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Studies on the Mechanism of Non-Specific Resistance to *Brucella* induced in Mice by Vaccination with BCG

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Summary. Mice vaccinated with living or killed BCG become resistant to infection with *Brucella abortus*, as evidenced by a lower bacterial count in the spleen and liver as compared to controls. Some of the observed resistance is attributed to the ability of the treated mice to mobilize quickly a large number of macrophages in the abdominal cavity, since these macrophages could phagocytose and subsequently kill a proportion of the injected bacteria. However, the main antibacterial effect appears to take place in the spleen and liver. These organs contained most of the *Brucellae* found in the animal at 6 hours after challenge and showed the most significant differences between vaccinated and non-vaccinated animals.

Sera of BCG-treated mice contain a factor capable of conferring to normal animals some protection against challenge with *Brucella*.

INTRODUCTION

The ability of mycobacteria to enhance the resistance of experimental animals to a variety of pathogens has been studied extensively. Guinea pigs were found to be more resistant to *Brucella* if infected simultaneously (Pullinger, 1936) or previously (Henderson, Lancaster, Packman and Peacock, 1956) with *Mycobacterium tuberculosis*. Enhanced resistance to anthrax bacillus was also observed (Henderson *et al.*, 1956). Mice pretreated with BCG had increased resistance to Staphylococci (Dubos and Schaedler, 1957) and Salmonellae (Howard, Biozzi, Halpern, Stiffel and Mouton, 1959). A fatal dose of virulent tubercle bacilli decreased greatly the susceptibility of mice to infection with *Pasteurella pestis* (Girard and Grumbach, 1958). Living BCG or their extracts were also effective (Weiss, 1960). Recently, Gledhill and Rees (1960) described the alteration in resistance of mice to *Ectromelia* induced by a tuberculous infection.

A number of mechanisms have been postulated, through which the mycobacteria might exert their resistance-enhancing activity. Probably the most striking of these is the stimulation of the reticulo-endothelial system, first observed by Lurie (1939). More recent studies with mice infected with tubercle bacilli described the proliferation of phagocytic cells of the spleen and liver of these animals, with subsequent increase in the rate of phagocytosis (Biozzi, Benacerraf, Grumbach, Halpern, Levaditi and Rist, 1954). An increase in the metabolic activity of the phagocytes was also observed (Howard *et al.*, 1959).

In addition to the stimulation of the RES, several other changes induced by mycobacteria might play a role in enhancing the resistance of the host. Among these, one would include the improved ability of mice to produce antibodies (Halpern, Biozzi,

Howard, Stiffel and Mouton, 1958) and the alterations in resistance at the cellular level described by Elberg (1960).

It is reasonably certain that the non-specific resistance induced by BCG is a complex phenomenon, and probably involves the simultaneous action of many of the factors enumerated above. The relative importance of these factors for any given host-pathogen combination cannot be predicted but must be determined experimentally. With this in mind, we have carried out a number of experiments, in order to obtain some information on the mechanism of the non-specific resistance to *Brucella* induced in mice by BCG. A preliminary communication on this work has been published (Bekierkunst and Sulitzeanu, 1958).

MATERIALS AND METHODS

ANIMALS. Random-bred white mice of both sexes were used.

BACTERIAL STRAINS AND MEDIA. A BCG strain of Danish origin was used in the immunization experiments (it was obtained through the courtesy of Dr. D. Weber of the department of Clinical Microbiology, Medical School, Jerusalem). The strain was grown and maintained in a liquid medium of the following composition: asparagine, 5 g., KH_2PO_4, 5 g., citric acid, 1.5 g., ferric ammonium citrate, 0.05 g., magnesium sulphate, 0.5 g., potassium sulphate, 0.5 g., glycerol, 0.5 g., tween 80, 0.5 g., distilled water to 1000 ml. The pH was adjusted to 6.8. Ten per cent bovine albumin was added after sterilization, to a final concentration of 0.25 per cent.

The virulent *Brucella abortus* 2308 was used as challenge strain. It was grown for 2 days on Trypticase Soy Agar, supplemented with 3 per cent glycerol and 1 ml./litre of 0.01 per cent thiamine solution.

PREPARATION OF ACETONE KILLED, RADIOIODINATED *Brucella* (^{131}I *Brucella*). The preparation of acetone-dried bacteria and the technique used in labelling them with radioactive iodine ^{131}I have been described (Sulitzeanu, 1959).

