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Chronic Aerogenic Tuberculosis in Mice* (33982)

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The intravenous injection of large numbers of virulent tubercle bacilli into mice usually results in death in 3 weeks or less, due to pulmonary insufficiency caused by tubercle formation and consolidation of the lung tissue. Mayer and Salamandra (1) reported that treatment of intravenously infected mice with isoniazid for 3 weeks prevented early death in these animals. The mice survived with chronic disease, and deaths occurred only after the seventieth day.

For the study of chronic mouse tuberculosis, it has been most useful to employ massive intravenous infection and treat with antituberculosis drugs which diminish the number of organisms, or to use attenuated virulent strains. Another approach is that of Gray and Mattinson (2) who infected mice by the intranasal instillation of fully pathogenic tubercle bacilli. By this technic Gray and Cheers (3) were able to define more clearly the "steady state," in which the number of viable units of tubercle bacilli remained constant for a long period of time. They proposed the term "immunological complaisance" for this phenomenon. In their experiments, 250 viable units of tubercle bacilli were introduced into the lungs of mice, which, considering the size of these lungs, seems a relatively large number.

The aerogenic infection route lends itself to controlled infection by small numbers of tubercle bacilli. We found that large numbers of mice may be infected simultaneously with approximately the same number of bacilli per mouse. The present report describes the natural history of chronic tuberculosis in mice infected by tubercle bacilli introduced in small numbers by this route.

Materials and Methods. The apparatus and the preparation of the single cell suspension used in aerogenic exposure of the mice to tubercle bacilli have been described elsewhere (4). The H37Rv strain, obtained from the Trudeau Institute, Inc., was subcultured in albumin Tween 80 liquid medium. Single cell suspensions were prepared and 1:100 dilutions were stored at —80° until needed. At

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this temperature the suspensions are stable and maintain their viability and pathogenicity (5). Exposure of guinea pigs to droplet nuclei from these suspensions containing 2.6 $\times 10^4$ bacilli/ml for 30 min gave highly reproducible results even after 6 months of storage.

For our experiment, 125 CF-1 mice 4–5 weeks old were simultaneously exposed to aerogenic infection. At certain time intervals the viable units of tubercle bacilli in the lungs and spleen of each of 10 mice were determined. The lobes of the lungs were removed aseptically without disturbing the trachea or esophagus. The lungs were free of extraneous organisms and decontamination was not necessary. The organs were homogenized with a Teflon-to-glass grinder in 2 or 5 ml of 0.2% albumin solution. Appropriate dilutions were planted on 7H10 oleic acid-albumin agar medium and incubated under 5% CO_2–air. Also, at each interval, 4 mice were killed for surface tubercle counts and for histological examination.

Results. Viable tubercle bacilli recovered from the lungs of 10 mice 1 day after infection showed that an average of 54 had been deposited. Average number of surface tubercles (4 mice) was: at 4 weeks, 7; 8 weeks, 17; 12 weeks, 17; 16 weeks, 21; 24 weeks, 57; 36 weeks, 41; and at 52 weeks, 40. Up to 16 weeks, the number of tubercles remained relatively stable, but at week 24 and later intervals there was an increase in the number of tubercles in some of the animals.

The average number of viable units of tubercle bacilli in the lungs and spleens of 10 mice is indicated for each interval in Fig. 1 by an open circle (top and bottom bars are maximum and minimum units, respectively); photographs of the lesions in a lobe of the lung are shown in Fig. 2. As shown in Fig. 1, the tubercle bacilli multiplied logarithmically for 3 weeks, reached a peak at week 8 and then maintained a "steady state" (in which the number of organisms remained relatively constant) until week 16. During this period there were no deaths due to tuberculosis or to any other cause. From weeks 16 to 24, there appeared to be a slight, inexplicable decline

