EFFECTS OF CELLULAR CONSTITUENTS OF MYCOBACTERIA ON THE RESISTANCE OF MICE TO HETEROLOGOUS INFECTIONS

II. Enhancement of Infection

By RUSSELL W. SCHAEDLER, M.D., and RENÉ J. DUBOS, Ph.D.

(*From The Rockefeller Institute for Medical Research*)

(Received for publication, July 22, 1957)

We have shown in the preceding paper that treatment with cellular materials derived from killed mycobacteria could, under the proper conditions, increase the resistance of mice to subsequent infection with either *Staphylococcus aureus* or *Mycobacterium fortuitum* (1). The results now to be presented demonstrate that the same crude materials, injected under other experimental conditions, are capable of increasing the susceptibility of mice to various types of bacteria, and indeed can convert chronic bacterial infections into acute processes that may result in death.

It must be emphasized that no information is available concerning the chemical nature of the substances responsible for these biological effects. Nor is it known whether the same, or different, constituents of the bacterial preparations used, were responsible for the protective effects reported in the preceding paper, and for the enhancement of infection observed in the present study. These aspects of the problem, as well as the relevance of the phenomena observed to the activation of natural infectious processes, will be discussed in subsequent publications.

Materials and Methods

The materials and techniques used in the present study were the same as those described in the preceding paper (1).

Experiments were also carried out with a strain of so called "atypical" mycobacterium listed in our collection as P_{17}. This strain, originally recovered from pulmonary lesions in a human patient, was kindly provided by Dr. Ernest H. Runyon of the Sunmount Veterans Administration Hospital, Sunmount, New York. It is pigmented, grows rather slowly in culture media, and does not cause progressive disease in guinea pigs (2). In our laboratory, this strain has been found capable of multiplying for a limited time in the liver and lungs of albino mice. The bacilli disappear progressively from these organs, although small numbers of them persist for several months.

The lipopolysaccharide endotoxin used in some of the experiments reported here had been prepared from *Serratia marcescens* in the Difco Laboratories, Detroit, and was kindly supplied by Mr. H. W. Schoenlein.

1. Enhancement of Heterologous Infections by Simultaneous Injection of Killed BCG Cells.—

It was found in the experiments reported in the preceding paper that prior treatment with killed BCG cells, injected by the intraperitoneal route, increased the resistance of mice to subsequent infection with *Staph. aureus* or *Myco. fortuitum*. In contrast, it will be presently shown that the intravenous

TABLE I

Enhancement of Staphylococcal Infection by Simultaneous Intravenous Injection of Killed BCG Cells

Killed BCG cells injected with staphylococci*	Age of mice	Cumulative deaths at indicated days (d.) after infection		
		3 d.	4 d.	7 d.
mg.	*wks.*			
			(out of 16 mice)	
0	5	1	4	11
0.1	"	5	8	14
0	9	1	3	9
0.1	"	14	16	

* Cell suspension heated at 90°C. for 10 minutes, injected intravenously with 0.05 ml. *Staph. aureus* (Giorgio).

TABLE II

Enhancement of Myco. fortuitum infection by Subcutaneous Administration of Killed BCG Cells

Amount of killed BCG cells injected s.c.*	No. of deaths within 10 days after infection
mg.	
	(out of 5 mice)
3	3
0.3	3
0.03	2
Saline	0

* Suspension of phenol killed, acetone-washed bacillary bodies injected subcutaneously in 0.3 ml. saline within 30 minutes after intravenous infection with 0.03 ml. *Myco. fortuitum*.

or subcutaneous injection of the same amount of BCG cells either at the same time as, or immediately after infection, markedly shortened the survival time of the infected animals.

Mice, either 5 weeks or 9 weeks old, were infected by the intravenous route with 0.05 ml. of an overnight culture of *Staph. aureus* (strain Giorgio). For half of each group the infective inoculum was mixed with 0.1 mg. of BCG cells killed by heating at 90°C. for 10 minutes; the other animals received the staphylococci alone. In all cases, the inoculum was made up to 0.2 ml. with physiological saline. The survival times following infection are presented in Table I.

As seen in Table I, mice receiving the infective inoculum of staphylococci in admixture with 0.1 mg. of killed BCG bacilli had a shorter survival time than mice infected with staphylococci alone.

In the preceding experiment, enhancement of infection was achieved by intravenous injection of the active material. A similar effect was produced in another experiment by administering *via* the subcutaneous route various amounts of BCG cells, killed by phenol and washed with acetone. An infective dose of 0.03 ml. of *Myco. fortuitum* had been injected 30 minutes before this treatment (Table II).