PREPARATION AND COLLECTION OF PERITONEAL EXUDATES. Exudates containing mostly mononuclear white cells were collected from mice as follows: the animals received an intra-abdominal injection of 3 ml. phosphate-buffered saline (pH 7.2), containing 3 units/ml. heparin and 0.2 per cent sodium acetate (PBSHA – Sulitzeanu *et al.*, 1960). They were immediately anaesthetized with ether and killed by cutting the neck blood-vessels. The abdominal cavity was opened, the fluid was collected with a siliconed Pasteur pipette and placed into chilled, siliconed tubes. The cavity was then washed with two to three 1-ml. portions of cold PBSHA, until the washings were free of macroscopically visible cells. The number of cells obtained was determined by counting in a standard blood-counting chamber.

INFECTION OF PERITONEAL MACROPHAGES WITH LIVING *Brucellae* FOR TRANSFER EXPERIMENTS. Peritoneal white cells infected with living *Brucellae* were obtained as follows: 2×10^8 bacteria in 0.2 ml. broth were injected into the peritoneal cavity of ten mice. Thirty minutes later, the cells were collected as described in the preceding section and pooled. They were washed twice in PBSHA solution and resuspended in 8 ml. of the same medium. A portion of the cells was frozen at $-20°$, in order to kill the phagocytes, then thawed. The effectiveness of this procedure was tested by examining the ability of the frozen cells to stain with trypan blue. If a large proportion of the cells was found unstained, the freezing-thawing procedure was repeated.

Appropriate quantities of the living or killed cell suspensions (undiluted, diluted 1/3 and 1/9) were then injected to groups of five mice, intra-abdominally, in a volume of 0.2 ml. Other mice received at the same time free *Brucellae*, in doses corresponding to the number of living bacteria expected to be found within the phagocytes. The number of living *Brucellae* found in the cells or adhering to them was determined by plating an aliquot of the frozen cell suspension. To ensure that the count thus obtained was not vitiated by the inclusion of extracellular bacteria left from the second washing, the bacterial count in the latter was also determined. Extracellular bacteria never accounted for more than a few per cent of the total number of organisms. About 1 per cent of the injected *Brucellae* were found in the phagocytes by this procedure.

The freezing and thawing technique was preferred to other methods of disintegrating the white cells (saponin, distilled water), as it gave higher counts of intracellular bacteria.

IMMUNIZATION PROCEDURE. Unless otherwise stated, living BCG were used in immunization experiments. The mice were injected intra-abdominally with a suspension of bacteria in physiological saline containing 0.04 mg. (dry weight) organisms. When a heat-killed vaccine was used, it was prepared by heating the suspension at 70° for 60 minutes.

At the desired intervals after vaccination, the mice were challenged with a suspension of 30,000 *Brucellae* in Trypticase Soy broth, administered intra-abdominally or intravenously. A week later, they were killed and the bacterial count in organs was determined by plating. The degree of immunity was measured as the protection index:

PI = (Mean spleen count of control animals)/(Mean spleen count of immune animals). An index of five or higher was considered as statistically significant (Sulitzeanu, 1955).

PASSIVE PROTECTION EXPERIMENTS. Sera from BCG-vaccinated mice were tested for their ability to protect mice against infection with *Brucella*, by the technique described by Sulitzeanu (1955). The animals (five in each group) were bled while under anaesthesia and the blood was collected in siliconed tubes, to prevent haemolysis. 0.1 ml. of each serum (undiluted or diluted 1/5) were immediately injected to groups of mice intramuscularly into the left hind leg. Sera from normal, untreated mice were injected to other groups to serve as controls. The animals were challenged the next day and the bacterial count was determined as described in the following section. Mice from the same batch as the serum donors were challenged at the same time, thus indirectly testing the resistance of the latter to *Brucella*.

COUNTING OF BACTERIA IN ORGANS. The spleens (or other organs) were removed aseptically and pooled. The mesenteric lymph nodes were obtained by detaching the whole mesentery from the intestines. The organs were triturated with sterile glass sand in porcelain mortars. Suspensions were prepared in trypticase soy broth, 5 ml. of broth being added for each organ. 0.2 ml. of each suspension or dilutions thereof were plated on Trypticase Soy agar plates. Colonies were counted after 3–4 days incubation.

