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EXPERIMENTAL IMMUNITY TO TUBERCULOSIS IN RABBITS IMMUNIZED WITH BCG MICROSONAL FRACTION.

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EXPERIMENTAL IMMUNITY TO TUBERCULOSIS IN RABBITS

IMMUNIZED WITH BCG MICROSONAL FRACTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

by

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INTRODUCTION

Despite gains in the management of tuberculosis, due to improved surgical and therapeutic measures, basic mechanisms regarding the nature of the disease processes and acquired host resistance need yet to be elucidated.

Although intensively studied, very little evidence has been forthcoming to assign a role for humoral antibodies in the acquisition of resistance to tuberculosis (1). However, numerous investigators have established that the most important resistance factor is the increased ability of host macrophages to inhibit intracellular multiplication of tubercle bacilli (2). It is well documented that the majority of microorganisms are handled effectively by phagocytic cells of the host, but pathogens which characteristically produce chronic diseases, are capable of surviving and multiplying within the intracellular environment of host macrophages. Among these pathogens are Mycobacteria, Brucella, Listeria, Salmonella, Pasturella, and certain fungi. These organisms have been classified together as "facultative intracellular parasites" (3).

Following prior experience with these agents, either infection or immunization, the host develops a relative resistance to reinfection which is mediated by an increased ability of host macrophages to inhibit intracellular multiplication of the organism or to resist
being destroyed by the parasites. Such cellular resistance constitutes the basis for the concept of acquired cellular immunity.

Practical immunization procedures for tuberculosis have employed non-virulent tubercle bacilli such as the attenuated bovine strain, Bacille Calmette Guerin (BCG) or the vole bacillus, *M. microti*. Limitations as to the appropriateness and effectiveness of these vaccines has prompted renewed efforts to not only identify the specific virulence and immunizing factors of the tubercle bacillus, but also to develop a more effective immunizing procedure. One of the more promising investigations is the study of Youmans and Youmans (4-13) in which particulate fraction, large, enzymatically active, labile particles recovered by 144,000 X G centrifugation of mycobacterial homogenates, or the ribosomes in adjuvant confer on mice significant immunity to tuberculous disease as measured by increased survival time when compared with untreated normal mice. Evidence also is suggestive that ribosomal RNA is the specific immunizing agent, and that ribosomes may function as specific virulence factors of the tubercle bacillus.

In the investigations reported here, the rabbit was employed as the test animal since its size offers obvious advantages over those of the mouse for serial bleedings and prolonged hematologic and serologic studies. In addition, this species is better suited for repetitive recovery of peritoneal macrophage for examining the various phenomena associated with acquired cellular immunity.

Particulate fraction (hereafter termed microsomal fraction) was extracted from BCG by the method of Youmans and Youmans (8,9,11)
and rabbits immunized with these preparations were tested for:
(1) both acquired cellular immunity and resistance to challenge infec-
tion; hematologic changes accompanying immunization; (3) production of
antibodies to RNA and microsomal fraction; and (4) development of
hypersensitivity—cutaneous and cellular.
LITERATURE REVIEW

Many questions regarding tuberculosis infection and immunity remain unanswered despite decades of intensive research and voluminous literature. However, numerous studies have endowed these topics with sound principles to serve for continued progress.

Many studies stem from the observation of Koch (14) who clearly demonstrated that tuberculous guinea pigs acquired resistance to reinfection. Subsequent to this classic demonstration (Koch phenomenon), numerous observations have amply confirmed the validity of this principle in small animals, such as guinea pigs, mice, and rabbits (1). Doubts lingered as to whether or not humans acquired resistance in a similar manner until the convincing report of Dahlstrom and Difs (15) established that BCG conferred a significant level of resistance to naturally acquired tuberculosis in a group of Swedish soldiers. These results were confirmed and the study extended. The British Medical Research Council (16) issued a report in which it was shown that BCG vaccination not only increases resistance to primary tuberculous infection, but also to secondary or tuberculosis of reinfection.

Humoral Factors in Immunity

In attempts to identify factors responsible for observed immunity to tuberculosis, early investigators logically attempted to establish an antibody-microbial relationship such as that typically associated with many bacterial diseases (1).
Various tests performed on sera from immunized humans and animals have included procedures of complement fixation, agglutination, agar diffusion, hemagglutination, hemolysis, Coomb's tests, and others where applicable. All efforts were directed toward the identification of antibodies specific for either surface bacillary antigens or isolatable components of bacilli (1). A measure of success was achieved in that antibodies were detected with specificities to a variety of bacillary proteins and polysaccharides. Despite the findings that immune sera fix complement in the presence of tubercle bacilli; precipitate with tuberculo-proteins and polysaccharides; agglutinate bacilli in vitro; and promote phagocytosis, none of these antibodies, even in the presence of complement, exerts a bactericidal or lytic effect on tubercle bacilli. Furthermore, although antibody promotes phagocytosis, tubercle bacilli continue to multiply within the phagocytes as rapidly as if antibody was absent (17). Thus, granting the possibility of an antibody involvement for which sufficient techniques for demonstration are lacking, there is no serologic evidence to support antibody involvement in acquired resistance to tuberculosis.

Additional evidence against a role for humoral factors is provided from experiments employing semipermeable capsules, as reported by Raffel (18). Measured numbers of tubercle bacilli were placed in these capsules which were impermeable to bacilli but not to plasma proteins. They, in turn, were implanted in the peritoneal cavities of immune and normal guinea pigs. At intervals up to three months, these capsules were removed and the bacilli were counted. No
difference was noted between the rate of multiplication of the bacilli subjected to immune humoral factors and those subjected to normal humoral factors.

More critical yet are the studies in which it was found impossible to passively transfer acquired resistance with serum from immune animals (19). Even when transfused sera comprised about 40% of the plasma volume of the recipient, no beneficial effects were demonstrable.

Finally, there is additional circumstantial evidence that antibody plays no role in immunity to tuberculosis since Zinneman and Hall (20) reported that hypogammaglobulinemic individuals rarely are infected with tuberculosis, and in these infrequent instances, the disease is usually self-limiting and easily manageable. Raffel et al. (21) found that guinea pigs subjected to whole body irradiation prior to vaccination with BCG, exhibited suppressed antibody formation without diminishing the acquisition of resistance to challenge infection.

In view of this evidence, it is still interesting to note the paradoxical situation acknowledged by Middlebrook (17). He recalled that Dienes and Schoenheit (22) discovered that infection with tubercle bacilli enhances host antibody response non-specifically. Subsequently, Freund (23) revealed that the incorporation of antigens in paraffin oil with non-viable tubercle bacilli enhanced the antigenicity of the antigens. Thus the paradox exists in which an organism markedly enhances humoral antibody production, but doesn't elicit a protective
humoral response against the disease for which it is responsible.

**Acquired Cellular Immunity**

With the failure to demonstrate a humoral mechanism, it becomes necessary to examine the cellular aspects of acquired resistance to tuberculosis.

Observation of the pathogenesis of tuberculous lesions led Lurie (24) in 1933 to the conclusion that the macrophage plays an essential role in resistance to tuberculosis. His subsequent ingenious experimentation provided the first classical evidence of the role of the macrophage. Macrophages from normal and immune rabbits were permitted to engulf equal numbers of tubercle bacilli *in vitro*, and these subsequently were injected into the anterior chambers of the left and right eye, respectively, of normal rabbits. After ten to twenty days' incubation, the numbers of bacilli recovered from each eye were compared. It was discovered that in the eyes injected with normal cells, the bacilli had proliferated, whereas their numbers were diminished in the eyes implanted with immune cells (25). It was thus concluded that a quantitative difference exists in the abilities of immune and normal macrophages to cope with tuberculous infection.

Lurie's original observations were confirmed *in vitro* in tissue culture studies by Suter (26) who found that peritoneal macrophages from BCG immunized rabbits and guinea pigs inhibited intracellular multiplication of tubercle bacilli, while the bacilli proliferated in normal macrophages. With the exception of a report by Mackaness (27), Suter's investigations have been successfully repeated and extended by numerous workers. Mackaness found no
difference in the intracellular multiplication of tubercle bacilli in normal and in immune macrophages, but his results were questioned by Suter and Hulliger (28) on the basis that the infective dose in Mackaness's experiments was 10 times that employed by Suter, and the overwhelming infection may have prevented any expression of difference between the normal and the immune cells.

Among those whose data concur with Suter's view are Abe (29) and Berthrong and Hamilton (30). The latter employed a plasma clot technique to compare responses of normal and immune guinea pig macrophages to challenge infection. If the multiplicity of infection was high, no difference in the inhibitory effect of the cells was found but if the initial infective dose was low, immune cells displayed a remarkable ability to inhibit intracellular bacilli as compared with normal cells.

Similar reports have confirmed the essential role of the macrophage in acquired resistance to all of the facultative intracellular parasites. Specific examples and details of procedure are extensively reviewed by Elberg (31), Lurie (32), Suter and Ramsier (2) and Mackaness and Blanden (33). It is not intended here to cite all cases, but rather to consider those which are pertinent to a general understanding of acquired cellular immunity and are applicable as well, to a consideration of tuberculosis immunity.

From the accumulated efforts of many investigators regarding acquired cellular immunity, it is possible to draw some general conclusions. Among the more notable features is the lack of specificity. The macrophages from an animal immunized against one of the
facultative intracellular parasites develop cross-resistance to others of this group (34). In another sense, however, the reaction is specific in that convalescent animals gradually lose their cellular immunity, but it returns at an accelerated rate following reinfection with the homologous organism. This accelerated recall cannot be elicited with heterologous organisms. In the latter instance, the immunity develops at a normal rate (35).

