Immunity and Intracellular Infection: Resistance to Bacteria in Mice Infected with a Protozoan

Abstract. *Mice infected with the intracellular parasite* Toxoplasma gondii *for periods of as long as 7 months were resistant to challenge with numbers of* Listeria monocytogenes *and* Salmonella typhimurium *that were uniformly lethal to normal mice. This resistance did not appear to depend on the strain of toxoplasma employed or the route of inoculation of either toxoplasma or bacteria. Onset of immunity to listeria was demonstrable as early as 1 to 2 days after infection with toxoplasma. Resistance to toxoplasma was not demonstrable in mice immune to listeria. Interferon did not appear to be a mediator of the immunity observed in toxoplasma-infected mice.*

Animals that survive infection with a species of bacteria that has a predilection for intracellular multiplication, for example, *Listeria monocytogenes,* acquire marked resistance to challenge with that organism (*1*), as well as to challenge with apparently unrelated intracellular bacteria [such as *Brucella abortus* (*2*) and *Salmonella typhimurium* (*3*)]. Immunity to both the original infecting bacterial species and to the unrelated bacteria appears to depend upon the development of a population of cells (for example, peritoneal macrophages) with enhanced yet nonspecific microbicidal activity (*3*). Animals that are infected with the intracellular protozoan *Toxoplasma gondii* acquire resistance to challenge with highly virulent strains of that parasite (*4*, *5*) and, as in the bacterial model, this resistance appears to be mediated by cell factors (*6*).

Since similar immune mechanisms may be operative in intracellular protozoan and bacterial infections, an attempt was made to determine whether cross-immunity is present in animals infected with these agents. The studies described in this report demonstrate that infection of mice with toxoplasma confers resistance to challenge with listeria and salmonella.

Female mice of the Swiss-Webster strain, weighing 22 to 24 grams, were infected intraperitoneally or subcutaneously with several strains of *Toxoplasma gondii* (Table 1). We used either the trophozoite form obtained from peritoneal exudate of acutely infected mice or the cyst form from brains of chronically infected mice. The majority of animals infected with toxoplasma for periods longer than 2 months were originally inoculated for the purpose of "storage" of the parasite. This is the reason for smaller numbers of animals in some experiments and for the use, in some experiments, of pools of animals infected with different strains at a similar time. We used a type 4a strain of *Listeria monocytogenes* isolated in 1967 from a case of human bacterial endocarditis [intravenous LD_{50} (lethal dose, 50 percent effective) was 10^5; intraperitoneal LD_{50}, 5×10^5], and streptomycin-sensitive strain C_5S of *Salmonella typhimurium* obtained from Dr. G. B. Mackaness [intravenous LD_{50} was 4×10^3; intraperitoneal LD_{50}, <10] (*7*). These strains were cultured for 16 to 18 hours in trypticase soy broth, dispersed by a vortex mixer, and then centrifuged for 15 minutes at 5000 rev/min. The resulting bacterial sediment was resuspended and appropriately diluted in Hanks balanced salt solution. Duplicate plate counts on tryptic soy agar were made from each dilution. Final suspensions were dispersed and were inoculated intraperitoneally or intravenously by way of the tail vein.

Mice infected intraperitoneally or subcutaneously for varying periods of time with each of seven strains of toxoplasma were found to be resistant to challenge with listeria. Results of representative challenge experiments are shown in Table 1. Mice infected 1.5 months with the C5 strain of toxoplasma were remarkably resistant to the almost uniformly lethal intravenous doses of 10^6 (experiment 1) and 10^7 listeria. Similar resistance to intraperitoneal challenge with listeria was noted in mice infected at different intervals with each of the other strains of toxoplasma employed (experiments 2 to 6; Fig. 1, a, b, and c). Mice infected with toxoplasma survived or exhibited delayed time to death even when inoculated intraperitoneally with 10^8 listeria, a dose uniformly lethal to 100 percent of normal mice in 2 days. Enhanced survival or prolongation of time to death of toxoplasma-infected animals

Table 1. Challenge of toxoplasma-infected mice with *Listeria monocytogenes*. The strains of toxoplasma were isolated from chicken (C), sheep (M), meat purchased commercially (ME), and humans (DH and Conly). When more than one strain is listed, mortality data represent a pool of the results obtained with each strain. No one animal received more than one strain. The route of inoculation of toxoplasma was intraperitoneal unless otherwise indicated. Varying numbers of mice infected with the different strains of toxoplasma were chosen at random and found to be immune to intraperitonal challenge with 10^3 organisms of the virulent RH strain. This challenge dose resulted in death of 100 percent of control animals. Abbreviations: i.v., intravenous; i.p., intraperitoneal.

