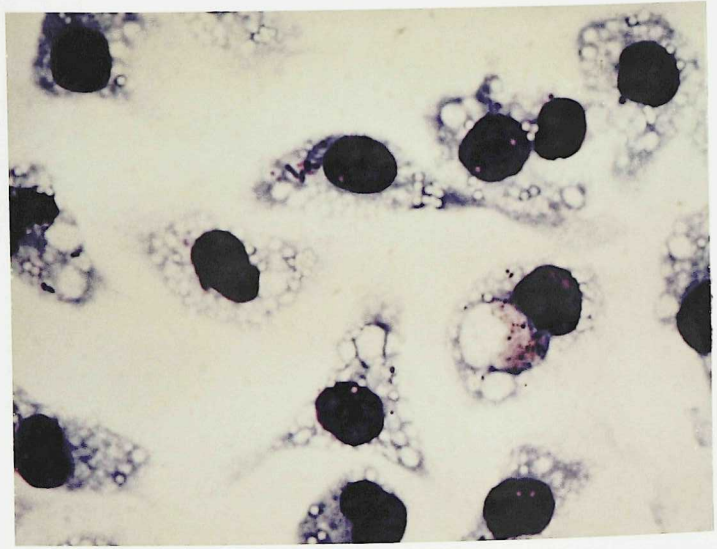


Figure 17a. Resident peritoneal macrophages from congenic C.D2Ity^r mice were infected *in vitro* with *S. typhimurium* strain TML. At 0, 4, and 24 hours after infection, latex particle ingestion by infected macrophages was assessed. Figure 17a. is representative of latex particle ingestion at 4 hours; cells examined at 100 X. Magnification shown = 975 X.

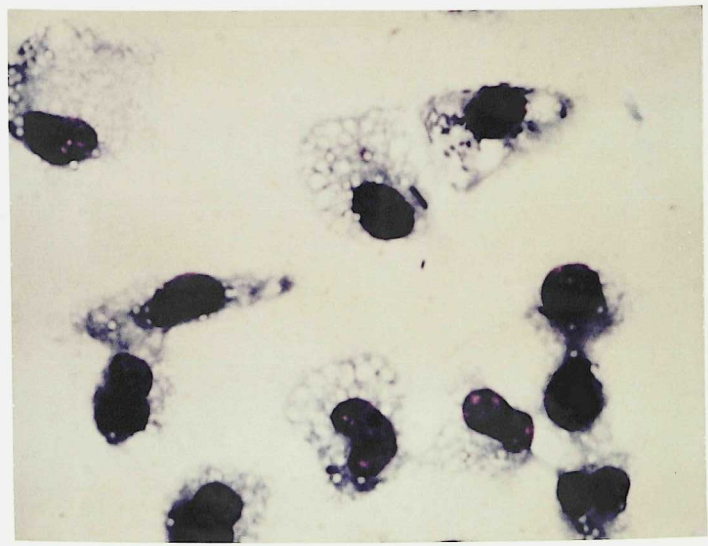


Figure 17b. Rresident peritoneal macrophages from BALB/cγ mice were infected *in vitro* with *S. typhimurium* strain TML. At 0, 4, and 24 hours after infection, latex particle ingestion by infected macrophages was assessed. Figure 17b. is representative of latex particle ingestion at 4 hours; cells examined at 100 X. Magnification shown = 975 X.

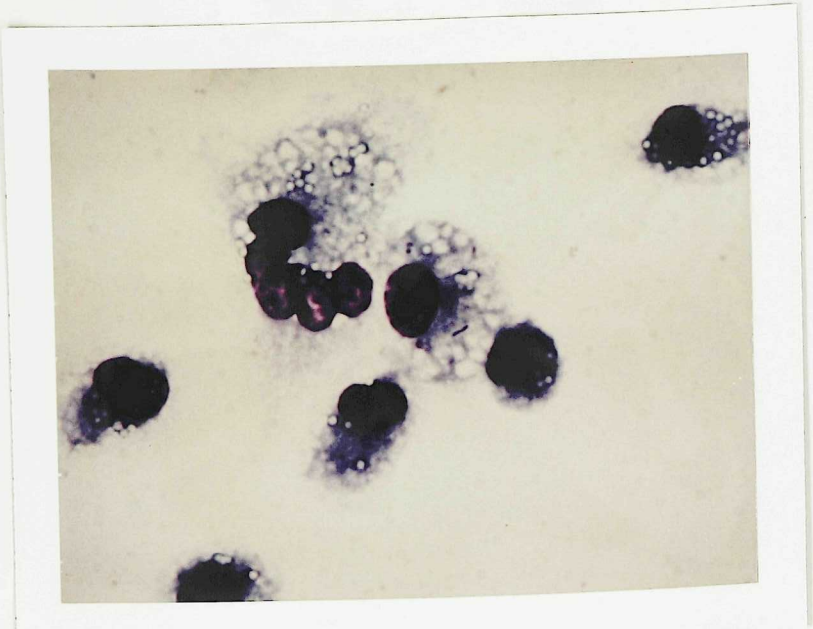


Figure 18a. Resident peritoneal macrophages from congenic C.D2Ity^r mice were infected *in vitro* with *S. typhimurium* strain TML. At 0, 4, and 24 hours after infection, latex particle ingestion by infected macrophages was assessed. Figure 18a. is representative of latex particle ingestion at 24 hours; cells examined at 100 X. Magnification shown = 975 X.

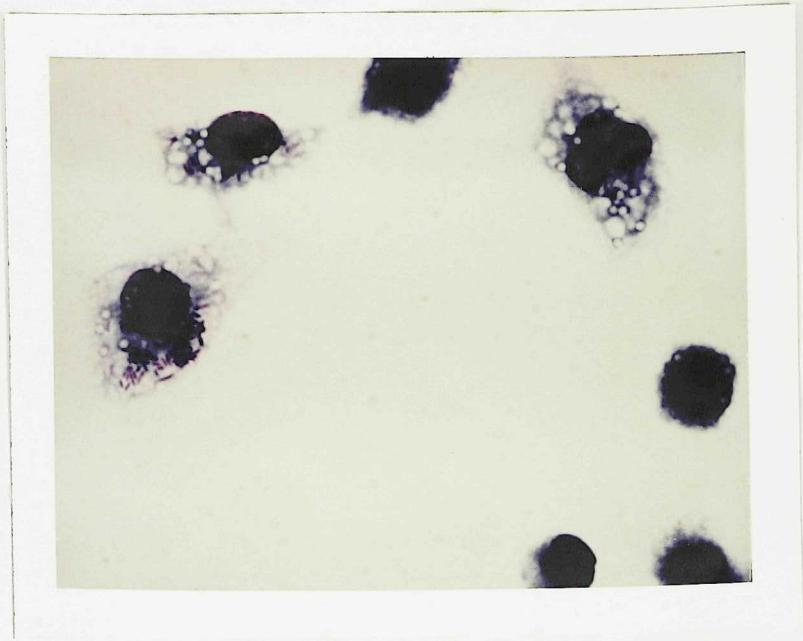


Figure 18b. Resident peritoneal macrophages from BALB/cη mice were infected *in vitro* with *S. typhimurium* strain TML. At 0, 4, and 24 hours after infection, latex particle ingestion by infected macrophages was assessed. Figure 18b. is representative of latex particle ingestion at 24 hours; cells examined at 100 X. Magnification shown = 975 X.

ingestion, at all times of observation. These results confirmed the validity of using the standard method of trypan blue exclusion *in situ* to measure the viability of macrophages during the *in vitro* assay.

VI. Expression of *Ity* phenotype by BALB/cn and congenic C.D2*Ity*^{r} macrophages, infected at three different infection ratios of *S. typhimurium*

To examine more specifically the effect of varying the ratio of *S. typhimurium* infection on the *in vitro* expression of *Ity* phenotype, resident peritoneal macrophages from BALB/cn and from congenic C.D2*Ity*^{r} mice were cultured (Method 2) and infected with *S. typhimurium* strain TML at infection ratios of 1:1, 5:1, and 50:1. The results of this experiment are shown in Figure 19. The findings from each of the different infection ratios suggested that: 1., the standard *in vitro* infection ratio of 5:1 best differentiated the *Ity* phenotype; 2., at the 1:1 ratio *Ity* phenotype was discernable over the first 4 hours of the *in vitro* infection; 3., at the 50:1 ratio the number of bacteria exceeded the capacity of even *Ity*^{r} macrophages to control net salmonellae growth, which eventually lead to the destruction/disruption of the macrophages.

Verification that the *In Vitro* Assay Reflects *Ity*-Controlled Early Phase Susceptibility to Murine Typhoid

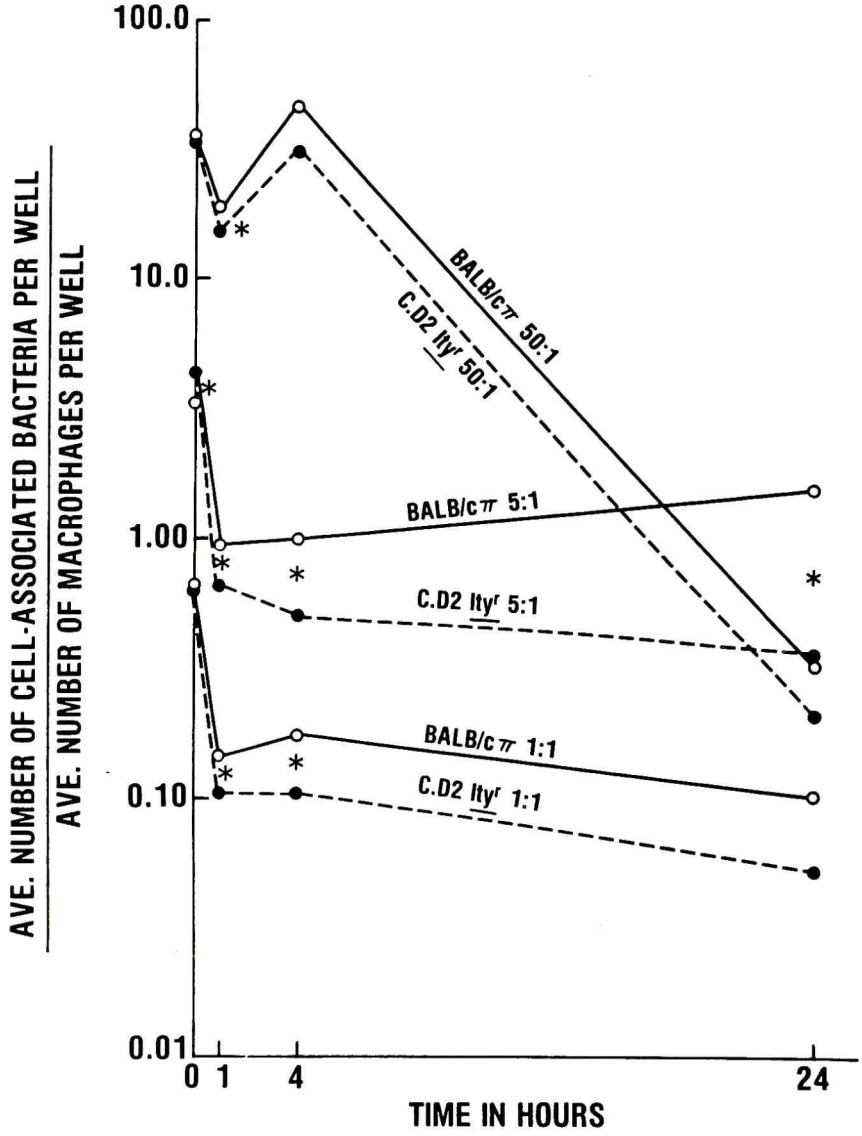


Figure 19. Resident peritoneal macrophages from congenic C.D2Ity^r and from BALB/cπ mice were infected at three ratios of infection, 1:1, 5:1, and 50:1, in vitro with S. typhimurium strain TML. The number of viable bacteria in macrophage lysates prepared at 0, 1, 4, and 24 hours after infection was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells. * indicates points for a particular ratio of infection are statistically significantly ($p < 0.05$) different, by Student's two-tailed t-test for unpaired samples.

To confirm that the results obtained with the *in vitro* infection assay were specific for *Ity* regulated, macrophage-mediated, early phase innate susceptibility to murine typhoid, the interaction of *S. typhimurium* with macrophages from a mouse strain that dies in the late phase of murine typhoid was examined. Resident peritoneal macrophages from late phase susceptible (CBA/N X DBA/2N) F1 Ity^r, *xid* male mice, from resistant (DBA/2N X CBA/N) F1 Ity^r male mice, and from Ity^s BALB/cn mice, used as a susceptible control, were cultured (Method 2) and infected with *S. typhimurium* strain TML. The results of this experiment are depicted in Figure 20. The results of this experiment verifed that the Ity^r phenotype of both resistant (DBA/2N X CBA/N) F1 male mice and susceptible (CBA/N X DBA/2N) F1 male mice was clearly discernable within the 24 hours of the *in vitro* assay, and the results showed that the *in vitro* assay was specific for early phase macrophage-mediated control of intracellular *S. typhimurium* growth.

Use of the In Vitro Assay to Determine the Ity Phenotype of Introgressively Backcrossed Mice

A specific aim of this work was to utilize the *in vitro S. typhimurium*-infected macrophage assay to determine the *Ity* phenotype of living mice derived from the continued introgressive backcrossing of Ity^r

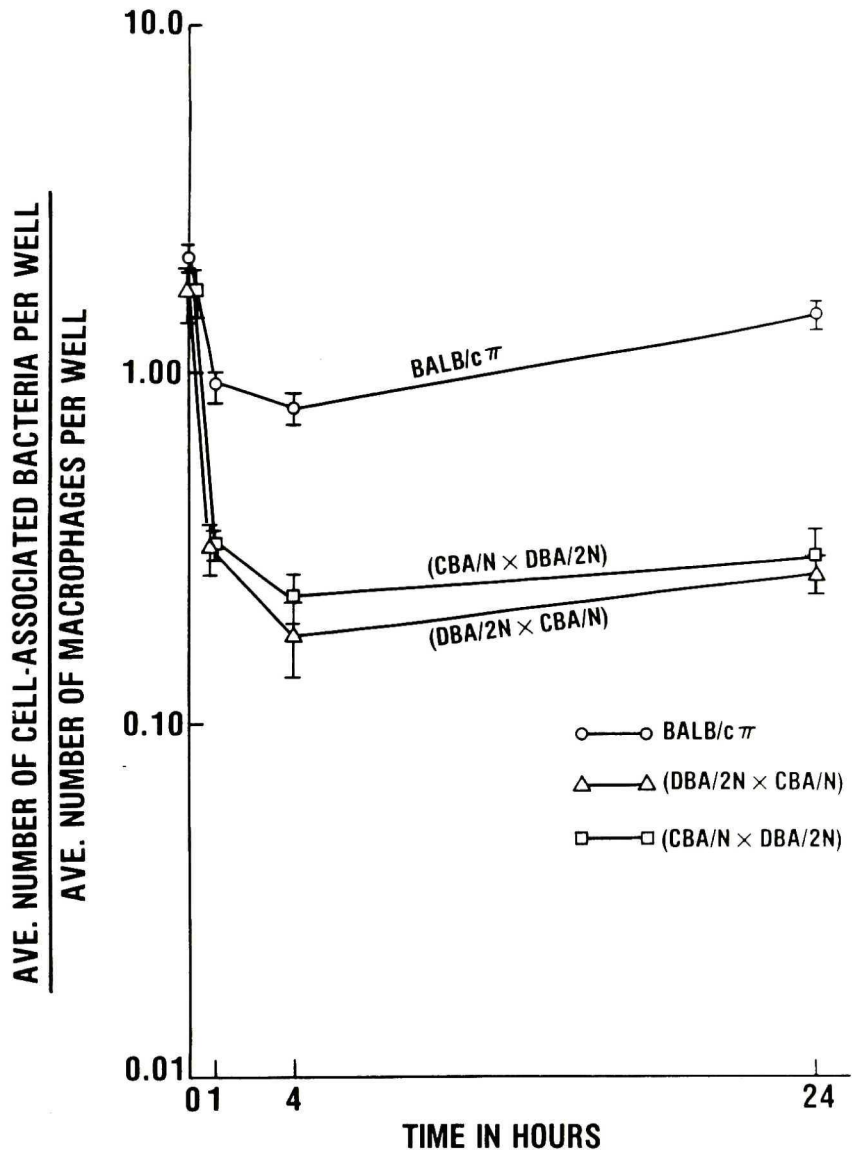


Figure 20. Resident peritoneal macrophages from (CBA X DBA/2N) ♂, (DBA/2N X CBA/N) ♂, and BALB/cπ mice were infected in vitro with S. typhimurium strain TML. Macrophage lysates were prepared at 0, 1, 4, and 24 hours after infection and the number of viable salmonellae was determined. The results are expressed as the number of viable cell-associated bacteria ÷ the number of macrophages counted. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

heterozygous protenitors onto the Ity^s BALB/cn progenitor background (Potter et al., 1983) (These mice are designated as NxFx, where "N" represents backcross breeding and "x" is the number of backcrosses, and "F" represents the generation of inbreeding and its "x" is the number of generations). The ultimate goal of such breeding is to construct a homozygous coisogenic Ity^r mouse strain.

In order to determine the Ity phenotype of live mice, peritoneal macrophages were collected (as described in Methods and Materials) and in vitro assays were performed. These experiments were hindered by the low number of macrophages that could be collected by peritoneal lavage of individual live mice. The lack of adequate numbers of peritoneal macrophages resulted in fewer replicate culture dish wells at each time point, fewer assay time points examined per mouse tested, and fewer macrophages per individual culture. Results from such in vitro experiments were tentatively reported as "suggestive" of Ity^r or Ity^s phenotype or as inconclusive.