Other cogent aspects relevant to this form of immunity have been summarized by Mackaness (34). Without exception, facultative intracellular parasites induce specific delayed-type hypersensitivity. This reactivity cannot be transferred with serum. The induction of both the immune response and the hypersensitive response requires the use of living vaccines. It is important to note here that the tubercle bacillus provides an exception to the last statement. Weiss and Dubos (36) have reported that non-viable tubercle bacilli are active as immunizing agents, and Rich (37) has demonstrated their ability to produce delayed type hypersensitivity. Mackaness (34) points to the latter as a fact significant to the ability of non-viable tubercle bacilli to function as adjuvants in the induction of delayed hypersensitivity.

The Nature of the Immune Macrophage

In view of the essential role of macrophages in the acquisition of resistance to chronic microbial diseases, the question arises as to what unique differences exist between immune and normal macrophages? It is well documented that for each of the facultative intracellular parasites, immunization is accompanied by an increased microbicidal
capacity in host macrophages (2). Stähelin et al. (38) found that peritoneal macrophages of guinea pigs infected with tubercle bacilli exhibit increased respiratory activity, as measured by increased CO₂ production and O₂ consumption. Correspondingly, increased synthesis of DNA in macrophages exposed to microbial antigens in vivo has been noted, and it is suggested that similar effects occur in vivo (39).

The mitotic rate of macrophages has been observed to increase dramatically following brucella and listeria infections of mice (40). These increased metabolic activities are accompanied by a concomitant increase in the enzymic content of the macrophages. Suter and Hulliger (28), Lurie (32) and Saito and Suter (41) all noted striking increases in the content of acid hydrolases in monocytes following tuberculous infection, and their findings are supported by those of North and Mackaness (42) in which immune macrophages were observed to have an increased number of lysosomes. Furthermore, it is interesting that the peritoneal macrophages of inbred rabbits with high native resistance to tuberculosis have increased metabolic activity in a variety of tests in comparison with macrophages of susceptible rabbits (43). Findings of increased enzymatic activity, associated with both immunization and genetic factors make it tempting to relate cellular immunity to the enzymatic activity of the cells, although there is no proof as yet that this activity is responsible for intracellular bacterial inactivation (34).

Following the demonstration that immune histiocytes contain a greater content of lysozyme and glycine, it was suggested that the glycine renders the bacterial cell walls susceptible to enzymatic
degradation and that the specific enzyme responsible for bacterial inactivation was lysozyme-like (31). This suggestion is not supported by the experiments of Weiser et al. (44), who found that lysozyme has little if any, effect on the in vivo growth of mycobacteraiae and brucellae. More recently however, it has been reported that although lysozyme is ineffective against tubercle bacilli alone, it does induce spheroplast formation of the bacilli when tested in combination with phospholipase C (45). This combined effect may play an important role in the limitation of intracellular growth of tubercle bacilli.

Studies of experimental salmonellosis by two groups of Japanese workers have suggested an additional property of peritoneal monocytes of immunized mice. In earlier papers (46,47) it was reported that mice hyperimmunized with viable but non-virulent Sal. enteritidis acquired resistance to fatal infection with virulent Sal. enteritidis. Additionally, the mouse monocytes cultivated in tissue culture resisted degeneration as initiated by challenge infection, and were inhibitory to intracellular bacterial multiplication. This immunity could not be transferred to normal mice with immune sera. In contrast, Turner et al. (48) found it possible to passively transfer immunity to experimental salmonellosis with immune serum. This latter finding again suggests that a humoral mechanism obviously is involved in acquired resistance to salmonellosis. In refutation, Kurashige et al. (49) reported that the Australian workers Rowley, Turner, and Jenkin have obtained recent unpublished data which concurs with that of the Japanese groups; thus again obviating the need for further consideration of humoral mechanisms here. Subsequent to their earlier reports, Kurashige et al. (50) and
Mitsuhashi et al. (51), using the techniques of immune transfer and immune adherence hemagglutination, were able to detect cell-bound antibody in the monocytes of mice hyperimmunized with viable *Sal. enteritidis*. More recently, Kurashige et al. (49) examined the extracted cellular antibody and found that in the presence of complement and lysozyme, it inhibited the growth of *Sal. enteritidis* on agar plates. By sucrose density centrifugation the cellular antibody was characterized as a 19S macroglobulin, in contrast to the established cytophilic antibody which is 7S globulin with a high affinity for cells (52). These studies were extended to include an investigation as to the origin of the cellular antibody (53). Homogeneous populations of normal mouse peritoneal monocytes were immunized in tissue culture with immune RNA from monocytes of mice immunized with *Sal. enteritidis*. Cellular antibody was detected in the homogenous monocyte population, suggesting that the monocytes, themselves, constitute a cell line responsible for antibody production. Should this phenomenon be re-affirmed by the studies of others, it may find widespread significance with respect to understanding immune responses to parasitism by many, if not all, of the intracellular parasites.

**Delayed Hypersensitivity and Specificity of the Immune Response**

Investigators have long been impressed by the apparent parallel which exists between resistance to tuberculosis and the extent of delayed-type hypersensitivity to tuberculoprotein (34). However, early considerations of tuberculin hypersensitivity as an important immune mechanism (54) became untenable when confronted with reports that
animals could be desensitized to tuberculin without a corresponding reduction in the level of immunity which, in fact, may even increase (55). Guinea pigs can be made hypersensitive with tuberculin-protein plus lipopolysaccharide without an accompanying increase in resistance (56) and, moreover, may be immunized with sub-cellular fractions of tubercle bacilli without developing cutaneous hypersensitivity (57). Thus, hypersensitivity and immunity were considered separate, but parallel events.

The recent studies of Mackaness et al. (34) have renewed interest in the possible role of delayed hypersensitivity in the development of cellular resistance.

The accelerated recall of acquired cellular immunity following re-infection with homologous organisms is reminiscent of a secondary antibody response, but this explanation is inconsistent with the non-specific nature of immunity following re-infection. For example, a BCG sensitized animal re-injected with BCG demonstrates accelerated resistance not only against BCG, but also against L. monocytogenes, B. abortus, and Sal. typhimurium (58). Resistance also can be augmented by re-injection of non-viable, antigentically related organisms, although these non-viable organisms alone are incapable of immunizing just as they are incapable of inducing delayed hypersensitivity. Further, it was demonstrated that the rate of inactivation of L. monocytogenes by mice infected with Sal. typhimurium was proportional to the number of Sal. typhimurium organisms present in the mice at the time of infection with L. monocytogenes (59). These observations led to the conclusion that acquired cellular resistance
requires specific sensitization of tissues by viable agents and sustained antigenic stimulation by either viable or non-viable organisms.

The transfer of specific delayed hypersensitivity to a non-sensitive recipient with lymphoid cells of a sensitive donor is well documented (60). It follows then, that if delayed hypersensitivity is involved in cellular immunity, resistance should be transferable to a normal recipient with immune lymphoid cells (58). When splenic lymphoid cells (free of macrophages) from BCG immunized mice were transferred to normal mice, the recipients became immune to BCG infection, but not to \textit{L. monocytogenes}. Thus, the transferred immunity was immunologically specific. If, however, an eliciting dose of BCG was intravenously injected along with BCG immune splenic lymphoid cells, the recipients became resistant to infection with \textit{L. monocytogenes} (58). This provides additional evidence that resistance to facultative intracellular parasites is a non-specific result of a specific immunologic reaction, dependent on specifically sensitized cells and sustained antigenic stimulation (58).

Mackaness (58) speculated that the relationship of delayed hypersensitivity to immune macrophages may be explained by the observations of David (61) and Bloom and Bennett (62) who reported that an interaction between sensitive lymphocytes and antigen (PPD) results in the production of a protein which inhibits the migration of macrophages in a tissue culture system. This protein is released in the supernate following the reaction, and it is likely that it would be released to the circulation following an \textit{in vivo} reaction. A mechanism of this kind might explain the acquisition of immunity.
by peritoneal macrophages which are protected from interaction with intravenously injected organisms.

Evidence against a hypersensitive involvement in the immune mechanism has been critiqued by Mackaness (34) as follows. Desensitization to cutaneous hypersensitivity may only temporarily remove from circulation those cells on which cutaneous hypersensitivity depends without affecting sensitivity at the cellular level. That skin sensitivity does not always reflect the true level of sensitivity is proven by the fact that skin desensitized rats and guinea pigs are fatally shocked by intravenous injection of tuberculin. The protection of animals by supposedly non-sensitizing extracts was explained on the basis that these extracts probably elicited a level of hypersensitivity which exists at the cellular level, but is below the threshold necessary for cutaneous demonstration.

Transfer of Immunity and Induction of the Immune Response

Many of the early studies of transfer phenomena employed cell populations which were impure, or mixtures of lymphocytes and macrophages. It is apparent that the degree of resistance achieved in cell transfer experiments varies considerably, but is influenced by the cell type, number of cells, route of injection, number of injections, and the interval between transfer of cells and challenge infection of the recipients (2).

Fong et al. (63) found that macrophages were more efficient than lymphocytes in the transfer of immunity, i.e., macrophage transferred immunity persisted for 40 days as compared to 20 days for
lymphocyte transfer and furthermore equal resistance was achieved with fewer macrophages. Macrophage lysates were effective for transfer while lymphocyte lysates were not. In later experiments the transfer factor from macrophage lysates was traced to ribosomal RNA (64).

In vitro transfer of cellular immunity was reported by Mitsuhashi and Saito (65) who found that tissue culture supernate of macrophages from mice immunized with viable *Sal. enteritidis* contained a transfer factor which confers immunity on normal macrophages after three days' incubation in tissue culture. This transfer agent was also identified as ribosomal RNA (66).