Exp.	Strain of toxoplasma *		*Listeria* challenge					
			Dose-route	Total No. dead mice / No. inoculated	Time to death †			
1	C5	(1.5)	10^6 i.v.	5/20	1(8)	1(14)	2(16)	1(21)
	0		10^6 i.v.	14/16	9(3)	2(5)	2(7)	1(10)
2	Conly, C56	(2.0)	10^8 i.p.	9/12	1(1)	5(2)	1(4)	2(5)
	0		10^8 i.p.	34/34	12(1)	22(2)		
3	ME49, C5‡	(12.5)	10^8 i.p.	6/6	3(2)	2(3)	1(4)	
	0		10^8 i.p.	10/10	10(2)			
4	C56	(2.5)	10^7 i.p.	5/26	1(4)	4(5)		
	0		10^7 i.p.	20/20	5(2)	9(3)	6(4)	
5	C37	(4.5)	10^7 i.p.	3/10	1(4)	1(5)	1(6)	
	0		10^7 i.p.	19/20	5(2)	9(3)	3(4)	2(5)
6	ME49, C5, C37	(6.5)	10^7 i.p.	12/19	1(2)	2(3)	7(4)	2(5)
	0		10^7 i.p.	22/25	5(2)	10(3)	4(4)	3(5)
7 §	C56	(48 hr)	10^7 i.p.	4/10	1(4)	2(6)	1(8)	
	0		10^7 i.p.	10/10	5(2)	3(3)	1(5)	1(6)
	C56	(4 days)	10^7 i.p.	5/10	2(5)	2(7)	1(9)	
	0		10^7 i.p.	10/10	1(2)	7(3)	2(4)	
	C56	(12 days)	10^7 i.p.	0/10				
	0		10^7 i.p.	10/10	1(2)	6(3)	2(4)	1(8)

* Figures in parentheses are intervals (in months, unless indicated otherwise) between toxoplasma infection and bacterial challenge; 0, normal mice. † Figures outside parentheses are the number of animals that died on the day (shown in parentheses) following bacterial challenge. ‡ Inoculated subcutaneously. § Experiment designed to determine onset of resistance to listeria in mice infected intraperitoneally with 10^4 organisms of C56 strain of toxoplasma.

was most striking, however, when an intraperitoneal dose of 10^7 organisms was used as the challenge inoculum.

Definite protection against listeria was demonstrable by 48 hours after the initial infection with toxoplasma in one experiment (Table 1, experiment 7) and by 24 hours in another experiment. No decrease in immunity was observed during the next 30 days—for example, on day 12 all toxoplasma-infected mice challenged with listeria survived, compared with 100 percent mortality in a control group. Although survival was notably increased in experiments where the interval between primary infection with toxoplasma and secondary bacterial challenge was shortest, resistance to listeria was observed in mice as long as 7 months after infection (Fig. 1a). Unfortunately, too few animals were available for adequate evaluation of resistance in mice infected for periods longer than 7 months. Pooling of data obtained from challenge of such animals resulted in what appeared to be a prolongation of time to death (Table 1, experiment 3). However, in other experiments not shown in the table, animals infected with toxoplasma for 12 and 13 months prior to challenge with 10^7 listeria did not appear to be significantly protected. Additional experiments are in progress to determine how long after initial infection with toxoplasma mice are resistant to challenge with this bacterium.

Figure 1d depicts representative results obtained in toxoplasma-infected mice that were challenged with *Salmonella typhimurium*. Although the doses of salmonella employed in our studies resulted in death of the majority of experimental as well as control animals, protection of the toxoplasma-infected animals was clearly evident when their time to death was compared with time to death of the controls. As with listeria, this immunity was present as long as 7 months (the longest interval tested) after primary infection with toxoplasma. Initial experiments to define the onset of resistance to salmonella produced less straightforward results. Although overall survival after challenge with this bacterium was prolonged or increased in mice infected with toxoplasma 12 days prior to bacterial challenge (but not in those infected for shorter periods of time), the majority of those mice that did not survive became ill and died 24 to 48 hours before comparably challenged normal animals. At the same time other mice from this toxoplasma-infected group were shown to be completely resistant to an LD_{100} dose of listeria. The reasons for the early deaths and apparent later onset of resistance to salmonella in these toxoplasma-infected mice is now being examined.

The induction of interferon production, which has been shown to occur after infection of mice with toxoplasma (*8*), did not appear to mediate the resistance to bacterial challenge observed in the toxoplasma-infected animals. Mice received three inducers of interferon—statolon (*9*); Pyran (*10*), a random copolymer of divinyl ether and maleic acid anhydride; and Newcastle disease virus (*11*). Both statolon and the copolymer were inoculated intraperitoneally into mice 18 hours prior to intraperitoneal challenge with 10^7 listeria. [Peak interferon level at 18 hours after inoculation of statolon was 1100 unit/4 ml, and after Pyran, 200 unit/4 ml (*12*).] There was a decreased survival and earlier time to death in animals inoculated with these compounds when compared with controls that did not receive these inducers. Newcastle disease virus was inoculated intravenously by way of the tail vein 6 hours prior to challenge with listeria and did not result in any demonstrable resistance. (Level of interferon at time of challenge was 600 unit/4 ml.)