Each experiment was performed at least twice, often several times, and the results were found to be reproducible.

RESULTS

IMMUNIZING EFFECT OF LIVING AND KILLED BCG

Groups of mice received intra-abdominally a suspension of 0.04 mg. living or 0.4 mg. killed BCG. At various intervals after immunization, five mice of each group were challenged with *Brucella*. The spleen counts showed a maximum protective effect at 7–14 days

after vaccination (Table 1). When the challenge was administered at longer intervals, good protection was often, but not always, obtained. Thus, in five separate experiments performed at 6 weeks after vaccination, the indices were 300, 15, 14, 6 and 1.

0.04 mg. living bacteria appeared to be the smallest quantity necessary to induce an increase in resistance. A dose of 0.004 mg. proved ineffective.

TABLE 1

IMMUNIZING EFFECT OF LIVING AND KILLED BCG AGAINST *Brucella abortus*

Immunizing antigen	*Spleen counts and protection indices at days after immunization*											
	2		7		14		28		42		56	
	Spleen count	*P.I*	*Spleen count*	*P.I.*	*Spleen count*	*P.I.*	*Spleen count*	*P.I.*	*Spleen count*	*P.I.*	*Spleen count*	*P.I.*
Living BCG	7.3×10^5	0	1.6×10^3	169	1.5×10^4	45	2.4×10^5	1.9	3×10^5	6	2.4×10^5	1.6
Killed BCG	1.9×10^5	0	1.5×10^4	18	3.6×10^4	19	1×10^5	4.5	1.3×10^5	14	7.4×10^4	5.3
None (Controls)	8.7×10^4		2.7×10^5		6.8×10^5		4.5×10^5		1.8×10^6		3.9×10^5	

The challenge consisted of 30,000 *Brucellae* administered intra-abdominally P.I.: Protection index: (No. of bacteria in spleens of BCG-treated mice)/(No. of bacteria in spleens of untreated mice) 0.04 mg. dry weight of living BCG or 0.4 mg. heat-killed BCG were used as immunizing antigens

DISTRIBUTION OF *Brucella* IN MICE PRETREATED WITH BCG

Previous studies in this laboratory (Sulitzeanu *et al.*, 1960) have shown that the distribution of ^{131}I *Brucella* injected intra-abdominally into mice can be altered by factors affecting the degree of phagocytosis. Treatment of mice with *Coli* lipopolysaccharide or anti-*Brucella* serum resulted in increased phagocytosis in the peritoneal cavity. Some of the engulfed bacteria were carried to the mesenteric lymph nodes, thus causing increased localization of radioactivity in these organs. There was a consequent reduction in the number of *Brucellae* reaching the spleen and liver.

Since vaccination with BCG is known to enhance phagocytosis (Biozzi *et al.*, 1954), it was considered worth while to examine the fate of ^{131}I *Brucella* in mice pretreated with BCG. The results were similar to those obtained after injection of lipopolysaccharide (Table 2).

TABLE 2

RADIOACTIVITY IN ORGANS OF MICE VACCINATED WITH BCG AND CHALLENGED WITH ^{131}I *Brucella*

Organ	*Percentage of radioactivity injected*		
	Normal mice	*Mice vaccinated with BCG*	*Mice passively immunized with* Brucella *antiserum*
Spleen	5.8	1.7	1.2
Liver	17.2	9.1	11.0
Mesenteric lymph nodes	4.2	4.7	5.1

The mice (five in each group) were challenged with ^{131}I *Brucella* 14 days after vaccination with BCG and the organs were removed for examination 4 hours after challenge

The possibility was now tested that, after infection, living bacteria might be distributed according to the same pattern. This was done by determining the bacterial count in the spleen, liver and mesenteric lymph nodes during the first hours after inoculation. Spleen counts of twelve groups of animals killed at various intervals after infection were obtained in five separate experiments. The results, given as protection indices, are summarized in Fig. 1 which shows that most indices were larger than 1. Most spleens of normal animals