The molecular intricacies whereby this transfer agent is capable of inducing immunity in normal cells are as yet not understood. Fong (64) suggested that immunization may activate a mechanism which originally received information from the microorganism's genetic code, but which is capable of self-replication after activation. This is similar to the modification of protein in *Drosophila melanogaster* in the absence of genetic material responsible for forming protein which prompted Fox et al. (67) to suggest the existence of an enzymatic system for the synthesis of RNA with preformed RNA as a primor.

This suggestion aids in explaining the results of Youmans and Youmans (10) who successfully improved the resistance of mice to tuberculous infection by injecting particulate fraction of H37Ra or ribosomes from this organism in adjuvant. The specific immunity factor subsequently was identified as ribosomal RNA (12). The results obtained by Venneman (68) in mice immunized against salmonellosis with ribosomes from *Sal. typhimurium* essentially concur with those of
Younans and Youmans. Thus, it is clearly established that bacterial RNA primers can initiate an immune response to facultative intracellular parasites in experimental animals. Of importance is the need for the presence of either membranous material or adjuvant to achieve immunization with bacterial ribosomes. This is not inconsistent with the view of Mackaness (34) who pointed up the role of hypersensitivity in cellular immunity, since it might be possible to achieve cellular hypersensitivity without the intact organism as long as the ribosomal RNA is provided the host in an acceptable state.
MATERIALS AND METHODS

Test Organisms

BCG (Tice vaccine strain) and *Mycobacterium tuberculosis* var. bovis (Trudeau 4228) were the organisms employed throughout the course of this investigation. These organisms were routinely cultivated on Lowenstein's medium and sub-cultured in Dubos Medium Albumin with or without Tween 80 as warranted by conditions of experimentation.

Experimental Animals

Young, adult, New Zealand white rabbits weighing 2.5 to 3.0 kilograms were immunized with either BCG or BCG microsomal fraction, or they served as non-immunized controls.

Prior to immunization procedures, the animals were bled by cardiac puncture to obtain control sera. The blood was allowed to clot at 37°C for 1 hour, and placed at 4°C overnight. The sera were recovered and stored at −20°C until needed.

BCG Immunization

Rabbits were injected with three intravenous (marginal ear vein) inoculations of 1.0 mg. of BCG each over the period of 1 week for a total of 3.0 mg. The organisms cultivated on Lowenstein's medium for 7 days, were harvested in weighing bottles. Sufficient unbuffered saline (0.85%) was added to effect the desired
concentration of 2.0 mg. per ml., and 0.5 ml. of this suspension was used for each injection.

Preparation of BCG Microsomal Fraction

Test rabbits were immunized with BCG microsomal fraction which was prepared by slight modification of the procedures of Youmans and Youmans (8,9,11) as follows. Batch cultures of BCG were grown for 2 weeks in Dubos Medium Albumin, without Tween 80 and the bacillary masses were collected on Millipore HA filters. The mass was washed twice with 0.1 M phosphate buffer, pH 7.0 and resuspended in a buffer of 0.44 M sucrose and 3 × 10⁻² M MgCl₂ in 2 × 10⁻₄ M phosphate buffer, pH 7.0. This suspension was blended for 3 minutes in a high speed, ice jacketed, micro-monel Waring Blender, after which the homogenous suspension was poured into a cold French Pressure Cell and subjected to a pressure of 15,000 to 17,000 psi applied with a Carver Laboratory Press. While maintaining constant pressure, the outlet nozzle of the French Press was opened, and the material was allowed to slowly leak out. This ruptured cell material was collected in another cold Waring Blender and blended for another 30 seconds at high speed. The material was then centrifuged at 15,000 rpm (27,000 × g) for 15 minutes in the SS-34 head of a Sorvall RC2B Refrigerated Centrifuge, and the supernate was carefully decanted and saved for a second centrifugation at 19,5000 rpm (46,000 × g). This final supernate, free of whole cells, cell walls, and debris then was centrifuged for 2 hours at 40,000 rpm (144,000 × g) in a number 40 head in a Spinco Model-L Ultracentrifuge. The supernate was discarded and the translucent, yellowish pellets were
considered to be microsomal fraction. The tubes and surfaces of the pellets were gently washed with a final diluent of $10^{-4}$M MgCl$_2$ in 0.01 M phosphate buffer, pH 7.0, and the washed tubes and pellets, drained free of excess moisture were weighed together. Then with the aid of a teflon stirring rod, the pellets were resuspended in the final diluent and collected in a vaccine bottle. The tubes were re-weighed and the difference determined the wet weight of BCG microsomal fraction. The microsomal fraction was diluted with the final diluent to a concentration of 200 mg. per ml., and 1.0 ml. of this suspension was immediately used to inoculate intramuscularly each rabbit (0.5 ml. in each hind leg). A small portion of each batch of microsomal fraction was saved for analysis.

Analysis of BCG Microsomal Fraction (MF)

Dry weights of the fractions were determined by spotting 0.1 ml. on planchets, weighing the planchets, drying in a hot air oven for 2 hours, and then re-weighing.

Protein determinations were accomplished with the Folin-phenol reagent by the method of Lowry et al. (69).

Ultraviolet absorption spectra were prepared by scanning diluted samples from 200 to 350 m$\mu$ in a Bausch and Lomb Spectronic 505 Recording Spectrophotometer.

Finally, samples were visually characterized by electron microscopy. Samples were spotted on carbon coated copper grids, allowed to dry and then shadow-cast with carbon-platinum in a Kinney Model DGB3 High Vacuum Evaporator. The shadowed specimens
were examined in a JEM Model 5-Y electron microscope, and the images were recorded on electron micrographs.

**Determination of Acquired Cellular Immunity**

After allowing at least 3 weeks post-immunization for the acquisition of resistance, the peritoneal macrophages of immunized rabbits were tested *in vitro* for cellular immunity as determined by their ability to resist destruction by a challenge infection with fully virulent *M. bovis* in comparison with cells from normal animals. The following methodology was severely modified from that of Fong *et al.* (70).

Rabbits were anesthetized with ether, and the resident population of peritoneal macrophages was recovered with Hank's Balanced Salt Solution (BSS). One hundred ml. of BSS, containing 2 units of heparin per ml., was injected into the peritoneal cavity at the mid-line. The rabbit's abdomen was then firmly massaged for 10 minutes to effect suspension of the cells. The fluid was removed from the lower left quadrant of the peritoneal cavity with a syringe and multi-perforated 18 ga. needle, and the cell suspension was transferred to screw-capped tubes and centrifuged at 200 X G for 3 minutes in an International Clinical Centrifuge. The supernate was discarded and the cells were washed once in a maintenance medium consisting of BSS supplemented with 40% normal rabbit serum. The cells were resuspended in 3 ml. of the medium and, transferred to a 3 oz. prescription bottle. Then the bottle was placed in a 37°C incubator for 1 hour to allow the macrophages to adhere to the glass surface, the medium was poured off
and the cell mass gently washed with 3 ml. of fresh medium to remove non-adhering cells, principally lymphocytes and erythrocytes. Three ml. of maintenance medium, additionally supplemented with 40 units of penicillin and 1 µg of ferric nitrate per ml., was added to the bottle and the macrophages were resuspended with a rubber policeman. After being counted in a hematocytometer, the cells were diluted to approximately $6 \times 10^5$ per ml. and the suspension was divided into two equal portions and placed in separate 3 oz. prescription bottles—one to be challenged with M. bovis and the other to remain as an uninfected control.

The challenge organism, M. bovis, was cultivated for 7 days in screw capped tubes of Dubos broth medium with Tween 80 at 37°C. The tubes were centrifuged for 1 minute at 125 X G to sediment clumps of bacilli and the resultant supernate contained a suspension of dispersed single bacilli. The bacilli were counted microscopically in a Petroff-Hauser Chamber and diluted with fully supplemented maintenance medium to contain approximately $4 \times 10^6$ bacilli per ml.

One portion of each macrophage suspension was challenged with a ratio of 5 bacilli per macrophage and incubated for 2 hours at 37°C to permit engulfment of bacilli. The macrophages were resuspended with a rubber policeman and washed once with the maintenance medium and then resuspended in 4 ml. of the medium containing 9 µg of streptomycin per ml. to inhibit extracellular multiplication of bacilli. As a result of the above procedures, 4 macrophage suspensions were prepared for each testing period, i.e., (1) normal macrophages,
(2) normal macrophages plus *M. bovis*, (3) immune macrophages and (4) immune macrophages plus *M. bovis*.

Each of the 4 suspensions was injected into three previously assembled Sykes-Moore Tissue Culture Chambers, and incubated for 1 hour at 37°C to allow the cells to adhere to the bottom coverslips. The chambers then were inverted and the number of macrophages in each of 10 consecutive fields was counted with a 40X objective on a Unitron phase contrast microscope. By marking the chambers and chamber holder, and noting the vernier stage settings on the microscope, approximately the same 10 fields could be counted on subsequent days. Cells were counted daily for 4 days, and for each macrophage preparation, the counts were averaged from the 2 chambers in closest agreement. The medium was renewed daily via the injection ports of the chambers.

The percentage of cells lost due to infection was determined in the following manner. Each day's count was subtracted from the previous day's count to determine the number and percentage of cells lost. Then for both normal and immune macrophages, the percentage of uninfected cells lost was subtracted from the percentage of infected cells lost to determine the loss attributable strictly to infection.

At different intervals during trial cellular immunity experiments, selected cover slips were removed from the chambers, washed carefully in BSS, air dried briefly, fixed in 95% ethanol for 10 minutes and air dried again. These preparations were stained with Kinyoun's carbol fuchsin for 5 minutes, washed in water, stained for 2 minutes in buffered Wright's stain, washed, and air dried. Permanent specimens
were obtained by mounting with a drop of piccolyte mounting medium on glass slides.