No resistance to toxoplasma could be demonstrated in mice shown to be immune to listeria. Thus, mice infected intraperitoneally 11 days previously with an immunizing dose (10^5) of listeria were challenged with varying doses of the virulent RH strain of toxoplasma (10^2, 10^3, and 10^4). Although mice that had received the immunizing dose of listeria resisted challenge with this same bacterium (in doses of 10^7 and 10^8),

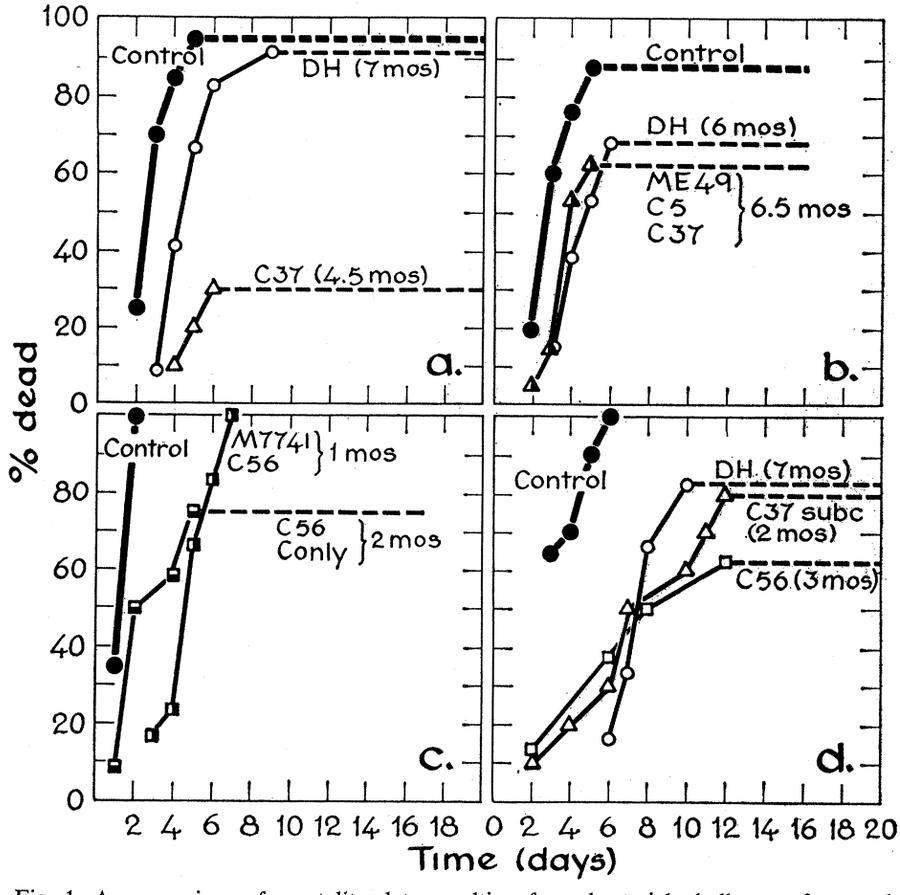

Fig. 1. A comparison of mortality data resulting from bacterial challenge of control mice and mice infected with various strains of toxoplasma. See Table 1 legend for source of strains. (a) Mice infected intraperitoneally with toxoplasma strains C37 for 4.5 months (Table 1, experiment 5) and DH for 7 months challenged intraperitoneally with 10^7 listeria. (b) Mice infected intraperitoneally with strain DH for 6 months and a pool of mice infected intraperitoneally with strains ME49, C5, and C37 for 6.5 months (Table 1, experiment 6); challenged with listeria as in (a). (c) A pool of mice infected intraperitoneally with strains M7741 and C56 for 1 month and a pool of mice infected intraperitoneally with strains C56 and Conly for 2 months (Table 1, experiment 2) were challenged intraperitoneally with 10^8 listeria. (d) Mice infected subcutaneously (*subc*) with strain C37 for 2 months, and intraperitoneally with C56 for 3 months and DH for 7 months challenged intraperitoneally with 10^5 salmonella.

there was no survival in any of these immunized animals or control animals after inoculation with different numbers of the virulent RH strain of toxoplasma.