To obviate the technical difficulties of using resident peritoneal macrophages, the in vitro assay was performed with thioglycollate-elicited peritoneal macrophages from BALB/cn and congenic C.D2Ity^r mice (animals in these initial studies were sacrificed) cultured by Method 2 and infected with S. typhimurium strain TML. The results of such an experiment are shown in Figure 21. By 24 hours of infection the elicited Ity^s macrophages contained approximately 3 times the number of viable

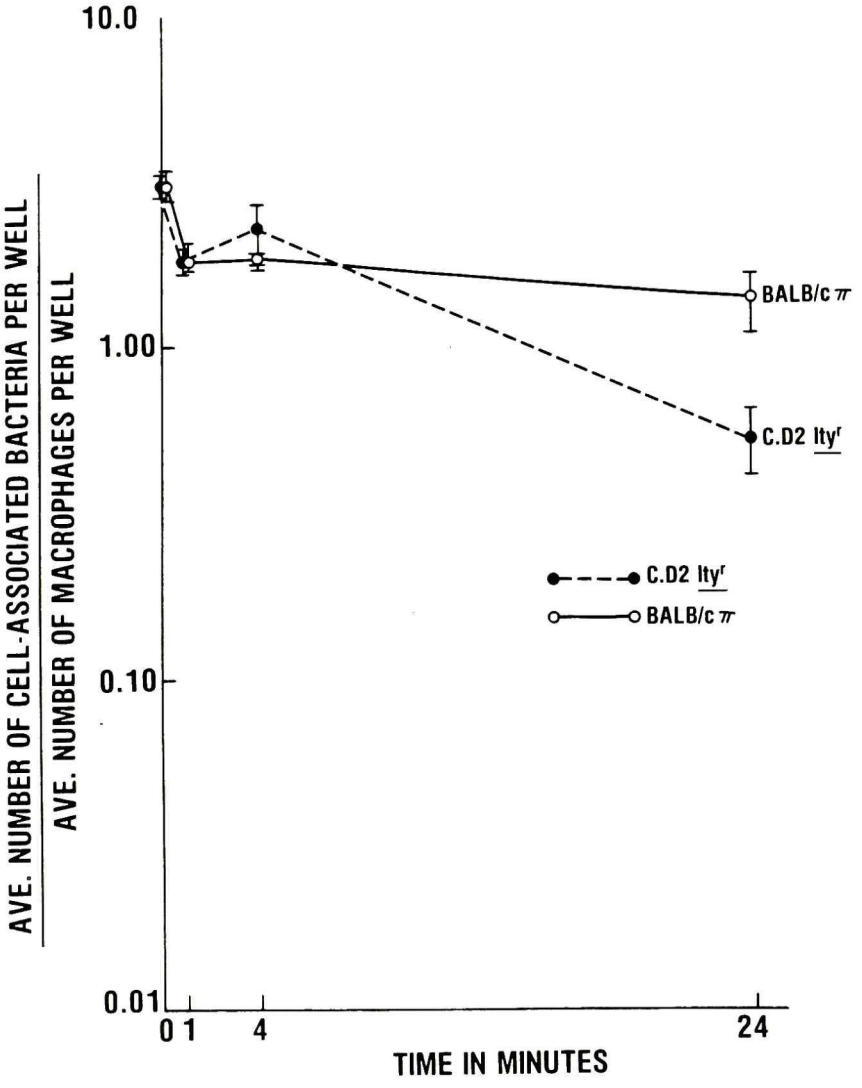


Figure 21. Thioglycollate-elicited peritoneal macrophages from congenic C.D2Ity^r and BALB/cπ mice were infected in vitro with S. typhimurium strain TML, and the number of viable bacteria in macrophage lysates prepared at 0, 1, 4, and 24 hours after infection was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

salmonellae than the elicited Ity^r macrophages ($<$ 0.05). Subsequently, (thioglycollate-elicited) peritoneal macrophages from 3 live N10F0 mice (strains #2915, 2918, 2929), F1 progeny of a (BALB/cn X N9F0) backcross of unknown Ity phenotype, from a live BALB/cn (Ity^s control) mouse, and from a live congenic C.D2Ity^r (Ity^r control) mouse, were cultured (Method 2) and infected with S. typhimurium strain TML. The results of this assay are depicted in Figure 22. The number of viable cell-associated salmonellae at 24 hours was significantly ($p < 0.05$) greater in BALB/cn macrophages than in the Ity^r control C.D2Ity^r macrophages. Strains 2915, 2918, and 2919 were phenotypically Ity^s. To increase the sample size an additional six (BALB/cn X N9F0) F1 mice were tested in another experiment. All of these animals, when compared to Ity^s and Ity^r controls, were phenotypically Ity^s (data not shown). The results obtained in these two experiments and the concomitant differential response of BALB/cn and congenic C.D2Ity^r controls suggested that the N9F0 male, from which the (BALB/cn X N9F) F1 mice were derived, was phenotypically Ity^s and, therefore, homozygous for the Ity^s allele. This assumption was tested by directly assaying thioglycollate-elicited macrophages from the N9F0 mouse (Figure 23). By 24 hours there were significantly ($p < 0.05$) more cell-associated salmonellae in the N9F0 macrophages than in the C.D2Ity^r macrophages, which had significantly ($p < 0.05$) fewer salmonellae than the Ity^s BALB/cn control macrophages.

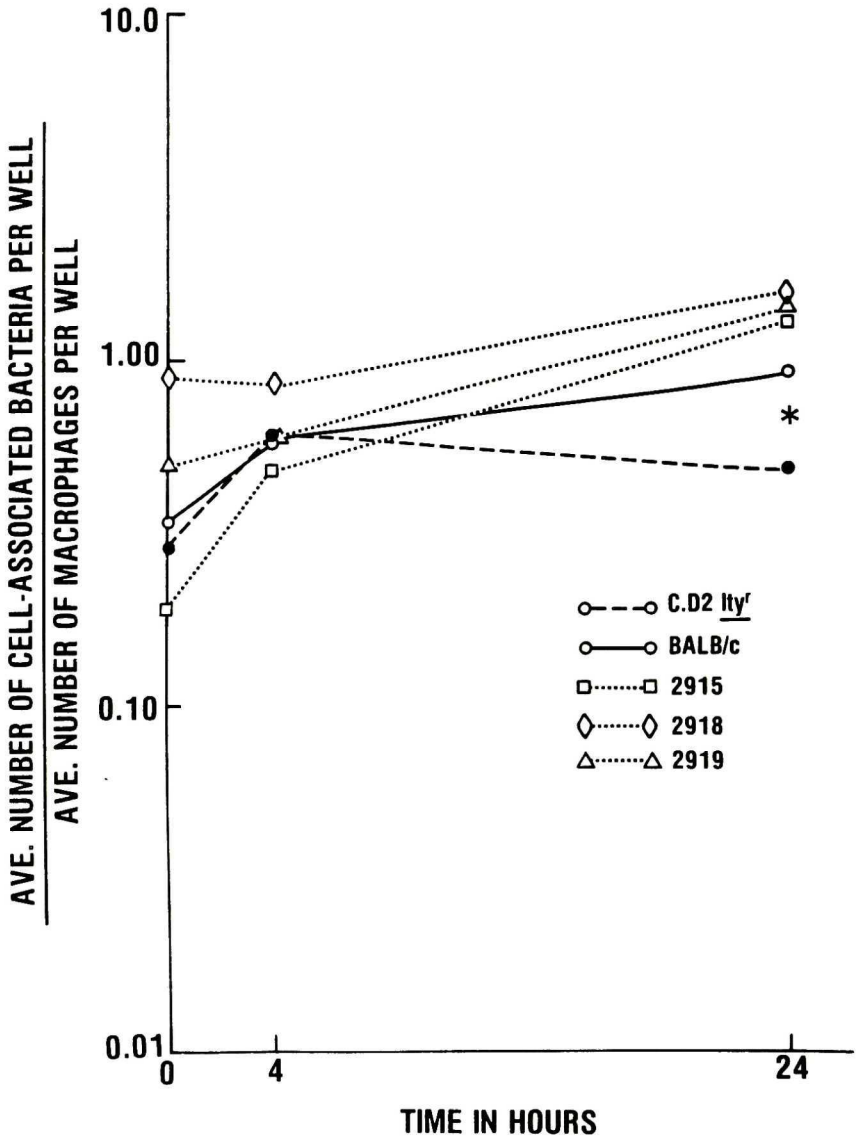


Figure 22. Thioglycollate-elicited peritoneal macrophages were collected from live mice: congenic C.D2Ity^r, BALB/cπ, and N10F0 strains # 2915, 2918, and 2919. Macrophages were infected *in vitro* with *S. typhimurium* strain TML, and the number of viable bacteria in macrophage lysates prepared at 0, 4, and 24 hours after infection was determined. The ordinate is a logarithmic scale. Each point is the average of 3 culture dish wells. * The number of viable cell-associated salmonellae in BALB/cπ macrophages was statistically significantly ($p < 0.05$) higher than in C.D2Ity^r macrophages at 24 hours.

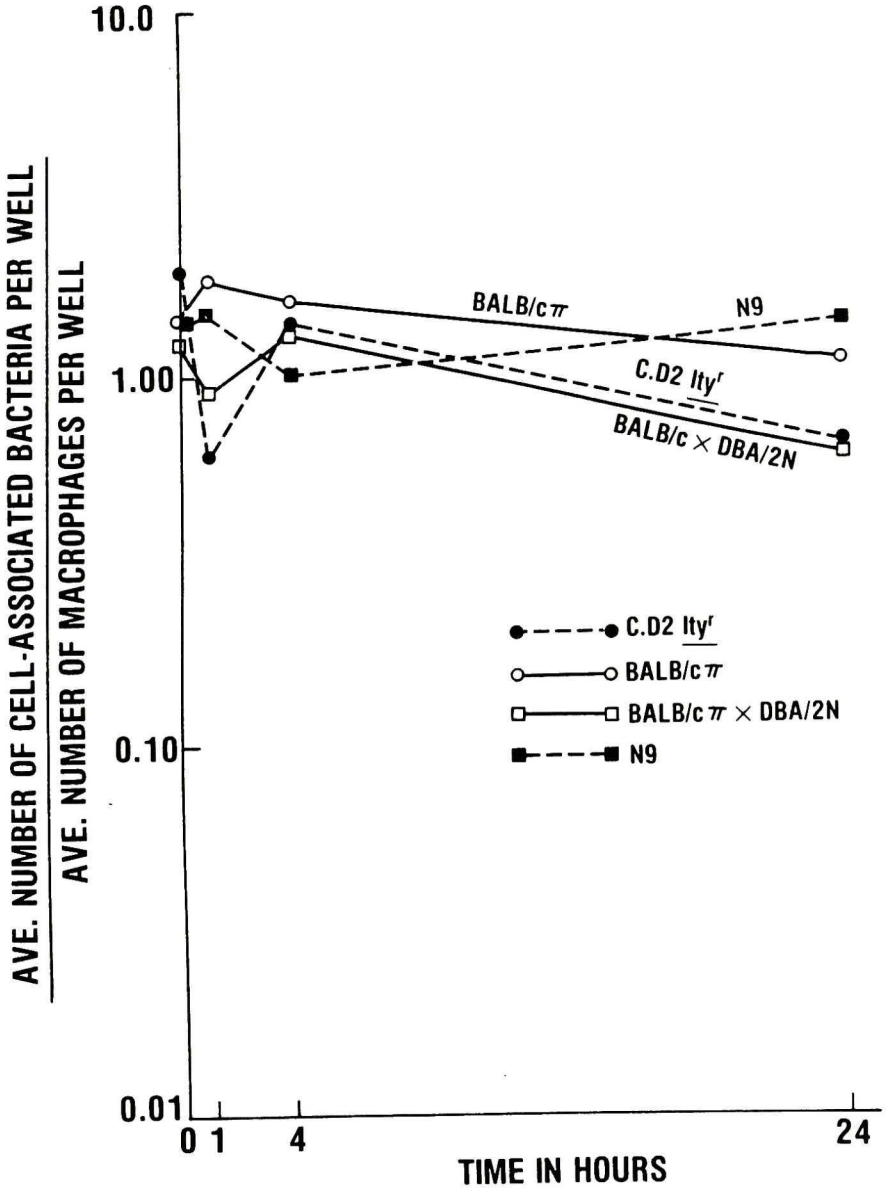


Figure 23. Thioglycollate-elicited peritoneal macrophages were collected from live mice: congenic C.D2Ity^r, BALB/cπ, F1 (BALB/cπ X DBA/2N), and an N9F0 backcross strain. Macrophages were infected *in vitro* with *S. typhimurium* strain TML, and the number of viable bacteria in macrophage lysates prepared at 0, 1, 4, and 24 hours after infection was determined. The ordinate is a logarithmic scale. Each point is the average of 3 culture dish wells. The number of viable cell-associated salmonellae in BALB/cπ macrophages was statistically significantly ($p < 0.05$) higher than in C.D2Ity^r macrophages at 24 hours.

These findings verified that the N9F0 mouse was an Ity^{s} animal. In addition, thioglycollate-elicited macrophages of a (BALB/cn X DBA/2N) F1 mouse (Figure 23) were phenotypically Ity^{r} *in vitro*, an observation which is consistent with the *in vivo* expression of the resistant phenotype by Ity heterozygous F1 animals (not shown).

Taken together the results obtained in this series of experiments lead to the following conclusions: 1., phenotype expression of the Ity^{s} and Ity^{r} alleles by thioglycollate-elicited peritoneal macrophages *in vitro* occurs by 24 hours of infection; 2., the use of thioglycollate elicitation is an effective method for collecting peritoneal macrophages from individual live mice; 3., heterozygosity at the Ity locus is expressed phenotypically as Ity^{r} in the *in vitro* assay.

Examination by Light Microscopy of Salmonella typhimurium-Infected and Uninfected BALB/cn and Congenic C.D2Ity^{r} Resident Peritoneal Macrophages

I. Cellular composition of macrophage cultures used in the *in vitro* infection assay

To determine the cellular composition of Ity^{s} and Ity^{r} resident peritoneal macrophage cultures, resident peritoneal cells from BALB/cn and C.D2Ity^{r} mice were cultured (Method 2) on plastic (Therminox) coverslips, sham-infected, and examined by light microscopy (as

described in Methods and Materials). Coverslips were examined at various time points and the number of macrophages, lymphocytes, basophils, neutrophils, and cells of indistinguishable morphology, termed "others," was determined in samples of 400 total cells counted per coverslip. Because there was no significant difference between the cellular compositions of BALB/cn and C.DIty^r cultures, the data for both strains were combined at each time point. The results of this procedure are shown in Table 8 and representative photomicrographs in Figures 24, 25, and 26. The results clearly showed that at all times of examination the peritoneal cell cultures contained at least 94% adherent macrophages and less that 5% lymphocytes. All other cell types were present in insignificant quantities ($\leq$ 1.6%) or not observed at all. These *in vitro* findings strengthen the conclusion that *Ity* phenotype expression is macrophage-mediated.

II. Fate of *S. typhimurium*, as assessed by light microscopy, in resident peritoneal macrophages from BALB/cn and congenic C.D2Ity^r mice

Resident peritoneal macrophages from BALB/cn and congenic C.D2Ity^r mice were cultured (Method 2) on glass coverslips and infected with *S. typhimurium* strain TML. At various times of the *in vitro* assay, coverslip cultures were prepared for light microscopy (as described in Methods and Materials). The percentage of infected macrophages and

Table 8. CELL TYPES PRESENT IN THE MACROPHAGE CULTURES OF THE IN VITRO INFECTION ASSAY a.

a. Resident peritoneal cells from BALB/cᵠ and from C.D2Ity^{r} mice were cultured on coverslips, sham-infected, and at various times were fixed, stained, and examined by light microscopy.

b. Time at which coverslips were fixed and the number of each cell types enumerated.

c. Results are expressed as the average percentage of 400 total cells counted per coverslip ± 2 standard errors of the mean, n = number of coverslips examined.

d. Macrophage cultures washed 1 time with RPMI 1640, representing the cultures present at the start of the 50 minute bacterial infection period.

Table 8. CELL TYPES PRESENT IN THE MACROPHAGE CULTURES OF THE IN VITRO INFECTION ASSAY [a].

TIME[b]	MACROPHAGES[c]	LYMPHOCYTES	BASOPHILS	NEUTROPHILS	OTHERS
– 50 minutes[d]	94.3 ± 1 , n = 4	4.8 ± 0.2	0.9 ± 0.8	0.0	0.0
0 hours	94.2 ± 1.3, n = 4	3.9 ± 0.8	1.6 ± 0.4	0.0	0.0
4 hours	96.9 ± 1.3, n = 4	3.0 ± 1.1	0.3 ± 0.2	0.0	0.0
24 hours	97.6 ± 0.4, n = 5	2.3 ± 0.3	0.2 ± 0.2	0.0	0.0

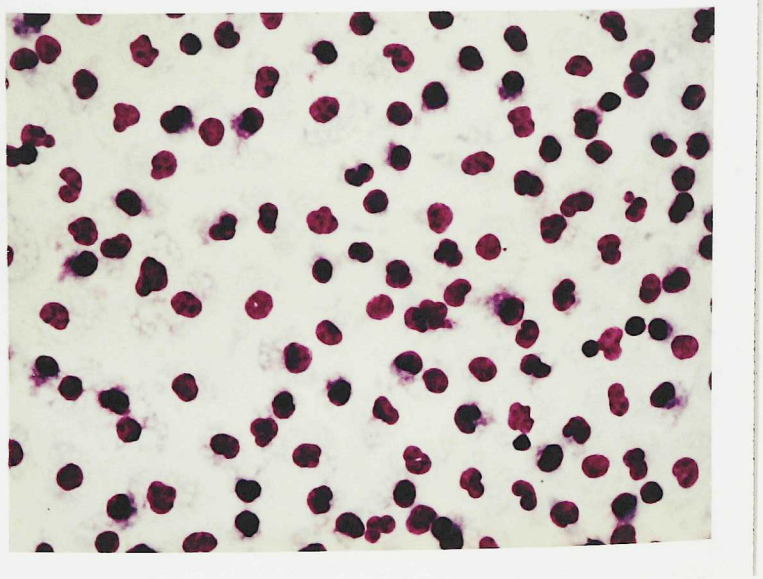


Figure 24a. Cell types present in uninfected C.D2Ity^{r} macrophage cultures were determined microscopically at 0, 4, and 24 hours of culture. Figure 24a. is representative of macrophage cultures at 0 hours; cells examined at 40 X. Magnification shown = 400 X.