In Vivo Test of Immunity

BCG immunized, MF immunized and normal rabbits were intravenously infected with 0.2 mg. of *M. bovis*. The organism was harvested from 14 day cultures on Lowenstein's medium and suspended for injection as described previously for immunization with BCG.

The animals were weighed prior to injection and at weekly intervals thereafter. On the 26th day post-infection, all animals were sacrificed by intracardial injection of 5 ml. of a saturated magnesium sulfate solution. Lungs, livers, spleens, and kidneys were removed to formalin solution for 48 hours and then examined for gross evidences of pathology. All of the organs were examined, but particularly the lungs and spleens where the number of tubercles per square cm. of surface area was counted.

Hematology

Selected MF immunized rabbits were bled at weekly intervals for 6 to 10 weeks post-immunization. Blood was collected in vacutainer tubes with EDTA anticoagulent on alternate weeks by cardiac puncture, or by pricking the marginal ear vein with a hemolet.

The blood values determined included hematocrit, hemoglobin, total red and white cell counts, and differential counts.

Serology

One pair of MF immunized animals was bled at bi-weekly intervals by cardiac puncture, and a second pair was bled at weekly intervals
post-immunization. The recovered sera were examined for antibodies to soluble RNA and MF by bis-diazotized benzidine (BDB) indirect hemagglutination as described by Bigley et al. (71).

BDB was prepared according to the method of Gordon et al. (72). To 0.23 g. of benzidine dissolved in 45.0 ml. of 0.2 N HCl, was added 0.175 g. of NaNO₂ contained in 5.0 ml. of distilled water at 0°C. The reaction was allowed to proceed for 30 minutes with intermittent stirring, after which 0.7 ml. aliquots of solution were placed in agglutination tubes, quick frozen at -70°C in a dry ice-acetone bath, and stored at -20°C until needed.

The antigens were prepared by coupling sRNA or MF with BDB to fresh human group O Rh positive red cells and was accomplished as follows: 0.1 ml. of washed packed red cells was added to 10.0 ml. of phosphate buffer (pH 7.0) containing 1000 μg. of sRNA or 1000 μg. of MF. Five-tenths ml. of 1:16 BDB was added, and the reaction was allowed to proceed for 10 minutes at room temperature with frequent shaking. The mixture was immediately centrifuged, and the supernate was discarded. After one washing with 1:100 rabbit serum buffer (1.0 ml. normal serum + 99.0 ml. phosphate buffer pH 7.3, 0.15 M Na₂HPO₄ - 0.15 M KH₂PO₄), the red cells were prepared as a 2% suspension in the rabbit serum buffer.

For the test procedure, serum samples in 0.7 ml. amounts were inactivated at 56°C for 30 minutes, diluted 1:2 with buffer, and absorbed with 0.4 ml. of washed packed red cells for 30 minutes at 37°C. Serial dilutions in 0.5 ml. amounts were prepared in duplicate (one for RNA; one for MF) for each serum sample with phosphate buffer
as the diluent. One-tenth ml. of the appropriate antigen suspension was added to each tube for the serial dilutions, and the tubes were incubated for 30 minutes at 37°C. After centrifugation for 30 seconds in an Adams Sero-Fuge, the tubes were examined for evidence of agglutination of the antigen-coupled red cells.

Specificities of the reactions were examined by enzymatically treating the antigens prior to coupling to red cells. RNAase (crystalline; 5X) from Nutritional Biochemicals Corporation and trypsin 1:250 from Difco Laboratories were the enzymes employed. RNAase was utilized in a 1:10 ratio of enzyme to substrate and the reaction proceeded overnight at 37°C. Trypsin was employed in a 1:5 ratio and the reaction was allowed to proceed for 1 hour at 37°C. Untreated antigens were subjected to identical conditions of incubation and served as controls in parallel tests.

Delayed Hypersensitivity

Immediately prior to immunization, and 4 weeks post-immunization, rabbits were routinely tested for delayed cutaneous hypersensitivity. The eliciting dose was 0.1 ml. of 2nd Test Strength Purified Protein Derivative, (P.P.D.) administered intradermally on the rabbit's flank.

Inhibition of Macrophage Migration

This test of cellular delayed hypersensitivity was patterned after the method of David et al. (73) and of Thor (74).

Rabbits were stimulated intraperitoneally with 25 ml. of light mineral oil. Four days later the peritoneal exudate cells were recovered as previously described in the methodology of testing for
acquired cellular immunity. The recovered fluid was placed in screw capped tubes and placed in an ice bath for 10 minutes. Residual oil floated to the top and was aspirated with a sterile capillary pipette. The cells were pelleted by centrifugation at 1,200 rpm for ten minutes in an International Clinical Centrifuge. The pellets were washed twice at 900 rpm for 5 minutes in BSS containing 40% rabbit serum and were finally made up to a 10% suspension in the fortified BSS. The cell suspension was introduced into a sterile capillary tube, one end of which was sealed by plunging it into a petri dish containing melted and cooled vaspar. The capillary tube was placed in a screw capped tube with a cotton plug at the bottom and centrifuged at 900 rpm for 5 minutes to pellet the cells. The capillary tube then was broken at the cell-fluid interface (a smooth break was effected by pre-scoring with a rasp). The portion with cells was attached to the bottom cover-slip of a Sykes-Moore Tissue Culture Chamber with a drop of melted vaspar and the chamber was assembled and filled by injecting either fortified BSS or fortified BSS containing 25 μg per ml. of P.P.D. The chamber was incubated at 37°C and examined at 24 and 48 hours by placing it on a Nikon Profile Projector and tracing the migration image on the ground glass screen. The area of migration was carefully cut out of the vellum tracing paper and the tracing was weighed on an analytical balance.

Migrations were prepared in triplicate in both the presence and absence of P.P.D. For each condition, the areas of migration in closest agreement were averaged. The percentage of migration in the
presence of antigen is defined by Thor (74) as the migration index (MI) which was determined as follows:

\[
\frac{\text{average area of migration with antigen}}{\text{average area of migration without antigen}} \times 100 = MI
\]

where the area of migration is proportional to the weight of its tracing.
EXPERIMENTAL RESULTS

Preparation and Analysis of BCG Microsomal Fraction

In order to conduct studies of rabbits immunized with BCG microsomal fraction (MF), it was necessary to develop procedures whereby large amounts of the fraction, of good quality, could be recovered.

After growing the BCG in batch cultures in Dubos Medium Albumin for 2 weeks, it was possible to recover about 1 gram (wet wgt.) of cells per 400 ml. of medium. If the cultures were incubated for an additional week, the yield of cells increased substantially, but was not accompanied by proportional increase in recoverable MF. Accordingly, two weeks was selected as the time of incubation. It was routinely possible to recover 50 mg. (wet wgt.) of MF per gram of ruptured cells.

Dry weights of the samples were 1/6 to 1/7 of the values for wet weights, and the Lowery protein test on a fresh sample revealed that 53% of the dry weight of the sample was protein. From the ultraviolet spectrum depicted in Figure 1, the 280:260 absorbance ratio was calculated as 0.55 which corresponds to 40% nucleic acid (RNA in this instance) on a standard curve comparing the per cent nucleic acid to the 280:260 ratio. The remaining 7% of dry weight was unaccounted for, but may be attributable to other substances such as lipid in the membrane-like material associated with the fraction, or to experimental
Figure 1. Absorption Spectrum for BCG Microsomal Fraction.
error. The spectrum shows strong absorption for nucleic acid at 260 mμ and is followed by a steep return toward the baseline, indicating that the preparation was relatively free of contaminating material. Slight displacement of the nucleic acid peak from 260 mμ is suggestive of some RNA degradation, probably due to one freezing and thawing of the sample prior to examination. With samples that had been repeatedly frozen and thawed, the nucleic acid peak was completely flattened.

After subjecting BCG cellular masses to 15,000 to 17,000 psi in a French Pressure Cell, acid-fast stains of the recovered material revealed greater than 90% rupture of the cells. As was anticipated, no acid-fast organisms or other recognizable forms were observed in stained samples of the final MF preparations.

Results of electron microscopic examination further attested to the purity of the preparations, since no whole cells, cell walls, membranes, or other contaminants were observed in scans of several shadowed specimens. The fraction, as it appears in Figure 2, consists of large particles, randomly arrayed, but often an obvious thread of continuity appears to link adjacent particles in some areas. The individual large particles have an average diameter of 257 mμ, and their particulate appearance suggests dense aggregates of ribosomes. The small sub-units within the large particles are of the appropriate size for bacterial ribosomes, averaging 184 angstroms in diameter.

**Acquired Cellular Immunity**

Early in the course of this investigation, efforts were directed toward establishment of a tissue culture test system employing
Figure 2. Low power electron micrograph of carbon-platinum shadowed BCG microsomal fraction. X 17,000
macrophages from BCG immunized rabbits. The relative resistance of BCG immune macrophages and normal macrophages to BCG infection was measured by numerical change in the organisms observed intracellularly in stained specimens taken at daily intervals. In this manner, it was possible to observe intracellular multiplication of bacilli, but differentiation of the abilities of immune and normal macrophages to cope with the intracellular bacilli was, at best, inconsistent. A tissue culture system prepared by the cover-slip method of Mabry (75) was supplanted by a system employing Leighton tubes, but neither showed to advantage in differentiating responses of the two cell types to BCG challenge. However the cultures prepared in loose capped Leighton tubes incubated in a 5% CO\textsubscript{2} in air atmosphere proved to be the better of the two methods for maintaining a healthy population of macrophages for a longer period.

The system which finally proved successful was one in which cellular immunity was determined by the ability of cells to resist destruction by infecting organisms, rather than by the unreliable method of determining their ability to inhibit intracellular multiplication (to be discussed later).

