Biological Effects of Poly rI:rC and Endotoxin in Mice Infected with *Mycobacterium* BCG: Interferon Induction and Toxicity (36308)

MITCHELL J. FRUITSTONE, JOSEPH W. SHANDS, JR., AND GEORGE E. GIFFORD

Department of Immunology and Medical Microbiology, College of Medicine, University of Florida, Gainesville, Florida 32601

Several investigators have noted a striking similarly in the biological effects elicited by bacterial endotoxins and synthetic polyribonucleotide complexes, such as poly rI:rC, following injection of these polymers into any of several animal species. Effects of endotoxin [reviewed in (1)] which are mimicked following administration of poly rI:rC include induction of interferon (2, 3); hyporeactivity to repeated administration (4, 5); a biphasic effect (depression followed by stimulation) on reticuloendothelial system (RES) clearance (6, 7) and antibody production (7); potent adjuvant activity (7, 8); ability to provoke the local Schwartzman phenomenon (9); and an increase in nonspecific resistance to infection by bacteria (10, 11), fungi (12), and protozoa (13). Like endotoxin, poly rI:rC causes hypoglycemia (14), and is pyrogenic (15, 16) and abortifacient (17). In addition, lethal toxicity of each polymer is greatly enhanced by adrenalectomy (14), or prior administration of lead acetate (9) or actinomycin D (18). Protection against both hypoglycemia and lethal toxicity can be achieved by administration of corticosteroids (14). The present report extends the comparison between the biological activities of endotoxin and polyribonucleotides to BCG-infected mice, which are known to be far more sensitive than normal mice to the lethal toxicity of (19) and interferon induction by endotoxin (21, 22).

Materials and Methods. Endotoxin. Salmonella typhimurium lipopolysaccharide was prepared by phenol–water extraction (20). Contaminating RNA was removed by treatment with pancreatic RNase (Worthington) followed by reextraction with phenol. The endotoxin was dissolved and appropriately diluted in nonpyrogenic isotonic sodium chloride.

Poly rI:rC. The homopolymer pair poly rI:rC was purchased from the Research Products Division of Miles Laboratories, Inc., Kankakee, IL and was dissolved and appropriately diluted in nonpyrogenic isotonic saline.

Mice. Pathogen-free female Swiss mice, strain CD-1, weighing 20–22 g, were obtained from Charles River Mouse Farms, Inc., Wilmington, MA.

BCG. Mycobacterium BCG, Phipps strain, originally obtained from the Geographic Medicine Branch, NIH, was maintained by weekly subculture in Dubos broth base (Difco) containing 0.5% bovine serum albumin (Armour, Cohn Fraction V). Mice were inoculated intravenously with 0.2 ml of a 14-day culture of BCG (approximately 10^8 bacteria per mouse).

Interferon production. Control and BCG-infected mice were randomized 14 days after BCG injection. Interferon was induced by intravenous injection of 0.2 ml of an appropriate dilution of poly rI:rC or endotoxin. Two hours after inoculation with inducer, the animals were anesthetized with sodium pentobarbital and bled via cardiac puncture.

Interferon assay. Sera from mice injected with poly rI:rC or endotoxin were assayed for interferon content in monolayer cultures of L-929 cells using a plaque reduction method with the Indiana strain of vesicular stomatitis virus (VSV). Interferon dilutions were made in Eagle's minimum essential medium (MEM) containing 10% calf serum. Two ml of each interferon dilution were then placed on each of 3 or 4 well-drained L cell cultures in 2-oz square tablet bottles. After incubation

of 18 hr at 37°, the supernatants were aspirated, and cultures were rinsed once with Gey's balanced salt solution (BSS) and challenged with approximately 150 plaque forming units (PFU) of VSV. Following a 1-hr adsorption period at room temperature, the virus inocula were aspirated, and cultures were rinsed once with BSS and overlaid with 5.0 ml of Eagle's MEM containing 10% calf serum and 1% methyl cellulose (1500 centipoise, Fisher Scientific Co.). The cultures were incubated for 56–60 hr at 37°, at which time the overlays were poured off, cultures stained with crystal violet, and plaques enumerated. Percent plaque reduction was plotted on log-probit paper against number of microliters of serum interferon contained in the 2.0 ml volume employed in pretreatment. The amount of interferon inhibiting the plaque number by 50% (PDD_{50}) and the interferon titer (PDD_{50} units per ml of mouse serum) were determined by interpolation using a computer program for linear regression analysis. Titrations of the NIH reference standard mouse interferon indicated that one interferon PDD_{50} unit in the L cell plaque assay system employed was approximately equivalent to 10 units of the NIH reference standard.