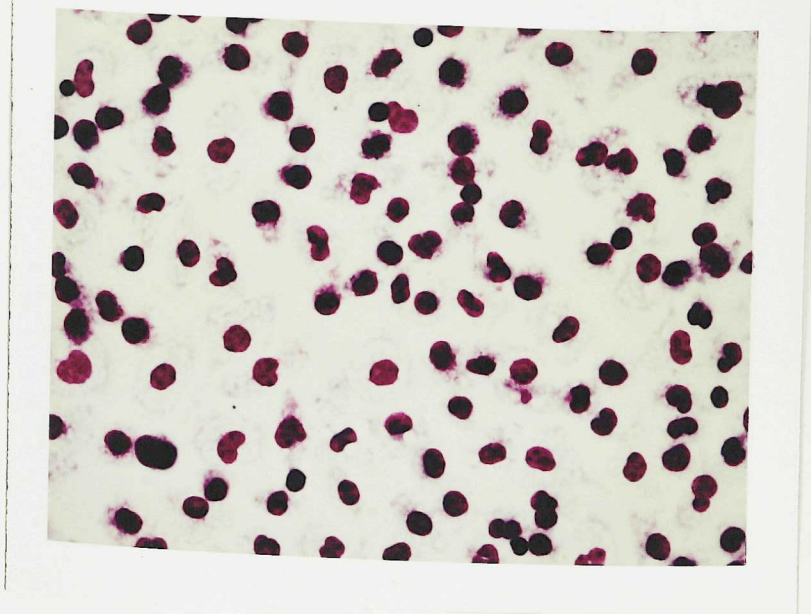


Figure 24b. Cell types present in uninfected BALB/c7 macrophage cultures were determined microscopically at 0, 4, and 24 hours of culture. Figure 24b. is representative of macrophage cultures at 0 hours; cells examined at 40 X. Magnification shown = 400 X.

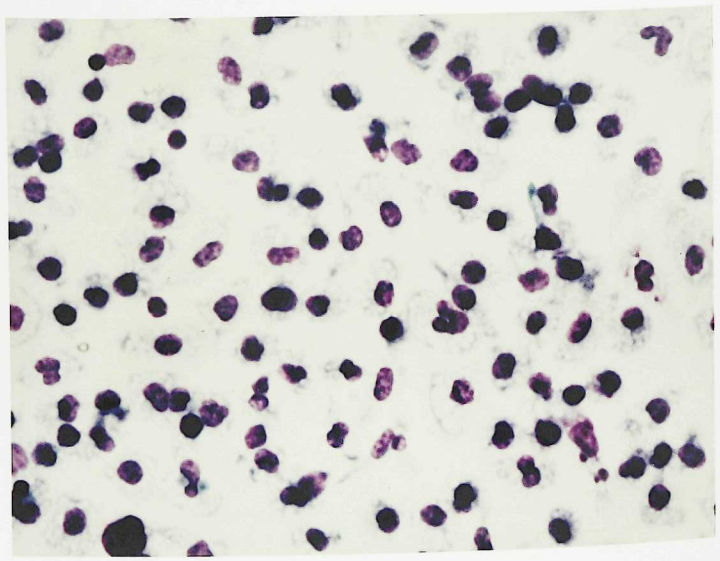


Figure 25a. Cell types present in uninfected C.D2Ity^{r} macrophage cultures were determined microscopically at 0, 4, and 24 hours of culture. Figure 25a. is representative of macrophage cultures at 4 hours; cells examined at 40 X. Magnification shown = 400 X.

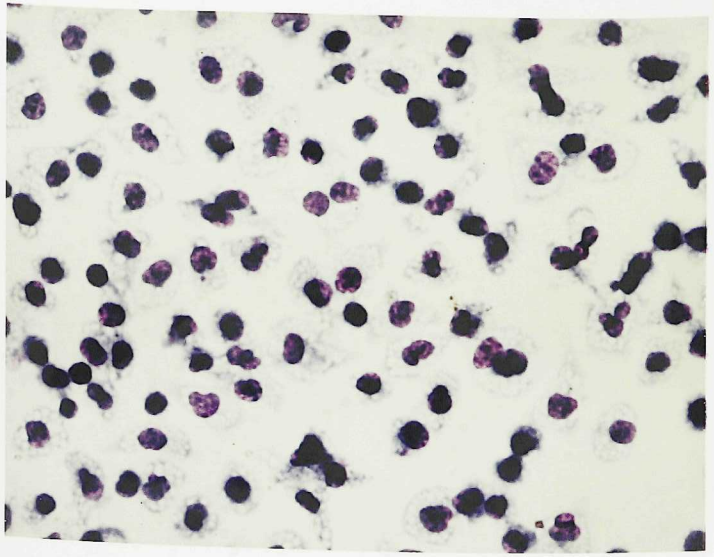


Figure 25b. Cell types present in uninfected BALB/cγ macrophage cultures were determined microscopically at 0, 4, and 24 hours of culture. Figure 25b. is representative of macrophage cultures at 4 hours; cells examined at 40 X. Magnification shown = 400 X.

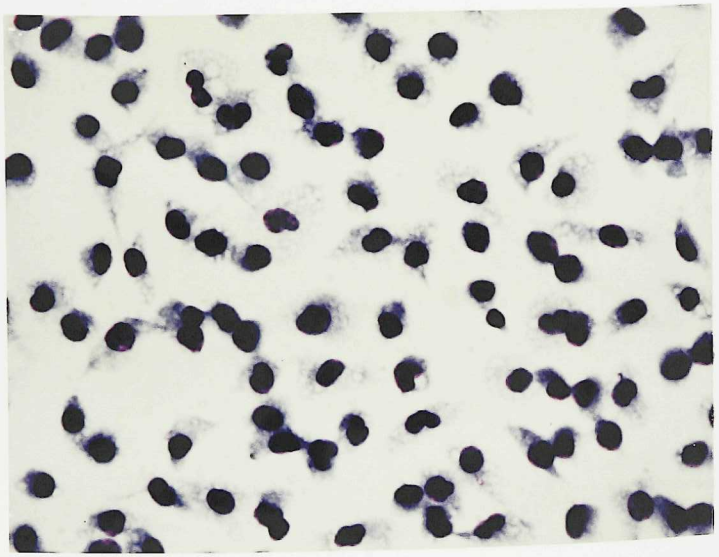


Figure 26a. Cell types present in uninfected C.D2Ity^{r} macrophage cultures were determined microscopically at 0, 4, and 24 hours of culture. Figure 26a. is representative of macrophage cultures at 24 hours; cells examined at 40 X. Magnification shown = 400 X.

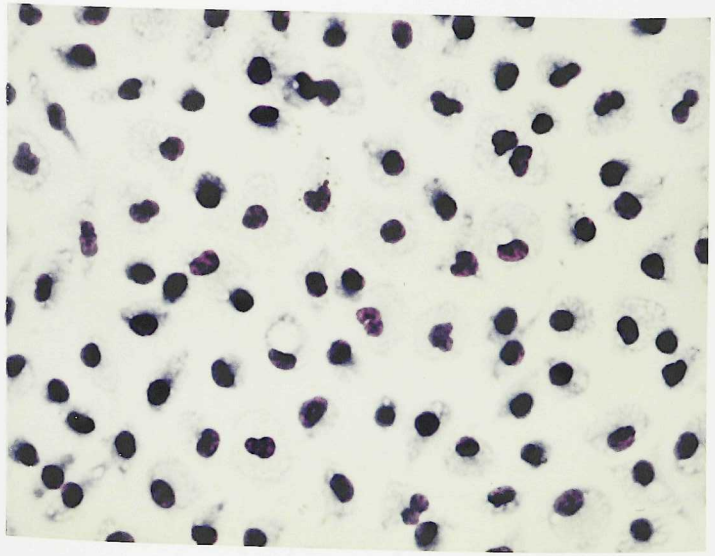


Figure 26b. Cell types present in uninfected BALB/cℵ macrophage cultures were determined microscopically at 0, 4, and 24 hours of culture. Figure 26b. is representative of macrophage cultures at 24 hours; cells examined at 40 X. Magnification shown = 400 X.

the number of cell-associated salmonellae per infected macrophage were determined. Because it was difficult to discern individual bacteria when macrophages contained > 20 salmonellae, such cells were scored as 21. Maintenance medium from 3 infected macrophage culture wells was combined at 4 and 24 hours, subjected to cytospin preparation, and examined in the same manner as coverslips. The results of this experiment are shown in Table 9 and representative photomicrographs are shown in Figures 27, 28, and 29.

The percentage of infected C.D2Ity^r coverslip macrophages significantly ($p < 0.05$) decreased at each point of observation after t_o. No significant difference in the number of salmonellae per infected C.D2Ity^r coverslip macrophage was observed. The percentage of infected BALB/cn coverslip macrophages remained statistically ($p > 0.05$) the same throughout the experiment. A significant ($p < 0.05$) increase in the number of salmonellae per infected macrophage occurred from 1 hour to 4 hours. The results obtained for Ity^s and Ity^r coverslip macrophage cultures were consistent with previous *in vitro* findings, which were based on macrophage lysates: 1., there was no difference in the number of salmonellae phagocytized by Ity^s and Ity^r macrophages at t_o; 2., by 24 hours of the *in vitro* infection there were significantly more bacteria in Ity^s macrophages than in Ity^r macrophages. Although live bacteria could not be differentiated from dead bacteria, by 24 hours the micro-

Table 9.

a. Resident peritoneal macrophages from BALB/cℸ and congenic C.D2Ity^r mice were cultured (Method 2) on coverslips and infected with S. typhimurium strain TML. The percentage of infected macrophages and the number of cell-associated Salmonella per infected macrophage were enumerated by light microscopy.

b. Time in hours of the in vitro assay at which samples were prepared for microscopy.

c. Macrophages examined were those freely floating in maintenance medium over infected coverslip cultures "fluid phase" or were those macrophages adhering to coverslips.

d. Results represent the average ± 2 standard errors of the mean. For fluid phase macrophages, values were obtained by pooling culture fluids over 3 infected coverslip cultures and by preparing 2 cytospin samples, 150 macrophages examined per sample, n = 2; for coverslip macrophages, 100 macrophages were examined per coverslip, n = 3.

e. Results represent the average number of cell-associated bacteria per infected macrophage ± 2 standard errors of the mean.

* Statistically significantly ($p < 0.05$) different, Student's two-tailed t-test.

Table 9. LIGHT MICROSCOPY OF BALB/cℸ ("Ity^s") AND CONGENIC C.D2Ity^r MACROPHAGES INFECTED IN VITRO WITH SALMONELLA TYPHIMURIUM a.

Table 9. LIGHT MICROSCOPY OF BALB/cπ (Ity^s) AND CONGENIC C.D2Ity^r MACROPHAGES INFECTED IN VITRO WITH SALMONELLA TYPHIMURIUM a.

MOUSE STRAIN	TIME[b.]	SOURCE OF MACROPHAGES[c.]	% INFECTED MACROPHAGES[d.]	AVERAGE NUMBER OF BACTERIA PER INFECTED MACROPHAGE[e.]
C.D2 Ity^r	t_o	Coverslip	46 ± 5	1.7 ± .4
BALB/cπ			45 ± 6	2.1 ± .4
C.D2 Ity^r	1	Coverslip	40 ± 2	2.2 ± 1.4
BALB/cπ			43 ± 2	2.1 ± .4
C.D2 Ity^r	4	Coverslip	22 ± 3 *	2.6 ± .6 *
BALB/cπ			32 ± 3 *	4.5 ± .9 *
C.D2 Ity^r	24	Coverslip	15 ± 2 *	2.7 ± 1.5 *
BALB/cπ			32 ± 12 *	7.1 ± 1.7 *
C.D2 Ity^r	4	Fluid Phase	30 ± 2	2.2 ± .4
BALB/cπ			33 ± 1	3.5 ± .3
C.D2 Ity^r	24	Fluid Phase	15 ± 2	6.9 ± 2.7
BALB/cπ			25 ± 8	11.3 ± .7

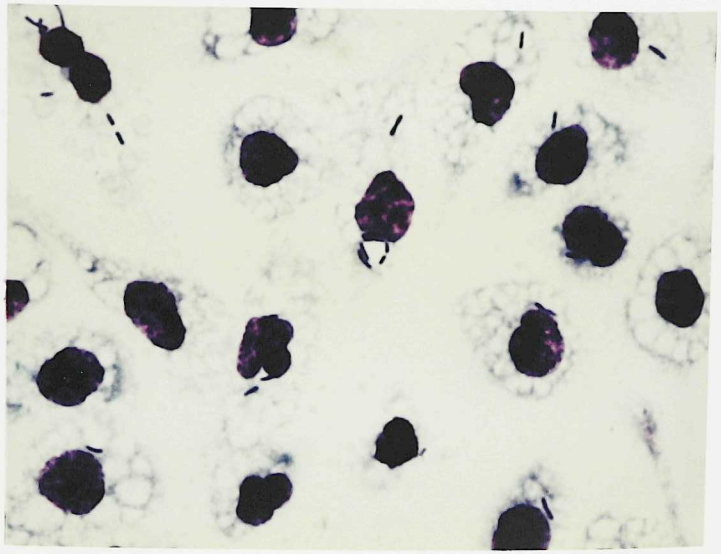


Figure 27a. Resident peritoneal macrophages from C.D2Ity^r mice were infected *in vitro* with *S. typhimurium* strain TML and the average number of salmonellae per macrophage was determined microscopically at 0, 1, 4, and 24 hours after infection. Figure 27a. is representative of infected C.D2Ity^r macrophages at 0 hours; cells examined at 100 X.
Magnification shown = 975 X.

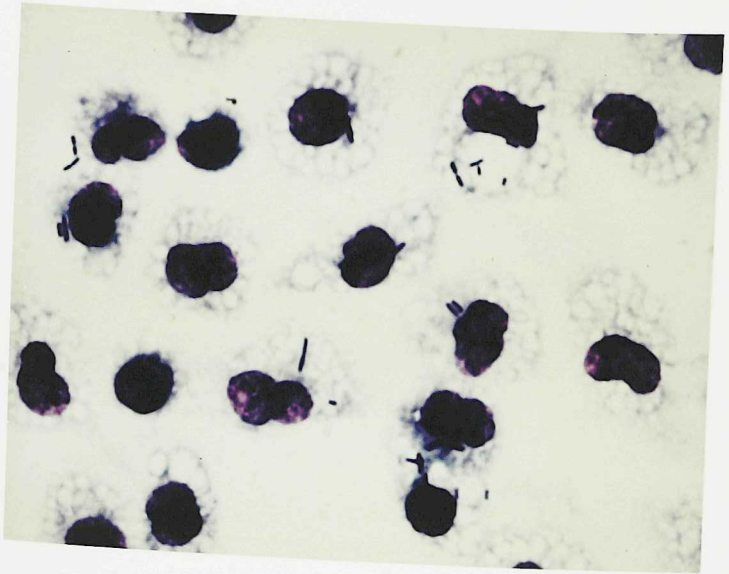


Figure 27b. Resident peritoneal macrophages from BALB/cn mice were infected *in vitro* with *S. typhimurium* strain TML and the average number of salmonellae per macrophage was determined microscopically at 0, 1, 4, and 24 hours after infection. Figure 27b. is representative of infected BALB/cn macrophages at 0 hours; cells examined at 100 X.
Magnification shown = 975 X.

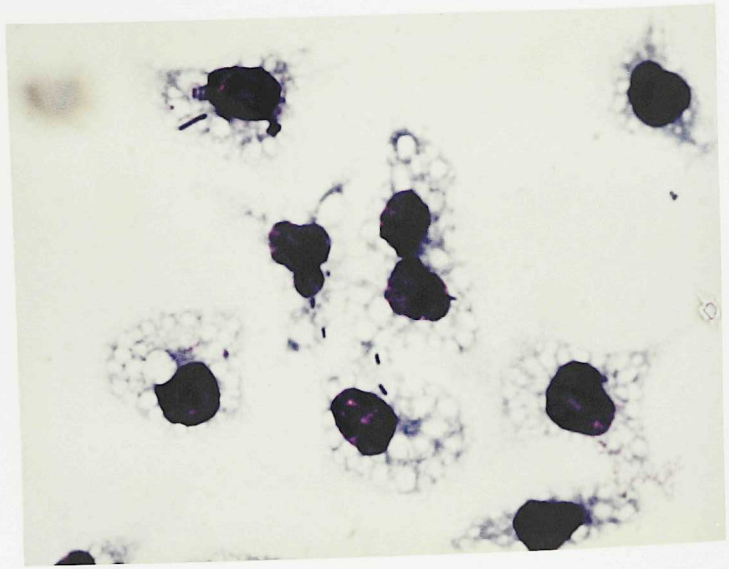


Figure 28a. Resident peritoneal macrophages from C.D2Ity^r mice were infected *in vitro* with *S. typhimurium* strain TML and the average number of salmonellae per macrophage was determined microscopically at 0, 1, 4, and 24 hours after infection. Figure 28a. is representative of infected C.D2Ity^r macrophages at 4 hours; cells examined at 100 X. Magnification shown = 975 X.