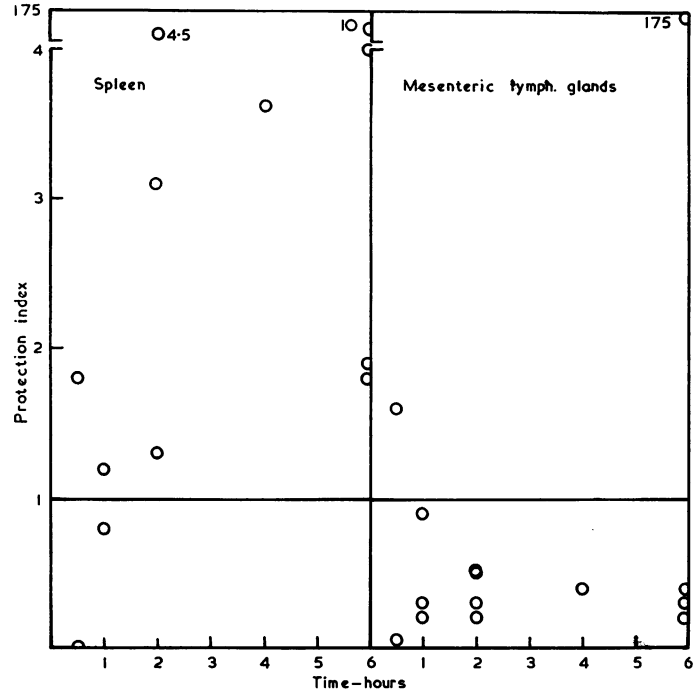

FIG. 1. Protection indices for spleens and mesenteric lymph glands of mice vaccinated with BCG. The bacterial counts were determined at various time intervals (1–6 hours) after challenge with 30,000 *Brucellae*. The points in the graph are logarithms of protection indices.
Protection index: (Bacterial count in organ of normal mice)/(Bacterial count in organ of BCG-treated mice).

(ten out of twelve) harboured, therefore, more bacteria than those of BCG-treated mice. Similar results were obtained for the liver.

The values obtained for the mesentric lymph nodes are also plotted in Fig. 1. Twelve out of fourteen groups tested gave indices smaller than 1, thus showing that the majority of the immune animals harboured more bacteria in their nodes than the corresponding controls. There were no significant differences in the number of *Brucellae* in the peritoneal cavity between normal and BCG-treated mice. These results indicated that the distribution of the living *Brucellae* during the first hours after inoculation was similar to that of the ^{131}I labelled organisms.

MOBILIZATION OF WHITE CELLS IN THE ABDOMINAL CAVITY OF MICE IMMUNIZED WITH BCG

The foregoing experiment suggested that the increased resistance of BCG-treated animals to infection with *Brucella* might be due (at least to some extent) to more efficient

phagocytosis in their abdominal cavities. Among the several ways in which this could be accomplished, the most straightforward seemed to be the rapid mobilization of large numbers of phagocytic cells. We, therefore, proceeded to compare the peritoneal cellular response of vaccinated and non-vaccinated animals, after stimulation with *Brucella.* 0.1 mg. acetone killed or 30,000 living *Brucellae* were injected into the abdominal cavities of normal and of BGC-treated mice. At various time intervals after inoculation, groups of ten mice were killed, the peritoneal cavities washed and the number of white cells in each

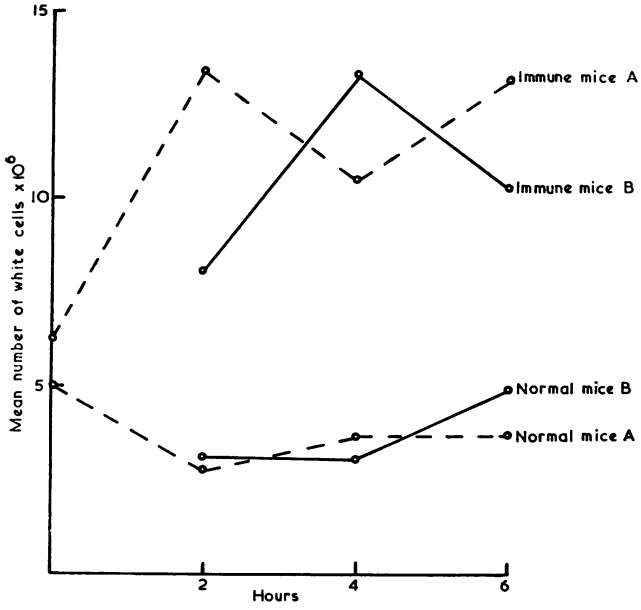

FIG. 2. Mean number of white cells in the peritoneal cavity of BCG-treated (immune) and normal mice, at various time intervals after intra-abdominal injection of living (B) or acetone killed (A) *Brucellae.* Each group consisted of ten mice.

washing determined. The results are given in Fig. 2. It is seen that both types of stimuli caused the appearance of a larger number of white cells in the BCG-treated mice.