Results. The results of two experiments comparing the interferon induction capabilities of poly rI:rC in BCG-infected and in normal mice are presented in Fig. 1. The interferon response of normal mice reached a plateau at a poly rI:rC dose of 200 μg per mouse. In contrast, the amount of interferon elaborated by BCG-infected mice following poly rI:rC doses of ≧200 μg per mouse was greater than that of normal mice, and increased with increasing amounts of inducer. At the highest concentration of poly rI:rC tested (2000 μg per mouse), serum interferon levels in BCG-infected mice were about four times greater than those in control mice. At low doses (2 or 20 μg) of poly rI:rC, however, BCG-infected mice produced less than half as much interferon as normal mice, but the low titers and small number of animals employed at these low doses of inducer make the significance of this observation questionable. Poly rI:rC at 2000 μg per mouse was lethally toxic and caused severe diarrhea and pronounced hemoconcentration (as determined by extremely small volume of blood obtainable by cardiac puncture, and yields of serum volumes of 10–20% from the resulting clots as compared with about 50% from blood of controls or animals injected with lower concentrations of poly rI:rC). At first it was considered possible that the increase in interferon titer in BCG-infected mice at high doses of inducer might be due to concentration of serum proteins. However, hemoconcentration following high doses of poly rI:rC occurred to approximately the same extent both in BCG-infected and in normal mice, so nonspecific concentration of serum proteins was probably not responsible for the elevated titers of interferon observed in BCG-infected mice. Clearly, although maximum attainable titers were higher, we did not observe a great increase in sensitivity to induction of interferon by poly rI:rC like that reported for BCG-infected mice challenged

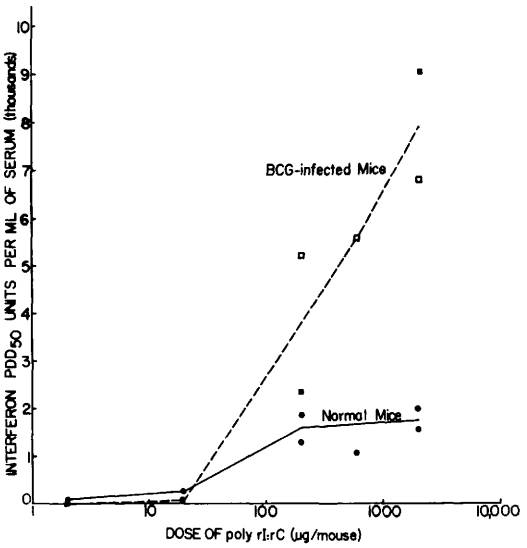

FIG. 1. Serum interferon response of normal and BCG-infected mice injected with poly rI:rC. Mice were bled 2 hr following intravenous injection of various doses of poly rI:rC. Lines are drawn through the means of two experiments. Solid symbols, Experiment 1, 2 mice per group. Open symbols, Experiment 2, 3 mice per group. Circles, normal mice; Squares, BCG-infected mice.

TABLE I. Interferon Titers in the Serum of Normal and BCG-infected Mice Following Induction by Poly rI:rC, and Following Induction by Endotoxin in Mice Injected 72 hr Previously with Poly rI:rC.

Dose of poly rI:rC (μg/mouse)	Poly rI:rC induction[a] of interferon in mice (no pretreatment)		Endotoxin induction[a] of interferon in mice injected 72 hr previously with poly rI:rC	
	Normal mice	BCG mice	Normal mice[b]	BCG mice[c]
2	90	<40	<25	160
20	280	90	<25	55
200	1875	2350	<25	25

[a] Interferon PDD_{50} units per ml of serum of mice bled 2 hr after injection of inducer (pooled serum from 2 mice per group).