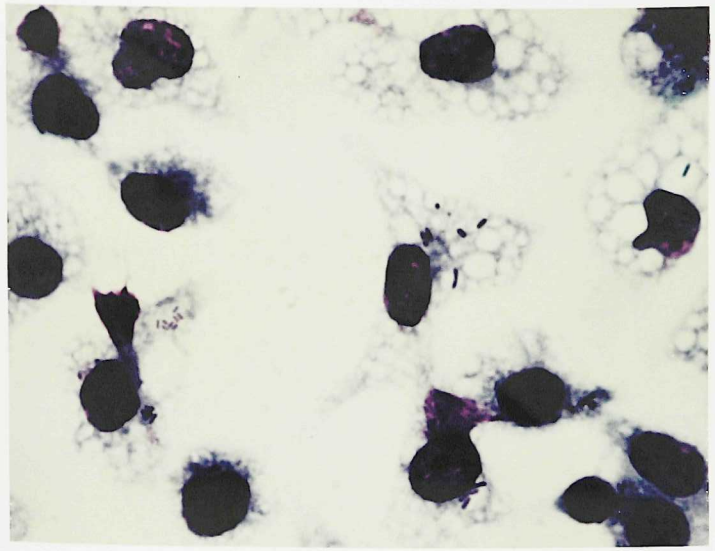


Figure 28b. Resident peritoneal macrophages from BALB/cℸ mice were infected *in vitro* with *S. typhimurium* strain TML and the average number of salmonellae per macrophage was determined microscopically at 0, 1, 4, and 24 hours after infection. Figure 28b. is representative of infected BALB/cℸ macrophages at 4 hours; cells examined at 100 X. Magnification shown = 975 X.

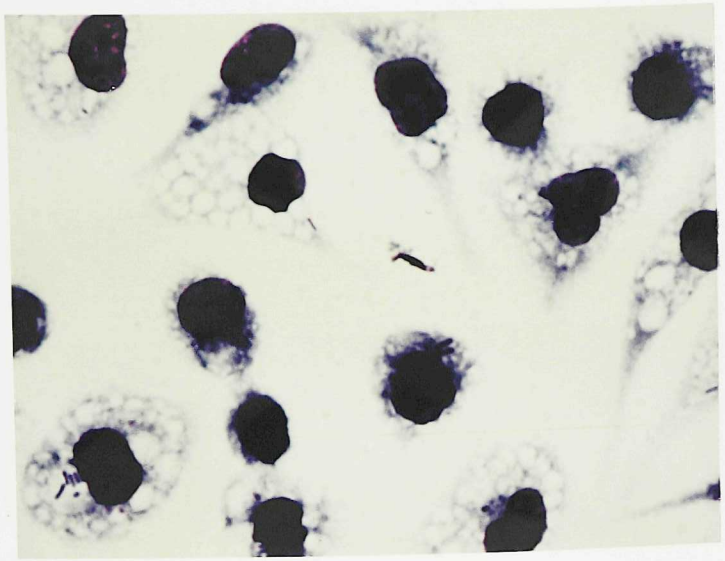


Figure 29a. Resident peritoneal macrophages from C.D2Ity^r mice were infected *in vitro* with *S. typhimurium* strain TML and the average number of salmonellae per macrophage was determined microscopically at 0, 1, 4, and 24 hours after infection. Figure 29a. is representative of infected C.D2Ity^r macrophages at 24 hours; cells examined at 100 X.

Magnification shown = 975 X.

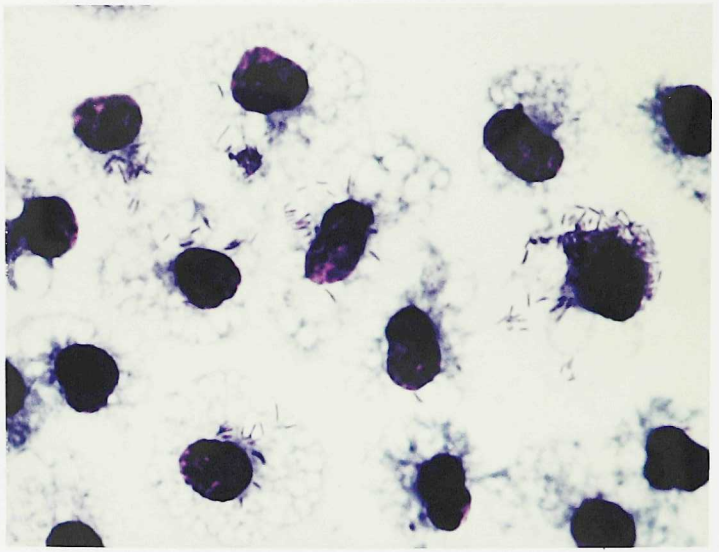


Figure 29b. Resident peritoneal macrophages from BALB/cn mice were infected *in vitro* with *S. typhimurium* strain TML and the average number of salmonellae per macrophage was determined microscopically at 0, 1, 4, and 24 hours after infection. Figure 29b. is representative of infected BALB/cn macrophages at 24 hours; cells examined at 100 X.

Magnification shown = 975 X.

scopic demonstration of a greater accumulation of bacteria in the Ity^s coverslip macrophages (Figures 28 and 29) and of a greater quantitative increase in the number of bacteria per infected macrophage in the Ity^s cells, substantiated that S. typhimurium can multiply within isolated adherent macrophages in the in vitro infection assay and that net multiplication is greater in Ity^s than in Ity^r macrophages.

The results obtained for fluid phase macrophages (Table 9) showed that a significant decrease in the percentage of infected C.D2Ity^r macrophages occurred from 4 to 24 hours; whereas, there was no significant change in the percentage of BALB/cπ macrophages over the same period. At 4 hours there was no difference ($p = 0.05$) between the number of bacteria per C.D2Ity^r or per BALB/cπ macrophages. Although there were numerically more infected Ity^s macrophages than Ity^r macrophages and the infected Ity^s cells had more bacteria per infected macrophage, statistically the fluid phase populations of BALB/cπ and C.D2Ity^r infected macrophages were the same. This absence of statistical significance may merely reflect the small sample size (n=2). Indeed, when the number and the percentage of heavily infected macrophages ( ) 20 S. typhimurium/cell), as shown in Figure 30, was calculated separately for fluid and coverslip cultures (Table 10), significantly more of the macrophages containing large numbers of salmonellae (shown in Figures 31 and 32) were found in BALB/cπ macrophage cultures than in C.D2Ity^r

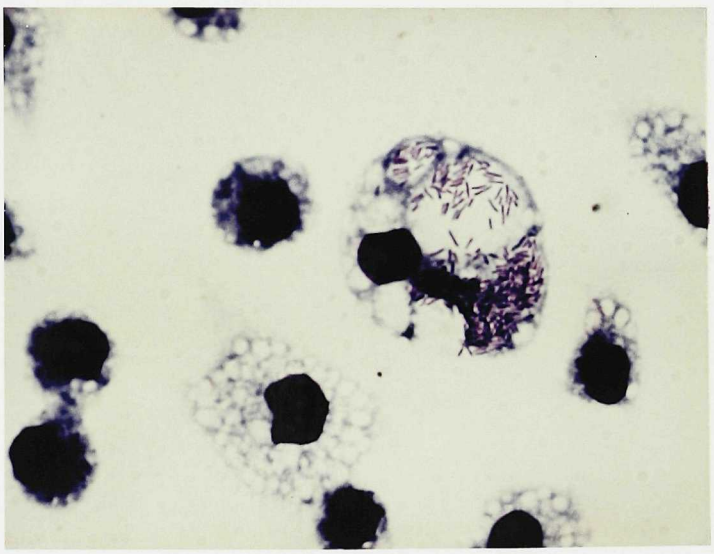


Figure 30. A representative resident peritoneal macrophage with > 20 intracellular S. typhimurium strain TML, heavily infected, after 24 hours of in vitro infection; cells examined at 100 X. Magnification shown = 975 X.

Table 10.

a. Resident peritoneal macrophages from BALB/cℸ and congenic C.D2Ity^r mice were cultured (Method 2) on coverslips and infected with S. typhimurium strain TML. The percentage of infected macrophages and the number of cell-associated salmonellae per infected macrophage were enumerated by light microscopy.

b. Macrophages examined were those freely floating in maintenance medium over infected coverslip cultures "fluid phase" or were those macrophages adhering to coverslips.

c. The average percentage of all macrophages examined which contained bacteria ± 2 standard errors of the mean. For Experiment 1, 150 fluid phase macrophages from 2 cytospin preparations, n = 2 and 100 macrophages per coverslip, n = 3 were examined; for Experiment 2, 150 macrophages per coverslip, n = 3 were examined.

d. The average percentage of all infected macrophages which contained > 20 bacteria per macrophage "heavily infected" ± 2 standard errors of the mean.

Table 10. PERCENTAGE OF HEAVILY INFECTED BALB/cℸ AND C.D2Ity^r MACROPHAGES AFTER 24 HOURS OF AN IN VITRO S. TYPHIMURIUM

Table 10. PERCENTAGE OF HEAVILY INFECTED BALB/cπ AND C.D2Ity^r MACROPHAGES AFTER 24 HOURS OF AN IN VITRO S. TYPHIMURIUM INFECTION [a].

	MOUSE STRAIN	SOURCE OF MACROPHAGES[b]	TOTAL % OF INFECTED MACROPHAGES[c]	% OF INFECTED MACROPHAGES HEAVILY INFECTED[d]
Experiment 1	C.D2 Ity^r	Fluid Phase	15 ± 2	16 ± 3
	BALB/c		25 ± 8	44 ± 4
	C.D2 Ity^r	Coverslip	15 ± 2	0
	BALB/cπ		32 ± 12	22 ± 9
Experiment 2	C.D2 Ity^r	Coverslip	44 ± 1	5 ± 2
	BALB/cπ		44 ± 1	17 ± 6

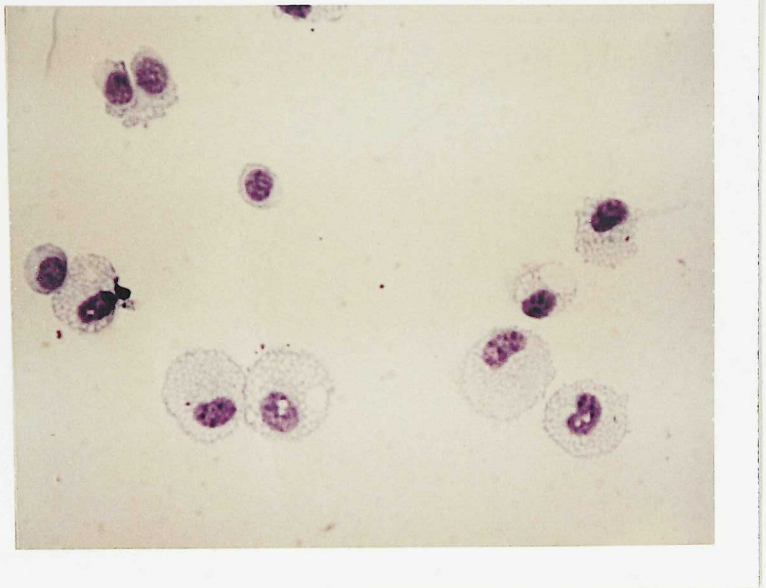


Figure 31a. This figure is representative of C.D2*Ity*^{r} fluid phase resident peritoneal macrophages after 24 hours of infection with *S. typhimurium* strain TML; cells examined at 40 X. Magnification shown = 400 X.

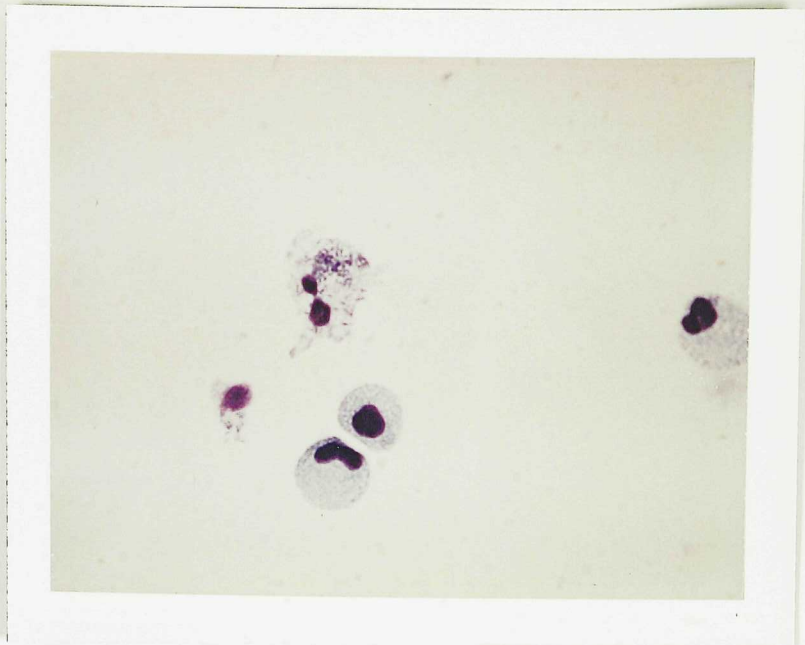


Figure 31b. This figure is representative of BALB/c$\mathcal{H}$ fluid phase resident peritoneal macrophages after 24 hours of infection with *S. typhimurium* strain TML; cells examined at 40 X. Magnification shown = 400 X.

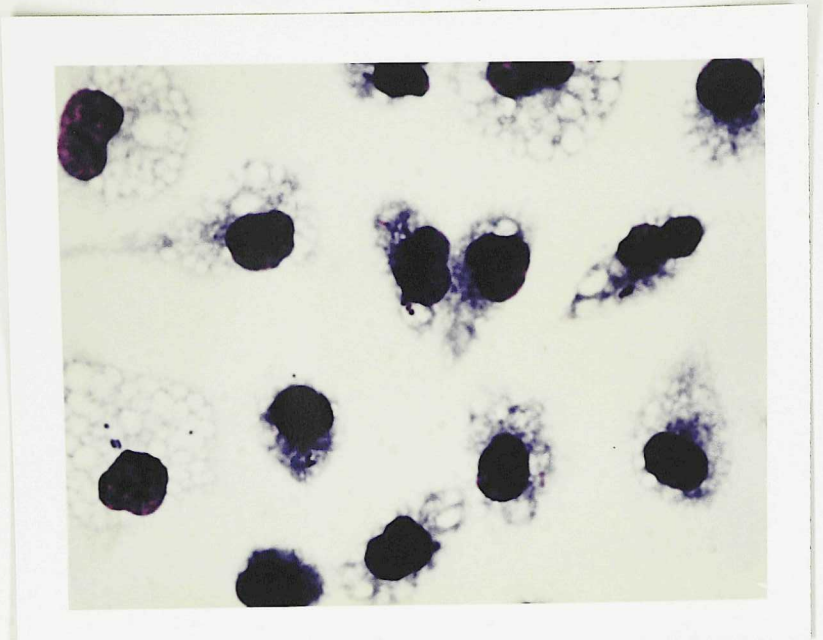


Figure 32a. This figure is representative of C.D2*Ity*^{r} adherent (coverslip) resident peritoneal macrophages after 24 hours of infection with *S. typhimurium* strain TML; cells examined at 100 X. Magnification shown = 975 X.

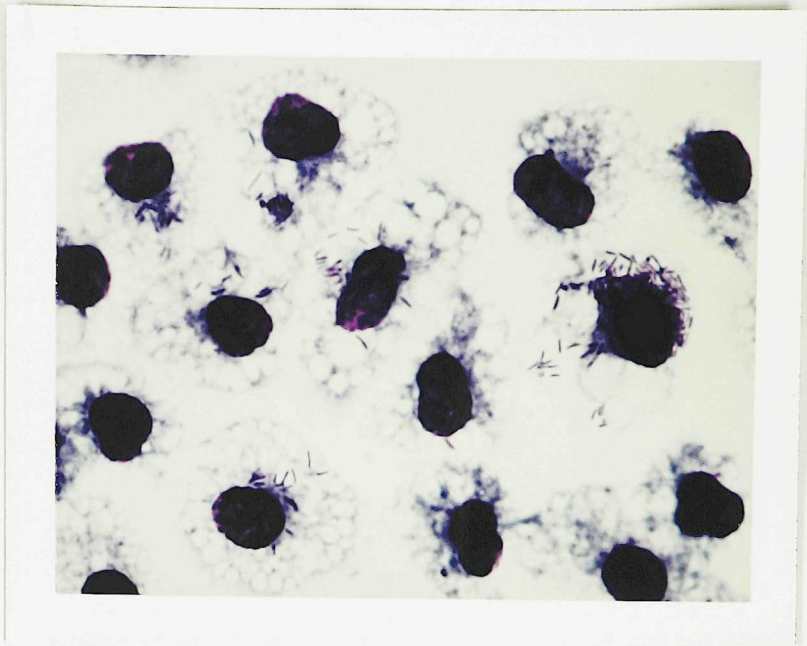


Figure 32b. This figure is representative of BALB/c adherent (coverslip) resident peritoneal macrophages after 24 hours of infection with *S. typhimurium* strain TML; cells examined at 100 X. Magnification shown = 975 X.

macrophage cultures. These heavily infected macrophages were not observed in either Ity^s or Ity^r macrophage cultures at t_o, and only rarely at 1 or 4 hours after infection. This analysis showed that although heavily infected macrophages may be found in Ity^r cultures, there are significantly more of these heavily infected macrophages in Ity^s cultures.

Transmission Electron Microscopic Examination of Salmonella typhimurium-Infected BALB/cπ and Congenic C.D2Ity^r Macrophages

S. typhimurium is a facultative intracellular parasite of macrophages (Ushiba, 1962; Blanden, 1968; Carrol, et al., 1979; Lowrie, et al., 1979). The purpose of this initial transmission electron microscopy (T.E.M.) study was to compare the cellular ultrastructure of Ity^s and Ity^r macrophages, which, as an expression of Ity phenotype, differ in their efficiency of killing S. typhimurium in vitro. In addition, the intracellular fate of S. typhimurium within the two macrophage types was compared.