The results presented in Tables I and II demonstrate that the infection-enhancing activity of killed BCG cells could be made evident by either the subcutaneous or the intravenous route of injection. As will appear from the results of other experiments presented in this paper, the intraperitoneal route was also effective. It will be seen, however, that the effect of the treatment on the course of infection varied greatly depending upon the dose of bacterial product injected, and the time of injection.

2. Effect of Dose and Time of Injection of Mycobacterial Constituents on Their Ability to Enhance Staphylococcal Infections.—

Mice were vaccinated by the intraperitoneal route with methanol extract of BCG, or with cell residue left after methanol extraction. These materials were prepared from bacilli previously washed with acetone and air dried twice in succession, which were then heated in 30 times their weight of boiling methanol, with constant stirring. The soluble fraction was separated as described under Materials and Methods in the preceding publication. The bacillary residue was air-dried. For injection, the proper amounts of methanol extract or of residue were resuspended in 0.2 ml. of pyrogen-free saline.

The animals were infected by the intravenous route 3 hours, 24 hours, or 7 days after injection of the BCG fraction. The survival times are recorded in Table III.

As seen in Table III, the mice which had received methanol extract, or bacterial residue, 3 hours before infection with staphylococci, died faster than infected animals treated with smaller amounts of these bacillary constituents or with saline alone. When the staphylococci were injected 24 hours or 7 days after administration of the methanol extract or bacillary residue, the infection-enhancing effect of these materials was no longer apparent. Furthermore, it can be surmised from the results of other experiments (as reported in the preceding paper) that a protective effect would have been observed had infection taken place several weeks later. The smaller amounts of bacillary residue exerted no detectable infection-enhancing effect, even when administered just before the staphylococci, but on the other hand, the animals infected 24 hours or 7 days after receiving this material survived longer than the controls.

3. Activating Effect of Bacterial Products on Chronic Infections.—

We have been able to establish in mice experimental infections with a chronic course by inoculating intravenously small doses of virulent cultures of *Staph.*

aureus, *Myco. tuberculosis*, *Myco. fortuitum*, or "atypical" mycobacteria. The infections so produced do not progress under usual circumstances, and the lesions may eventually heal. However, the animals continue for many months to harbor small numbers of virulent bacilli—in the spleen and lungs with *Myco. tuberculosis*, in the kidneys with *Myco. fortuitum* and *Staph. aureus*. As shown by the following results, this chronic type of infection could be rapidly brought into a state of activity by administering to the infected animals small amounts of cellular constituents of Gram-negative bacilli or of mycobacteria.

TABLE III

Relation of Dose and Time of Administration on the Effect of Constituents of BCG Cells on Resistance of Mice to Staphylococcal Infection

Materials injected (i.p.)		Interval between treatment and infection	Cumulative deaths at indicated days (d.) after infection 3 d.	4 d.	6 d.	8 d.
	mg.		(out of 8 mice)			
Methanol extract	5	3 hrs. before	8			
Bacterial residue	5	" " "	6	8		
" "	1.5	" " "	4	5	7	7
" "	0.5	" " "	1	5	6	8
Saline		" " "	4	5	6	6
Methanol extract	5	24 hrs. after	6	6	7	7
Bacterial residue	5	" " "	7	7	8	
" "	1.5	" " "	5	5	7	7
" "	0.5	" " "	3	3	4	4
Saline		" " "	8			
Methanol extract	5	7 days after	3	3	4	7
Bacterial residue	5	" " "	2	3	4	4
" "	1.5	" " "	0	1	2	2
" "	0.5	" " "	0	2	4	6
Saline		" " "	1	2	4	8

Mice were infected by the intravenous route with 0.002 ml. of a 7 day old culture of *Myco. fortuitum*. None of them died of the infection. One month later they received by the intraperitoneal route either pertussis vaccine (approximately 10 billion cells), or killed cells of BCG (2 mg.), or physiological saline (0.2 ml.). Some of the animals were sacrificed 5 days after this treatment, others 9 days. The numbers of viable bacteria in their organs were determined by quantitative bacteriological techniques (Table IV).

As seen from the results presented in Table IV, injection of pertussis vaccine or of killed cells of BCG brought about an explosive multiplication of *Myco. fortuitum* in the organs of mice which had been infected with a small dose of this organism 1 month before. Injection of 50 μg. of lipopolysaccharide fraction prepared from *Serratia marcescens* caused within a few days the death of 5 out of 8 infected animals. Because the enumeration of colonies in the organs

of the surviving three animals gave erratic results, the bacteriological results of this particular test will not be presented.

In another experiment similar in design, the interval between the establishment of the chronic infection and the injection of bacterial product was extended to 3 months. Killed cells of *Myco. fortuitum* were compared with killed cells of BCG with regard to their ability to activate the chronic infection.

