# REACTIVITY TO BACTERIAL ENDOTOXIN AND TOXIC ACTION OF MYXOVIRUSES IN MICE INFECTED WITH BCG

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## INTRODUCTION

In recent years Suter *et al.* have reported that infection of mice with BCG, human tubercle bacilli and so on induces increased sensitivity of these animals towards endotoxin derived from gram-negative bacteria.[1)2)3)]

On the other hand, it has been reported for myxoviruses to manifest toxicity when introduced intraperitoneally, intravenously, subcutaneously and intranasally in a large quantity to animals, and this toxicity in which imply intestinal haemorrhage, hepatic haemorrhage, necrosis, pulmonary haemorrhage, fever, and so on,[4)5)6)] has to do much with that of bacterial endotoxin, although resistance of bacterial endotoxin and myxoviruses to physical and chemical agents such as temperature, ether etc., are extremely different from each other.[7)]

Furthermore, Ogasawara *et al.* have inferring from their studies that the toxicity of myxoviruses consists in components or constituents of these, as bacterial endotoxin in components of the bacterial cell.[8)9)] Mims, the other hand, suggested in his report that viruses and bacterial endotoxin share a final common path of action representing the haemorrhagic enteritis Syndrome in mice.[10)]

Experiments therefore have been carried out to study whether similar hyperreactivity to myxoviruses, comparing in case of bacterial endotoxin is observed in mice infected with BCG.

## MATERIALS AND METHODS

### *Myxoviruses*

The PR 8 strain of influenza A virus and the Miyadera strain of Newcastle Disease Virus (NDV) which were maintained in this laboratory were used. The viruses were obtained by inoculation of 9-day-old embryonated eggs by the chorioallantoic route with 0.2 ml of infected allantoic fluid diluted $10^{-5}$.

After 48 hours incubation at 37°C in case of the PR 8 strain and 72 hours in case of the Miyadera strain, the eggs were chilled at 4°C and killed, thereafter allantoic fluids were collected, clarified by preliminary centrifugation at 2,000 r.p.m. for 20 min., then centrifuged at 25,000 r.p.m. for 30 min.

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The pellets were resuspended in 0.01 M-Phosphate Buffered Saline (PBS) concentrated in one-tenth of the volume of original allantoic fluids. The virus suspensions were finally centrifuged at 2,000 r.p.m. for 20 min. to be free from larger particulates.

*Haemagglutination titration tests (HA tests)*

HA titrations were carried out by the pattern method of Salk. 0.5 ml of serial two-fold dilutions of the virus preparations made in 0.01 M-PBS and the same volume of 0.5 per cent chicken red cell suspensions were added and the mixtures were incubated at 4°C for 1 hour and results were recorded according to the patterns of the sedimented red cells.

The end-point was represented as the highest dilution giving complete agglutination. HA titres were expressed as values per 0.5 ml.

*Bacterial endotoxin*

Crude lipopolysaccharide was prepared from cultures of E. coli O-111 which was maintained in this laboratory, according to the phenol-water extraction procedure of Westphal[11].

Eighteen-hour-old cultures of E. coli O-111 on agar media were collected, suspended in a distilled water, sedimented by centrifugation at 3,000 r.p.m. for 30 min., and resuspended in a distilled water. This clarification method by centrifugation and resuspension technique was carried out three times. The final pellets were then resuspended in aceton, sedimented after 1 hour by centrifugation at 3,000 r.p.m. for 20 min. and then resuspended in aceton. The aceton suspensions were then aspirated using a water jet pump for 10 hours, leaving white powder.

The aceton powder of E. coli resuspended in a distilled water was heated to a temperature of 75°C in a water bath and mixed little by little finally with the same volume of 90 per cent phenol heated at the same temperature.

After complete mixing having been done, the mixture was further blended for 20 min. at the same temperature in a water bath. Then the mixture was cooled to a temperature of 5°C using a chilled water and segregated to a water and a phenol layers by centrifugation at 2 000 r.p.m. for 10 min.

The upper water layer was separated by pippeting from the under phenol layer. The water layer was dialysed through a cellophane tube against running tap water for 72 hours. After dialysis, ethylalcohol was added finally to 76 per cent, precipitated crude lipopolysaccharide. The crude lipopolysaccharide was dissolved in a distilled water and precipitated with ethanol again. The lipopolysaccharide sedimented by centrifugation was more purified three times by the precipitation and resolution cycle using ethanol and distilled water.

The final precipitates were resolved in a small quantiy of a distilled water and freezed and dryed. About 10 mg. of final crude lipopolysaccharide was obtained from 12 g. of aceton powder of E. coli.

When required, dryed lipopolysaccharide was resolved in 0.01 M-PBS and placed for use.

*BCG*

The in this laboratory maintained strain was used. Thirty-day-old cultures of the strain on Ogawa media were collected, desiccated using sterilized filter papers as possible as can and weighed. Then these were made to be suspended in a distilled water by shaking method using crystal balls and sedimented by centrifugation at 3000 r.p.m. for 30 min. The procedure of clarification was carried out three times to remove possible mixing of components of media. The last pellets, resuspended in 0.01 M-PBS,were used.

