Resistance to Virus Challenge in Mice Infected with Protozoa or Bacteria* (34066)

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Recently we reported that mice acutely or chronically infected with an intracellular protozoan (*Toxoplasma gondii*) were resistant to ordinarily lethal inocula not only of toxoplasma but also of phylogenetically unrelated intracellular bacteria (*Listeria monocytogenes* and *Salmonella typhimurium*) (1). In parallel experiments, animals infected with listeria and challenged with toxoplasma showed significantly less mortality and a prolonged time to death compared to controls. The general nature of this heterologous resistance in the toxoplasma-infected animals was shown in experiments using animals chronically infected with the intracellular protozoan *Besnoitia jellisoni*; these animals were resistant to toxoplasma as well as to the bacterial species (2, 3).

Attempts were made to determine the mechanism(s) underlying the observed "immunity" in the mice infected with toxoplasma or besnoitia (2, 3). They did not reveal enhanced reticuloendothelial clearance in these animals or any protective effect of "immune" serum in passive transfer experiments. Preliminary studies in which high levels of circulating interferon were induced in animals by pyran copolymer, Newcastle disease virus, or statolon did not suggest a role for this material. In some instances, however, animals receiving the interferon inducers died before controls raising the possibility of toxic effects of the inducers when they are employed in close proximity to the bacterial challenges. The most encouraging results were obtained with *in vitro* cultures of peritoneal macrophages of infected and normal mice. Macrophages from toxoplasma- and besnoitia-infected animals appeared to be capable of more rapid killing of the challenge bacteria, and monolayers of these cells resisted destruction when compared with normal cell monolayers (2).

These findings suggest the possibility that a common protective mechanism against intracellular organisms may exist in animals infected with either facultative or obligate intracellular parasites. It was therefore considered of interest to determine if the resistance against bacterial and protozoal challenge present during chronic infection with these intracellular organisms could be demonstrated to be active against ordinarily lethal virus challenge as well.

Materials and Methods. Female mice of the Swiss-Webster strain were used in all experiments, and they weighed 22–24 g at the time of their original bacterial or protozoal infection. Multiple strains of *Toxoplasma gondii* were used, and their derivation and methods of preparation and inoculation have been described elsewhere (1). These strains were all relatively avirulent at the inoculum size employed, and toxoplasma cysts could be demonstrated in the brain of recipient mice during the entire life span of the animal. The strain of *Besnoitia jellisoni* and methods for preparation of inocula have been described elsewhere (3). Inocula calculated to contain 10 trophozoites of this strain were lethal to 100% of mice within 10 days. Sulfadiazine in the drinking water during the first 2 weeks of infection prevented death, and the organisms thereafter could be found encysted in multiple organs and in the omentum.

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The method of preparation of the Mengo virus has been described elsewhere (4). Each mouse received 10^8 pfu by the intraperitoneal route. The mortality in the different control groups was between 62 and 93% within 3–6 days after this inoculum. Assay of antiviral antibody in mouse sera was performed using a neutralization test; 1 part serum was mixed with 9 parts Eagle's minimal essential medium, and after 1-hr incubation at 38° residual virus was measured by assay of serial tenfold dilutions of this mixture for plaque-forming ability on L cells. To the agar overlay was added 0.05% protamine sulfate (5) to increase the size of plaques which were read by neutral red staining of monolayers at 3 days. Assay of interferon levels was performed as described elsewhere (4), employing a plaque-inhibition assay on L cell monolayers with bovine vesicular stomatitis virus as the challenge virus. Both a standard mouse interferon control and an aliquot from a pool of serums obtained from unstimulated control mice were titered in each assay.

Results. In Table I are the results of Mengo virus challenge in animals previously infected with phylogenetically unrelated intracellular organisms. Toxoplasma and besnoitia each conferred a significant resistance to Mengo virus challenge as demonstrated by both decreased mortality and prolongation of time to death in the protozoal-infected mice when compared with the control animals. Remarkable resistance to viral challenge was demonstrable in each of the three groups of mice infected for 1 year or more. Mice infected with besnoitia for periods longer than 3 months were not available. In contrast to the prolonged resistance demonstrable in the protozoal-infected animals, mice infected with listeria were resistant to viral challenge for a period of only 1–3 months, and the resistance appeared to be less than that conferred by the protozoa.