Resident peritoneal macrophages from BALB/cπ and from congenic C.D2Ity^r mice were cultured (Method 2) on plastic (Therminox) coverslips and infected with S. typhimurium strain TML. Sham-infected macrophage coverslip cultures were included as uninfected controls. Coverslip cultures were prefixed in situ immediately after aspiration of infection or maintenance media. The

coverslips were then transfered to 35 mm culture dishes and processed for T.E.M. (as described in Methods and Materials).

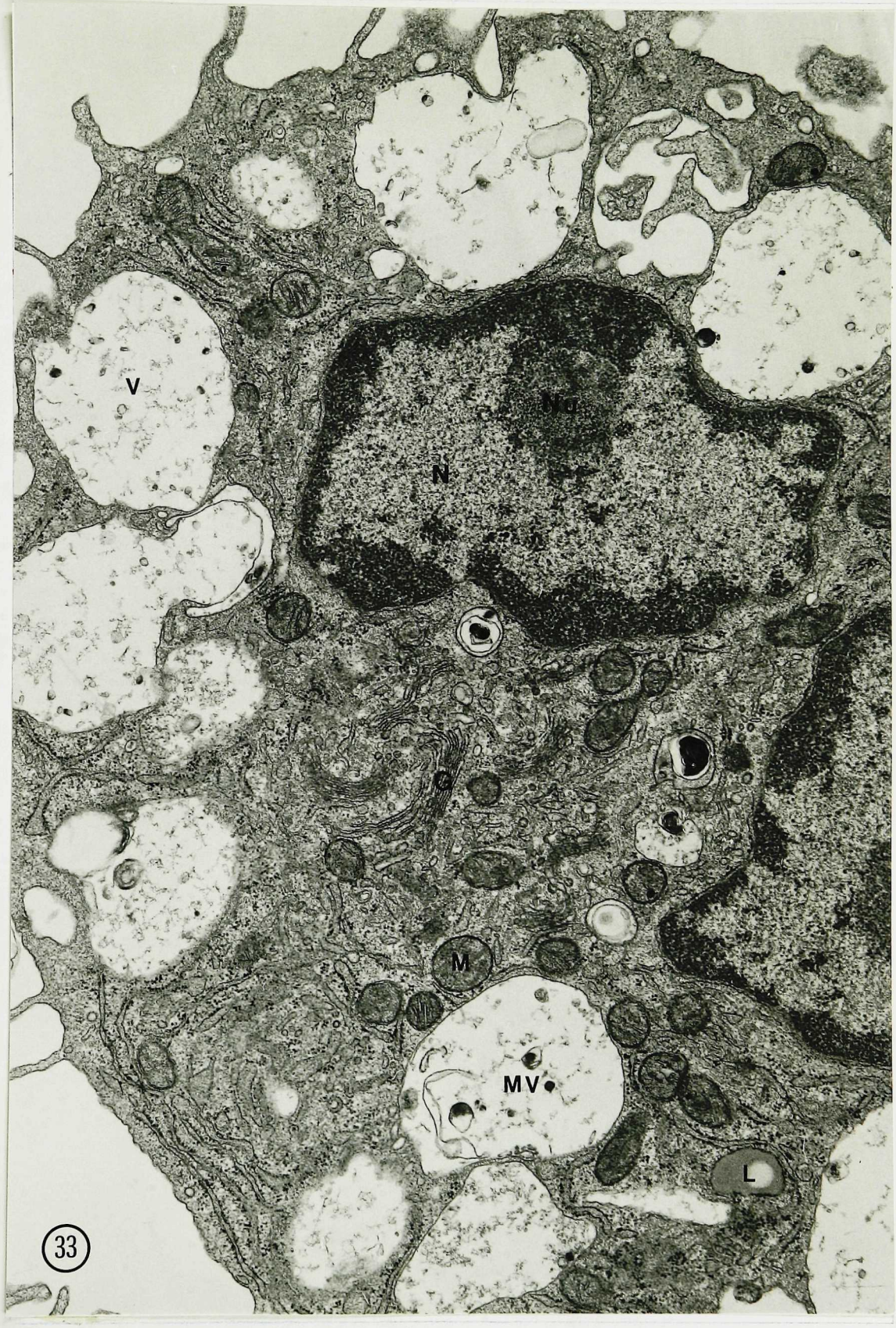
I. Uninfected macrophages

Figure 33 is a representative uninfected resident peritoneal macrophage from a BALB/cn culture at t_o. No differences between BALB/cn and C.D2Ity^r uninfected macrophages were noted at this or any time point over the 24 hours of the *in vitro* assay. The nucleus is typical for macrophages with condensed peripheral chromatin and a nucleolus. All of the major structures and organelles in the cytoplasm appeared normal: ribosome-studded rough endoplasmic reticulum; a well developed Golgi apparatus with flattened cisternae and small vessicles; primary and secondary lysosomes; vacuoles, some of which contained vesicles, lipid granules, or amorphous electron opaque material; and numerous mitochrondria. Pseudopod-like projections were routinely seen along the periphery of the cell.

Primary and secondary lysosomes, phagosomes, and phagolysosomes were identified in this study according to the descriptions of Daems (Daems, 1980b.) and Alberts *et al.* (Alberts *et al.*, 1983). A primary lysosome is defined as a small vesicle (with a 50 nm limiting membrane) which arises from the Golgi apparatus and contains specific acid hydrolases and other lytic enzymes. A phagosome is a

Figure 33.

A representative uninfected resident peritoneal macrophage (BALB/cℱ) at t_o. The nucleus (N), with a nucleolus (Nu), is well defined by a unit membrane. Major structures and organelles appear normal in the cytoplasm: these include a well developed Golgi apparatus (G); numerous mitochondria (M); rough endoplasmic reticulum; large vacuoles (V), some of which contain small vesicles, multivesicular vacuoles (MV); and a lipid granule (L). Pseusdopod-like projections appear along the periphery of the cell. No difference between C.D2Ity^r and BALB/cℱ uninfected macrophages were noted at this in vitro assay time point or any other assay time point. Magnification = 20,000 X.


V
Nu
N
M
MV
L
33

structure formed by phagocytosis of particulate material from the cell surface and subsequent enclosure of such material within a unit membrane-bound intracellular vacuole. A phagolysosome is produced by the fusion of primary and/or secondary lysosomes with a phagosome. A secondary lysosome is formed by the fusion of primary lysosomes with each other and other intracellular vesicles. It is limited by a unit membrane. A multivesicular body is a large vacuole containing many vesicles, including lysosomes. An autophagic vacuole is a large unit membrane-limited vacuole in which the cell's own membranes and organelles are actively being degraded. Primary and secondary lysosomes, multivesicular bodies, and phagolysosomes may contribute to an autophagic vacuole.

II. Infected macrophages

The data presented in Figures 34 and 35 show S. typhimurium at three stages of parasite-macrophage interaction at the end of the 50 minute infection period (t_o): 1., contained within intact phagosomes; 2., within phagosomes about to fuse or fusing with lysosomes; 3., within true phagolysosomes. No difference in these bacteria-macrophage interactions were observed between Ity^s and Ity^r macrophage cultures.

Figure 34a. (C.D2Ity^r) and Figure 34b. (BALB/cn) show S. typhimurium that have just been phagocytized. In both the Ity^r and the Ity^s cells, the bacteria are

Figure 34a. and Figure 34b.

Figure 34a. (C.D2Ity^r) and Figure 34b. (BALB/cη) are representative of infected resident peritoneal macrophages at t_o. S. typhimurium (S) is contained within phagosomes (P). The salmonellae within these phagosomes are intact and have an even dense granular cytoplasm. The internal structures and organelles of both the Ity^r and the Ity^s macrophages are unperturbed. Magnification = 16,000 X.


P
S
34 a

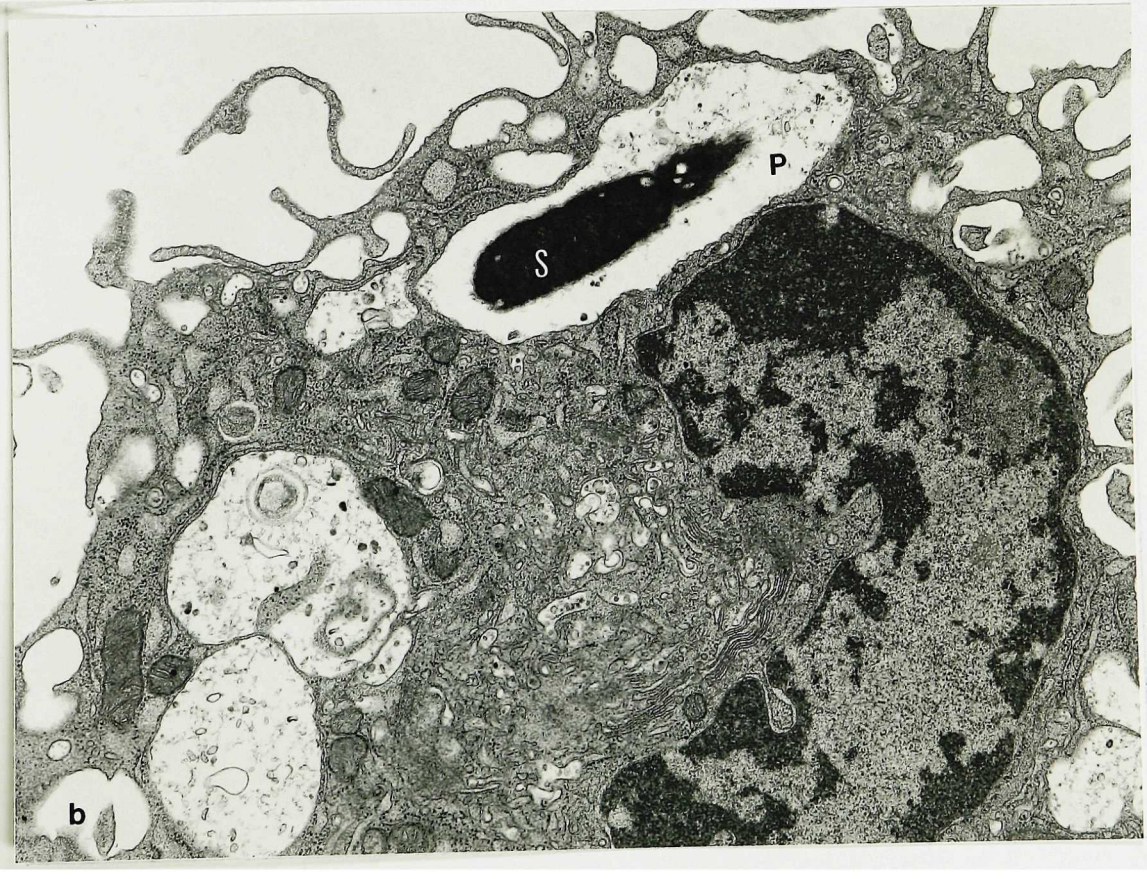

P
S
b

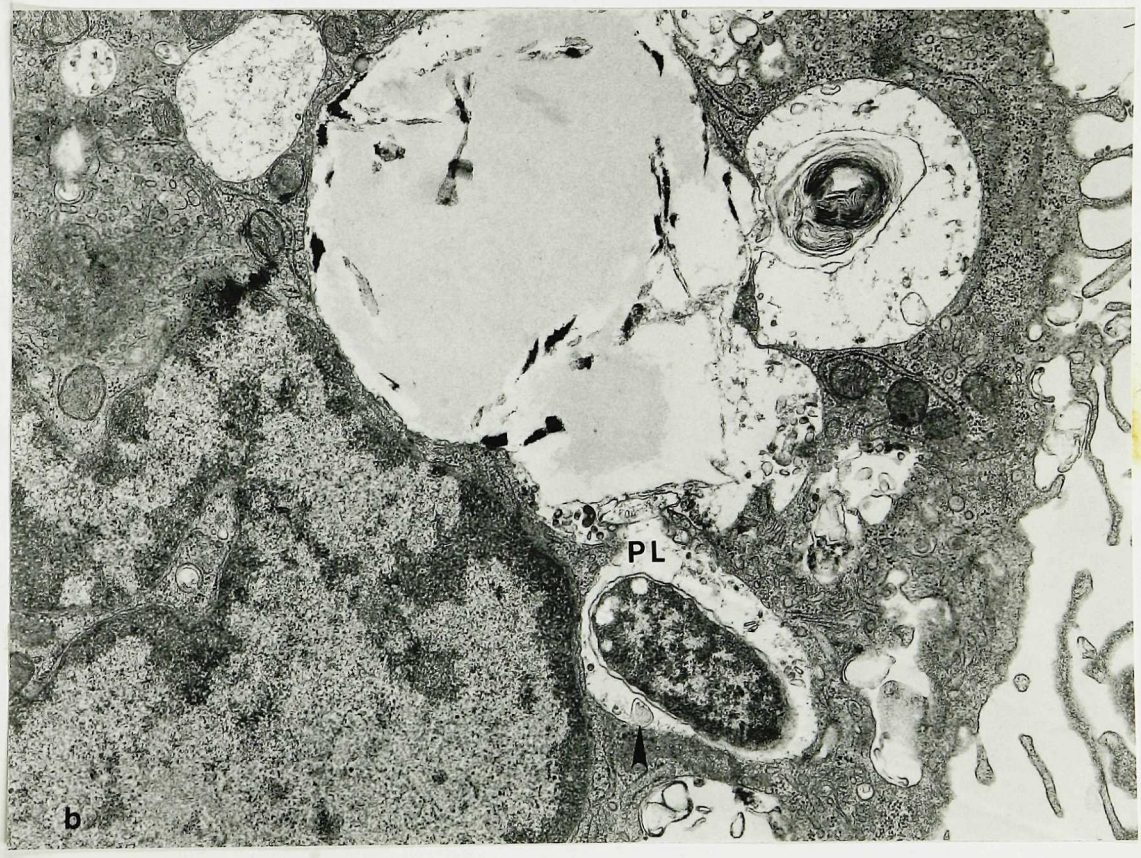
Figure 35a.

This figure is representative of a S. typhimurium-infected C.D2Ity^r resident peritoneal macrophage at t_o. The salmonellae are intact and contained within phagosomes (P) or phagolysosomes (PL). The cytoplasm and intracellular structures of the macrophage show no signs of disruption. Several multivesicular vacuoles (MV) are present in the cytoplasm. Magnification = 10,000 X.

Figure 35b.

This figure is representative of a S. typhimurium-infected BALB/cγ resident peritoneal macrophage at t_o. The bacterium is within a phagolysosome (PL) in which a secondary lysosome (arrow head) is in the process of fusion. Magnification = 16,000 X.


MV
PL
P
35 a


PL
b

contained within phagosomes with defined, continuous borders. The cytoplasm and intracellular organelles of both the Ity^r and Ity^s macrophages appear to be unperturbed. In both Figures 34a. and 34b. the salmonellae are intact and have an even, dense, finely granular cytoplasm. The outer membranes can just be discerned at these magnifications, but they appear to be intact when observed in cross section. No ultrastructural differences in the initial interaction and phagosome formation were observed between BALB/cn and C.D2Ity^r macrophages.

Figures 35a. and 35b. are representative of infected Ity^r and Ity^s macrophages, at t_o, in which fusion of lysosomes with phagosomes is occurring. In Figure 35a. (C.D2Ity^r) three salmonellae are in phagosomes which are about to fuse or have fused with lysosomes to form phagolysosomes. At higher magnification (Figure 35b. [BALB/cn]) a phagolysosome containing a single bacterium is seen in greater detail. Lysosome fusion has occurred, as evidenced by the patchy opaque material within the phagolysosome, which contains many small intact vesicles and an intact secondary lysosome. The cellular architecture, cytoplasm, and organelles of the macrophages (Figures 35a. and 35b.) reveal no signs of disruption. In cross section the salmonellae within either phagosomes or phagolysosomes are intact.