Peritoneal macrophage populations from rabbits were divided into two equal aliquots, one of which was challenged with fully virulent \textit{M. bovis} in a 5:1 ratio of bacilli to macrophages. The infected macrophages were injected into Sykes-Moore Chambers where they were counted to determine the numbers of macrophages degenerated at 24, 48, and 72 hours. Degeneration was determined by the loss of macrophages from the bottom cover-slip. This invariably was preceded
by vacuolization of the cells, loss of refractility, and the appearance of "ghost" forms.

Data from two experiments with MF immunized rabbits is presented in Table 1, where degeneration of macrophages is represented by the average percentage of cells lost from duplicate cultures of each macrophage type. The percentage of cells lost to infection at each interval was calculated by subtracting the percentage of uninfected cells lost from the percentage of infected cells lost, and these results are plotted in Figures 3 and 4. The reader is referred to the appropriate section in the Materials and Methods for the details of calculation, but it is noteworthy that approximately 800-1000 macrophages were initially counted for each cell type.

In the first experiment, marked degeneration of infected normal cells had already occurred at the 24 hour interval and maximum differentiation between infected and uninfected cells was noted at that time for both immune and normal systems. By 48 hours, all of the cell populations had undergone extensive degeneration, and at 72 hours degeneration of the infected and uninfected cells from each animal were nearly equal. At no time during the period of observation in the second experiment was degeneration as great as noted in the first. Maximum differentiation between infected and uninfected cells occurred at 48 hours, and as in the first case, this was reflected in a fourfold difference between the percentages of infected and immune cells lost to infection. Thus it was established that rabbits immunized with MF developed an acquired cellular resistance to the degenerative effects of virulent *M. bovis*; this resistance was demonstrable at
<table>
<thead>
<tr>
<th>Experiment †</th>
<th>Source of Macrophages</th>
<th>Average Per Cent Cells Lost</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 hr</td>
</tr>
<tr>
<td>I</td>
<td>Uninfected Normal</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Infected Normal</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Uninfected MF Immune</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Infected MF Immune</td>
<td>17</td>
</tr>
<tr>
<td>II</td>
<td>Uninfected Normal</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Infected Normal</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Uninfected MF Immune</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Infected MF Immune</td>
<td>12</td>
</tr>
</tbody>
</table>

† In experiment I the immune macrophages were tested 3 weeks post-immunization. In experiment II they were tested 3 months post-immunization.
Figure 3. Acquired Cellular Immunity in an MF Immunized Rabbit 3 Weeks Post-Immunization.
Figure 4. Acquired Cellular Immunity in an MF Immunized Rabbit 3 Months Post-Immunization.
3 weeks and 3 months post-immunization; a maximal four-fold differentiation between normal and immune cellular resistance occurred at 24 to 48 hours post-infection; and these effects were noted without the presence of immune serum in the test system, as employed by Fong et al. (70).

In acid fast-Wright's stains of infected macrophages prior to infection into Sykes-Moore Chambers, 18 to 20% of the macrophage were observed to contain intracellular bacilli. Throughout the test period the percentage of infected cells remained within about 5% of the initial figure. After incubation in the presence of streptomycin, macrophage supernates seldom contained any acid-fast bacilli. This suggests that the extensive degeneration of cell populations cannot be completely accounted for by either mechanical rupture by the proliferating intracellular bacilli, or by external bacillary influences.

In observations of stained preparations such as those in Plate I, the bacilli in healthy macrophages were initially enclosed in vesicles (phagosomes) near the periphery of the cells. As the incubation increased, the bacilli were generally observed in closer proximity to cell nuclei. In healthy cells, the intracellular bacilli exhibited uniform acid-fast staining characteristics and were observed to elongate prior to growth. To the contrary, bacilli within damaged cells (those with "rosy" cytoplasm) exhibited polar staining, shortening, and fragmentation.

The phase contrast appearance of infected macrophages in
Plate I. Rabbit peritoneal macrophages after infection with *M. bovis*. X 2,500

1. 0 time
2. 3 days post-infection
3. 5 days post-infection
Sykes-Moore Chambers is shown in Figure 5. Counts of intracellular bacilli as viewed by phase microscopy compared favorably with those of stained preparations, but here too, differentiation of immune and normal macrophages proved unreliable.

In Vivo Test of Immunity

Macrophages from MF immunized animals appeared as efficient as those from BCG immunized animals in resisting cellular destruction in the tissue culture experiments. However, it was decided that a better measure of the effectiveness of the two immunogens would be afforded by a comparison of in vivo responses of immunized rabbits to an infective dose of _M. bovis_. Accordingly, 2 BCG immunized, 4 MF immunized and 4 unimmunized rabbits were infected intravenously with 0.2 mg. of _M. bovis_.

At two weeks post-infection, the unimmunized rabbits had begun to show signs of respiratory distress, and by the 26th day they had deteriorated so rapidly that it was decided to terminate the experiment. Interestingly, neither of the BCG immunized rabbits and only one MF immunized rabbit (#1337) displayed obvious signs of respiratory distress as characterized by wheezing, rales, and a purulent discharge from the nose. Two rabbits, on the terminal date of the experiment are pictured in Plate II. The healthy appearance of the MF immunized rabbit is shown in marked contrast with the deteriorated condition of the unimmunized rabbit. Not evident in the picture was the very significant weight loss of the unimmunized animals.

After sacrificing the rabbits, lungs, spleens, livers and
Figure 5. Infected rabbit peritoneal macrophages. Phase contrast X 4,500

Mb - intracellular M. bovis
Plate II. Experimentally infected rabbits 26 days after intravenous inoculation with *M. bovis*.

1. normal rabbit
2. MF immunized rabbit
kidneys were removed to formalin for 48 hours for subsequent gross post mortem examination. Pertinent data from this experiment are summarized in Table 2. The rabbits are grouped according to their treatment, and the elapsed period post-immunization at the time of infection is given for each animal. Rabbit #1337 died on the 25th day and was discarded because bloating and putrefaction occurred prior to discovery of its death.

The weight losses for immunized animals, excepting #1337, were, in general, considerably less than those exhibited by the normal animals. The appearance of livers and kidneys from the normal rabbits was very inconsistent, and at the recommendation of Hassan Gaafar, M.D., only the lungs and spleens were subjected to critical evaluation. Since the normal control rabbits manifested most consistent disease in these organs, it was felt that only these should properly serve as experimental references.

The lungs of all normal and MF immunized rabbits were studded with yellowish-gray miliary tubercles. These lungs all were markedly distended and when grossly sectioned with a knife revealed that almost all of the lung tissue had been replaced by areas of conglomeration. In contrast, the lungs of BCG immunized rabbit #1320 displayed only a few miliary tubercles, mostly on the left lower lobe which appeared congested. Additionally, one area of conglomeration was noted on the anterior border of the right lower lobe, but in general, there was much more lung tissue remaining in the other two groups of animals. Similarly, the lungs of #1321 were relatively free of disease with
### TABLE 2

EXPRESSION OF TUBERCULOUS DISEASE IN IMMUNIZED AND UNIMMUNIZED RABBITS
CHALLENGED WITH 0.2 mg. M. BOVIS

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Immunogen</th>
<th>Months Post-Immunization</th>
<th>Wgt. Loss</th>
<th>Lung Tubercles/cm²</th>
<th>Spleen Tubercles/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1303</td>
<td>-</td>
<td>-</td>
<td>620 g.</td>
<td>44</td>
<td>26</td>
</tr>
<tr>
<td>1304</td>
<td>-</td>
<td>-</td>
<td>320</td>
<td>31</td>
<td>36</td>
</tr>
<tr>
<td>1306</td>
<td>-</td>
<td>-</td>
<td>970</td>
<td>33</td>
<td>55</td>
</tr>
<tr>
<td>1307</td>
<td>-</td>
<td>-</td>
<td>660</td>
<td>42</td>
<td>72</td>
</tr>
<tr>
<td>1320</td>
<td>3 mg. BCG</td>
<td>19</td>
<td>0</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>1321</td>
<td>3 mg. BCG</td>
<td>16</td>
<td>110</td>
<td>0-1</td>
<td>0</td>
</tr>
<tr>
<td>1325</td>
<td>200 mg. MF</td>
<td>16</td>
<td>110</td>
<td>33</td>
<td>3</td>
</tr>
<tr>
<td>1333</td>
<td>200 mg. MF</td>
<td>4</td>
<td>0</td>
<td>44</td>
<td>22</td>
</tr>
<tr>
<td>1337</td>
<td>200 mg. MF</td>
<td>2</td>
<td>530</td>
<td>Not Done</td>
<td>Not Done</td>
</tr>
<tr>
<td>1342</td>
<td>200 mg. MF</td>
<td>2</td>
<td>330</td>
<td>48</td>
<td>2</td>
</tr>
</tbody>
</table>

*Wet wgt. is approximately 6-7 X dry wgt.*
only 3 tubercles observed at the base of the lower right lobe. All of the infected animals exhibited splenomegaly, but the spleens from the BCG immunized animals were completely free of macroscopic tubercles. Spleens of normal animals and MF immunized rabbit #1333 were studded with miliary tubercles, but the spleens of MF immunized animals were notably more resistant than the normal spleens from the non-immunized control rabbits as reflected in the counts of macroscopic tubercles per square cm. of surface area. Thus, in comparison with normal rabbits, it was demonstrated that MF immunized animals developed relative resistance to tuberculous disease, as determined by overt signs of respiratory distress, weight loss, and the extent of splenic lesions. This resistance, although demonstrable up to 16 months post-immunization, was not nearly as remarkable as that exhibited by the BCG immunized rabbits. Further, the data suggest that splenic macrophages from MF immunized rabbits are more capable of coping with infection than are their alveolar macrophages. (See Discussion).

Examples of lungs and spleens from infected animals are illustrated in Figure 6. A lung and spleen from an uninfected animal are included for comparative purposes.