Mice were infected by the intravenous route with 0.005 ml. of a 7 day old culture of *Myco. fortuitum*. None of them died of the infection. Three months later they received by the intraperitoneal route either 2 mg. of killed cells of BCG, or 2 mg. of killed cells of *Myco. fortuitum*, or physiological saline. The animals were sacrificed 1 day, 3 days, or 6 days after

TABLE IV

Activation of Chronic Myco. fortuitum Infection by Intraperitoneal Injection of Pertussis Vaccine or Killed BCG Cells

Materials injected (i.p.)	Time between treatment and sacrifice	Numbers of bacterial colonies recovered from							
		Liver*				Kidney*			
	days								
Pertussis vaccine‡	5	20	27	600	700	3×10^4	2×10^5	5×10^5	5×10^6
Killed BCG§	"	13	20	180	600	4×10^4	5×10^4	7×10^4	5×10^5
Saline	"	0	1	25	45	16	1×10^4	2×10^4	6×10^4
Pertussis vaccine‡	9	1	2	3	280	3×10^4	5×10^4	2×10^5	5×10^5
Killed BCG§	"	25	600		2000	4×10^4	6×10^4	5×10^5	—
Saline	"	0	1	6	60	0	20	4×10^4	8×10^4

* Figures to be multiplied by 1000 for whole organ.

‡ Approximately 10 billion cells, diluted to 0.2 ml. saline.

§ 2 mg. injected in 0.2 ml. saline.

receiving the bacterial suspensions and the numbers of viable cells of *Myco. fortuitum* present in their organs at the time of sacrifice were determined by quantitative bacteriological techniques. The results are summarized in Table V, which also presents the changes in weight of individual animals before sacrifice.

The results presented in Table V confirm and extend those of the preceding experiment. In this case again, intraperitoneal injection of 2 mg. of killed mycobacteria brought about a rapid activation of the chronic infection established in mice 3 months before with *Myco. fortuitum*. As can be seen, extensive multiplication of these organisms in the liver and kidneys was apparent within 3 days after injection of the killed bacillary bodies.

Table VI presents the results of a similar experiment in which a chronic infection, established 56 days before by the intravenous injection of 0.02 ml. of the atypical mycobacteria P_{17}, was activated by treatment with killed cells of

Myco. fortuitum or BCG. It can be seen that extensive multiplication of the bacteria in the liver and lungs was evident as early as 3 days after administra-

TABLE V

Activation of Chronic Myco. fortuitum Infection by Intraperitoneal Injection of Killed Cells of BCG and Myco. fortuitum

Materials injected (i.p.)		Cumulative weight change at indicated days (d.) after injection			Time between treatment and sacrifice	Numbers of bacterial colonies recovered from	
		1 d.	3 d.	6 d.		Liver*	Kidney*
		gm. per mouse			*days*		
Myco. fortuitum	2.0 mg.	+1.1			1	0	3
		−1.5			"	0	9×10^2
		−3.1			"	38	5×10^4
BCG	2.0 mg.	−1.6			"	0	1×10^2
		−2.3			"	0	4×10^2
		−3.5			"	2	5×10^4
Saline	0.2 cc.	−.2			"	0	9
		+.8			"	0	25
		−.9			"	0	1×10^4
Myco. fortuitum	2.0 mg.	−3.6	−4.3		3	1×10^3	5×10^2
		−2.1	−1.4		"	6×10^3	4×10^3
		−2.1	−1.8		"	5×10^4	5×10^3
BCG	2.0 mg.	−0.5	−3.8		"	?	5×10^4
		−2.0	−2.3		"	?	1×10^5
		−2.3	−2.5		"	?	?
Saline	0.2 cc.	0	−0.6		"	0	34
		+0.2	+0.9		"	0	1×10^2
		+0.1	+0.5		"	0	3×10^2
Myco. fortuitum	2.0 mg.	−2.8	−2.8	−3.1	6	1×10^3	5×10^3
		−2.8	−2.7	−0.8	"	3×10^3	9×10^3
		−4.2	−5.4	−6.1	"	4×10^3	1×10^4
		−3.6	−4.2	−3.6	"	5×10^3	1×10^5
BCG	2.0 mg.	−1.4	0	+1.0	"	0	50
		−4.1	−4.7	−1.5	"	1	2×10^3
		−2.6	−1.6	0	"	70	1×10^4
Saline	0.2 cc.	+.9	+2.1	+4.1	"	0	0
		+.7	+1.0	+1.6	"	0	6
		−0.5	−0.4	−0.6	"	0	4×10^3
		+0.7	+0.7	+0.7	"	1	8×10^3

* Figures to be multiplied by 1000 for whole organ.

tion of the killed mycobacterial cells. In preliminary experiments with chronic staphylococcal infections—not reported here—clear evidence of activation of the infectious process in the liver and kidneys was observed 24 hours after injection of the killed mycobacteria.