When compared to uninfected 4 hour controls, both infected Ity^r and Ity^s macrophages exhibited similar ultrastructure, including normal undamaged

intracellular organelles and Golgi apparati; however, in both Ity^r and Ity^s infected cells, large multivesicular vacuoles and autophagic phagolysosomes were frequently found. No qualitative differences between Ity^s and Ity^r infected macrophages were observed (Figures 36a. and 36b.). Both Ity^r and Ity^s macrophages at 4 hours contained many phagolysosomes in which more than two salmonellae were present. This was not observed in t_o specimens. It was not possible to exclude multiple phagolysosomal fusion events, between t_o and 4 hours, as the origin of these more "populated" phagolysosomes; on the other hand, intraphagosomal replication of S. typhimurium could not be ruled out. Figures 36a. (C.D2Ity^r) and 36b. (BALB/cπ) show, after 4 hours of infection, salmonellae within phagolysosomes. In Figure 36a. the four salmonellae in one large phagolysosome show internal disruption, indicated by the condensation of bacterial cytoplasm and by protrusions of the outer membrane. A fourth bacterium, seen in transverse cross section, appears to be less damaged internally but does exhibit shrinkage away from the outer membrane. Within this phagolysosome are numerous vesicles and lysosomes, as well as, a region of apparant autophagy. A multivesicular body appears to be in the process of fusing with the phagolysosome. Figure 36b. shows three salmonellae at various stages of degradation within a peri-nuclear phagolysosome. The bacterium seen in complete longitudinal cross section is swollen and remnants of its cytoplasm have been condensed along the

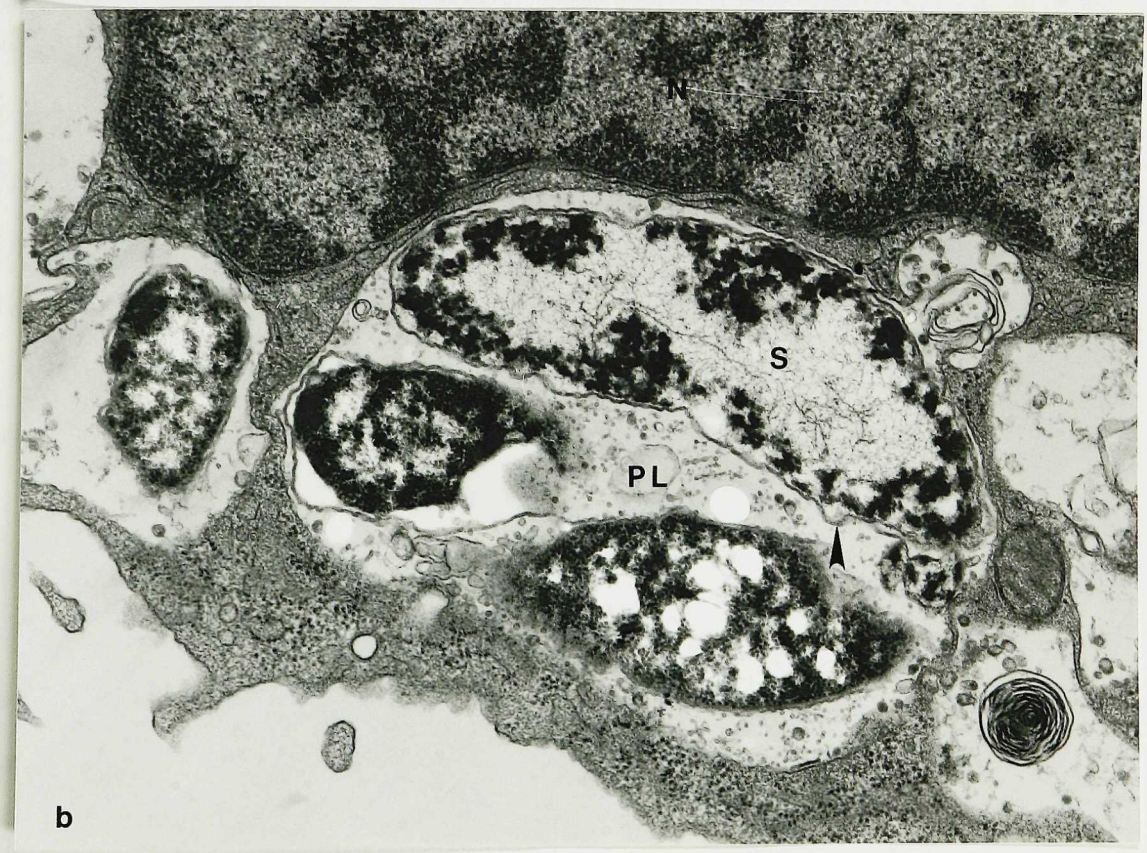
Figure 36a.

This figure shows a portion of a representative S. typhimurium-infected C.D2Ity^r resident peritoneal macrophage after 4 hours of infection. A large phagolysosome (PL) contains salmonellae which show signs of internal disruption. The phagolysosome also contains numerous vesicles and lysosomes, as well as, an area of autophagy (A). Magnification = 16,000 X.

Figure 36b.

This figure shows a portion of a representative S. typhimurium-infected BALB/cγ resident peritoneal macrophage after 4 hours of infection. A perinuclear phagolysosome (PL) contains salmonellae at various stages of internal degradation. A bacterium (S) in complete cross section is swollen and remnants of the cytoplasm have condensed along the inner periphery of the cell. Evidence of damage to the cell wall and outer membrane of the bacterium is evidenced by protrusions (arrow head). Magnification = 28,000 X.


36 a
PL
A


N
S
PL
b

periphery of the cell. The outer membranes of all three bacteria show protrusions. A multivesicular vacuole appears to be fusing with this phagolysosome. In both Figures 36a. and 36b. the macrophage cytoplasm around the phagolysosomes is rich in free and membrane associated ribosomes.

Figure 37a. (BALB/cn) is presented to illustrate that, in terms of the visible degree of bacterial damage, S. typhimurium in phagolysosomes represented a heterogeneous population. The bacterium in Figure 37a. is clearly within a phagolysosome, surrounded by an area of intense autophagy. This bacterium, while showing some perturbation of the outer membrane, exhibits the finely granular, evenly distributed cytoplasm observed in bacteria contained in phagosomes at t_o. The two bacteria in Figure 37b. show some internal damage and the outer membranes do not show complete integrity; nevertheless, these bacteria may potentially still be viable and capable of replication. No difference in this apparant heterogeneity in the degree of bacterial damage was noted between Ity^s and Ity^r macrophage cultures.

BALB/cn and congenic C.D2Ity^r macrophages examined after 24 hours of infection (data not shown) exhibited S. typhimurium in phagolysosomes and, as described above for the 4 hour time point, showed the same heterogeneity in the population of intra-phagosomal bacteria. No differences between the Ity^s and Ity^r macrophages could be discerned; and, while both populations



Figure 37a. and Figure 37b. (inset)

These figures depict portions of two S. typhimurium-infected BALB/cγ resident peritoneal macrophages after 4 hours of infection. The salmonellae are within phagolysosomes and appear to be intact with little or no apparant damage (compare with Figure 36a. and Figure 36b.). The phagolysosome with a S. typhimurium (S) in Figure 37a. is surrounded by areas of intense autophagy (A). These figures are representative of similar findings with S. typhimurium-infected C.D2Ity^r macrophages after 4 hours of infection. Figure 37a. magnification = 28,000 X. Figure 37b. magnification = 20,000 X.


S
A
37 a

of infected macrophages were seen to be actively responding to the intracellular parasite, neither *Ity*^{s} nor *Ity*^{r} macrophages exhibited cytoplasmic disruption nor organelle damage. All bacteria remained within the phagolysosomes.

III. Summary

The findings of these T.E.M. survey experiments showed that: 1., there were no observable differences between *S. typhimurium*-infected BALB/cn and C.D2*Ity*^{r} resident peritoneal macrophages in the initial bacterium-macrophage interaction, i.e., the formation of the phagosome or phagolysosome; 2., at later time points of the *in vitro* assay, salmonellae were contained within phagolysosomes, and if salmonellae were qualitatively ranked by signs of external and/or internal damage, a heterogeneous population ranging from nearly undamaged to nearly complete degradation was present similarly in *Ity*^{s} and *Ity*^{r} macrophages; 3., phagolysosomes at 4 hours and 24 hours of infection contained more salmonellae/phagolysosome than at t_{o}, however, these differences could be attributable to phagolysosome-phagolysosome fusion, bacterial survival and multiplication within the phagolysosome, or to both of these possibilities; 4., T.E.M. can be used to examine *S. typhimurium* infected *Ity*^{s} and *Ity*^{r} macrophages as processed during the *in vitro* infection assay and, therefore, other labeling techniques may be utilized, which may better detect, at the

ultrastructural level, the basis for the more efficient killing of S. typhimurium in vitro by Ity^r rather than by Ity^s macrophages.

Killing of Avirulent Strains of Salmonella typhimurium by BALB/cn and C.D2Ity^r Resident Peritoneal Macrophages

The finding that more S. typhimurium accumulate in Ity^s than in Ity^r macrophages by 24 hours of infection could reflect more rapid killing of the bacteria by Ity^r macrophages or a slower rate of bacterial division within Ity^r macrophages or both. To compare the microbicidal efficiency of Ity^r and Ity^s macrophages, the assay was performed (Method 1) with two mouse avirulent strains of S. typhimurium: SL3235 which undergoes only slight net growth in the tissues of mice (A. D. O'Brien, personal communication) due to a defect in aromatic acid metabolism (Hoiseth and Stocker, 1981), and TML/TS27 which is unable to replicate at 37° C because of a non-lethal temperature sensitive mutation (Swanson and O'Brien, 1983). When BALB/cn and C.D2Ity^r resident peritoneal macrophages were infected with S. typhimurium strain SL 3235, the results shown in Figure 38 were obtained. At t_o there was no difference observed between the initial number of viable salmonellae in BALB/cn and C.D2Ity^r macrophages ($p > 0.05$). The magnitude of these initial values was considerably reduced (nearly 10 fold) from those t_o values obtained with virulent strain TML for reasons as yet

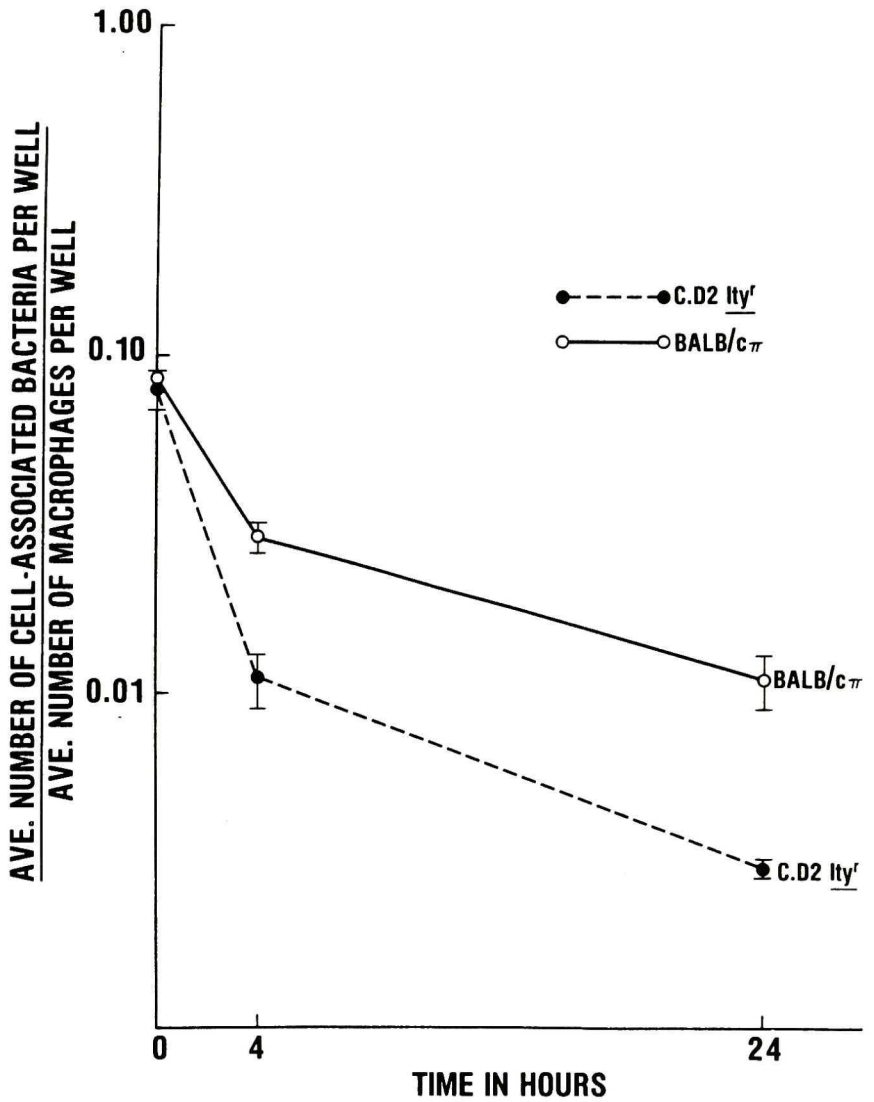


Figure 38. Resident peritoneal macrophages from congenic C.D2Ity^r and BALB/cπ mice were infected in vitro with the mouse avirulent (Hoiseth and Stocker, 1981) S. typhimurium strain SL3235. The number of viable bacteria in macrophage lysates prepared at 0, 4, and 24 hours after infection was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean. (from Lissner et al., 1983)

unclear. At 4 hours the number of viable salmonellae had declined significantly ($p < 0.05$) for both strains; nevertheless, BALB/cn macrophages had 2.9 times the number of bacteria found in C.D2Ity^r macrophages ($p < 0.05$). Over the next 20 hours of infection the number of viable salmonellae in both BALB/cn and C.D2Ity^r macrophages continued to decline significantly ($p < 0.05$) relative to the number at 4 hours. Although the Ity^s macrophages did show a reduction of intracellular bacteria over the entire 24 infection period, the number of bacteria in these macrophages at 24 hours was 3.7 fold higher ($p < 0.05$) than in Ity^r macrophages. When S. typhimurium strain TML/TS27 was used to infect resident peritoneal macrophages from BALB/cn and C.D2Ity^r mice, the results shown in Figure 39 were obtained. Because TML/TS27 does not grow at 37° C, this experiment was performed in the absence of gentamicin. Time points at 1 hour and at 12 hours were included. Macrophages from each mouse strain were able to significantly ($p < 0.05$) reduce the number of viable salmonellae during the first 12 hours of infection. From 12 hours to 24 hours there was no significant ($p > 0.05$) change in the number of intracellular bacteria in either Ity^r and Ity^s macrophages. The ratio of average number of viable salmonellae in BALB/cn macrophages to average bacterial counts in C.D2Ity^r macrophages was 4.5 ($p < 0.05$) at 1 hour; 6.1 ($p < 0.05$) at 4 hours; 5.3 ($p < 0.05$) at 12 hours; and 5.7 ($p < 0.05$) at 24 hours. Thus, C.D2Ity^r macrophages appeared to kill TML/TS27 more

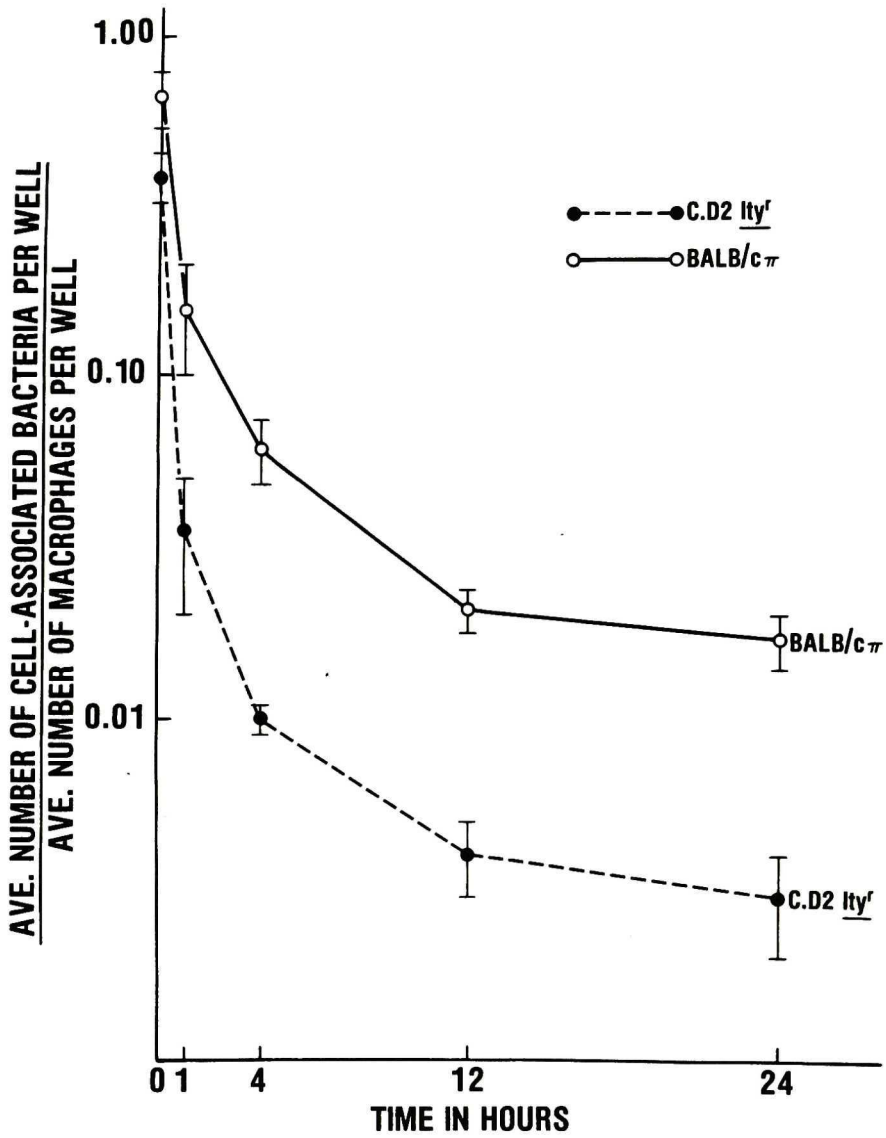


Figure 39. Resident peritoneal macrophages from congenic C.D2Ity^r and BALB/cπ mice were infected in vitro with TML/TS27, a non-replicating temperature sensitive mutant of S. typhimurium (Swanson and O'Brien, 1983). The number of viable bacteria in macrophage lysates prepared at 0, 1, 4, and 24 hours was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean. (from Lissner et al., 1983)

efficiently than did BALB/cn macrophages.

Comparison of the Bactericidal Capacities of BALB/cn and Congenic C.D2Ity^r Macrophages Infected In Vitro with Bacterial Species other than Salmonella typhimurium

The findings that Ity^r macrophages killed avirulent strains of S. typhimurium more efficiently than did Ity^s macrophages in vitro lead to a general question: Was the difference in bactericidal activity restricted to S. typhimurium or was the difference less specific and not confined to S. typhimurium? To address this question in vitro assays were performed with other bacterial species.