**Hematology and Serology**

Youmans and Youmans (10) have suggested that the membrane-like material associated with the microsomal fraction of the tubercle bacillus has an adjuvant effect which accounts for the ability of this fraction to immunize equally as well as ribosomes which have been incorporated in Freund's Incomplete Adjuvant. Dodd et al. (76)
Figure 6. Lungs and spleens of rabbits 26 days after intravenous infection with 0.2 mg. *M. bovis*.

1. Uninfected control
2. Infected control
3. BCG Immunized
4. MF Immunized
reported that rabbits immunized with rat liver ribosomes developed autoimmune disease characterized by hemolytic anemia and by the presence of autoantibodies to ribosomes and to RNA. In view of the above, it became important to examine MF immunized rabbits for evidence of autoimmune response as revealed by blood studies and antibody directed to RNA and MF measured by the BDB sensitized erythrocyte technique.

Weekly blood samples from two pairs of MF immunized rabbits were examined for the following values: hematocrit, hemoglobin, total leucocyte, total erythrocyte and differential counts. Normal values for rabbits had been previously experimentally determined in these laboratories. The pertinent hematologic data are presented in Table 3. The leukocyte and differential counts are omitted since they were included in the study only for purposes of completeness and did not deviate from normal values at any test period with but one exception. In the 5th week rabbit #194 developed changes appropriately associated with the appearance of a respiratory disorder. This condition cleared following a 2 week course of aureomycin and was, therefore, not considered to be associated with prior MF immunization. The hematology experiment was terminated after 5 weeks for the pair of rabbits, #176 and #194, however, since they were cagemates, and it seemed probable that the respiratory disease might well be communicable.

All of the rabbits experienced a slight depression of hematocrit, hemoglobin and erythrocyte count at the 1 week post-immunization test, but this was considered of little significance since it more than likely represented an early physiologic response to the immunizing
### TABLE 3

HEMATOLOGIC VALUES FOR RABBITS IMMUNIZED WITH BCG MICROSONAL FRACTION

#### I. NORMAL VALUES FOR RABBITS:
- Hematocrit——37-40%
- Hemoglobin——12-16 gms%
- Erythrocytes—4.0-6.0 X 10^6

#### II. EXPERIMENTAL VALUES FOR IMMUNIZED RABBITS:

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Value</th>
<th>Base</th>
<th>Weeks Post-Immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1332</td>
<td>Hematocrit</td>
<td>38</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Erythrocytes</td>
<td>5.18</td>
<td>4.95</td>
</tr>
<tr>
<td>1333</td>
<td>Hematocrit</td>
<td>38</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Erythrocytes</td>
<td>5.10</td>
<td>4.50</td>
</tr>
<tr>
<td>176</td>
<td>Hematocrit</td>
<td>43</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Erythrocytes</td>
<td>5.53</td>
<td>5.06</td>
</tr>
<tr>
<td>194</td>
<td>Hematocrit</td>
<td>42</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Erythrocytes</td>
<td>5.00</td>
<td>4.17</td>
</tr>
</tbody>
</table>
procedure. The depressed values subsequently returned to the normal ranges, and it was concluded that MF immunization had produced no blood changes suggestive of acquired hemolytic anemia.

Initial serologic studies were performed with the sera of 2 rabbits which had been placed on a bi-weekly bleeding schedule. The recovered sera were stored at -20°C and were tested simultaneously following the last bleeding. Passive hemagglutination titers against sRNA and MF are presented in Table 4. Reference baseline serum from rabbit #1332 was slightly reactive for both antigens, thus invalidating conclusions relevant to effects of the immunization for that particular animal. Sera from #1333 reacted with both antigens at 2 weeks post-immunization and displayed maximum reactivity at 6 weeks. No particular pattern of reactivity was observed with the sera of either rabbit against either antigen.

In view of the invalidating effects of the baseline reactivity in the sera of rabbit #1332, and because no particular pattern of reactivity was established, a 2nd pair of MF immunized rabbits was placed on a weekly bleeding schedule. The sera from both animals was shown to be non-reactive prior to immunization. Titers for these rabbits also are in Table 4. The patterns of reactivity were remarkably similar for both rabbits. At one week post-immunization, titers for sRNA were already significantly elevated at 1:128, suggestive of a secondary antibody response. Titers of both rabbits for sRNA remained within one dilution factor at 2 weeks, dropped sharply in the third week, and disappeared by the end of the 4th week. Titers for MF were more typical of a primary antibody response, starting out low after
<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Antigen</th>
<th>Base</th>
<th>Weeks Post-Immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1332</td>
<td>sRNA</td>
<td>4</td>
<td>4 4 - - - 8</td>
</tr>
<tr>
<td></td>
<td>MF</td>
<td>8</td>
<td>8 8 4 4 32</td>
</tr>
<tr>
<td>1333</td>
<td>sRNA</td>
<td>-</td>
<td>8 4 32 4 4</td>
</tr>
<tr>
<td></td>
<td>MF</td>
<td>-</td>
<td>8 8 16 8 8</td>
</tr>
</tbody>
</table>

**II. WEEKLY BLEEDING SCHEDULE**

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Antigen</th>
<th>Base</th>
<th>Weeks Post-Immunization</th>
</tr>
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<tr>
<td>176</td>
<td>sRNA</td>
<td>-</td>
<td>128 256 4 - - -</td>
</tr>
<tr>
<td></td>
<td>MF</td>
<td>-</td>
<td>8 32 32 64 8 -</td>
</tr>
<tr>
<td>194</td>
<td>sRNA</td>
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<td>128 64 4 - - -</td>
</tr>
<tr>
<td></td>
<td>MF</td>
<td>-</td>
<td>16 32 64 64 64 -</td>
</tr>
</tbody>
</table>

*Titer expressed as reciprocal of highest serum dilution showing a positive reaction.*
the 1st week and reaching a maximum about 4 weeks post-immunization. The MF titer for rabbit #176 had declined sharply from 1:64 to 1:8 after the 5th week, but the titer remained at 1:64 for rabbit #194. The experiment was interrupted at that time again because of the previously noted respiratory disease which was contracted by rabbit #194. After the infection was eradicated, the rabbits were bled again at 8 weeks post-immunization and their sera proved to be non-reactive for both antigens.

Partial characterization of the anti-RNA and anti-MF antibodies from rabbits #176 and #194 was achieved by pre-treating the antigens with RNAase and trypsin prior to coupling them to the red cells. Results of hemagglutination against enzymatically treated antigens are presented in Table 5.

Serum samples from the 2 weeks post-immunization test were quite reactive for the conventional sRNA antigen while those from the 4th week were equally highly reactive for MF. The sera from both rabbits, as expected, were significantly less reactive for sRNA when the sRNA had been pretreated with RNAase, but the titers were undiminished by pretreating sRNA with trypsin. Sero-activity for the MF also was substantially reduced by pretreating the MF with either RNAase or trypsin. These results indicate that the anti-RNA was, in fact, specific for RNA, while the anti-MF is either comprised of 2 antibodies (one specific for RNA and the other for protein) or 1 antibody with specificity for an RNA-protein complex.

**Delayed Hypersensitivity**

Twelve tuberculin negative rabbits were immunized with single
<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Weeks Post-Immunization</th>
<th>Antigen</th>
<th>Enzyme</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>Titer</th>
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<td>1+</td>
<td>-</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNAase</td>
<td>3+</td>
<td>2+</td>
<td>1+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trypsin</td>
<td>4+</td>
<td>4+</td>
<td>3+</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
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<td>MF</td>
<td>-</td>
<td>3+</td>
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<td>Trypsin</td>
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<td>8</td>
</tr>
</tbody>
</table>
injections of 200 mg. (wet wgt.) of BCG microsomal fraction. None demonstrated cutaneous skin reactivity to an intradermal injection of 0.1 ml. of 2nd Test Strength P.P.D. at 4 weeks post-immunization. Some of those rabbits had been skin tested 3 weeks post-immunization and failed to evidence cutaneous sensitivity at that time as well. As was discussed by Mackaness (34) lack of cutaneous hypersensitivity does not necessarily preclude sensitivity at the cellular level. Consequently, peritoneal exudate cells from an MF immunized rabbit five weeks post-immunization were tested for hypersensitivity by their ability to migrate from capillary tubes onto cover-slips in the absence or presence of P.P.D. This was accomplished in comparison with exudate cells from BCG immune and normal rabbits.

Cell pellets, contained in capillary tubes, were sealed in Sykes-Moore Chambers which were filled with medium containing either no antigen or 25 µg per ml. of P.P.D. After 24 hours and 48 hours incubation, the areas of migration were traced and the tracings were weighed. Since the weights of the tracings were proportional to the area of migration, the migration index was expressed as follows:

\[
\frac{\text{average wgt. of migration tracing with antigen}}{\text{average wgt. of migration tracing without antigen}} \times 100
\]

The experimental values are shown in Table 6 and the migrations are pictured in Plate III. All of the cells migrated well in the absence of antigen, and those from the unimmunized animal migrated nearly as well in the presence of P.P.D. Migration of cells from a tuberculin sensitive BCG immunized animal was almost completely inhibited. Most interesting though, was the inhibition of migration
<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Immunogen</th>
<th>Inhibitor</th>
<th>Wgt. of Migration Trace</th>
<th>Avg. Wgt.</th>
<th>Migration Index</th>
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<td></td>
<td></td>
<td></td>
<td>24 hr</td>
<td>48 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td>1370</td>
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<td>None</td>
<td>.0267g</td>
<td>.0149</td>
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<td></td>
<td>PPD</td>
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<td></td>
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<td>.0017</td>
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<td>.0142</td>
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<tr>
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<td>.0239</td>
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<tr>
<td></td>
<td></td>
<td>PPD</td>
<td>.0106</td>
<td>.0094</td>
<td>.0214</td>
</tr>
</tbody>
</table>
Plate III. Effect of PPD on the migration of peritoneal exudate cells after 48 hr. incubation.