[b] Intravenous injection of 200 μg endotoxin in 0.2 ml nonpyrogenic saline; normal mice not previously injected with poly rI:rC produced titers of about 400 units per ml of serum.

[c] Intravenous injection of 1 μg endotoxin in 0.2 ml nonpyrogenic saline; BCG-infected mice not previously injected with poly rI:rC produced titers of about 90 units per ml of serum.

with endotoxin (21, 22).

Table I shows the results of an experiment examining the development of hyporeactivity to endotoxin induction of interferon 72 hr after administration of poly rI:rC. Complete hyporeactivity to interferon induction by endotoxin was observed in normal mice pretreated with poly rI:rC, even when the dose of poly rI:rC was insufficient to elicit more than a minimal interferon response. In contrast, in BCG-infected mice, the extent of hyporeactivity to endotoxin induction of interferon was directly related to the dose of poly rI:rC previously injected.

Table II shows pooled data from several experiments designed to test the sensitivity of BCG-infected and of normal mice to the lethal toxicity of poly rI:rC. In contrast to the well known hyperreactivity to the lethal effects of endotoxin following BCG infection (usually a 1000-fold increase in sensitivity (19)) we observed no increased sensitivity to the lethal effects of poly rI:rC in BCG-infected mice. The LD_{50} of endotoxin for mice employed in these experiments was >250 μg for normal mice and <0.1 μg for BCG-infected mice, so hypersensitivity to the lethal toxicity of endotoxin had been induced in the BCG mice.

Discussion. Although there are many similarities in the biological effects elicited by endotoxin and by poly rI:rC, the data presented show that marked differences also exist. The hyperreactivity of BCG-infected mice to the lethal effects of endotoxin, and to interferon induction by endotoxin, have been well documented (19, 21, 22). This hyperreactivity apparently does not extend to poly rI:rC. In BCG-infected mice the doses of poly rI:rC required for lethal effects, or for the induction of interferon, appear to be comparable to those required in normal mice. These observations, and those of others (6), suggest that the mechanisms of action of the two polymers differ from one another.

The observation that the capacity of en-

TABLE II. Lethal Toxicity of Poly rI:rC in Normal and BCG-infected Mice.

Dose of[a] poly rI:rC (μg/mouse)	Mortality at 48 hr[b]	
	Normal mice	BCG mice
1	0/5	0/5
2	0/5	0/5
3	0/5	0/5
10	0/5	0/5
20	0/5	0/5
30	0/5	0/5
100	0/5	0/5
200	0/4	0/5
300	0/5	0/5
1000	2/5	2/5
2000	5/5	5/5

[a] Intravenous injection in 0.2 ml nonpyrogenic saline.

[b] Number dead/number injected.

dotoxin to induce interferon in normal mice is greatly diminished 72 hr following poly rI:rC administration could be interpreted in several ways. First, because of changes in the functional activities of the RES, a phenomenon known to occur after poly rI:rC (6, 7), the injected endotoxin might be "handled" in a manner such that it is diverted from the interferon-releasing cells. Second, it is possible that both polymers act upon the same cells, and that prior interaction of cells with poly rI:rC renders them refractory to endotoxin induction of interferon. Since it was not necessary for the interferon system to be maximally induced by poly rI:rC in order to develop complete hyporeactivity to endotoxin induction of interferon, it is possible that endotoxin affects a more restricted population of cells than does poly rI:rC, and that those cells which are susceptible to endotoxin induction (probably limited to RES) are the primary, but not exclusive, targets of poly rI:rC.