*Mice*

Male and female white mice of the SM strain were kindly offered from the inbred experimental animal laboratory in this school of medicine. Initially the mice weighed approximately 10–12 g. Having been observed for 2 days, the mice were used.

*Outline of experimental methods*

The mice were devided into two groups of the same number respectively. The mice of one group were injected intravenously with 2 mg. of BCG suspended in 0.25 ml. of 0.01 M-PBS. The mice of another group which were nothing done, were used as normal controls.

Nine days after the infection of BCG, the mice of two groups were challenged by intravenous injection of bacterial endotoxin or virus suspensions. After the challenge, the mice were observed for 5 days and death was immediately followed by necropsy for macroscopical observation of pathological lesions. Toxicity of endotoxin or viruses was expressed as $LD_{50}$ calculated by Finney's probit analysis[12].

## RESULTS

1) Sensitivity to bacterial endotoxin of normal and BCG-infected mice

Nine days after the infection of BCG, ten-fold dilutions of crude lipopolysaccharide were injected intravenously in a solution of 0.25 ml. of 0.01 M-PBS into the normal and BCG-infected mice devided into five subgroups respectively. The results of two experiments are given in Table 1.

The $LD_{50}$s in the Table were calculated as total of the two experiments. The difference of the two groups, normal and BCG-infected, to crude LPS was about 25-fold. Thus, BCG-infected mice exhibited marked hyperreactivity to endotoxin in this case, too. Using, therefore, normal and BCG-infected mice in the same way, next studies were carried out.

2) Sensitivity to influenza A virus and NDV of normal and BCG-infected mice

In these cases, as a preliminary examination using 10-fold dilutions of viruses exhibited no significant difference in the sensitivity of the two groups of mice, two-fold dilutions of viruses were used. Likewise 9 days after the inoculation of BCG, the mice of the two groups were devided into five subgroups of 4 mice respectively and challenged intravenously with 2-fold dilutions of virus suspensions in a volme of 0.25 ml. of 0.01 M-PBS. The animals

TABLE 1. Toxicity of Crude Lipopolysaccharide (LPS) Derived from E. Coli for Normal and BCG-Infected Mice

| LSP dose in μg. | | Total injected | Deaths: Days after injection 0~1 | 1~2 | 2~3 | 3~4 | 4~5 days | Per cent mortality (%) |
|---|---|---|---|---|---|---|---|---|
| | | | Normal mice | | | | | |
| Exp. No. 1 | 600 | 4 | 3 | 0 | 0 | 0 | 0 | 75 |
| | 60 | 4 | 1 | 0 | 0 | 0 | 0 | 25 |
| | 6 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0.6 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0.06 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Exp. No. 2 | 600 | 4 | 4 | 0 | 0 | 0 | 0 | 100 |
| | 60 | 4 | 1 | 0 | 0 | 0 | 0 | 25 |
| | 6 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0.6 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0.06 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | $LD_{50}$≒150 μg./mouse | | | | | | |
| | | BCG-infected mice | | | | | | |
| Exp. No. 1 | 600 | 4 | 4 | 0 | 0 | 0 | 0 | 100 |
| | 60 | 4 | 4 | 0 | 0 | 0 | 0 | 100 |
| | 6 | 4 | 1 | 1 | 0 | 0 | 0 | 50 |
| | 0.6 | 4 | 1 | 0 | 0 | 0 | 0 | 25 |
| | 0.06 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Exp. No. 2 | 600 | 4 | 4 | 0 | 0 | 0 | 0 | 100 |
| | 60 | 4 | 4 | 0 | 0 | 0 | 0 | 100 |
| | 6 | 4 | 2 | 0 | 0 | 0 | 0 | 50 |
| | 0.6 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 0.06 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | $LD_{50}$≒6 μg/mouse | | | | | | |

were thereafter observed for 5 days.

The results of two experiments are given in Table 2 and 3.

The $LD_{50}$s in these Tables are likewise given as the total of two successive experiments.

The died animals exhibited slight intestinal petechiae or haemorrhages. Most death occured between 18 and 72 hours after injection of the two strains of viruses, while between 6 and 24 hours in case after that of bacterial endotoxin.

## DISCUSSION

The emergence of hyperreactivity to bacterial endotoxin was distinctly ascertained in the case of the present author, too. On the contrary, that to two strains of myxoviruses was not exhibited at all, moreover slight hyporeactivity was observed. The difference of reactivity to myxoviruses from to bacterial endotoxin indicates that a discrepancy in the toxic mechanisms of

TABLE 2. Toxitcity of the PR 8 Strain of Influenza A Virus for Normal and BCG-infected Mice.