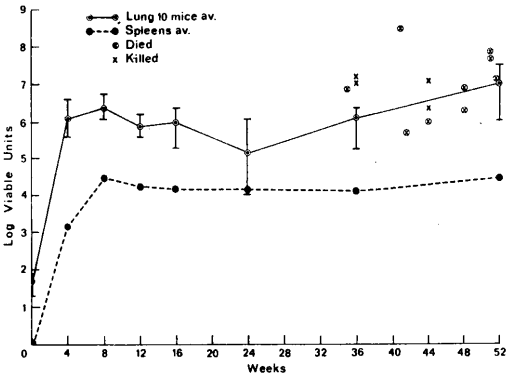

FIG. 1. Viable unit counts of tubercle bacilli following aerogenic infection of CF-1 mice with *M. tuberculosis* H37Rv.

in the number of viable units of tubercle bacilli. However, from weeks 24 to 52 the increase in the number of viable units was highly significant ($p<0.005$). From weeks 24 to 36 there was an increase in the number of viable units ($p<0.05$). One mouse died at week 35 and its lungs contained 8.6×10^6 viable units of tubercle bacilli; 2 mice of the 10 killed at week 36 contained 12.0×10^6 and 17.0×10^6 viable units, respectively. These latter 2 were not averaged in the usual manner and are shown as separate star points. The mice in this group killed for analysis were not picked randomly from the original pool but were chosen because they appeared to be ill. It was hoped that the remaining mice might survive for the 1-year interval of analysis. From weeks 36 to 52 the number of viable units increased ($p<0.05$). During this period 11 animals died; culturable units were recovered from the lungs of 8 (Fig. 1). In these animals, and some of those killed at week 52, a count of the number of tubercles was not possible because the lungs contained diffuse tuberculous tissue. At the time of killing, simply by observation of the symptoms of anoxia and emaciation, we could select animals which had very little normal lung tissue remaining.

The number of viable units of tubercle bacilli in the spleens remained constant after 8 weeks. However, the animals that died or had large numbers of tubercle bacilli in the lungs also had a larger number of viable units of tubercle bacilli in the spleens.