DESTRUCTION OF *Brucellae* PHAGOCYTOSED BY PERITONEAL WHITE CELLS AFTER TRANSFER OF THESE CELLS TO NORMAL MICE

The experiments to be described in this section were undertaken in order to provide more direct proof of the destruction of *Brucellae* after phagocytosis by mouse peritoneal phagocytes. The experimental set-up was designed so as to reproduce as nearly as possible the conditions obtaining in the intact mouse after challenge with *Brucellae.* Peritoneal phagocytes carrying living intracellular *Brucellae* were obtained from BCG-treated mice as described in methods. A portion of the cell suspension was frozen in the deep freezer (−20°) in order to kill the phagocytes. Three-fold dilutions of the living and of the frozen cells were prepared in PBSHA and each dilution was injected into five mice. Groups of other mice received at the same time *Brucellae* in doses close to the assumed number of

bacteria present in the phagocytes. The results of a typical experiment are shown in Table 3. The spleen counts of the mice receiving free *Brucellae* or *Brucellae* contained in killed phagocytes were higher than those of the animals injected with bacteria contained in, or adhering to, living phagocytes. The differences, however, are small and would not be considered significant, except for the fact that similar results were obtained in six out of seven groups, tested in three separate experiments. We conclude that phagocytosis of *Brucellae* in the peritoneal cavity results in some reduction in the number of injected bacteria, with a consequent reduction in the spleen count.

TABLE 3

SPLEEN COUNTS OF MICE INJECTED WITH FREE OR PHAGOCYTOSED *Brucellae*

Challenge dose	*Mice injected with*		
	Phagocytosed Brucellae *contained in*		*Free* Brucellae
	Living phagocytes	*Frozen phagocytes*	
1300	4.5×10^3	5.6×10^3	
3300			5×10^4
4000	2.8×10^4	5.8×10^4	
10,000			1.1×10^5
12,000	4.3×10^4	1×10^5	
30,000			2.6×10^5

Macrophages containing phagocytosed *Brucellae* obtained from peritoneal cavities of mice injected intra-abdominally with 2×10^8 bacteria. A portion of these macrophages was killed by freezing, a procedure which left the intracellular bacteria unharmed. Appropriate dilutions of the living and killed macrophage suspensions containing *Brucellae* were injected to groups of five mice. Other groups received non-phagocytosed (free) bacteria in quantities corresponding to those of the intracellular *Brucellae*

EFFECT OF ROUTE OF INOCULATION ON THE BACTERIAL COUNT IN THE ORGANS

The results described so far, while pointing to the role played by peritoneal phagocytes in protecting the mouse against *Brucella*, did not allow any conclusions to be reached regarding the function of the RES rich organs (spleen, liver) in this protection. To obtain information on this point, a comparison was carried out between the level of infection attained after intra-abdominal and intravenous routes of inoculation. It was argued that, if the peritoneal cavity played a major role in the elimination of bacteria from the organs, by-passing the cavity (through intravenous injection) should lead to a high level of infection in the organs. If, on the other hand, the major destruction of bacteria took place outside the peritoneal cavity, the route of inoculation should make little difference as regards the final level of infection.

This argument was valid only in so far as it could be shown that changing the route of inoculation did not cause any drastic alterations in the overall distribution of bacteria. If after intravenous challenge the bacteria accumulated at completely different sites, comparisons of the level of infection in spleen and liver only would have been misleading. Bacterial counts were, therefore, performed in a large number of organs, after both intra-abdominal and intravenous challenge. The results at 6 hours and 7 days after challenge are given in Tables 4 and 5, respectively.