Assays of serums of mice infected with besnoitia for 2 and 6 months or with toxoplasma for 1, 2, and 8 months prior to viral challenge revealed no neutralizing antibody for Mengo virus. Interferon was studied as a

TABLE I. Results of Mengo Virus Challenge in Mice Infected with Toxoplasma, Besnoitia, or Listeria.[a]

Organism	Strain[b]	No. mice	% Survival	Time to death[c]
Besnoitia	(1)	10	100	
	(3)	10	100	
	Controls	15	7	8(4) 6(5)
Toxoplasma	C56 (1)	10	90	1(12)
	C56 (4)	9	89	1(10)
	C56 (16)	10	80	2(6)
	C56 (9)	10	60	1(6) 2(7) 1(8)
	C56 (12)	5	80	1(8)
	ME49 (14)	10	40	1(5) 1(6) 2(7) 2(10)
	H44 (5)	10	60	1(4) 2(6) 1(9)
	C37 (6)	10	80	1(6) 1(12)
	Controls	15	7	8(4) 6(5)
Listeria	(.25)	10	50	1(4) 2(5) 1(6) 1(8)
	Controls	15	7	8(4) 6(5)
	(1)	10	60	2(5) 1(6) 1(12)
	Controls	10	20	5(5) 1(6) 1(7) 1(13)
	(3)	10	20	3(5) 2(6) 1(7) 1(8) 1(10)
	Controls	8	37.5	1(4) 1(5) 3(6)

[a] Mice received 1.2×10^8 pfu of Mengo virus by the intraperitoneal route.

[b] Figures in parentheses = duration of infection in months.

[c] Figures outside parentheses = number of mice dying on day shown in parentheses.

possible mediator of the observed resistance in the mice chronically infected with toxoplasma or besnoitia, and the levels of this substance in serum, peritoneal fluid or washings, and spleens of such animals are shown in Table II. Interferon could be demonstrated in samples of serum only during the acute stages of the protozoal infection.

TABLE II. Titers of Interferon in Mice.

Organism and strain[a]	Material assayed[b]	Interferon titers (units/4 ml)	Control
Toxoplasma			
RH (3 days)	S	10	
	PF	33	
(4 days)	S	7	<3 units
	PF	65	
H44 (8 mo)	S	<10	
C37 (8 mo)	S	<10	
C37 (2 wk)	S	17	
C56 (1 yr)	S	<10	
C56 (2½ mo)	S	<10	
C56 (8 mo)	PW	<10	
	Spleen[c]	<10	
Besnoitia			
(7 days)	S	22	
	PF	100[d]	<18 units
(9 days)	S	30	
	PF	100	
(2 mo)	S	17	
(6½ mo)	S	<10	
(9 days)	PF	30	
	Spleen[c]	<10	
(3 mo)	PW	<10	
	Spleen[c]	<10	
Uninfected control	PW	<10	<10 units
	Spleen	<10	

[a] Figures in parentheses = time after infection.

[b] S = serum; PF = peritoneal fluid; PW = peritoneal cavity was washed with 1 ml of minimum essential medium (Eagle's).

[c] The entire spleen was triturated in Eagle's minimum essential medium (1:10 weight/volume) with mortar and pestle, centrifuged, and the supernatant fraction was assayed for interferon activity.

[d] Activity measured in VSV assay and found to be inactivated by incubation with trypsin, not sedimented during 2 hours of 100,000*g* ultracentrifugation and cell species specific in that it was not active in a similar plaque assay on chick embryo fibroblasts (21).

Freund's complete adjuvant was employed to investigate the role of chronic immunologic stimulation. Ten mice were injected with 0.1 ml of Freund's complete adjuvant intraperitoneally and 0.1 ml subcutaneously. Ten control mice received incomplete adjuvant by the same routes. Five months later both groups received 2×10^8 pfu of Mengo virus intraperitoneally. Control mice were all dead by Day 5 after challenge. Only one mouse died in the group which received complete adjuvant; the experiment was terminated on Day 14.