Apparently BCG-infected mice have a greater capacity to release interferon upon stimulation with poly rI:rC. This is evident from the data presented in Fig. 1. Whereas the threshold for induction of interferon by poly rI:rC was approximately the same for normal and for BCG-infected mice, the response of normal mice reached a plateau at the three highest doses tested. In contrast, BCG-infected mice elaborated additional interferon with increasing doses of poly rI:rC. This increased response following BCG infection may be the result of either an increase in total number of RES cells, or an increase in the interferon releasing capacity of individual cells, or both. The increase in capacity to elaborate interferon might also explain the data dealing with hyporeactivity to endotoxin after poly rI:rC (Table I). Normal mice were refractory to interferon induction by endotoxin 72 hr after poly rI:rC at all doses of poly rI:rC tested, whereas only the highest doses of poly rI:rC rendered BCG-infected mice refractory to endotoxin induction. This suggests that BCG-infected mice may have a larger pool of cells capable of elaborating interferon upon induction by either poly rI:rC or endotoxin.

Summary. Infection of mice with BCG failed to enhance sensitivity to poly rI:rC, as assayed by the amount of polymer required for interferon induction or for lethal toxicity. The amount of interferon induced in normal mice reached a plateau at a poly rI:rC dose of 200 μg per mouse. In contrast, the amount of interferon elaborated by BCG-infected mice following poly rI:rC doses of $\geq$200 μg per mouse was greater than that of normal mice, and increased with increasing amounts of inducer. Complete hyporeactivity to interferon induction by endotoxin was observed in normal mice pretreated with poly rI:rC, even when the dose of poly rI:rC elicited only a minimal interferon response, whereas a poly rI:rC dose-related hyporeactivity was observed in BCG-infected mice.

The research for this paper was supported by National Institute of Health Grants 5TI AI0128-12 and AI07257-06.

1. Nowotny, A., Bacteriol. Rev. **33**, 72 (1969).

2. Youngner, J. S., and Hallum, J. V., Virology **35**, 177 (1968).

3. Herman, R., and Baron, S., Nature (London) **226**, 168 (1970).

4. Park, J. H., and Baron, S., Science **162**, 811 (1968).

5. Ho, M., Breinig, M. K., Postic, B., and Armstrong, J. A., Ann. N.Y. Acad. Sci. **173**, 680 (1970).

6. Berry, L. J., Smythe, D. S., Colwell, L. S., Schoengold, R. J., and Actor, P., Infec. Immunity **3**, 444 (1971).

7. Chester, T. J., DeClercq, E., and Merigan, T. C., Infec. Immunity **3**, 516 (1971).

8. Braun, W., Yajima, Y., and Ishizuka, M., J. Reticuloendothel. Soc. **7**, 418 (1970).

9. Absher, M., and Stinebring, W. R., Nature (London) **223**, 715 (1969).

10. Weinstein, M. J., Waitz, J. A., and Came, P. E., Nature (London) **226**, 170 (1970).

11. Remington, J. S., and Merigan, T. C., Nature (London) **226**, 361 (1970).

12. Regelson, W., and Munson, A. E., Ann. N.Y. Acad. Sci. **173**, 831 (1970).

13. Jahiel, R. I., Nussenzweig, R. S., Vilček, J., and Vanderberg, J., Amer. J. Trop. Med. Hyg. **18**, 823 (1969).

14. Zedeck, M. S., Marquardt, H., Sternberg, S. S., Fleisher, M., and Hamilton, L. D., Proc. Nat. Acad. Sci. U.S.A. **67**, 180 (1970).

15. Lindsay, H. L., Trown, P. W., Brandt, J., and

Forbes, M., Nature (London) **223**, 717 (1969).

16. Hilleman, M. R., J. Infect. Dis. **121**, 196 (1970).

17. Adamson, R. H., and Fabro, S., Nature (London) **223**, 718 (1969).

18. Pieroni, R. E., Bundeally, A. E., and Levine, L., J. Immunol. **106**, 1128 (1971).

19. Suter, E., and Kirsanow, E. M., Immunology **4**, 354 (1961).

20. Westphal, O., Lüderitz, O., and Bister, F., Z. Naturforsch. **7B**, 148 (1952).

21. Youngner, J. S., and Stinebring, W. R., Nature (London) **208**, 456 (1965).

22. Youngner, J. S., and Stinebring, W. R., Virology **29**, 310 (1966).

23. Huang, K., and Landay, M. E., J. Bacteriol. **100**, 1110 (1969).

Received Nov. 18, 1971. P.S.E.B.M., 1972, Vol. 139.