1. normal
2. normal + PPD
3. BCG immune
4. BCG immune + PPD
5. MF immune
6. MF immune + PPD
of cells from MF immunized rabbits in the presence of P.P.D. Although not as low as for BCG immunized rabbits, the migration indices of 43 and 36 were significantly lower than those of 81 and 93 for the non-immunized rabbit. Thus, it was demonstrated that MF immunized rabbits do develop hypersensitivity demonstrable at the cellular level.
DISCUSSION

These studies were initiated in an attempt to improve understanding of immunity to tuberculosis, in particular, acquired cellular immunity. As such, it seemed that more meaningful examination of the immune mechanisms could be accomplished in host cells, free of the influences of viable bacilli in the immunizing preparations, because of the possibility of their persistence in host cells. Although the likelihood of intravenously injected viable BCG appearing in the peritoneal exudate cells employed here in the studies of cellular immunity is slight, this possibility is not without precedent. In reports of cellular transfer of resistance to salmonellosis, Saito et al. (77) found that the peritoneal macrophages of mice intravenously injected with *Sal. enteritidis* always contained small numbers of viable salmonellae. It seemed advisable, therefore, that studies of immunity would be facilitated by employing immunogens consisting of subcellular components of tubercle bacilli such as the particulate (microsomal) fraction or ribosomes as successfully employed by Youmans and Youmans (10) in improving the resistance of mice to tuberculous disease.

Another consideration was the desire to study the acquisition of immunity in a circumstance devoid of the complicating influences of delayed hypersensitivity, and again the immunogenicity of subcellular particles seemed to offer this advantage since Youmans (78) did not
detect delayed cutaneous hypersensitivity in mice immunized with ribosomes.

It was decided principally to employ the microsomal fraction (MF) of tubercle bacilli in these studies, because of ease of recovery (eliminating approximately 6 hours additional procedure in the preparation of the ribosomes by treatment of MF with sodium dodecyl sulfate), and in proper dosage, this fraction immunizes as efficiently as ribosomes without the need of Freund's Adjuvant, which further can complicate the interpretation of results. In retrospect, the choice of immunogen proved unsound with respect to the influences exerted by delayed hypersensitivity. Although cutaneous hypersensitivity was not demonstrable in MF immunized rabbits, capillary tube migration studies of immune macrophages indicated that the rabbits indeed were hypersensitive at the cellular level. Thus, the influences of the allergy must be appropriately considered in interpreting the results.

Rabbits were employed as test animals due to the facility of serial bleedings for the hematologic and serologic studies. Also, the rabbit is notably susceptible to tuberculous infection with bovine strains of tubercle bacilli, and accordingly MF from BCG, an attenuated bovine strain was employed as the immunogen, and M. bovis 4228 served as the challenge organism for in vitro and in vivo tests.

In vitro tissue culture studies afford a means for determining acquisition of resistance by peritoneal macrophages without sacrificing the experimental animal, which after suitable rest can be tested repeatedly. Traditionally, cellular immunity has been tested by either counting intracellular bacilli to determine the ability of immune
macrophages to inhibit intracellular multiplication of bacilli (26) or by counting the macrophages to determine their ability to resist destruction by challenge infection (70).

The previously noted problems which were encountered in attempting to enumerate intracellular bacilli were, by no means, original to this laboratory. Hanks (79) has extensively reviewed the difficulties involved in the assay of intracellular mycobacteria. Chief among these is the failure of investigators to cope with the preferential loss of heavily infected cells, thus, probably ignoring to count an important proportion of the bacilli. Another problem with which one must contend is the inconsistency with which individual macrophages engulf bacilli. For example, some cells may be initially heavily infected with bacilli, whereas others will not contain any organism. Hanks further suggests that the only accurate method of enumerating mycobacteria in cell cultures is to perform total counts of all the bacilli by combining supernates after each renewal of media with remaining intracellular bacilli at the end of the test period.

Considering the experiences reported from this investigation, as well as those of others in counting intracellularly located organisms, it was decided to concentrate on another method of determining acquired cellular immunity, i.e. resistance of macrophages to destruction.

The methodology of counting the peritoneal exudate cells in micro-chambers was patterned after that of Fong et al. (70), but differed in several essential respects. The tissue culture medium was Hank's Balanced Salt Solution, rather than Tyrode's; Sykes-Moore
Chambers were employed instead of Mackaness-type chambers; and peritoneal cells were not trypsinized prior to incubation in the chambers. A most critical difference involved the use of pooled normal rabbit serum as a medium supplement for cultivation of both normal and immune cells. Pong et al. (70) were unable to detect differences in resistance when immune cells were not incubated with immune serum, while the results of experiments reported here clearly indicated the superiority of immune over normal cells in resistance to destruction even in the absence of immune serum. Perhaps the major difference lies in the extent of infection which was 5 bacilli per macrophage, whereas Fong employed a 10:1 ratio. As Suter and Hulliger (28) have suggested, the inability to demonstrate differences in the presence of a 10:1 multiplicity of infection may have been due to an overwhelming infection of both cell types, thereby prohibiting moderate expression of difference. The systems examined in the present studies were more compatible to the visualizing of modest but still significant differences. As previously mentioned, Berthrong and Hamilton (30) also have clearly established the relationships of low and high infective doses to expression, or lack of expression, of cellular immunity to tuberculosis. Further, it was suggested (28) that the need for immune serum in Fong's experiments might be attributable to an opsonizing effect which somehow prepares the bacilli for destruction within the activated immune macrophages. Although opsonization of bacilli would also occur in situations in which normal macrophages are incubated with immune serum, the unactivated normal cells would be unable to cope with the intracellular bacilli.
A combination of the above observations and explanations suggests that the activated immune macrophages are independently capable of resisting a limited infection with tubercle bacilli, but their fullest measure of resistance is expressed following interaction of bacilli with immune serum.

Another aspect worthy of consideration is the possibility that the macrophages, themselves, from animals immune to tuberculosis, may produce a cellular antibody such as that demonstrated on salmonellae immune macrophages (49). This cellular antibody was shown to render salmonellae susceptible to the inactivation by lysozyme in the presence of complement.

If a similar cellular antibody can be demonstrated for the systems examined here, it will be necessary to consider three factors in the fullest expression of immunity: the activated macrophage, immune serum, and cellular antibody. It is important to note that the only one of these which functions independently in the expression of resistance is the activated macrophage.

It is difficult to account for the extent of degeneration of macrophages observed in the tissue cultures. Here, as in Fong's experiments, the number of infected macrophage was not proportional to the extent of degeneration. Considering the slow multiplication of intracellular tubercle bacilli, it is unlikely that much, if any, degeneration is attributable to mechanical rupture by proliferating organisms. Further, it has been shown that in suitable medium, macrophages can remain viable when infected with hundreds of mycobacteria (79). A hypersensitive involvement is not pertinent, here,
since normal cells, which were not sensitized, were even more susceptible to degenerative effects than were sensitive immune cells. An alternative possibility is that a toxic product of the bacilli is responsible for the observed effects. Fong et al. (70) demonstrated that a 7 day culture filtrate of H37Rv duplicated the deleterious effects of bacilli on macrophage cultures. Only the immune cells in the presence of immune serum proved resistant to degeneration by this filtrate. All other combinations of immune and normal cells and serum were susceptible. The effect was not due to either Old Tuberculin (O.T.) or P.P.D., both of which proved toxic for immune, but not normal cells. Again it seems that neutralization of the toxic effect required immune serum in combination with activated macrophages. In the experiments reported here, the immune serum was missing. However, the possibility remains that a cellular antibody substituted and accounted for the effects Fong attributed to immune serum. If such an antibody exists at the surface of tuberculous immune macrophages, it was probably destroyed in Fong's experiments, since all of his cell cultures were trypsinized prior to incubation in micro-chambers. Resolution of these controversial concepts ultimately will depend on whether or not the cellular antibody actually exists on macrophages from animals immune to tuberculosis.

Although it was clearly established that MF immunized rabbits acquired resistance demonstrable at the cellular level, it was decided that an additional measure of the degree of resistance would be reflected in the response of these rabbits to in vivo challenge with virulent M. bovis.
The results of intravenous challenge of these rabbits indicated that resistance had been acquired when compared with unimmunized rabbits, but the level was not of the magnitude afforded by immunization with BCG. This was true regardless of the criteria applied. The possibility that observed differences between the effectiveness of the two immunogens is attributable to the intensity of immunization with MF merits further experimentation. The dosage of a single intramuscular injection of 200 mg. (wet wgt.) was chosen empirically, referring only to the reports of Youmans and Youmans who employed 20 mg. (wet wgt.) to improve resistance of mice (10). Despite the low intensity of MF immunization in rabbits, the MF did prove to be a very effective immunogen in the sense that resistance persisted up to 16 months post-immunization. A stronger resistance may have developed in these rabbits if they would have been immunized with 2000-3000 mg. of MF which would have been equal on a weight basis to the dosage given mice.

The absence of overt signs of respiratory distress in MF immunized animals is puzzling in view of the extent of disease revealed in their lungs on autopsy. Perhaps, despite the number of lesions, the tubercle bacilli were more efficiently localized than in the lungs of unimmunized rabbits. Bacteriological cultures of these organs may have shown quantitative differences in the numbers of recoverable bacilli, but the unequal distribution of lesions would have required homogenization of whole lungs. This is an unwieldy, dangerous process that could better be accomplished with a smaller animal such as the mouse.
The differences between the resistance of spleens and of lungs of MF immunized animals suggests that splenic macrophages are more efficient than alveolar macrophages in coping with tuberculous infection. However, conclusions in this regard, must be tempered by the realization that the infecting organisms were administered via the marginal ear veins of the rabbits, so the lungs were naturally exposed to the infecting inoculum before the spleens. It may well be that because of pooling of blood in the lungs, the alveolar macrophages were more severely challenged than were the splenic macrophages. Further, it should be pointed out that some workers consider the alveolar macrophages to be different constitutionally from macrophages located in other tissues (80).