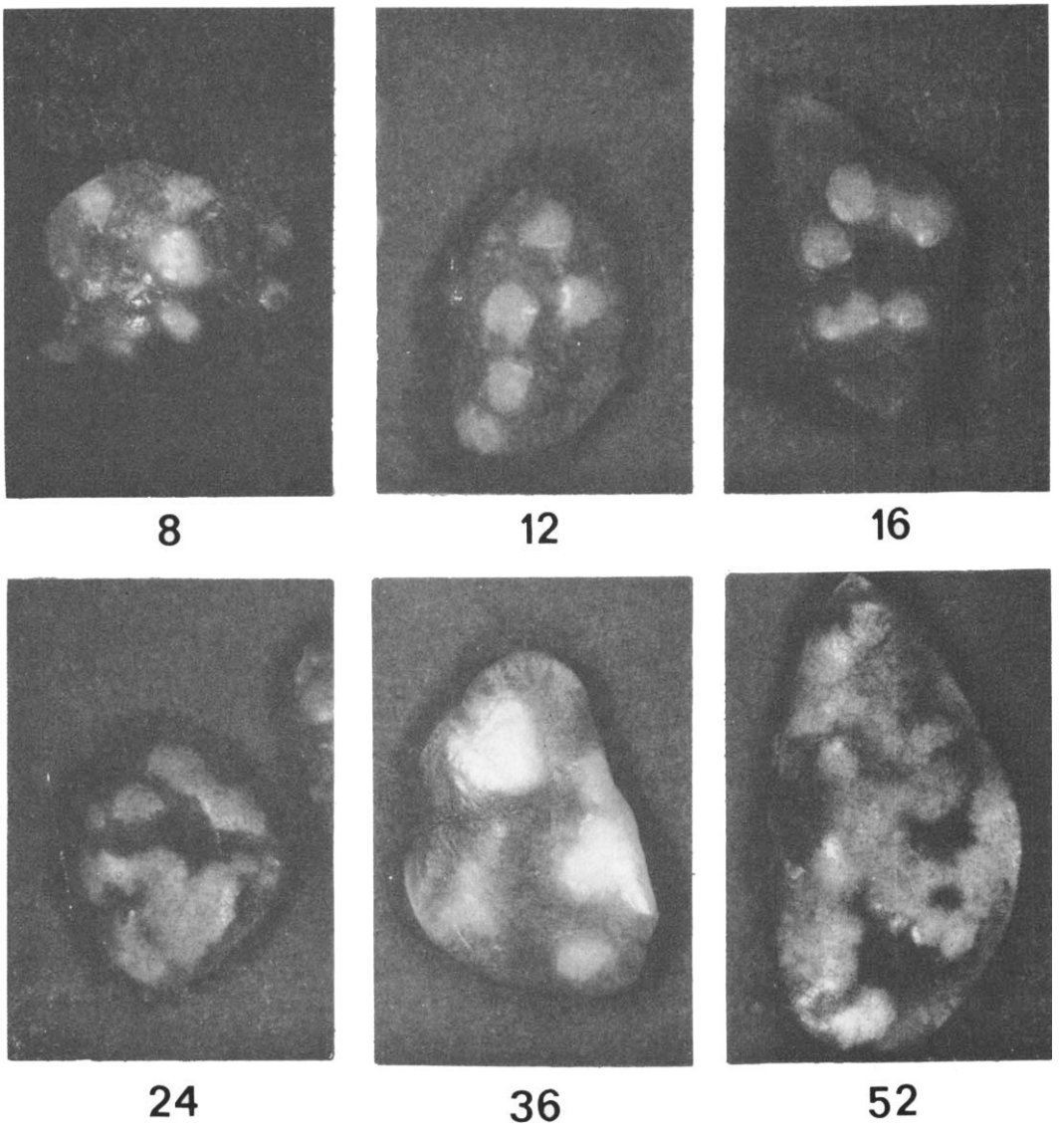

FIG. 2. Lobes of the lung (3.4×) illustrating development of tubercles; numerals indicate weeks after infection.

Figure 2 shows that the tubercle developed and appeared circumscribed until week 16, at which time there was some suggestion that the lesions had begun to coalesce. This effect was more pronounced by week 24. By determination of the area surface occupied by the tubercles it was found that 28% of the surface was involved at 16 weeks and 55% at 24 weeks. Besides the coalescence of tubercles, an increase in the number of lesions appeared to occur in some of the mice. In those animals almost complete tuberculous involvement of the lungs occurred, resulting in death of the animals.

Histopathology. At 4 weeks, the tubercles in the lungs varied in size from microscopic to 1.5 mm. The smaller tubercles consisted of epithelioid cells, lymphocytes, and many polymorphonuclear leukocytes with some nuclear fragmentation. A few foam type macrophages were also present in the larger tubercles. Acid-fast bacilli were present in tuber-

cles and were seen within the epithelioid and foam cell macrophages.

From week 4 on, the tubercles increased in size and the foam type macrophages constituted the greater part of the lesions. There were fewer epithelioid cells and relatively few polymorphonuclear leukocytes in the tubercles. Acid-fast bacilli occurred within the lesions, and, more particularly, within the foam cell macrophages. As the tuberculous process became more extensive, there was more replacement of the normal lung tissue. Eventually, as the foam cells at the edges of proximate lesions merged to form homogeneous tubercles, there was coalescence of tubercles. At no time was there any evidence of necrosis, encapsulation, or connective tissue. Giant cells first appeared at week 36. Plasma cells were then seen admixed with the lymphocytes contained within the tuberculous areas; acid-fact bacilli were still present within the epithelioid and foam cells.

Between weeks 36 and 52 there was a marked increase in epithelioid cells and an increase in the size of the lungs of the anoxic mice. Numerous acid-fast bacilli were observed in both the foam and epithelioid cells. In the nonanoxic mice, more normal lung tissue was present; these animals were no different pathologically from those killed at 36 weeks.

Discussion. The tubercle in the mouse is quite different from the tubercle in the guinea pig, rabbit, or man. It contains no necrotic tissue or calcification. There is no encapsulation or connective tissue. It differs also in one cellular constituent: the presence of the foam cell macrophages, which usually contain tubercle bacilli. These cells have been reported in mouse tuberculosis, but their origin and function have not been elucidated. This type of cell, laden with leprosy bacilli, has been reported in human leprosy (Virchow cells), and its fine structure has been investigated by Brieger and Allen 6). The results of fat staining (by the scharlach R stain) of frozen sections of the mouse tubercle indicated that the foam cell vacuoles contain lipid material.