Table 4 shows the bacterial count in the spleen, liver, mesenteric nodes and peritoneal exudate. The other organs tested (see Table 5) contained few organisms and are, therefore, not included. It can be seen from Table 4 that 6 hours after challenge:

(*a*) The immune animals harbour fewer bacteria than the normal mice, probably less than 50 per cent of the total injected (even if allowance is made for losses in recovery).
(*b*) The two routes of inoculation lead to approximately the same level of infection in the organs. The only large difference is seen in the peritoneal exudate.

TABLE 4

BACTERIAL COUNT OF NORMAL AND BCG-TREATED MOUSE ORGANS AT 6 HOURS AFTER CHALLENGE WITH *Brucella*

Organ	*Bacterial count after*			
	Intra-abdominal challenge		*Intravenous challenge*	
	Normal mice	*BCG-treated mice*	*Normal mice*	*BCG-treated mice*
Spleen	9600	5300	6000	3800
Liver	4800	2100	3800	2600
Mesenteric nodes	180	650	0	0
Peritoneal exudate	9000	8400	0	0

These data indicate that the BCG-treated mouse injected intravenously with *Brucella* can rid itself of a large number of bacteria even without the benefit of the peritoneal macrophages. It appears then that the main antibacterial activity takes place outside the peritoneal cavity.

Table 5 shows the level of infection at 7 days. Again, no striking differences are noted

TABLE 5

BACTERIAL COUNT OF NORMAL AND BCG-TREATED MOUSE ORGANS AT 7 DAYS AFTER CHALLENGE WITH *Brucella*

Organ	*Bacterial count after*			
	Intra-abdominal challenge		*Intravenous challenge*	
	Normal mice	*BCG-treated mice*	*Normal mice*	*BCG-treated mice*
Spleen	1.1×10^6	6.8×10^4	8.6×10^5	1.5×10^5
Liver	1.0×10^5	1.2×10^3	5.0×10^4	3.3×10^3
Kidneys	8.2×10^3	2.3×10^2	1.6×10^3	5.0×10
Mesenteric nodes	8.8×10^4	Contaminated†	3.0×10^4	5.6×10^3
Other lymph nodes*	4.8×10^4	9.3×10^2	6.7×10^4	3.8×10^4
Lungs	5.1×10^3	5.5×10^2	8.0×10^2	1.6×10^3
Bone marrow	3.8×10^4	2.1×10^4	9.0×10^3	5.5×10^4
Peritoneal exudate‡	4.8×10^3	2.2×10^2	0	4×10^1

* These included: axillary and inguinal nodes and as many nodes as could be found in the neck region.

† It was found in other experiments that mesenteric nodes of BCG-treated mice contained five to twenty times fewer bacteria than nodes of untreated mice.

‡ Peritoneal exudates were not counted in this experiment. The figures are taken from another experiment.

between the two routes of challenge. It can be seen in addition that the spleen was always the most heavily infected organ. The number of *Brucellae* in the spleen was, therefore, a true index of the immune state of the animal.

HUMORAL FACTORS INVOLVED IN THE NON-SPECIFIC RESISTANCE AGAINST *Brucella*

A great deal of work was devoted to the question as to whether humoral factors play a role in the increased resistance of mice due to BCG. Two possibilities were considered: first, that BCG vaccination might induce the appearance of humoral anti-*Brucella* factors and secondly, that at least part of the increased resistance might be due to enhanced production of anti-*Brucella* antibody.

TABLE 6

PROTECTION OF MICE AGAINST *Brucella* INFECTION BY INJECTION OF SERUM TAKEN FROM BCG-IMMUNE ANIMALS

	Protection indices									
Experiment no.	1		2				3			
*Serum taken at days**	10		10		15		10		15	
Pretreatment of mice	*IT*‡	*RT*§	*IT*	*RT*	*IT*	*RT*	*IT*	*RT*	*IT*	*RT*
BCG serum 1/1	180	—	1	—	0	2	90	72	7	3
BCG serum 1/5	44	57	16	1	7	0	30	24	4	10
NM serum† 1/1	0	—	0	—	4	0	3	2	0	0
NM serum 1/5	0	2	2	0	2	2	5	8	10	0
Brucella antiserum 1/5	60	160	60	90	90	520	440	120	120	40
Living BCG	25	—	8	—	150	—	1	—	5	—

* Days after immunization with living BCG.
† Normal mouse (NM).
‡ Immediate test.
§ Repeated test.