I. In vitro assays with other gram negative bacilli

Resident peritoneal macrophages from BALB/cn and congenic C.D2Ity^r mice were cultured (Method 2) and infected with S. typhi strain Quailes or with E. coli strain HS. When S. typhi was used to infect macrophages in vitro (Figure 40), both Ity^s and Ity^r cultures significantly ($p < 0.05$) reduced from t_o to 1 hour and over the next 20 hours of infection ($p < 0.05$) the number of viable cell-associated salmonellae. This finding is in concert with the non-pathogenicity of S. typhi in the murine host (Collins and Carter, 1978; O'Brien, 1982). However, when the ratio of viable cell-associated S. typhi in BALB/cn macrophages to C.D2Ity^r macrophages was

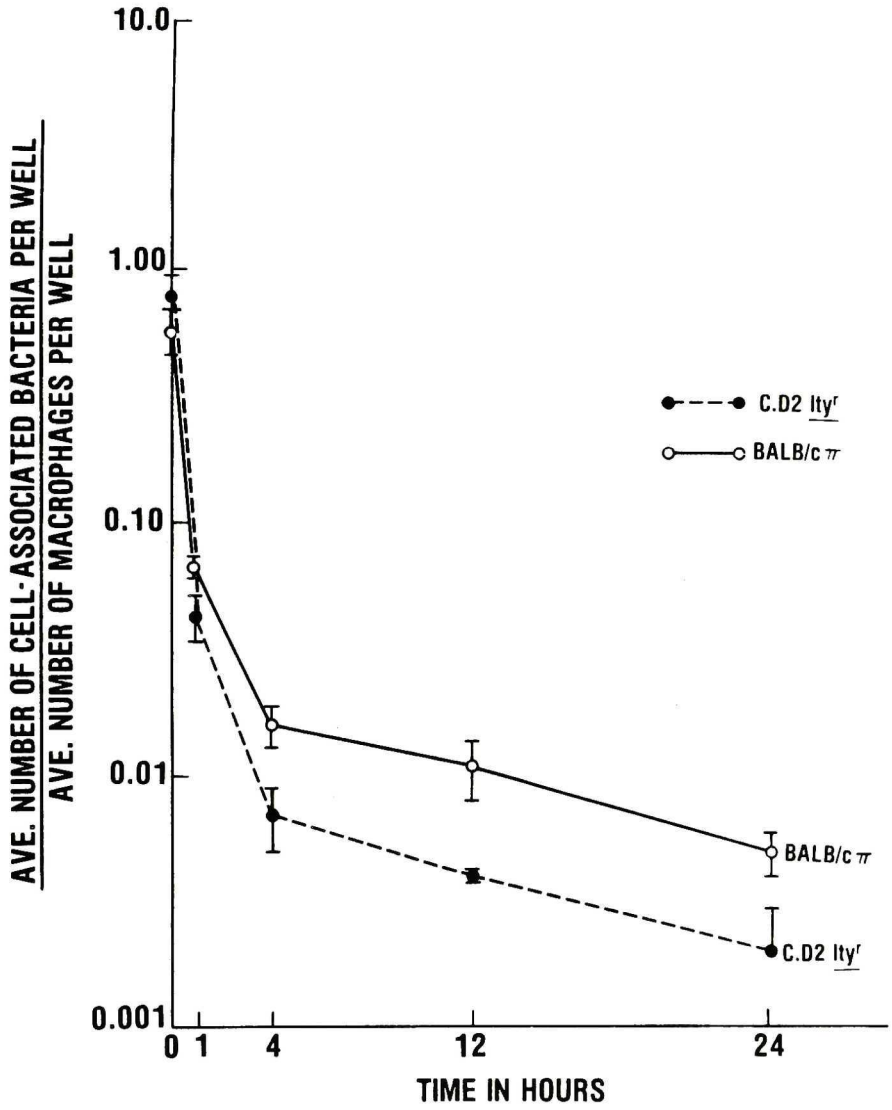


Figure 40. Resident peritoneal macrophages from congenic C.D2Ity^r and BALB/cπ mice were infected in vitro with mouse avirulent S. typhi. The number of viable bacteria in macrophage lysates prepared at 0, 1, 4, 12, and 24 hours after infection was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

compared, the ratio was: 0.73 ($p > 0.05$) at t_o; 1.58 ($p < 0.05$) at 1 hour; 2.29 ($p < 0.05$) at 4 hours; 2.75 ($p < 0.05$) at 12 hours; and 2.50 ($p < 0.05$) at 24 hours. Similar significant findings were obtained when the amount of macrophage lysate protein was used to calculate the index of S. typhi infection (Figure 41). Infection of BALB/cn and C.D2Ity^r macrophages with E. coli strain HS (Figure 42) gave results which paralleled the results obtained with S. typhi: i.e., Ity^r macrophages killed E. coli more efficiently than did Ity^s macrophages. Comparison of the ratio of cell-associated E. coli in BALB/cn macrophages to C.D2Ity^r macrophages was: 0.78 ($p > 0.05$) at t_o; 1.74 ($p < 0.05$) at 1 hour; 3.58 ($p < 0.05$) at 4 hours; and 5.00 ($p < 0.05$) at 24 hours. Considered together, the findings of in vitro assays performed with S. typhi and with E. coli suggest that the more efficient killing of S. typhimurium strains by Ity^r macrophages is not restricted to this single species of gram negative bacilli.

II. In vitro assays with gram positive bacteria

Resident peritoneal macrophages from BALB/cn and C.D2Ity^r mice were cultured (Method 2) and infected with a human isolate of Staphylococcus aureus or with the non-toxin producing Corynebacterium diphtheriae strain C7 (macrophage disruption for these assays was accomplished with 0.01% BSA in water). When S. aureus was used to

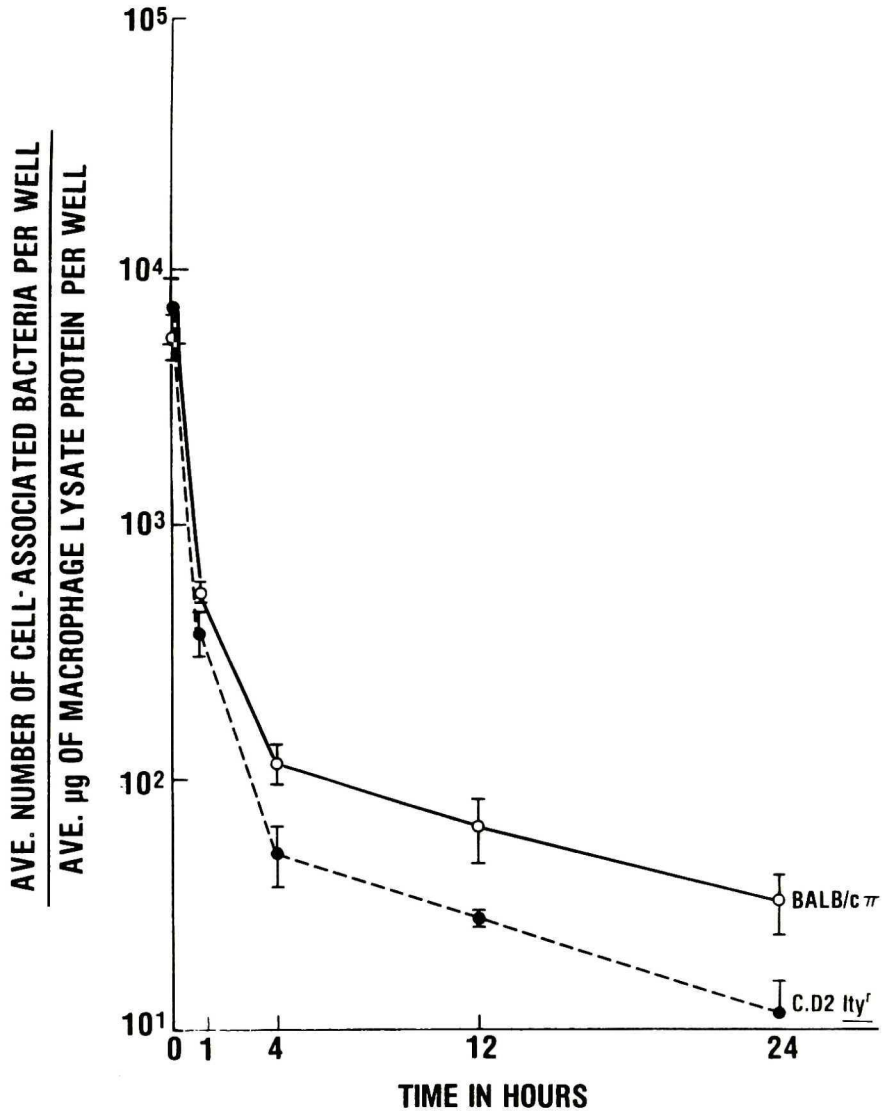


Figure 41. Resident peritoneal macrophages from congenic C.D2Ity^r and BALB/cπ mice were infected in vitro with mouse avirulent S. typhi. The number of viable bacteria in macrophage lysates prepared at 0, 1, 4, 12, and 24 hours after infection was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

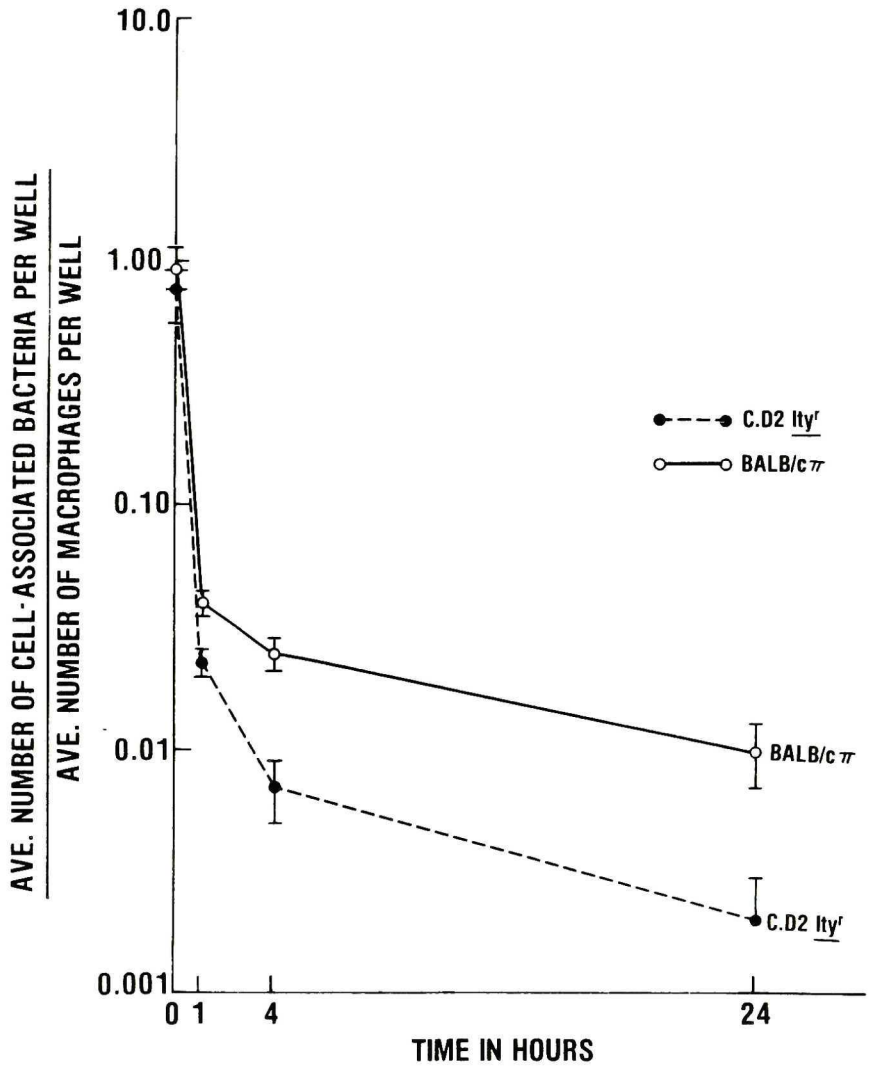


Figure 42. Resident peritoneal macrophages from congenic C.D2Ity^r and BALB/cπ mice were infected in vitro with E. coli strain HS. The number of viable bacteria in macrophage lysates prepared at 0, 1, 4, and 24 hours was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

infect macrophages (Figure 43), quantification of the number of viable bacteria was difficult due to the cluster-like growth characteristic of this gram positive coccus. The results depicted in Figure 43 were viewed with this technical difficulty in mind. The ratio of the number of viable cell-associated S. aureus (colony forming units) in BALB/cn macrophages to C.D2Ity^r macrophages was: 3.40 ($p < 0.05$) at t_o; 1.47 ($p < 0.05$) at 1 hour; 1.18 ($p > 0.05$) at 4 hours; and 1.89 ($p < 0.05$) at 24 hours. Although the number of viable bacteria at t_o was signif-cantly higher in Ity^s macrophages, which may reflect more efficient bactericidal activity by Ity^r macrophages during the infection period, at 4 hours there was no difference between the Ity^s and Ity^r cells. By 24 hours, however, the Ity^r macrophages had significantly fewer viable S. aureus than the Ity^s macrophages. When C. diphtheriae strain C7 was used to infect BALB/cn and C.D2Ity^r macrophages, the results shown in Figure 44 were obtained. As was observed with S. aureus, both Ity^s and Ity^r macrophages significantly ($p < 0.05$) reduced the number of viable cell-associated C. diphtheriae over the 24 hours of in vitro infection. At t_o and at 4 hours there was no difference ($p > 0.05$) in the ratio of viable bacteria in BALB/cn macrophages to C.D2Ity^r macrophages. At 24 hours the Ity^s macrophages had 32 times ($p < 0.05$) the number of viable C. diphtheriae than did the C.D2Ity^r macrophages. Although the courses of the in vitro infections of Ity^s and Ity^r macrophages were different

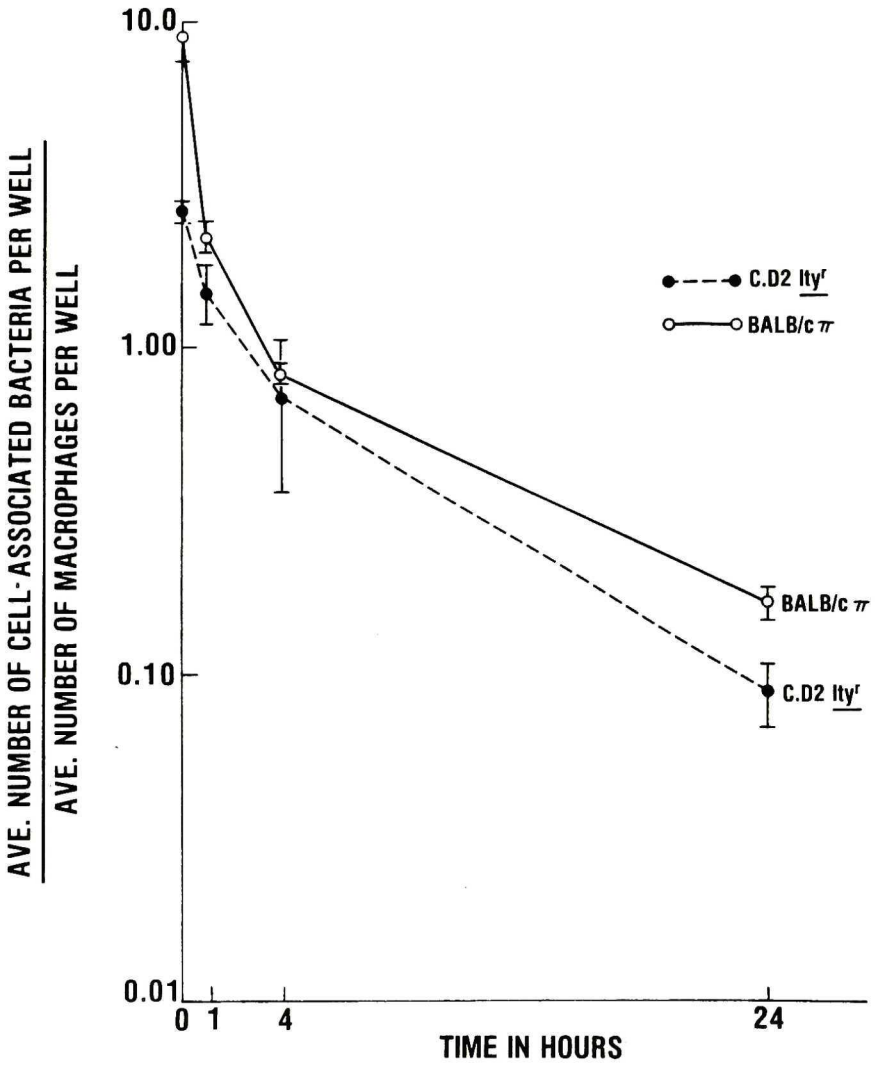


Figure 43. Resident peritoneal macrophages from congenic C.D2Ity^r and BALB/cπ mice were infected in vitro with S. aureus. The number of viable bacteria in macrophage lysates prepared at 0, 1, 4, and 24 hours was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

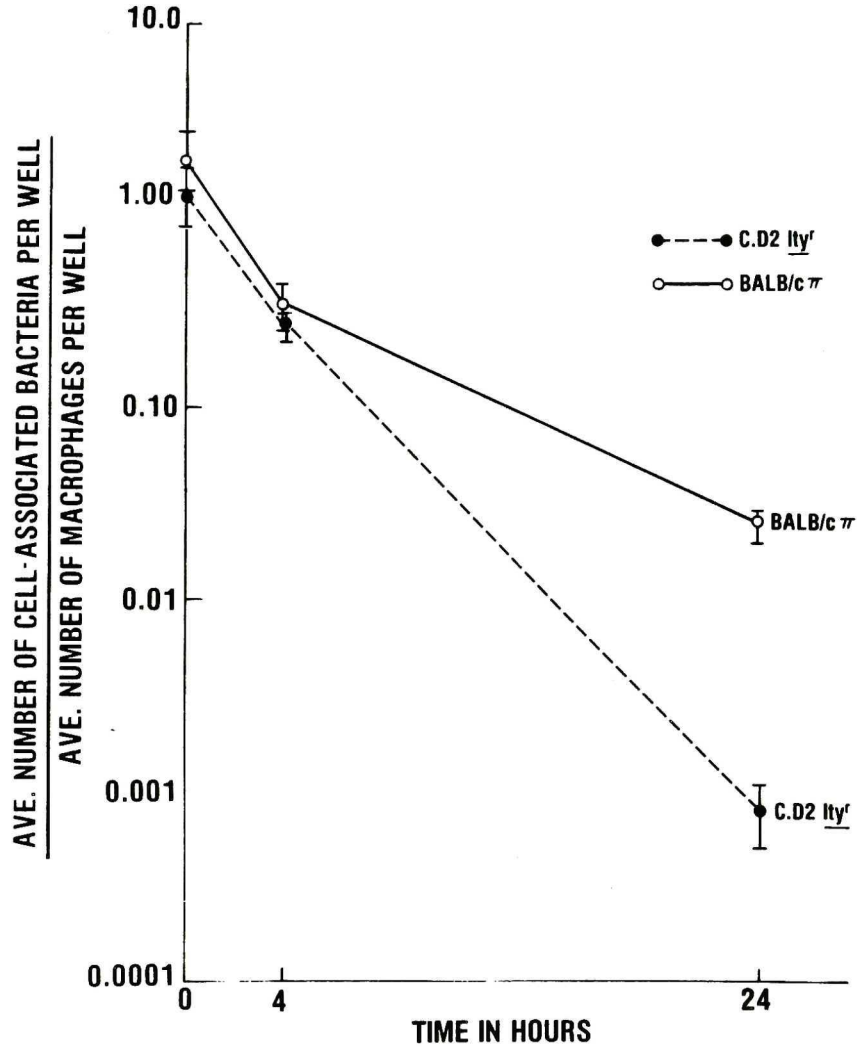


Figure 44. Resident peritoneal macrophages from congenic C.D2Ity^r and BALB/cπ mice were infected *in vitro* with *C. diphtheriae* strain C7. The number of viable bacteria in macrophage lysates prepared at 0, 4, and 24 hours was determined. The ordinate is a logarithmic scale. Each point represents the average of 4 culture dish wells ± 2 standard errors of the mean.

for the gram positive coccus and the gram positive bacillus, two conclusions could be drawn: 1., the number of viable intracellular bacteria were significantly lowered during the assay by both *Ity*^{s} and *Ity*^{r} macrophages; 2., by 24 hours *Ity*^{r} macrophages had significantly fewer viable cell-associated gram positive bacteria than did *Ity*^{s} macrophages.