| PR 8 dose in HA titre /0.5 ml. | | Normal mice: Total injected | Deaths: Days after injection 0~1 | 1~2 | 2~3 | 3~4 | 4~5 days | Per cent mortality (%) |
|---|---|---|---|---|---|---|---|---|
| Exp. No. 1 | 4000 | 4 | 2 | 2 | 0 | 0 | 0 | 100 |
| | 2000 | 4 | 1 | 1 | 1 | 0 | 0 | 75 |
| | 1000 | 4 | 0 | 1 | 1 | 0 | 0 | 50 |
| | 500 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 250 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Exp. No. 2 | 4000 | 4 | 1 | 2 | 1 | 0 | 0 | 100 |
| | 2000 | 4 | 0 | 2 | 1 | 0 | 0 | 75 |
| | 1000 | 4 | 0 | 1 | 1 | 0 | 0 | 50 |
| | 500 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 250 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | $LD_{50}$ ≒ 1000 HA/0.25 ml = 500 HA/0.5 ml/mose | | | | | | |
| | | BCG-infected mice | | | | | | |
| Exp. No. 1 | 4000 | 4 | 1 | 2 | 1 | 0 | 0 | 100 |
| | 2000 | 4 | 0 | 1 | 1 | 0 | 0 | 50 |
| | 1000 | 4 | 0 | 1 | 0 | 0 | 0 | 25 |
| | 500 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 250 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Exp. No. 2 | 4000 | 4 | 1 | 1 | 1 | 0 | 0 | 75 |
| | 2000 | 4 | 0 | 1 | 1 | 0 | 0 | 50 |
| | 1000 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 500 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 250 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | $LD_{50}$ ≒ 2000 HA/0.25 ml = 1000 HA/0.5 ml/mouse | | | | | | |

TABLE 3. Toxicity of the Miyadera Strain of NDV

| Miyadera HA titer /0.5 ml. | | Normal mice: Total injected | Deaths: Days after injection 0~1 | 1~2 | 2~3 | 3~4 | 4~5 days | Per cent mortality (%) |
|---|---|---|---|---|---|---|---|---|
| Exp. No. 1 | 2000 | 4 | 1 | 2 | 1 | 0 | 0 | 100 |
| | 1000 | 4 | 1 | 3 | 0 | 0 | 0 | 100 |
| | 500 | 4 | 1 | 2 | 1 | 0 | 0 | 100 |
| | 250 | 4 | 0 | 1 | 1 | 0 | 0 | 50 |
| | 125 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |
| Exp. No. 2 | 2000 | 4 | 1 | 3 | 0 | 0 | 0 | 100 |
| | 1000 | 4 | 2 | 1 | 1 | 0 | 0 | 100 |
| | 500 | 4 | 0 | 2 | 1 | 0 | 0 | 75 |
| | 250 | 4 | 0 | 1 | 1 | 0 | 0 | 50 |
| | 125 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | $LD_{50}$ ≒ 250 HA/0.25 ml = 125 HA/0.5 ml/mouse | | | | | | |

TABLE 3. (*Continued*)

| Miyadera HA titer /0.5 ml. | | Normal mice | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Total injected | Deaths | | | | | Per cent mortality |
| | | | Days after injection | | | | | |
| | | | 0~1 | 1~2 | 2~3 | 3~4 | 4~5 days | |
| | | BCG-infected mice | | | | | | |
| Exp. No. 1 | 2000 | 4 | 2 | 2 | 0 | 0 | 0 | 100 |
| | 1000 | 4 | 2 | 1 | 1 | 0 | 0 | 100 |
| | 500 | 4 | 0 | 1 | 1 | 0 | 0 | 50 |
| | 250 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 125 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Exp. No. 2 | 2000 | 4 | 1 | 3 | 0 | 0 | 0 | 100 |
| | 1000 | 4 | 1 | 2 | 1 | 0 | 0 | 100 |
| | 500 | 4 | 0 | 1 | 0 | 0 | 0 | 25 |
| | 250 | 4 | 0 | 0 | 1 | 0 | 0 | 25 |
| | 125 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | $LD_{50} \fallingdotseq 500$ HA/0.25 ml = 250 HA/0.5 ml/mouse | | | | | | |

the two, bacterial endotoxin and myxoviruses, would exist in spite of many analogous phenomenon.

In this connection, Dougherty and his collaborator's study in 1957 is interesting[13], which reports that prior intravenous infection of bacterial endotoxin protected mice against subsequent inoculation of not only the same endotoxin but also influenza virus, while previous infection of influenza virus failed to protect mice against challenge with endotoxin administered by the same route, although made these tolerated with the same influenza virus.

According to this one-way-traffic phenomenon of Dougherty, the present anthor's study and so on[14], it would be suggested that similar and dissimilar parts of mechanisms exist in the toxic action of the two, bacterial endotoxin and myxoviruses, and that BCG-infection in mice participates in one of the parts of response mechanism to bacterial endotoxin and this part is not related to myxoviruses.

## SUMMARY

Marked hyperreactivity to bacterial endotoxin was observed in mice infected with BCG. The mice however, did not show any hypersensitive response to the toxic action of influenza virus or NDV.

This result would represent that the toxic mechanisms of bacterial endotoxin and myxoviruses are different at least partially notwithstanding many analogous phenomena.

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