Cohn and Benson's (7) work suggests that the foam cell is a differentiated mononuclear phagocyte such as may be obtained by the intraperitoneal injection of bacterial lipopolysaccharide. The fine structure of the phagocyte was extensively studied by Cohn *et al.* (8), who also showed that these cells produce abnormally large amounts of acid phosphatase, beta-glucuronidase, and cathepsin. It seems to us that, although they are intimately concerned with defense against infectious agents, their function, other than phagocytosis, is not fully understood.

Multiplication of tubercle bacilli in these macrophages is extremely slow; sometimes it does not occur for long periods, accounting for the slow progression of mouse tuberculosis when the initial infection is minimal. Despite no apparent increase in the number of organisms in the lungs, the viability of the bacilli is not impaired.

Rees and Hart (9) stated that "in chronic murine tuberculosis the bacilli are in a static or resting phase rather than in a dynamic equilibrium between multiplication and destruction." Our studies confirm this opinion.

Summary. Aerogenic tuberculous infection of mice with small numbers of tubercle bacilli resulted in chronic tuberculosis which persisted in some of the animals for over a year. The lesions produced by this infection contained a constant number ("steady state") of culturable units of tubercle bacilli after 8 weeks, but there was enlargement and coalescence of the lesions because of an increased number of foam cell macrophages. In consequence, there was an increased involvement of normal lung tissue. Thirty-six to 52 weeks after infection, the number of culturable units of tubercle bacilli had increased beyond that of the steady state; deaths continued to occur during this period.

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Hyperpotentiation by Synthetic Double-Stranded RNA of Antibody Responses to Influenza Virus Vaccine in Adjuvant 65 (33983)

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The previous reports by our group (1–11) described the development and evaluation of a new immunologic adjuvant named adjuvant 65, which was applied to killed influenza vaccine. The adjuvant preparation consisted of a water-in-peanut oil emulsion of the aqueous vaccine employing mannide monooleate (Arlacel A) as emulsifier and aluminum monostearate as stabilizer. Use of the adjuvant effected great increase in the height of antibody compared with aqueous vaccine and this differential was maintained for several years following the last dose of vaccine.

Braun and Nakano (12) demonstrated that certain oligodeoxyribonucleotides and ribonucleotide homopolymers, especially complexed double-stranded (e.g., rA:rU) molecules may function as potent stimulators of the immune mechanism. The question was raised, therefore, whether addition of complexed RNA homopolymers to adjuvant 65 might provide a potentiation of antibody response which would exceed that of adjuvant 65 or of the polynucleotides alone. It was found that complexed polynucleotides Poly I:C (rI:rC) and Poly A:U (rA:rU) effected a very marked hyperpotentiation of the antibody responses to influenza antigens. These same polymers were found previously by our group (13, 14) to serve as highly potent inducers of interferon and of protection against viral infections *in vitro* and *in vivo*. The present report presents the findings in qualitative and quantitative studies to measure the potentiation by the polynucleotides of the antibody responses to influenza viral antigens in adjuvant 65.

Materials and Methods. Vaccines: Quadrivalent, trivalent, or monovalent influenza virus vaccines containing the kinds and amounts [chick cell agglutinating (CCA) units] of formalin-killed influenza viral antigens shown in Table I were employed. All vaccines were prepared according to procedures ordinarily used for commercial influenza vaccine production. The physiochemically purified (5) and the filter-purified (8) vaccines were described previously. The vaccines were prepared in 0.15 *M* phosphate buffered saline solution, pH 7.2. Certain of the vaccines were precipitated with potassium aluminum sulfate (alum) and contained 8.0 mg of alum/ml of aqueous vaccine.

Polynucleotides. The polyinosinic (Poly I or rI), polycytidylic (Poly C or rC), polyadenylic (Poly A or rA), and polyuridylic (Poly U or rU) acid ribonucleotides which were used to potentiate immune responses (see Table I) were obtained from Miles Laboratories. The individual nucleotides were dissolved in phosphate buffered saline and sterilized by filtration through UF sintered glass. Complexes of Poly I:C (rI:rC) and of Poly A:U (rA:rU) were prepared by mixing equimolar solutions of the complementary polymers and allowing them to react at room temperature. The complexing was measured