Sera were taken from BCG-vaccinated and untreated control mice at 10 (first experiment) or 10 and 15 days after immunization (second and third experiment). They were immediately injected to normal animals (immediate test). Other animals received anti-*Brucella* serum for the purpose of comparison. The challenge was administered the next day and the spleen counts performed 1 week later. BCG-immune mice and their controls were challenged at the same time, thus testing the resistance of the serum donors.

The tests were repeated after the sera had been kept a few days at −5° (repeated test).

Protection indices are given in whole numbers. Indices smaller than 1 are given as zero.

The first possibility was tested in passive protection experiments, in which sera from BCG-treated animals were used. The results are given as protection indices in Table 6. The sera in the first experiment were taken from mice killed at 10 days after immunization; in the subsequent experiments samples were also obtained from animals killed at 15 days after immunization; each serum was retested after storage for several days at −5°. Mice (BCG-treated or controls) from the same batch as the serum donors were challenged with *Brucella* at the same time as the passively protected animals.

Table 6 shows that sera from BCG-immune mice conferred a significant degree of protection against *Brucella* in eight out of ten experiments, in at least one of the two dilutions tested. In several instances the protection was quite high. Normal mouse sera were effective in only three out of ten experiments and the protection afforded was minimal. One can therefore conclude that sera of BCG-vaccinated mice contain a factor active against

Brucella. There was no correlation between the activity of the serum factor and the resistance of the donor mice to *Brucella*.

Before testing the second possibility mentioned above, namely that BCG vaccination increased the resistance to *Brucella* by stimulating the antibody response, it was necessary to find out whether administration of anti-*Brucella* serum would modify a pre-existing *Brucella* infection. This was done by giving daily injections of rabbit anti-*Brucella* serum to mice previously infected. No effect whatsoever was obtained. A similar experiment in which mouse antiserum was employed was equally unrewarding. These negative results suggest that the enhanced antibody production plays little, if any, role in the non-specific resistance induced by BCG against *Brucella*.

ENHANCEMENT OF SPECIFIC ANTI-*Brucella* IMMUNITY THROUGH PRETREATMENT WITH BCG

The findings described so far suggested that BCG might act synergistically with *Brucella* in raising the level of anti-*Brucella* immunity. This was fully confirmed in several experiments, one of which is detailed below. Mice were immunized with a single dose of 0.04 mg. BCG. Two weeks later, 0, 0.1, 1 or 10 μg. acetone-killed *Brucella* were injected to

TABLE 7

ENHANCEMENT OF SPECIFIC ANTI-*Brucella* IMMUNITY BY BCG

Type of pretreatment	*Spleen counts of mice injected with acetone-killed* Brucella abortus (μg.)			
	0	0.1	1	10
None	1.5×10^6	6.3×10^4	2.6×10^4	1.2×10^4
BCG	1.1×10^5	7.6×10^3	1.3×10^2	3.6×10^3

Mice vaccinated with 0.04 mg. BCG. A fortnight later, 0, 0.1, 1 or 10 μg. acetone-killed *Brucella* were injected to groups of five mice. Challenge administered after another 14 days.

groups of five of these mice and to other groups of untreated control animals. After another 14 days all mice were challenged.

The results are seen in Table 7. The spleen counts of the doubly treated mice are much lower than those of the mice vaccinated with either BCG or *Brucella* alone.

DISCUSSION

Three defence mechanisms have been examined for their possible role in the observed resistance of *Brucella* after BCG injection into mice: the peritoneal macrophages, the spleen and liver and the blood serum. The participation of peritoneal macrophages was suggested by the results of experiments on the distribution of *Brucella* in the mouse during the first hours after challenge. More bacteria were found in the mesenteric lymph nodes and less in the spleen and liver of BCG-treated mice, as compared to controls. Since this distribution was assumed to result from increased phagocytosis in the peritoneal cavity (with consequent transfer of *Brucellae* to mesenteric lymph nodes, Sulitzeanu *et al.*, 1960) a mechanism for enhanced phagocytosis was sought in the vaccinated animals. It was found

that these animals responded with a rapid mobilization of macrophages to stimulation with *Brucella*, a response which could hardly fail to affect the degree of phagocytosis. Evidence on destruction of bacteria engulfed by macrophages was obtained in the experiments with transferred cells. In these experiments, a three-fold reduction in the dose of free bacteria resulted in a two-fold reduction of the spleen count. The difference in the level of infection produced by *Brucellae* contained in living phagocytes and those contained in frozen phagocytes was about two-fold. A significant amount of bacteria contained in living cells must, therefore, have been killed, either directly or after transfer to the mesenteric lymph nodes.