Hematologic studies did not indicate any untoward side effects resulting from the MF immunizations and certainly did not suggest any acquired hemolytic anemia sometimes associated with autoimmune disease (81). Antibodies to sRNA might well be considered autoantibodies because of the established fact that anti-nucleic acid antibodies generally lack species specificity (82). However, in the results reported here, these antibodies did not persist long after immunization. This, coupled with hematologic data and the fact that rabbits survived up to two years, led to the conclusion that no autoimmune disease was induced by MF immunization. These results agree favorably with those of others (83) who have reported anti-nucleic acid antibodies in rabbits free of disease. The possibility still exists that a greater dosage, or that BCG ribosomes incorporated in Freund's Adjuvant, might elicit such a response.
In the production of autoimmune disease in rabbits injected with rat and rabbit liver ribosomes, Dodd et al. (76) employed immunizing inocula of approximately 200 mg. (dry wgt.) of ribosomes in Freund's Adjuvant. This dosage was 6 to 7 times greater than the dry weight dosages of MF employed in the present experiments. Thus, it was not anticipated that the hematologic data would be comparable.

The serologic data from the two MF immunized rabbits on bi-weekly bleeding schedules is neither impressive, nor conclusive, but the sera from the two rabbits bled at weekly intervals, exhibited strikingly similar patterns of reactivity. The early high titer to sRNA resembles a booster response which is not unlikely considering that experimental animals are constantly exposed to micro-organisms and their sub-cellular constituents. Another explanation for this pattern of responsiveness would be that it represents a characteristic primary response (the term primary is used in the generally accepted sense) with the production of IgM antibody, but not IgG. This would permit an accounting for the disappearance of activity between the 3rd and 4th weeks, which is a typical observation for IgM antibody (84). Short-lived appearance of the antibody further argues against a booster effect unless the antibody is absorbed onto cells as rapidly as it is produced. The results of enzymatic treatment of sRNA prior to interaction with anti-sRNA sera confirmed the directed specificity of the antibody to sRNA. The activity remaining after pretreatment of the antigen with RNAase may be accounted for by residual nucleosides and nucleotides, because specificities of anti-RNA antibodies exist at that level (71).
The patterns of MF reactive antibodies correspond to typical responses involving both IgM and IgG. The substantial reduction of MF sero-activity by pre-treatment of the MF antigen with either RNAase or trypsin suggests the presence of two antibodies (one specific for RNA and the other specific for protein). Assuming an anti-RNA antibody against MF, the low titers in comparison to sRNA titers after one week could be explained by the fact that the red cells employed in preparing the hemagglutination antigen were sensitized with 1000 µg (dry wt.) of MF, only 40% of which was RNA. Consequently the red cells may not have been optimally sensitized for expression of anti-RNA activity. However, since MF titers remained high after disappearance of sRNA reactivity, it is unlikely that anti-RNA was involved in sero-activity against MF. A more plausible explanation would be that anti-MF activity was a result of antibody specific for ribonucleoprotein.

In view of the early antibody response to sRNA, it is interesting to recall the resistance-lowering effect of ribosomes and MF (13). If mice are immunized against tuberculosis with MF or ribosomes and challenged 28 days later, they exhibit significant resistance to tuberculous disease; but if they are challenged at the time of immunization their resistance is less than that of unimmunized controls, and they develop a more fulminating disease. It was inferred from the results that ribosomes may be important virulence factors of tubercle bacilli. Since the ribosome is an important immunizing constituent of the mycobacterium, the immune mechanism might be directed at that portion of the mycobacterial cell which is most injurious to the host.
The rapidity of anti-RNA production following MF immunization, noted here, may aid in providing explanation for the resistance lowering effect of MF immunization reported by Youmans and Youmans (13). See above. If anti-RNA could react with and inactivate the transfer agent (RNA) responsible for cell to cell transfer of immunity, this would result in lowering resistance of the host to disease. Anti-RNA was shown here to disappear after three to four weeks post-immunization, and thus, would exert no resistance lowering effect if the challenge were administered after four weeks post-immunization. This hypothesis could be tested in MF immunized rabbits by infecting them at 7 to 14 days (time of maximum anti-RNA production) and then comparing their resistance with that of unimmunized controls. The resistance lowering effect might also be transferable with serum recovered from an MF immunized rabbit 7 to 14 days post-immunization. The proposed mechanism would require the availability of transfer agent for the antigen-antibody reaction. Elberg (85) has suggested that the cell to cell transfer of immunity occurs via cytoplasmic bridges which have frequently been observed in tissue cultures. However, it is difficult to explain the acquisition of cellular immunity by peritoneal macrophages following intravenous injection of viable bacilli unless the transfer agent is, in fact, elaborated into the circulation (58) where it would be available for reaction with anti-RNA. On the other hand, Sato and Mitsuhashi (66) can impart resistance to salmonellosis in mice injected with ribosomes from immune monocytes. The mechanism of this conversion is unexplained. Nevertheless, a mechanism involving transfer agent inactivation would be an important factor contributing to the chronicity
of diseases associated with facultative intracellular parasites. In this regard it is interesting to re-examine data previously obtained in these laboratories (86). Sixteen rabbits were chronically infected with tuberculosis and their serially collected sera were examined for anti-sRNA during a twelve week evaluation. Sera from thirteen of the rabbits were positive for anti-sRNA by the criterion of a 1:8 titer in at least one of the serum samples. In general, reactivity appeared four to six weeks post-infection, reached a maximum at six to eight weeks and declined thereafter. These results were originally interpreted as being suggestive of an autoimmune involvement in which autoantibodies are formed in response to host tissue destruction and, in turn, contribute to further tissue destruction. The mechanism proposed here also is one of autoimmunity, differing only in the subtly of the mechanism, i.e. an indirect effect which interferes with host defenses so that bacilli proliferate more readily. This would suggest a dual role for mycobacterial ribosomal RNA: one as an immunogen (primer) capable of initiating macrophage activation and the other as a virulence factor responsible for the production of antibody (anti-RNA) which inactivates the transfer agent (RNA) responsible for the transfer of resistance from cell to cell. A "two-edged sword" of this type would be compatible with the facts associated with delayed hypersensitivity which may be either beneficial to the host by facilitating bacillary localization, or detrimental by virtue of the exacerbation of the disease accompanied by spreading of bacilli (17).

Thus far, the data discussed concur with and extend the views
expressed by Youmans and Youmans in that MF from BCG was successfully employed to improve resistance to tuberculosis in another animal species, the rabbit. In addition, the *in vitro* studies proved that MF immunization induces acquired cellular immunity as well. However, the macrophage migration studies also confirm the viewpoint of Mackaness (34) who states that resistance to tuberculosis is accompanied by, or anteceded by delayed-type hypersensitivity. Although the migration of peritoneal cells from MF immunized rabbits was significantly inhibited by P.P.D. as compared with control macrophages, the effect was not nearly as pronounced as that observed with peritoneal cells from BCG immunized rabbits. This difference in sensitivity corresponds to the proportional difference seen in the *in vivo* resistance afforded by the MF and the BCG immunizations.

Significantly, the MF immunized rabbits whose macrophages were inhibited from migration by P.P.D. failed to demonstrate skin reactivity to P.P.D. This same phenomenon was noted in all animals immunized with MF under the conditions stated here, i.e. evidence of acquired resistance was not accompanied by skin reactivity. This suggests that the threshold for hypersensitivity demonstration at the cutaneous level is higher than that necessary for demonstration at the cellular level.

It should be noted, however, that more intensive immunization might better amplify these data. Moreover it would seem appropriate to extend these studies to include immunization with ribosomes or bacterial RNA incorporated in Freund's Adjuvant and to test these animals in the parameters described here in order to determine whether the lipoprotein of the MF fraction was responsible for the induction
of the cellular hypersensitivity observed in this investigation.

Should immunization with sub-cellular fractions provide effective levels of resistance and not be accompanied by the development of skin hypersensitivity, another very practical dimension would be apparent. The tuberculin skin test would remain of value in identifying cases or rapid converters.
SUMMARY

1. Microsomal fraction (MF) recovered from homogenates of BCG by differential centrifugation was analyzed spectrophotometrically and chemically and visualized by electron microscopy prior to being employed as immunogens in the study of resistance to tuberculosis.

2. By employing BCG immunized rabbits, methodology was developed to satisfactorily quantitate the acquisition of resistance by peritoneal macrophages from immunized rabbits.

3. Macrophages from MF immunized rabbits resisted degenerative effects of in vitro challenge by M. bovis more efficiently than control macrophages. This resistance was demonstrated in the absence of immune serum.

4. MF immunized rabbits occupied an intermediate position between BCG immunized rabbits and unimmunized rabbits in resistance to intravenous challenge with virulent M. bovis.

5. Immunization of rabbits with MF resulted in the rapid development of antibodies with specificities directed to sRNA and MF. Accompanying hematologic data failed to reveal changes frequently associated with the development of autoimmune disease.

6. Rabbits immunized with MF were not skin reactive to P.P.D., but MF immune macrophages were inhibited from migration from capillary tubes by P.P.D. Migration inhibition was not as great as that demonstrated for BCG immune macrophages.
7. Mechanisms were proposed for some of the observed phenomena and further study along these lines was indicated.


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