Discussion. The results described above demonstrate a remarkable resistance against challenge with ordinarily lethal numbers of Mengo virus in mice infected with the obligate intracellular protozoa, *Toxoplasma gondii* and *Besnoitia jellisoni*, and the facultative intracellular bacterium, *Listeria monocytogenes*. The duration of this resistance in the protozoal-infected animals was demonstrated at a significant level for at least 1 year, whereas the effect of bacterial infection was more transient. Direct interference by the protozoa was considered as a possible mechanism for the observed resistance. During the chronic stage of infection, however, recognizable forms of toxoplasma or besnoitia in their intracellular habitat are virtually never demonstrable in histologic sections as they are during the acute stage of infection. Microscopic examination of tissues reveals only occasional cysts of the parasites, usually without a cellular reaction about them. Evidence of continued activity of toxoplasma has, however, been demonstrated during the chronic infection (6, 7). For instance, toxoplasma is easily isolated from the blood of chronically infected animals (7) and probably persists within circulating white blood cells (8), since the parasitemia persists in the presence of high levels of neutralizing antibody. This continued activity of the parasite may account for the continued "activation" of macrophage populations noted in previous experiments (2, 3) and may also result in continued low level interferon production.

In contrast to the persistence of the protozoal species, listeria disappears rapidly from the tissues of mice after primary infection

(9). The resistance to bacterial challenge which develops during the primary listeria infection is most marked on approximately the seventh day and diminishes rapidly thereafter. Old *et al.* (10) also reported resistance to Mengo virus infection in mice infected with Bacillus Calmette-Guerin and mentioned that the protective effect was still apparent 3.8 months after BCG. In listeria-infected animals some resistance to challenge with the homologous infecting strain is still demonstrable for several months after the bacteria of the primary infection have been eliminated (9). The absence of viable listeria in the tissues for prolonged periods may be related to the failure of such animals to resist Mengo virus challenge.

Gorhe (11) observed inhibition of foot and mouth disease virus in animals previously inoculated with Freund's complete adjuvant and attributed the inhibition to stimulation of the reticuloendothelial system. Similarly, Old *et al.* (10) noted a significant degree of resistance to Mengo virus in mice that received prior treatment with intravenous injections of zymosan, a substance which is also known to influence the functional activity of the reticuloendothelial system. Our results demonstrate that Freund's complete adjuvant confers longstanding resistance against Mengo virus, whereas incomplete adjuvant does not. It is possible that the complete adjuvant activated and enhanced functions of the same cells for resistance to virus that are involved in the resistance we observed in animals chronically infected with the intracellular protozoa and bacteria. Similarly, the early production of interferon noted with foot and mouth disease virus (11) may have occurred after virus infection in the protected animals described in this paper, and their resistance may be due to this cellular enhancement. Studies are presently in progress to determine the interferon response in these animals.

In previous studies performed in an attempt to determine the mechanisms underlying the resistance to bacterial challenge in mice chronically infected with these same protozoa (2), carbon clearance was employed to evaluate the possibility of enhanced reticuloendothelial activity. There was no demonstrable difference in carbon clearance between the protozoal-infected and control animals. Such enhanced clearance would therefore be unlikely as an explanation for the resistance against virus in these protozoal-infected mice.

More recently we have reported that the peritoneal macrophage is the effector arm of the resistance in protozoal-infected animals against bacterial challenge and *vice versa* (2, 3). Attempts were made to perform similar studies with peritoneal macrophages, Mengo virus, and bovine vesicular stomatitis virus. Unfortunately, yields of virus in the cultures of peritoneal macrophages obtained from normal mice were insufficient for employment of this system as an experimental model. The recent study by DuBuy and Johnson employing LDH virus in macrophage cultures reported significant replication of this virus (12). Their results suggest the possibility that LDH virus might be useful to determine the role of the macrophage in the resistant mice.

The finding in our laboratories (4) and in those of Rytel and Jones (13) that toxoplasma can induce interferon production in acutely infected mice suggests the possibility of an interferon-associated mechanism in the observed resistance. We noted that acute infection with this organism protected against ordinarily lethal viral challenge (4) and interpreted our findings as suggesting that the protection was mediated by toxoplasma-induced interferon. Others have reported the ability of listeria to induce interferon production in the mouse (14), and the findings in this paper represent the first demonstration of interferon production during besnoitia infection. Although measurable interferon levels were not demonstrable at late times after infection in mice chronically infected with the protozoal species, there is evidence that very small doses of exogenous interferon not likely to give sustained or significant circulating levels can confer an antiviral effect *in vivo* (15).