The findings in the liver are of special interest since *Myco. fortuitum* and *Staph. aureus* are always rapidly eliminated from this organ in normal mice,

TABLE VI

Activation of Chronic Mycobacterial (P_{17}) Infection by Intraperitoneal Injection of Killed Cells of BCG and Myco. fortuitum

Materials injected (i.p.)		Cumulative weight change at indicated days (d.) after injection				Time between treatment and sacrifice	Numbers of bacterial colonies recovered from	
		1 d.	3 d.	6 d.	10 d.		Liver*	Lungs*
		gm. per mouse				*days*		
Myco. fortuitum	2.0 mg.	−1.7	−2.4			3	1×10^4	16
		−1.3	−1.0			"	8×10^2	20
		−1.6	−0.7			"	1×10^5	1×10^5
BCG	2.0 mg.	−1.0	−0.6			"	6	3
		−0.4	+1.0			"	13	0
		−3.0	−1.3			"	23	5
Saline	0.2 cc.	+0.2	+1.5			"	0	0
		+0.6	+1.5			"	0	0
		+1.0	+1.6			"	0	0
Myco. fortuitum	2.0 mg.	−2.2	−2.6	−1.4		6	1×10^4	8
		−3.0	−0.6	−1.0		"	0	0
		+0.7	+1.6	+3.9		"	3×10^3	8
BCG	2.0 mg.	−2.6	−1.4	−0.1		"	1	2
		−2.7	−1.7	−2.1		"	25	1×10^4
		−2.9	−1.8	+0.2		"	40	1
Saline	0.2 cc.	+1.3	+2.5	+2.5		"	0	0
		0	+0.2	0		"	0	0
		0	+1.4	+1.5		"	0	0
Myco. fortuitum	2.0 mg.	−1.0	−0.8	+1.0	+1.7	10	1×10^5	5
		−0.9	+0.2	+1.5	+1.9	"	1×10^5	1×10^4
		−3.9	−2.1	−2.8	−1.9	"	1×10^4	0
BCG	2.0 mg.	−2.2	−0.5	+1.3	+2.2	"	1	0
		−2.8	−2.9	−1.9	−0.5	"	26	0
		0	−0.4	−0.3	+0.7	"	0	0
Saline	0.2 cc.	+1.3	+1.2	+1.1	+1.0	"	0	0
		+0.6	+0.7	−0.2	−0.3	"	0	0
		+0.2	+1.9	+1.9	+2.6	"	0	0

* Figures to be multiplied by 1000 for whole organ.

whereas they multiplied rapidly in the treated animals. It is worthy of mention, furthermore, that the livers of several of the animals which had received the killed mycobacteria yielded on numerous occasions large numbers of colonies of organisms belonging to species other than the bacteria injected. This fact suggests that the physiological disturbances resulting from the injection of the killed mycobacteria interfered with the clearing power of the liver cells

and thus allowed the colonization in this organ of organisms derived from the intestinal tract.

SUMMARY

Injection of certain cellular constituents of mycobacteria into mice simultaneously with, or shortly after, infection with *Staph. aureus* or with *Myco. fortuitum*, markedly shortened the life of the infected animals. This infection-enhancing effect could be achieved by injecting the cellular constituents by either the intravenous, the intraperitoneal, or the subcutaneous route.

Suspensions of killed mycobacteria were injected into mice which had been infected several months before with small doses of various bacterial pathogens and which still harbored small numbers of living organisms in their organs. Under these conditions, a marked increase in the numbers of living bacteria in the organs of the treated mice could be detected within a very few days after treatment with the mycobacterial products, and a certain percentage of the animals died rapidly.

One of the first and most constant manifestations of the change in the infectious process from the chronic to the acute state was the appearance of a large microbial population in the liver. This happened even though *Staph. aureus* and *Myco. fortuitum* are rapidly cleared from the liver of normal mice.

In addition to killed cells of BCG and of *Myco. fortuitum*, pertussis vaccine and the purified lipopolysaccharide (endotoxin) of Gram-negative bacilli also proved capable of converting chronic bacterial infections into acute infectious processes.

BIBLIOGRAPHY

1. Dubos, R. J., and Schaedler, R. W., Effects of cellular constituents of mycobacteria on the resistance of mice to heterologous infections. I. Protective effects, *J. Exp. Med.*, 1957, **106**, 703.
2. Runyon, E. H., Veterans Administration–National Tuberculosis Association Cooperative Study of Mycobacteria, *Am. Rev. Tuberc.*, 1955, **72**, 866; and private communication.