III. Summary

The findings of these *in vitro* assays performed with bacteria other than *S. typhimurium*, though limited to four species representing three families (*Enterobacteriaceae*, *Micrococcaceae*, *Actinomycetaceae* [Coryneform Group]), address the question initially proposed in this section regarding the bacterial specificity of the *Ity* gene/locus. These findings strongly support the answer that this gene is of general and fundamental importance in the intracellular microbicidal activity of murine macrophages or in the processing of phagocytized bacteria by murine macrophages. Therefore, the expression of the gene is not limited to *Salmonella typhimurium*.

DISCUSSION

At the time these studies were initiated the innate resistance of inbred mice to Salmonella typhimurium during the early phase of infection had been demonstrated to be under host-gene control. The locus designated Ity had been mapped to mouse Chromosome 1, and the dominant resistance allele and the recessive susceptibility allele at that locus had been respectively designated Ity^r and Ity^s. By midway through these experiments, in vivo genetic analysis revealed that Ity mapped to a position between the Idh-1 and Pep-3 genes at a position either identical to or closely linked to the loci which controlled early, innate resistance to L. donovani (Lsh) and to M. bovis strain BCG (Bcg). Because S. typhimurium, L. donovani, and M. bovis replicate within the RES and are facultative or obligate intracellular parasites of macrophages, this laboratory and several other groups of investigators proposed that the Ity-Lsh-Bcg locus is expressed by resident RES macrophages. Indirect in vivo data supported this working hypothesis. A major goal of these studies was to develop an in vitro assay which would make it possible to both infect isolated populations of resident peritoneal Ity^r and Ity^s murine macrophages with a virulent strain of S. typhimurium and to follow the intracellular bacterium-macrophage interaction for at least 24 hours. An in vitro assay was successfully developed and appeared to meet the criterion for biological relevancy:

that is, the results obtained with the assay mimicked *in vivo* findings. The *in vitro* assay was used to study, at the cellular level, the nature and mechanism of *Ity*-controlled, macrophage-expressed, early phase innate resistance or susceptibility of the murine host to *S. typhimurium*.

Throughout the developmental phase of the *in vitro* assay, test conditions were designed to maximize differences between Ity^r and Ity^s expression and were also scrutinized for potential artifactual influence on experimental observations. Six assay parameters were particularly assessed for potential artifactual invalidation of results. These six parameters were the cellular composition of the resident peritoneal cells from different mouse strains, the viability of macrophages, the enumeration of macrophages, the disruption of infected macrophages, the control of extracellular bacterial replication, and the specificity of the *in vitro* system for early phase expression of murine resistance to *S. typhimurium*. These parameters were experimentally tested and the following conclusions were reached: 1., No differences were noted in the cellular composition of resident peritoneal cells among the several mouse strains used and, in particular, between the congenic *Ity* strains BALB/cn and C.D2Ity^r. 2., Viability of Ity^r and Ity^s macrophages in peritoneal cell suspensions and in both uninfected and infected adherent macrophage populations was routinely $\geq$ 95% for both *Ity* phenotypes. The sustained

viability of adherent infected macrophages was verified by latex particle ingestion. 3., Lidocaine-trypan blue releasing solution was used to quantify the number of viable adherent macrophages. This, in turn, permitted a determination of the ratio of infecting bacteria to macrophages at t_o, as well as a relative index of infection for Ity^r and Ity^s macrophage cultures at each assay time point. 4., Na-desoxycholate was used to completely disrupt adherent macrophage cultures and was shown to have no adverse effect on the viability of S. typhimurium, whether or not the bacteria had been exposed to the intracellular milieu of the macrophages. 5., Control of extracellular bacterial replication was achieved by incorporating gentamicin into macrophage maintenance medium at a concentration near the MBC of the antibiotic for S. typhimurium strain TML. Results obtained from RIA analyses of uninfected and infected Ity^r and Ity^s macrophages indicated that no significant amount of gentamicin was taken up during the in vitro assay. Furthermore, the ability to distinguish the Ity^r and Ity^s phenotypes either in the presence of antibiotic or in its absence (when no antibiotic was required, i.e., S. typhimurium strain TML/TS27) indicated that the low concentration of gentamicin present during most of the in vitro assays did not affect either the intracellular fate of S. typhimurium or the expression of the Ity locus and did meet the criteria for in vitro antiobiotic use as proposed by Cole and Brostoff (Cole and Brostoff, 1975). 6., Resident

peritoneal macrophages from late phase Salmonella susceptible xid mice (CBA/N X DBA/2N) F1 males were tested in the assay to assess the specificity of the in vitro system. When compared to Ity^r and Ity^s control macrophages, xid macrophages expressed the Ity^r phenotype of the CBA/N and DBA/2N mouse strains. Therefore, the assay appeared to be specific for expression by resident macrophages of early phase S. typhimurium resistance/susceptibility.

When the in vitro assay was used to follow the fate of virulent S. typhimurium in resident peritoneal macrophages from Ity^r and Ity^s inbred mouse strains, macrophages of the Ity^s strains had significantly more viable cell-associated salmonellae than did macrophages of the Ity^r strains by 24 hours of infection. Moreover, net multiplication of S. typhimurium occurred from 4 to 24 hours of infection only in Ity^s cells. Repetition of these experiments with Ity congenic mice conclusively demonstrated that by 24 hours of infection the Ity locus is responsible for the difference in the number of viable intracellular S. typhimurium and the effector cell for Ity expression is the resident macrophage. These conclusions coincide with the in vivo definitions of the Ity^r and Ity^s phenotypes (Plant and Glynn, 1976; Plant and Glynn, 1977), and have been corroborated by recent in vitro findings with splenic macrophages infected with S. typhimurium (Lissner et al., 1983), with peritoneal macrophages infected with M. bovis (BCG) (Stach et al.,

1984), and with liver macrophages infected with L. donovani (Crocker et al., 1984).

Expression of the Ity phenotype by macrophages was not limited to those infected with the highly mouse virulent TML strain of S. typhimurium. Other less virulent strains of S. typhimurium were processed by Ity^r and Ity^s resident peritoneal macrophages in a quantitative manner that paralleled the relative ranking of the in vivo virulence (net growth rate in the RES) of each S. typhimurium strain (data not shown; D. L. Weinstein et al., 1984). Thus, Ity^r macrophages consisently reduced the number of cell-associated salmonellae more efficiently than did Ity^s macrophages, but, the less virulent the S. typhimurium strain, the fewer viable organisms were present by 24 hours in both Ity^s and Ity^r macrophages. These in vitro findings are consistent with those obtained in vivo by Hormaeche (Hormaeche, 1981), who showed that the extent to which a strain of S. typhimurium can replicate in the RES of Ity^r and Ity^s mice reflects the virulence of the bacterial strain.

Several possible mechanisms can be proposed to explain the in vitro observation that Ity^s macrophages have more viable cell-associated Salmonella typhimurium by 24 hours of infection than do Ity^r macrophages. Conceivable explanations for the differential net growth of salmonellae in Ity^r versus Ity^s macrophages include a difference in phagocytosis/uptake of the bacterium, a difference in the bactericidal activities of the macro-

phages, a dissimilarity in the nutrients available for bacterial growth, a variation in the intracellular location of the bacterium, or a combination of these factors. The following discussion will address the analysis of each of these potential mechanisms based on the data gathered during these studies.

Various experimental approaches, specifically uptake of radiolabeled S. typhimurium, IFA, and light microscopy, demonstrated that the Ity locus does not determine differential phagocytosis/uptake by Ity^r or Ity^s macrophages. These in vitro findings are in complete agreement with in vivo studies which have shown that there is no difference in the uptake of S. typhimurium by the RES organs of Ity^r and Ity^s mouse strains (Groschel, 1970; Swanson and O'Brien, 1983). Furthermore, adaptation of the in vitro assay for T.E.M. examination of infected Ity^r and Ity^s macrophages showed that there were no discernable differences at the ultrastructural level between the Ity^r and Ity^s phagocytized S. typhimurium. In each instance, phagosomes were formed which subsequently fused with lysosomes to become phagolysosomes. At later time points, both Ity^r and Ity^s macrophages contained large phagolysosomes with multiple salmonellae. These salmonellae were a heterogeneous population in that many bacteria were observed at various stages of degeneration, along with (and often in the same phagolysosome) salmonellae apparantly unscathed by the microenvironment of the phagolysosome. None escaped the phagolysosomes.

The differential expression *in vitro* of the *Ity* alleles could be attributed to more efficient bactericidal activity of *Ity*^{r} macrophages or to a nutritionally richer, growth promoting intracellular environment in *Ity*^{s} macrophages. To determine which of these alternatives better explain why more virulent *S. typhimurium* are invariably found in *Ity*^{s} macrophages by 24 hours after infection than in *Ity*^{r} macrophages, avirulent strains of *S. typhimurium* (SL3235 and TML/TS27) were used to infect macrophages *in vitro*. The finding that these avirulent salmonellae strains were killed by both *Ity*^{r} and *Ity*^{s} macrophages over the 24 hours of infection was expected, but, the observation that the strains were killed more efficiently by *Ity*^{r} macrophages than by *Ity*^{s} macrophages was not anticipated. Such observations strongly support the conclusion that the differential expression of the *Ity* locus relects greater or more efficient bactericidal activity by *Ity*^{r} macrophages than by *Ity*^{s} macrophages (Lissner *et al.*, 1983).

One problem with the conclusion that *Ity*^{r} macrophages kill *S. typhimurium* more efficiently than do *Ity*^{s} macrophages is the discrepancy between certain results obtained *in vivo* and *in vitro*. *In vivo* studies by Hormaeche (Hormaeche, 1981) and Swanson and O'Brien (Swanson and O'Brien, 1983) failed to demonstrate that *Ity*^{r} animals eliminate temperature sensitive mutants of *S. typhimurium* more rapidly than do *Ity*^{s} animals. There are several factors critical to *in vitro* analysis of *S.*

typhimurium infection that are less clearly defined in vivo and may account for this discordance between the in vivo and in vitro studies with temperature sensitive mutants of S. typhimurium. These uncontrolled in vivo factors (Lissner et al., 1983) include the proportion of extracellular bacteria to intracellular bacteria, the heterogeneity of the cell population (polymorphonuclear cells, endothelial cells, lymphocytes, etc.), and the ratio of salmonellae to macrophages.

On the basis of in vivo studies, O'Brien (O'Brien, 1982) proposed that S. typhi is avirulent for mice because it can not divide within murine tissues. The in vitro assay was used to test this supposition and to determine whether Ity^s and Ity^r macrophages process S. typhi differently. As anticipated, S. typhi strain QS did not undergo net growth in mouse macrophages in vitro: both Ity^r and Ity^s macrophages cleared the bacterium. However, C.D2Ity^r macrophages killed S. typhi more rapidly than did BALB/cn Ity^s cells. This observation indicated that expression of the Ity phenotype can be discerned whether or not the infecting Salmonella species is pathogenic for mice. To ascertain whether the differential kill by S. typhi-infected Ity^r versus Ity^s macrophages merely reflected the fact that S. typhi belongs to the genus Salmonella, E. coli strain HS was used in the in vitro assay. The results of this study indicated that the enhanced killing by Ity^r macrophages is not Salmonella specific. The data from S. typhi and E. coli in

in vitro assays suggested that the *Ity* gene may mediate killing of a broad group of bacteria. To test this theory, the interaction of Ity^s and Ity^r macrophages with mouse avirulent, gram positive bacteria *S. aureus* and *C. diphtheriae* was evaluated with the assay. It was possible to distinguish the *Ity* phenotype of the macrophages by the enhanced rate at which the Ity^r phagocytes killed these microbes; but, for these gram positive bacteria, the differential kill was only statistically ($p < 0.05$) evident after 24 hours of infection. When viewed collectively, the results obtained with gram positive bacteria and with gram negative bacteria, other than *S. typhimurium*, clearly indicate that *Ity* gene expression is not specific for a particular bacterium. That such a conclusion has not previously been reached may reflect an inadequacy, as discussed by Lissner *et al.* (Lissner *et al.*, 1983), of *in vivo* analyses in discriminating between Ity^s and Ity^r mice infected with non-replicating bacteria. The diversity of bacteria that are more rapidly killed *in vitro* by Ity^r than by Ity^s macrophages suggests that the Ity^r allele may encode for a structural or regulatory product which affects macrophage microbicidal activity, and Ity^s macrophages either synthesize less of that product or the Ity^s product is less active. The possibility that the Ity^r gene product is induced by a component of the infecting organism was recently proposed by Crocker *et al.* (Crocker *et al.*, 1984) in a discussion of their *in vitro* observations of murine liver macrohages infected with *L.*

donovani. Perhaps the observation that Ity^r macrophages more rapidly display enhanced killing when infected with gram negative than with gram positive bacteria reflects the presence of the LPS on the surface of S. typhimurium, S. typhi, and E. coli. Alternatively, the Ity gene product may be expressed constitutively, but the length of the lag before it is expressed may depend on such factors as whether the particular infecting organism is a mouse pathogen and how fast the microbe replicates in vivo.

Further studies are required to elucidate the exact mechanism by which Ity^r macrophages are better able to kill such taxonomically diverse microorganisms (e.g., C. diphtheriae, L. donovani, S. typhimurium, and S. aureus) than are Ity^s macrophages. Possible mechanisms for the enhanced non-organism-specific expressed microbicidal activity by Ity^r macrophages may evolve from the five considerations that follow. First, microbicidal reduced oxygen species may play a role after S. typhimurium has been completely phagocytized, as has been demonstrated for oxygen-dependent killing of Toxoplasma gondii by macrophages (Murray et al., 1979), although Blumenstock and Jann (Blumenstock and Jann, 1981) observed no difference in the early phase production of reduced oxygen intermediates by Ity^r and Ity^s macrophages. Second, the lysosomes of Ity^r and Ity^s macrophages may exhibit an identifiable difference in the presence and/or activity of a specific microbicidal enzyme. The use of cytochemical staining and examination of stained macrophages by both light microscopy

and T.E.M. (Edelson and Cohn, 1974) could provide potential screening methods for evaluating lysosomal enzymes. Additionally, lysosomes from Ity^r and Ity^s macrophages could be isolated and compared by biochemical and physical methods. In conjunction with these kinds of studies, the acidity of intact phagolysosomes could be examined *in situ* (Akporiaye *et al.*, 1983) for any difference in the pH of these structures in Ity^r and Ity^s cells. Third, while the T.E.M. in this study revealed no apparant ultra-structural difference between the macrophage phenotypes, an electron dense substance such as thorium dioxide (Kielian and Cohn, 1980) could be used to label macrophage lysosomes which would permit comparative studies of phagosome-lysosome fusion in Ity^r and Ity^s macrophages. This type of T.E.M. study might be coupled with assessment of bacterial viability in infected macrophage cultures that have been treated to prevent phagolysosomal fusion (D'Arcy Hart and Young, 1975). Fourth, an Ity gene product may bind to intracellular microorganisms and cause a lethal lesion (Elsbach and Weiss, 1983) and/or a structural or configurational change on the surface of the parasite that permits degradative phagolysosomal enzymes to gain access to the parasite. Bacteria might be isolated from infected Ity^r and Ity^s macrophage cultures and examined for such a product. Fifth, the Ity gene may code for an inducible product or function (Crocker *et al.*, 1984); thus, it may be possible by inhibiting macrophage protein synthesis before and/or during the *in vitro* assay to prevent Ity

expression.

Whatever the specific mechanism by which Ity^r macrophages more effectively kill bacteria than do Ity^s macrophages, the global nature of Ity expression suggests that this Chromosome 1 gene plays a pivotal role in the final outome of the interaction between a variety of virulent and avirulent parasites and the murine host. The findings presented in these studies have addressed the genetic control, as expressed by macrophages in vitro, of this host-parasite relationship.

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