This is, to our knowledge, the first demonstration of immunity to intracellular bacteria induced by an intracellular protozoan. Of interest in relation to this observation are the few previously reported experimental examples of "interference" between other heterologous organisms [fungus-rickettsia (*13*), virus-bacteria (*14*), bacteria-virus (*15*), and virus-virus unrelated to interferon production (*16*)]. In these latter systems, however, the demonstration of immunity has often been dependent upon the choice of specific routes of administration of the infecting agents and upon the strict selection of intervals between primary infection with one agent and secondary challenge with the other. Despite lack of these restrictions in the toxoplasma-bacteria system and what appears to be a more lasting and perhaps more striking immunity in the experiments described here, the underlying mechanisms of resistance in all these experimental models may be similar.

Note added in proof: Since acceptance of this report, additional experiments have been performed which demonstrate that mice infected with listeria may in fact resist lethal toxoplasma challenge. The resistance observed in these experiments was elicited with a strain of toxoplasma less virulent than the RH strain previously employed in challenge studies.

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Recombination of Influenza A Viruses of Human and Animal Origin

Abstract. *Simultaneous infection of the allantoic sac of the chick embryo with influenza A/equine 1/56 and any of three recombinants derived from human influenza viruses produced stable hybrids with antigens from each parent strain. These hybrids contain the hemagglutinin protein of the equine virus and the neuraminidase of the human strains. The experiments demonstrate genetic homology of human and equine influenza A viruses and suggest the possibility of their recombination in nature.*

Although the source of interpandemic outbreaks of influenza in man is probably man himself (*1*), the origin of the pandemics remains a mystery. Studies of the pandemic of 1957 reinforced earlier indirect evidence that pandemics are associated with the emergence of antigenically novel influenza A viruses. The sudden appearance of such viruses, without evidence of continuous mutation from prior human strains, has suggested that they may have come from animal reservoirs (*2*). Serologic evidence points to the etiologic role of a virus similar to the swine influenza virus of the 1918 pandemic (*3*), although the virus may have been transmitted from man to swine rather than in the other direction (*4*). Recently, new influenza A viruses have been isolated from several avian species (*5*), and one such strain is antigenically related to human influenza A_2 subtype virus (*6*). Two antigenically distinct equine influenza A viruses, equine 1 and equine 2 were isolated in 1956 and 1963, respectively. One (A/equine 2) may be related antigenically to a human influenza A virus of the last century (*7*) and is capable of infecting man (*8*).

Genetic recombination of human and animal influenza A viruses in nature could provide an immediate exchange of markers and, in effect, an instant "multistep mutation." Hybridization of human (*9, 10*) and of human and avian strains incorporating antigens from each parent (*11*) has been accomplished in the laboratory. I now describe a hybrid derived from influenza A/equine 1/56 virus and an A_2 recombinant virus (X-1L), the latter having been derived from the human strains A_0/NWS/33 and A_2RI/5$^+$/57 (Fig. 1). Recombinant X-1L was selected for recombination with the equine virus because its capacity to form plaques in a unique cell line, clone 1-5C-4 (*12*), and its high neuraminidase activity (E) provided distinctive markers potentially useful in the isolation of recombinants of equine serotype.

Chick embryos 10 days of age (four per group) were infected via the allantois with a mixture of 10^4, EID_{50} (egg infective doses 50 percent effective) of A/equine 1 and X-1L viruses. Control groups received each virus singly in equivalent dosage. After a 40-hour incubation period at 35°C, hemagglutinating virus demonstrated in allantoic fluid harvest was inoculated [diluted in decimal series (10^{-1} to 10^{-6})] onto clone 1-5C-4 cell monolayers in plastic petri dishes (*12*). Monolayers were then covered with a layer of agar containing A_2e antibody selective against the major (A_2) antigen of X-1L, but not against its neuraminidase (E) (Fig. 1). This antiserum was produced by immunization of rabbits with a recombinant, X-9, that has A_2 hemagglutinin but A_0 neuraminidase (*10*). One 0.5-mm plaque was detected in a dish inoculated with the 10^{-2} dilution. A plug of agar immediately overlying the plaque was inoculated into eggs and clone 1-5C-4 monolayers, for passage and isolation of an equine E recombinant (Fig. 1). Poorly visible plaques were only sporadically produced in subsequent passage so that further cloning and isolation of this recombinant (X-15) were carried out in eggs by the limiting dilution technique. The methods used for viral neuraminidase assay and for characterization of the hemagglutinin by hemagglutination inhibition have been described (*13, 14*). Individual allantoic fluids positive for hemagglutinating virus were tested for viral neuraminidase activity with a fetuin substrate. Fluids with high activity were retested in the presence of antiserum specific for A_2 neuraminidase (*15*). Viruses in those fluids in which activity was inhibited were identified in hemagglutination inhibition tests as being simi-