As a result of our previous findings of cross protection in mice infected with phylogenetically unrelated intracellular organisms, a common mechanism of resistance to all in-

tracellular organisms was postulated. It appears that the "activated" macrophages first described in the bacterial-bacterial model from the laboratories of Elberg (16), Mackaness (17) and Mitsuhashi (18) are also the mediators of resistance in the protozoal-bacterial and bacterial-protozoal models. Whether this is true also for these same organisms and viral challenge remains to be determined. We do not consider that our results exclude a role for interferon either acting to promote resistance by being present in low quantities not detectable by presently available methods or being produced more readily by "activated" cells after virus challenge. In a similar manner, the absence of neutralizing activity in the serums of the resistant mice does not rule out the presence of other serum factors in these animals, which may play role in defense of the host as described in the bacterial model by Fong *et al.* (19) and by Gelzer and Suter (20).

Summary. To determine if the resistance to intracellular bacteria observed in mice chronically infected with *Toxoplasma gondii* or *Besnoitia jellisoni* extends also against viruses, mice infected with these protozoa were challenged with Mengo virus. Significantly greater survival and/or prolonged time to death were noted in the protozoal infected animals when compared with controls. This resistance to viral challenge persisted in some groups of mice for longer than 1 year. Resistance to viral challenge was also noted in mice infected with *Listeria monocytogenes* but less marked and persisted for a shorter period of time than in protozoal-infected animals. To define a possible role for interferon, serum, peritoneal fluid, and spleens of the protozoal-infected mice were assayed for interferon levels at various intervals following initial infection. Interferon was demonstrable only during the acute stage of the infections, whereas resistance to viral challenge in the same animals persisted in some instances for more than 1 year. Freund's complete adjuvant also conferred resistance to Mengo virus for a period of 5 months. The role of persistent active infection and of humoral and cellular mechanisms as well as of interferon in the observed resistance are discussed.

1. Ruskin, J. and Remington, J. S., Science **160**, 72 (1968).

2 Remington, J. S. and Ruskin, J., Clin. Res. **17**, 156 (1969).

3. Ruskin, J. and Remington, J. S., Antimicrobial Agents and Chemo. 1968 (in press).

4. Freshman, M. M., Merigan, T. C., Remington, J. S., and Brownlee, I. E., Proc. Soc. Exptl. Biol. Med. **123**, 862 (1966).

5. Campbell, J. B. and Colar, J. S., Ann. Med. Exptl. Biol. Fenniae (Helsinki) **44**, 282 (1966).

6. Remington, J. S., Melton, M., and Jacobs, L., J. Infect. Diseases **108**, 163 (1961).

7. Remington, J. S. and Hackman, R., J. Immunol. **95**, 1023 (1966).

8. Miller, M. J., Aronson, W. J., and Remington, J. S., Ann. Internal Med. 1969 (in press).

9. Coppel, S. and Youmans, G. P., J. Bacteriol **97**, 121 (1969).

10. Old, L. J., Clarke, D. A., and Benacerraf, B., Federation Proc. **19**, 208 (1960).

11. Gorhe, D. S., Nature **216**, 1242 (1967).

12. DuBuy, H. G. and Johnson, M. L., Proc. Soc. Exptl. Biol. Med. **128**, 1210 (1968).

13. Rytel, M. W. and Jones, T. C., Proc. Soc. Exptl. Biol. Med. **123**, 859 (1966).

14. Lukas, B. and Hruskova, J., Folia Microbiol. Prague **12**, 157 (1967).

15. Finter, N., J. Gen. Virol. **1**, 395 (1967).

16. Elberg, S. S., Bacteriol. Rev. **24**, 67 (1960).

17. Mackaness, G. B., J. Exptl. Med. **116**, 381 (1962).

18. Mitsuhashi, S., Sato, I., and Tanaka, T., J. Bacteriol **81**, 863 (1961).

19. Fong, J., Chin, D., Akiyama, H. J., and Elberg, S. S., J. Exptl. Med. **109**, 523 (1959).

20. Gelzer, J. and Suter, E., J. Exptl. Med. **110**, 715 (1959).

21. Merigan, T. C., Winget, C. A., and Dixon, C. B., J. Mol. Biol. **13**, 679 (1965).

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