With the exception of mesenteric lymph nodes, spleens and livers, there were no significant differences in the number of bacteria in the organs of BCG-treated mice and controls, 6 hours after inoculation. Animals immunized with BCG had, however, fewer organisms in the spleen and liver; this could be attributed to destruction of bacteria taking place:

(*a*) Mainly in the peritoneal cavity
(*b*) Mainly in the spleen and liver
(*c*) More or less equally in the three critical sites.

If the destruction of *Brucellae* took place mainly in the peritoneal cavity (while the spleen and liver had little anti-bacterial activity) bacteria injected directly into the blood should have caused a higher level of infection in the spleen and liver, as compared to those injected into the peritoneal cavity. A similar result should have been obtained even if the antibacterial activity was equally divided between the three critical sites. As the infection resulting from intravenous challenge was no higher than that resulting from intra-abdominal challenge, one must conclude that most bacteria passed through the cavity relatively unharmed and reached the blood-stream, and thence the organs, almost as if injected intravenously. It appears, therefore, that the major destruction of *Brucellae* in the BCG-treated mice took place outside the peritoneal cavity, most likely in the spleen and liver.

The fate of *Brucella* in the mouse can now be tentatively described as follows: after intra-abdominal inoculation, a small number of *Brucellae* are phagocytosed by macrophages. Many of these are probably killed, but some are first brought to the mesenteric nodes and possibly to other lymphatic locations. Most of the bacteria reach the blood through the lymphatic channels and are trapped in the spleen and liver. A fair proportion of them is destroyed in these organs, particularly in mice treated with BCG. However, having once gained a foothold in suitable cells they start multiplying. The lower level of infection observed in immune animals at 7 days after challenge is the result of the lower initial level from which multiplication started.

There seems to be an inconsistency between the postulated killing of *Brucella* in the spleen and liver on the one hand, and the subsequent multiplication of bacteria in these organs on the other; this would require the presence of cells of different degree of resistance towards *Brucellae*, but experimental evidence on this point is not available for our system. The selection of monocytes with enhanced resistance following infection, as postulated by Holland and Pickett (1958), might provide a reasonable explanation for these findings.

The role of the serum factor in the overall increase in resistance to *Brucella* induced by BCG vaccination is difficult to assess. The factor was unquestionably effective in passive

protection experiments, but its presence in the blood of the vaccinated animals was not correlated to their resistance to infection. The factor could actually be recovered from mice which themselves were fully susceptible to *Brucella*. This discrepancy might have been due, among other reasons, to variability of the experimental animals. Thus, if one mouse in a group of five had a high concentration of the serum factor but the group as a whole responded poorly to vaccination, the pooled serum would have shown protective ability while the donors themselves would have had high spleen counts.

The nature of the serum factor remains yet to be elucidated. References can be found in the literature regarding substances in the serum active in non-specific immunity (Rowley, 1960; Elberg, 1960) but their relationship, if any, to the factor described in this communication is not known.

A small number of experiments in which the BCG were injected subcutaneously showed that this route was ineffective against intra-abdominal challenge. A similar failure in evoking non-specific immunity because of unsuitable route of administration was encountered by Henderson (1960), when both the *Mycobacteria* and the *Brucellae* were administered by the respiratory route.

Additional insight into the problem of the mechanism of non-specific resistance induced by BCG will certainly be gained by *in vitro* studies. It has been amply demonstrated by Elberg (1960) that monocytes from BCG-immune rabbits are less vulnerable to *Mycobacterium* or *Brucella* infection than normal monocytes. It will remain to be seen whether these differences can be reproduced with mouse monocytes. *In vitro* techniques might also help in deciding whether the protective effect exerted by the spleen and liver is due to qualitative differences in their histiocytes or simply to numerical advantage due to BCG stimulation.

The use of BCG to enhance immunity to subsequent vaccination with *Brucella* could find practical applications, particularly with a view to replacing living with non-living preparations. Pretreatment of sheep with BCG might enhance the otherwise poor response of these animals to vaccination with *Brucella melitensis*.

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